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Proteus mirabilis Swarming

O-antigen, Surface Sensing, and the Rcs System

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A dissertation submitted to Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

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Proteus mirabilis is a Gram-negative bacterium that exists as a short rod when grown in liquid media. During growth on surfaces, *P. mirabilis* undergoes a distinct physical and biochemical change that culminates in the formation of a swarmer cell. Swarmer cells are elongated, polyploid, and hyper-flagellated cells that up-regulate virulence factors. How *P. mirabilis* senses a surface is not fully understood; however, the inhibition of flagella rotation and accumulation of putrescine have been proposed to be sensory mechanisms. Our lab has isolated a transposon insertion in *waaL*, encoding O-antigen ligase (PM942), that results in loss of swarming, but not swimming motility. Upon further examination, it was shown that the swarming decfect in the *waaL* mutant stemmed from a failure to activate *flhDC,* the class 1 activator of the flagellar cascade, when grown on solid surfaces. The swarming defect could be returned to the *waaL* mutant by overexpression of *flhDC* in trans or by making a mutation in the response regulator *rcsB.* We propose that surface sensing is relayed by O-antigen, to the Rcs phosphorelay, a known repressor of *flhDC*. In order to test this hypothesis, mutations were made in *rcsC*, *rcsB*, *rcsF*, and *umoB* (*igaA*), and *umoD* in wild-type and *waaL* backgrounds. By comparing the swarming phenotypes of the single and double mutants, along with overexpression strains, we have begun to establish a working model for the role of O-antigen in surface sensing and the Rcs pathway in *P. mirabilis*. We have shown that along with RcsF, UmoD acts on the Rcs system, and that UmoD is activated by solid surfaces.

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Table of Contents

Figures and Tables

Chapter 1: Introduction

Proteus

"A Greek god of the ocean who took many shapes to escape questioning" motivated Hauser, in 1885, to name this genus of bacteria *Proteus* (62, 64). The two bacteria that Hauser studied, *Proteus mirabilis* and *Proteus vulgaris*, each exhibit a form of motility, termed swarming, for which the name *Proteus* was given. In order to swarm, *Proteus* must differentiate from peritrichously flagellated, small, swimmer cells, into elongated, hyper-flagellated swarmer cells which can move over solid surfaces (143). There are now five named species of the genus *Proteus*: *P. mirabils, P. vulgaris, P. penneri, P. myxofaciens,* and *P. hauseri*, along with three unnamed genotypes (128). The following work will focus on *P. mirabilis* due to its ability to cause diseases in humans.

P. mirabilis is found as a normal inhabitant of the gut in many animals as well as in sewage, water, and soil (81, 128, 133, 162). Each of these environments comes with a unique set of challenges for survival which may have been an evolutionary cause for the development of swarming. Typically in healthy individuals, *P. mirabilis* does not cause infections (126). This raises the question of whether; animals are a normal niche for *Proteus,* or if they were originally intended to be used only as a vehicle for transmission. Studies have shown *Proteus* is found in the fecal matter of animals, possibly explaining its presence in sewage and other water sources (133, 162). However, Stahl and Williams showed that *Proteus* species were not found in fresh water samples near farm land or sewage treatment plants (162). It is hard to say however, if this

is because of sampling error or because there were no bacteria in those locations. Nevertheless, it is clear *Proteus* has now evolved to live inside animal hosts. It has been proposed that the natural reservoir of the human gut leads to self-contamination of the urinary tract (32). *Proteus* makes a variety of virulence factors that allow it to survive in humans as well as cause infections in compromised humans (126).

Virulence

Disease

P. mirabils is an opportunistic pathogen of the human urinary tract that can escape through capillaries to cause more systemic infections. While *Escherichia coli* is the most common cause of uncomplicated urinary tract infections (UTIs), *Proteus* produces a third of these types of infections, especially in hospital-acquired cases (128, 163). Unlike *E. coli*, where only specific strains can cause UTIs, all strains of *P. mirabilis* are infective (155, 158). However, in complicated UTIs involving patients with abnormal urethras, decreased immune functions, or undergoing extended catheterization, the percentage of infections caused by *P. mirabilis* is greatly increased. In the cases of long-term catheterization, 44% of cases are caused by *P. mirabilis* (189). Along with the symptoms of either a lower UTI (cystitis) or an upper UTI (pyelonephritis), *Proteus* can cause the formation of kidney or bladder stones (96, 121). It is also possible for the minerals to encrust the catheter, requiring surgery to remove it (165). The activity of a urease enzyme (see below) causes polyvalent cations, such as Mg^{2+}

and Ca^{2+}, to precipitate out of the urine and form struvite and carbonate hydroxyapatite crystals (56). The mineral structures also provide bacteria a habitat to hide from antibiotic treatment and the host immune cells (96).

Proteus can escape from the urinary tract through capillaries to allow bacteria to be present in the blood stream (bacteremia), which can lead to a variety of diseases (81). In infants, along with causing urinary tract infections, *P. mirabilis* can also cause neonatal meningoencephalitis (55, 159). *P. mirabilis* has also been shown to be involved in empyema and osteomyelitis (72, 109). *Proteus* has recently been determined to play a role in rheumatoid arthritis (RA). Patients with this disease show an increase amount of antibodies against *P. mirabilis* in the bloodstream, while antigens to other microbes are not increased in the sera (141, 142). It appears that *P. mirabilis* is able to mimic a human epitope expressed in patients who are genetically predispositioned to develop RA, causing an autoimmune effect (46).

Virulence Factors

Urease

Urease activity has been described in over 200 bacteria, including those found in UTIs, however, *E. coli* does not exhibit urease activity, while *P. mirabils* does (117, 120). The urease enzyme is a predicted trimer of trimers made of UreA, UreB, and UreC, together with a nickel coenzyme (76, 120). Induced upon contact with urea (through de-repression of the repressor UreR), urease causes the breakdown of urea to ammonia and carbon dioxide (125). The ammonia

raises the pH and causes minerals to precipitate, which can lead to kidney and bladder stones, along with formation of crystalline biofilms along an indwelling catheter (56, 164). *P. mirabilis* is especially adept at making these biofilms because of their capsule, which not only aids in cell adherence, but also in the formation of crystal structures (43, 148). These stones have an affect on *P. mirabilis's* virulence in multiple ways. First, the increase of ammonia can cause host cell lysis resulting in an increase of nutrients for the bacteria (38). Second, the stones create a safe haven for the bacteria to hide and evade not only host immune cells, but also antibiotics (38, 96). Crystalline material in the host is not healthy, and can lead to disease. Kidney/bladder stones and crystalline biolfims on catheters can block urine flow in the ureters possibly causing greater problems such as pyelonephritis and eventually septicemia (166).

Urease activity increases in swarmer cells and is necessary for a *P. mirabilis* infection (5, 47, 75, 77). While swarmer cells do up-regulate the production of urease, swarming is not needed to form stones or crystalline biofilms (77). During an infection urease activity is very important. Studies have shown when structural genes of the enzyme are mutated; there is a large competitive disadvantage (74, 75). When mutations are introduced into one of the urease genes, *P. mirabilis* fails to colonize the bladder or kidneys as well as wild-type, in a CBA mouse model of ascending UTIs (75). Another study shows that when urease is mutated, *P. mirabilis* exhibits a 1000-fold increase in infectious dose (74).

IgA Protease

There are a few bacteria along with *P. mirabilis* that can produce an extracellular enzyme capable of cleaving IgA. Some of these include the *Neisseria*, *Haemophilus influenza*, and *Streptococcus pneumoniae*, which cause infections at mucosal membranes (124). The protease *P. mirabilis* produces is a metalloprotease of the serralysin family of zinc proteases, encoded by *zapA* (190). The C-terminal end of ZapA contains a motif indicating it is probably exported by a member of the ABC transporter superfamily of transporters (190). Alkaline pH is optimal for the activity of many of these types of proteases, which is often present due to the urease activity of *P. mirabilis* (see above) (151). Unlike most IgA proteases, which only cleave IgA at the hinge region, ZapA completely degrades IgA (88, 152). ZapA has also been shown to be able to cleave many other proteins found in the urinary tract, including: complement components, cytoskeletal elements, and antimicrobial peptides (17). This work was done *in vitro* and may not represent the natural function of this enzyme, but is interesting to imagine the role of a protein that can protect the bacterial cell from a variety of host proteins

Similar to other toxins produced by *P. mirabilis*, *zapA* production is increased in swarmer cells (5, 183). During infection, ZapA is produced and active, causing the degradation of IgA *in vivo* (152). ZapA has also be shown to be needed for infection in the ascending UTI model of infection (183). When *zapA* was mutated, the numbers of recovered bacteria were specifically decreased in the urine and the bladder, with a 100,000 and 10,000 fold decrease

respectively (183). These data represent the first time an IgA protease has been specifically shown to be involved in virulence.

Hemolysin

Hemolytic activity is commonly found amongst Gram-negative and Gram-positive bacteria. *P. mirabilis* exhibits hemolytic activity encoded by the *hpmAB* genes (180). HpmA is a calcium-independent secreted toxin, which is both activated through cleavage of its N-terminus and transported by HpmB (180, 194). The function of hemolysins is to form pores in target host cells. It has been purposed that hemolytic activity helps *P. mirabilis* spread into the kidneys during infection (38). This is probably mediated through the increased ability of hemolytic *P. mirabilis* cells to invade host tissue (138, 147)

Hemolysin is not as critical for infection as urease, however, similar to urease and IgA protease, it is overexpressed in swarmer cells (5, 135). In the ascending mouse model of UTIs, no difference is found between wild-type cells or a *hpmA* mutant during colonization of the urinary tract (171). However, the same study also showed that when administered intravenously, the lethal dose for the hemolytic mutants is six times higher than the wild-type, indicating hemolytic activity may play a role in pathogenesis under some conditions (171). Anther study also indicates that strains lacking hemolytic activity are less virulent (137).

Proteus toxic agglutinin

A more recently discovered toxin, Pta, produced by *P. mirabilis* has both cytotoxic and agglutination ability (1, 2). Pta is a calcium-dependent exported protein that remains at the cell surface. Cytotoxic activity is associated with Pta when it is cell-associated or in a purified form. At alkaline pH and under high cell density Pta also showed the ability to cause *P. mirabilis* to aggregate (2).

While the role of Pta during swarming has not been studied, some work has been done to address its relevance during infection. It was shown that Pta is expressed *in vivo* during an infection and that the ID_{50} is 100-fold greater in a Pta mutant (1, 127). In a mouse infection, bacteria lacking Pta have less severe disease symptoms in the kidneys (1). These data suggest an important role of Pta in *P. mirabilis* pathogenesis.

UTI

P. mirabilis is a natural member of the human flora as well as being found in soil. It is hypothesized in order for infection to occur, self-contamination allows access to the periurethral area. This does not explain the high prevalence of *Proteus* infections in catheterized patients, or people with abnormal urethras. These factors are believed to inhibit normal washout of *P. mirabilis* from the urinary tract, allowing infection to occur (38). It has also been shown that *P. mirabilis* can adhere to certain catheters (depending on material), perhaps making it harder to wash the bacteria away (145). After entering the periurethral tissue, the bacteria must pass through the urethra to gain entry into the bladder,

where they can colonize. From the bladder, it is then possible to ascend into the kidneys, where the bacteria can replicate and invade cells. It is also possible for the bacteria to escape into the bloodstream and cause bacteremia (38).

Flagella and Swarming

P. mirabilis exists as both a peritrichously flagellated swimmer cell and a hyper-flagellated swarmer cell (143). It is assumed this ability to swim and swarm using flagella is needed for the bacteria to move from the urethra to the bladder and kidneys. However, there is contradictory evidence for the role of swimming and swarming during infection. There have even been clinical isolates found that were non-flagellated (201). It is important to note, as stated above, most of the main virulence factors of *P. mirabilis* are induced during swarming (5).

There are two ways that one can study the role of swarming during infection. Firstly, one can use direct observation to see if swarmer cells are present in infected animals or tissues. This is possible because swarmer cells are elongated compared to swimmer cells. Secondly, mutations can be made in the bacteria that inhibit swarming, which can be introduced into a mouse and assayed for virulence. Using a direct visualization approach two different studies came to the same conclusion: swarmer cells are rarely present during mouse infections (73, 200). However, other studies were able to see swarmer cells present in the ureters or kidney cells, but not in the surrounding pus (4, 38). From these studies it is difficult to make a definitive statement about the role of

swarming during infection. Mutational analysis coupled with pathology and colonization data might provide more insight.

Unfortunately, genetic analysis, coupled with pathology and colonization data, does not provide a definitive answer either. Allison et al. used two different infection models to study the effects of swarming during infection. Using a motile but non-swarming mutant, in comparison to a motile partial-swarmer, they showed during a systemic infection both strains have less kidney abscesses than wild-type bacteria (4). However, during an ascending UTI, the partial-swarmer was able to colonize the bladder, albeit not to the degree of wild-type, but not the kidneys, while the non-swarmer was unable to colonize either the bladder or the kidneys (4). These data seem to suggest that swarming is important during infection. On the other hand, Zunino et al. demonstrated that a non-flagellated clinical isolate had the same infectivity as flagellated clinical isolates in both an ascending UTI and hematogenous infection (201). This would suggest flagella and therefore swarming are not needed for infection.

Fimbriae

Fimbriae are bacterial surface appendages used for adherence. The recent sequencing of the *P. mirabilis* genome revealed there are 17 different fimbrial operons, spanning 5 different classes of fimbriae (136). Only a few of these have been shown to play a role in virulence**.** Fimbriae are normally expressed inversely to flagella, indicating adherence and motility are needed during different times of the life/infective cycle. It has been shown that the

mannose-reistant *Proteus*-like (MR/P) fimbrial operon encodes a protein, MrpJ, which can repress flagella expression during fimbrial expression (13, 95, 134).

MR/P fimbriae are probably the best studied of the *Proteus* fimbriae. Like many virulence factors, MR/P are controlled by a phase variation mechanism (199). When cells are collected from a mouse during infection, they are always in the phase-on state, indicating a role for the MR/P during infection (199). The MR/P class of fimbria has been shown to be expressed *in vivo* (11). Not only are these fimbriae needed for adherence to cells *in vitro*, but they cause a higher frequency of cortical abscesses than cells expressing mannose-resistant *Klebsiella*-like (MN/K) fimbria (154). In a CBA mouse model of ascending UTI, it was shown the cells lacking the MR/P were less able to colonize the urine, bladder, or kidneys of mice, although the cells were still present. In correlation with the colonization defect, there was less renal damage to the uroepithilium, and there was no pyelonephritis (kidney infection) present (12).

To further elucidate the role of MR/P during infection, studies were done where the phase variation mechanism was removed, so that cells were locked in either a phase-on or phase-off state (94). In this model, phase-off mutants were out-competed by wild-type cells in a mouse model, but when infected alone were able to cause infection equal to wild-type. If the cells were phase-on for MR/P expression they colonized the bladder and kidneys better than wild-type cells when co-infected or during single infection (94). These data show that MR/P are needed for both bladder and kidney infection, but are more important once the

infection reaches the kidneys. It is possible that MR/P mutants can still colonize the kidney because other fimbriae help with colonization.

Other fimbriae that have been shown to be involved in infection are the *Proteus mirabilis* fimbriae (PMF) and uroepithelial cell adhesion fimbriae [UCA, also known as non-agglutinating fimbriae (NAF)]. PMF have been shown in the ascending mouse model of UTIs to be involved with bladder infection. Mutants lacking PMF cannot colonize the bladder, but have equal numbers of cells compared to wild-type in the kidneys (110). A direct role for UCA has not been shown *in vivo*, but these fimbriae have been shown to bind different host factors and uroepithial cells, providing evidence for a possible role of UCA during infection (6, 92, 198).

As a whole, the data presented above indicated more research is needed to fully understand a *P. mirabilis* infection. This organism makes a variety of toxins that appear to be sufficient but not absolutely necessary for infection. One of the better-studied aspects of *P. mirabilis* biology is its ability to swarm. While swarmer cells are known to up-regulate toxin production, their role in infection is not as well understood. Data from different studies demonstrate conflicting roles for swarming cells during infection. Non-flagellate variants have even been found clinically. Other structures needed for infection are fimbriae. It appears *P. mirabilis* makes a variety of fimbriae that are needed for different aspects of infection. The roles of all the fimbriae have not been looked at *in vivo*. Once the roles of all these structures are further characterized, it might be possible to produce novel antimicrobials that target them and inhibit infection.

Motility

Bacteria, like all organisms, must obtain nutrients from the environment to sustain growth. There are many mechanisms different organisms have evolved to this. Some organisms are sessile and rely on the environment itself to bring nutrients toward them. Other organisms, such as *P. mirabilis*, are able to move in their environments to obtain nutrients they might not otherwise be able to use. Bacteria, in general, have devised a number of different ways to move in their environment (motility), while being able to sense the concentrations of nutrients to adjust their movements accordingly (chemotaxis).

In one of the first reviews of bacterial motility, Henrichsen reported six different forms of bacterial motility (67). These can be separated into different classes of motility based on their mechanisms. Swimming and swarming are both rely on flagella for motility, while twitching requires Type-IV pili for movement (67, 112). However, not all forms of motility need external appendages. Sliding and darting both make use of growth as the force generator and either slime or expulsion from capsules to facilitate spreading instead of appendages (67). It has been proposed that during gliding motility, slime is extruded from the cell to power movement, but recently it has been proposed that gliding is powered by intracellular motor complexes (118, 196). Because *P. mirabilis* undergoes both swimming and swarming motility, these topics will be discussed in more detail along with a brief survey of swarming motility among Gram-negative bacteria.

Flagella

Structure

Flagella are external bacterial appendages made of the flagellin protein that rotate in a counter-clockwise direction to power the cell forward (103). Flagella can exist on the bacteria's surface in multiple patterns. Bacteria can have one polar flagellum (monotrichous) or multiple flagella at the pole (lophotrichous). If flagella are present at both poles, the bacteria are said to be amphitrichous, while the presence of flagella all around the cell is called peritrichous. *P. mirabilis* is peritrichously flagellated.

The flagella complex spans the entire cell envelope. In Gram-negative bacteria the complex starts at the inner membrane and continues through the periplasm and outer membrane. Construction of the complex begins with the inner membrane components and works outward, with the flagella filament and cap being the last pieces assembled. The flagella complex is homologous to a type-III secretion apparatus, allowing the external components to be assembled by secretion through the flagella apparatus itself, rather than going through the periplasm. The first parts of assembly are the MS ring in the cytoplasm and the export proteins. The C-ring is then connected to the MS ring on the periplasmic side of the inner membrane. These proteins constitute the type-III secretion apparatus and the rest of the exported proteins, except for the P and L rings go through this channel. A basal body is formed to which the P (periplasm) and L (outer membrane) rings can attach, and anchor the structure in the cell envelope.

Next the hook is attached, followed by junction proteins, and then the flagella filament and cap (103). There is also a motor complex that is attached to the MS ring that appears to be able to attach at any point during construction (22).

The motor complex powers flagella rotation through the use of the proton motive force. In some bacteria, including *P. mirabilis,* the motor is bi-directional, allowing for both clockwise and counter-clockwise rotation (173). The motor connects to the rotor attached to the MS ring located in the cytoplasm. The MS ring is torque generating, which powers flagella rotation. The direction of rotation determines if the bacteria will swim (counter-clockwise), or tumble (clockwise) and is controlled by the chemotaxis system in most bacteria through binding of FliM in the motor complex (21).

Regulation

Regulation of flagella synthesis in *P. mirabilis* appears to be conserved with that of *E. coli* and *Salmonella typhimurium*. It consists of a multi-tiered regulatory cascade that includes three different promoter classes (31). The first and only Class I promoter is needed for the transcription of two genes: *flhD* and *flhC*, which are the master regulators of flagella synthesis. These two genes encode a heterodimeric protein complex in *P. mirabilis*, $FlhD_2C_2$ (33). During swarming, expression of *flhD* and *flhC* are induced leading to the large increase of flagella that is a hallmark of swarmer cells (34). The importance of $FlhD_2C_2$ can be inferred from the level of control the cell exhibits over their expression. There are many different proteins that play a role in the control of *flhDC*

expression that all must act in concert to obtain a functional swarmer cell (see *P. mirabilis* swarming). During swimming motility, these regulators work in conjunction to ensure that only four-eight flagella are made for a peritrichous bacterium such as *P. mirabilis*. However, during swarming, flagella are highly up-regulated, especially in *P. mirabilis* (123). Thus, to swarm, the cell must reprogram itself to allow for an increase in *flhDC* expression.

There are two other tiers of gene regulation involved in flagella synthesis. The Class II promoters are controlled transcriptionally by $FlhD_2C_2$ and σ^{70}, and include genes involved in flagellar basal body and hook assembly as well as an alternative sigma factor, σ^{28}. Active σ^{28} is used to control the expression of Class III gene promoters, which includes the flagella filament itself (130). There are some operons in *Salmonella* that are controlled by both Class II and Class III promoters (197). It was shown the expression of hook-associated proteins from the Class III promoter was important during swarming motility.

The early proteins of flagella assembly form a type III secretion system that is used by the later proteins for export and assembly. Because of this, the cell has devised a way to link the construction of flagella with gene regulation. One of the Class II genes encodes an anti-sigma factor, FlgM, which holds σ^{28} inactive until early assembly is complete (131). FlgM inhibits the activity of σ^{28} as long as there is a high concentration of FlgM in the cell. To lower the concentration of FlgM, the cell exports FlgM from the partially-constructed flagellar apparatus (70, 89). In this way, completion of the type III secretion

complex of the flagella acts as a checkpoint for flagella synthesis. Once FlgM is exported from the cell, σ^{28} is free to activate Class III promoters.

Chemotaxis

As bacteria move through their environment, they need a way to sense the conditions around them and respond accordingly. Chemotaxis is a way for organisms to sense chemical stimuli from the environment and change their direction of movement. Bacteria control their direction of movement through a regulated series of runs and tumbles. When nutrients are sensed, the flagella rotate in a counter-clockwise direction, propelling the bacteria forward. However, when toxins are sensed or the concentration of nutrients decreases, the flagella spin in a clockwise direction causing the bacteria to tumble randomly before beginning to swim again (177, 181). If the new random direction is away from the toxins, then the bacteria will continue to swim, but if the concentration does not decrease then the bacteria will tumble again until they go in a direction of decreasing concentration (181).

There are five classes of methyl-accepting chemotaxis proteins (MCPs) that are used to sense a variety of classes of molecules (66). Each MCP is a homodimer regardless if a ligand is bound (119). These dimers cluster at a cell pole and have been crystallized as a trimer of dimers (82, 83, 104). Interestingly, because of homology between the contact points in each class of MCPs, the trimer groups can be made up of any combination of the five MCP classes (169). The MCP acts as a sensor, and in conjunction with CheA, through a CheW

bridge, forms a sensor kinase of the two-component system family of regulators to relay the information it obtains from the environment to the cell and control the frequency of motor switches.

The flagella of bacteria are naturally set to spin counter-clockwise causing the bacteria to be propelled forward. Only as toxin concentration goes up or nutrient concentration goes down does the cell switch to clockwise rotation (182). The sensor kinase composed of the MCP-CheW-CheA complex senses small changes in ligand concentration and relays that information through transfer of a phosphate group to a response regulator CheY (68, 104). Upon phosphorylation, CheY can bind FliM of the flagella motor complex and cause a switch to clockwise rotation (193). In order to return to swimming, CheY must be dephosphorylated. This is done by the constitutive cytoplasmic protein CheZ (116).

As they move through the environment bacteria must also adapt to changing levels of stimuli. Through a process termed adaptation, bacteria can "reset" the chemotaxis machinery to sense new concentrations of stimuli as they move. This allows the cells to sense higher concentrations of the same signal, hence ensuring they move up a concentration gradient. Along with CheY, there is another response regulator that competes for CheA binding, CheB (93). CheB is a methylesterase that removes methyl groups from the MCP and competes with the methyltransferase CheR, which is constitutively active (80, 160). When methylated, the MCPs activate CheA, mimicking a toxin binding to the MCP (23). When an attractant is bound to the MCP, CheA activity is inhibited which biases

the cell toward runs by decreasing phosphorylated CheY levels. At the same time, CheB is held inactive allowing CheR to methylate the MCP, which turns on the MCP allowing CheA to be phosphorylated. This in turn, activates both CheY and CheB activity, essentially reseting the system prior to attractant being sensed (102).

The role for chemotaxis during swarming is not well known. Early reports indicated there was no role for chemotaxis during *P. mirabilis* swarming, however, recent date have indicated a possible role (15, 24, 195). The only known genes to be involved in both chemotaxis and swarming in *P. mirabilis* are CheW (the scaffold protein that connects the MCP to CheA) and CheA. When there is no CheW, the MCP cannot signal to CheA. Mutants in both CheW and CheA are unable to swarm under standard conditions (24). Unfortunately the exact roles for CheW and CheA in swarming are not known. It is possible that it is not chemotaxis itself, but the ability for the flagella to switch directions which is important for swarming and is a phenotype affected in these strains.

The role of chemotaxis has also been examined in other swarming organisms (see below). In accordance with the idea of flagella switching as the important factor involving chemotaxis and swarming, in *S. enterica* chemotaxis is proposed to be needed to induce motor switches, which promote wetness on the swarming surface (107). In *E. coli*, when a MCP was saturated to inhibit chemotactic behavior, swarming was unchanged. Mutations that bias the direction of rotation frequency did not effect swarming, indicating MCP sensing might be important, but not chemotaxis itself, and the MCP might be sensing new

signals that are relayed independent of the normal chemotaxis relay (25). Chemotactic signaling has also been shown to be important in hyper-flagellation of *E. coli* swarmer cells, suggesting a role for the chemotaxis machinery in surface-sensing (61). These data suggest in *E. coli* and *S. enterica* the chemotactic system is important, not chemotaxis itself. A screen for swarming mutants found many mutations in the chemotaxis genes. All of these mutants displayed a different surface pattern, but were able to swarm, indicating a non-critical role for chemotaxis or flagella switching during swarming (52). In *Serratia marcescens*, chemotactic mutants are unable to swarm (129). It is not known if this is because of chemotaxis directly or if just the chemotaxis machinery is needed. Another swarming organism, *Vibrio parahaemolyticus* needs chemotaxis to achieve proper swarming motility, but the study did not address the question of the role of the chemotactic system versus chemotaxis itself (149).

Swarming

Swarming is a flagella based form of surface motility that involves the differentiation of cells into elongated cells with extra flagella. Swarming is a form of social motility, as opposed to swimming, which is performed by the individual bacterium. In swarming, bacteria form “rafts” of cells aligned along their long axes and as a group move out from a central inoculum (40, 60, 61, 78).

While most bacteria need specialized soft agar media to swarm, *P. mirabilis* swarms on agar at concentrations ranging from 0.6% to over 2.0%. *P. mirabilis* swarmer cells are characterized by being elongated (10-40X) and

hyper-flagellated (over 50X) compared to swimmer cells, which are small, and only have a few flagella (15). Below is a brief description of swarming in other Gram-negative bacteria highlighting key differences between these species and *P. mirabilis* or highlighting important intellectual ideas, followed by a more detailed analysis of swarming in *P. mirabilis*.

E. coli and *Salmonella*

While *E. coli* and *S. enterica* are two of the most widely studied Gram-negative organisms, it was not until 1994 that they were shown to exhibit swarming motility (61). When inoculated onto Eiken agar at a concentration between 0.5 and 0.8%, *E. coli* and *S. enterica* exhibit swarming motility that involves elongated, polyploid cells, with increased flagellation (2X) (61, 174). The swarm of both these organisms fails to show consolidation rings, but does move out in all directions (178). Interestingly, unlike with *P. mirabilis,* in *S. enterica* there is no *flhDC* increase during swarming, only an increase in the flagella filaments (184).

Unlike *P. mirabilis*, which can swarm on 1.5% Difco agar, *E. coli* can only swarm on Eiken agar, while *S. enterica* can swarm on either types of agar. It is possible that Eiken agar has properties different than Difco agar, such as increased wettabilty, that allows for swarming when Difco does not. In congruence with this idea is the fact that *E. coli* and *S. enterica* need a wetting agent to swarm. When the O-antigen is not made in *S. enterica*, swarming is inhibited on Difco agar, but can be restored with the addition of surfactin (174).

Surfactin also restores swarming on Difco agar to an *E. coli* strain that lacks O-antigen (174). It is hypothesized that the surfactin provides a lubricant that is normally supplied by the LPS and is absent in those mutants. To further understand what supplies the wetness (the amount of fluid contained in the swarm), Chen et al. tested the fluid surrounding *S. enterica* cells to determine its chemical properties (30). They show the wetness is not caused by a surfactant, but by an osmotic agent (possibly LPS) that can increase the wettability (ability to wet a nonpolar surface) of the swarm (30). This raises the questions: how does LPS get removed from the cell surface in order to wet the agar, and does it need to be removed at all? It has been proposed flagellar motor reversals caused by the chemotaxis system (see above) is used for LPS removal (107). Another important question relates to the ability of the bacterial cell to sense the wetness of the agar. It has also been proposed that the flagella can be used by at least one bacterium to act as a wetness sensor affecting its own gene expression on solid surfaces (186).

Recent technological advances have allowed detailed studies of the *E. coli* swarm raft. Using microscopy either alone or coupled with flagella staining dyes, researchers have been able to observe individual cells and even individual flagella on these cells in swarm rafts (39, 40, 178). These approaches have revealed similarities between the flagella of swimming and swarming bacteria. Both types of cells exhibit motor switching resulting in flagella that spin in either a clockwise or counter-clockwise manner, however, unlike in swimming motility, swarming cells do not appear to run and tumble (39, 40, 178). Cell orientation is

instead based on collisions with neighboring cells (40, 178). The flagella of swarming cells can form bundles that propel the bacteria, but unlike with swimming cells, the flagella of neighboring cells in a raft can bundle together; although, this is rare (39, 178). A unique feature of swarming cells is their ability to reverse direction. Swimming bacteria use tumbles and Brownian motion to change direction, but swarmer cells can change direction relative to the cell body by reorienting the direction of the flagella (178). There is also an apparent flexibility of swarmer flagella which have been seen to bend at 90^0 angles (39).

These techniques have also shown an apparent difference between the outer edge of a swarm front and the interior of the colony. At the edge the cells either slow down or stop moving completely while the flagella continue to rotate (39, 40). The flagella appear to face outward from the swarm into the un-colonized agar, perhaps allowing the cells to spread a wetting agent onto the area (39, 40). Once at the edge, cells either reverse direction and recede back into the swarm raft, or allow the rest of the swarm to catch up with them. In the interior of the colony cells are packed tightly leading to many collisions, which align the cell body to move outward. Once free, the cell can move quickly outward. As a group, cells are not quicker, but rather more resistant to being pushed (40).

Classical genetics and microarray analysis have been other approaches taken to study both *E.coli* and *S. enterica* swarming, providing researchers with a list of new motility genes as well as new roles for previously known genes (52, 71, 184, 185). Both organisms show a need for outer-membrane structures such

as lipopolysaccharide (LPS), oligopolysaccharide (OPS), and enterobacterial common antigen (ECA) during swarming (52, 71, 184). As discussed above, this is likely due to a role for these structures in wettability. However, it is possible to find suppressor mutations that restore swarming in outer-membrane mutants suggesting a possible signaling role for them. O-antigen mutants can be suppressed by mutations in the Rcs two-component system, suggesting a link between the LPS and gene expression through control of the Rcs system (52).

In both *E. coli* and *S. typhimurium*, there is a need for an increase in iron acquisition and metabolic changes in the cell during swarming (71, 85, 184). It is not known if swarming cells need more iron or if swarm media is more iron depleted. It is believed that during growth on surfaces certain nutrients are harder to obtain due to a hindered ability to diffuse (114). Wang et al. showed that iron genes were only induced when grown on 0.6% swarm media compared to 1.5% agar plates, discounting a role for iron diffusion because there should be less diffusion in the plate with a higher agar concentration (184). Perhaps an increased need for iron is based on a change in cellular metabolism. *E. coli* and *S. enterica* need parts of or the entire TCA cycle for swarming as well as other ATP producing genes because swarming is an energy expensive process (71, 85). In a detailed study of the metabolic differences between *Salmonella* swarmer and swimmer cells, it was shown that there are different nutrient needs by the cell during differentiation opposed to during swarming. Swarmer cells showed an increase of de novo biosynthetic pathways while showing a decrease

in outer-membrane permeability, most likely due to a decrease in porin expression and a change in the charge of the outer-membrane (84-86).

A change in outer-membrane permeability has potential consequences during infection. Many organisms exhibit an increase in antibiotic resistance during swarming, suggesting an advantage for these organisms during a human infection. There are two different thoughts on what confers antibiotic resistance to swarmer cells. One group believes that changes in the outer-membrane properties of swarmer cells through regulation of OMPs and the *pmr* operon contribute to antibiotic resistance (84-86). An opposing view is that antibiotic resistance is an intrinsic property of cells during swarming due to an increase in cell density, and high speed mobility (26, 90).

Another important aspect of swarming, in a clinical setting, is the regulation of pathogenicity. For a bacterium to infect a person it must first be able to enter the body. For *Salmonella*, this happens through the consumption of contaminated food, such as fruits and vegetables. A connection has been shown between genes needed for the colonization of plant seeds and swarming, providing an early role for swarming in human disease (14). Once the bacterium enters the body, it normally increases virulence factors necessary for host survival and/or colonization. In *Salmonella*, many of these genes can be found on SPI2 (*Salmonella* Pathogenicity Island), which is regulated by the Rcs system, a two-component system involved in regulating flagella synthesis and therefore swarming motility (187). It appears swarming plays a role in infection at the

earliest stages (food contamination) and during an active infection (control of SPI-2).

Vibrio and *Pseudomonas*

Vibrio and *Pseudomonas* species are monotrichously flagellated Gram-negative bacteria, which can cause human disease and swarm. They are unique because most swarming bacteria, like *P. mirabilis*, are peritrichously flagellated. *V. parahaemolyticus* and *P. aeruginosa* are the species where swarming motility is best understood. While swarming in *V. parahaemolyticus* has been studied since the 1970s, it was not until 2000 that swarming was observed in *P. aeruginosa* (87, 140, 153, 179). The following will be a short description of the unique aspects of swarming from monotrichous bacteria, as well as a discussion of the important concepts learned from these bacteria.

Upon surface contact, *V. parahaemolyticus* produce an elongated (~30X), aseptate cell that is hyper-flagellated. Like *Proteus*, *V. parahaemolyticus* is able to swarm on a range of agar concentrations up to over 2% (115). *Vibrio* is rare among swarming bacteria because it produces two distinct types of flagella during their life. As a swimmer cell, *V. parahaemolyticus* produce a single, sheathed, polar flagellum made of multiple flagellar proteins. Upon growth on surfaces, a new lateral flagella system is expressed, consisting of non-sheathed flagella made from a single protein (113, 115). These two flagellar systems do not share any components, as it has been impossible to find mutations that disable both, and both are coupled to different ions for rotation (10, 115).

A prevailing hypothesis in the swarming field is that bacteria use their flagella to sense surfaces. It is not known exactly what is being measured by the flagella-surface interactions. It is possible the cell is measuring rotation inhibition, external pressure on the flagella, or 'swimming speed'. This idea originates from the work done in *V. parahaemolyticus*, which showed that the polar flagellum acts as a surface sensing organelle (19, 79, 113). When *V. parahaemolyticus* is grown in a viscous medium or is incubated with anti-flagellar antibodies, lateral flagella synthesis is induced (19, 113). Mutations in FlaC (one of the polar flagellar components) remove the requirement for solid surfaces in lateral flagellar expression (113). By using ion channel inhibitors and viscosity it was shown that lateral flagellar synthesis was similarly controlled under both conditions, indicating the need for polar flagellum rotation (79). This indicates the flagellum is not measuring force, but most likely rotation speed. Mutations in the motor components of the flagella also induce lateral flagella synthesis (113). However, it is still possible that swimming speed is being measured, because speed is reduced in all cases.

Work in *V. parahaemolyticus* has revealed another signal necessary for swarming initiation. Along with polar flagellum signaling, *V. parahaemolyticus* also senses ion concentrations, with low iron needed for swarming. The lack of iron was not able to induce without simultaneous inhibition of the polar flagella (114). Originally the only ion suspected of being a signal for differentiation was iron, however, recently, a need for calcium ions has also been shown to induce lateral flagella (54).

As with *Vibrio*, *Pseudomonas* also has one polar flagellum, but upon surface contact *Pseudomonas* does not become peritrichously flagellated, but instead produces an extra polar flagellum (87, 140). During swarming, *P. aeruginosa* double in size and possibly form a swarming raft, which can only move on media between 0.5-0.7% agar (87, 140). On this media *P. aeruginosa* form a tendril shaped swarm pattern. The tendrils radiate out of a central inoculum and never touch each other (27). While the role for pili in swarming in this organism is contested, it is known rhamnolipid production is necessary for swarming (27, 41, 87, 132, 140, 176).

Many swarming bacteria need to make wetting agents to overcome surface friction and swarm (see *E. coli* and *Salmonella* above, and *Serratia* below). *P. aeruginosa* produce extracellular glycolipids, termed rhamnolipids, that are required for swarming and controlled by quorum sensing. Rhamnolipids are produced by the addition of a TDP-L-rhamnose molecule by the *rhlB* gene product to 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAAs), produced by the *rhlA* gene product (41, 176). Rhamnolipids, in either a mono or di state, are excreted from the cells along with HAAs. Cells with mutations in *rhlA* (needed for HAA production) are unable to swarm, while mutations in *rhlB* (needed for rhamnolipid production) produce cells that can swarm, indicating HAAs are needed for swarming, not rhamnolipids themselves (41, 87). Further examination of rhamnolipids showed they inhibited tendrils from interacting with each other (27). In fact, purified rhamnolipids can inhibit swarming, contradictory to their role in promoting swarming. It was hypothesized the HAAs were needed for surface

wetting, while the rhamnolipids themselves were used to modulate swarming activity, giving rise to tendrils (27). Recently, it has been demonstrated HAAs and di-rhamnolipids have a chemotactic effect on swarming, while mono-rhamnolipids act solely as a wetting agent (176). HAAs act as a repellent and di-rhamnolipids act as an attractant, with each molecule having a different diffusion rate. As HAA concentration increases near the central inoculum it forces the bacteria to swarm away from it, during which they form tendrils and are attracted to di-rhamnolipids that diffused more quickly away from the colony.

Serratia

A genus closely related to *Proteus*, *Serratia* are also able to swarm. Unlike *P. mirabilis*, *Serratia* can only swarm on agar less than 0.9% (3). When *Serratia* swarm, they form swarming rafts composed of elongated, aseptate cells, with more flagella than their vegetative counterparts (3, 44). The swarming pattern in *Serratia* is radial, similar to *Proteus*, however *Serratia* does not consolidate (45). As with other swarming bacteria, differentiation occurs in liquid media when a thickening agent is added, possibly showing a role for flagella inhibition in surface sensing (3). An interesting aspect of swarming in *Serratia* is temperature control. *Serratia* only swarm at 30° but not 37°C (3, 101).

Control of *flhDC* expression is essential for swarming. Two different species of *Serratia* are well studied and it appears they regulate *flhDC* differently during swarming. *S. marcescens* exhibits an increase of *flhDC* during swarming that relates to an increase in flagella synthesis (157). However, *S. liquefaciens*

does not appear to upregulate *flhDC* (175). The swarmer cells do have extra flagella but because they are also elongated, it does not appear that they are hyper-flagellated. It is possible, through the artificial induction of *flhDC*, to cause the formation of swarmer cells in liquid media. This suggests on surfaces there might be post-translational control of *flhDC* (175). Interestingly, while an artificial increase of *flhDC* causes a hyper-swarming phenotype in *S. marcescens*, it inhibits swarming in *S. liquefaciens* (157, 175). It has been shown that at 37°C *flhDC* expression is decreased in *S. marcescens* most likely causing the temperature regulation of swarming (101).

All *Serratia* species secrete surfactants termed serrawettins, that are cyclic lipopeptides used to promote wetness on surfaces (111). Serrawettin production is necessary for swarming and is controlled through quorum sensing, indicating a need for high cell density during swarming (100, 111). This is plausible because a high cell density would be necessary to form swarming rafts. If the quorum sensing system is mutated, swarming can be fully restored upon addition of purified signal, indicating serrawettin may be the only quorum sensing controlled gene needed for swarming. It is also of note that serrawettin production is much higher on surfaces than in liquid even after the addition of exogenous signal, demonstrating a potential role for surface interactions as well as quorum sensing in their production (100).

P. mirabils

Surface Sensing

P. mirabilis is one of the more thoroughly studied organisms in the swarming field. As with other swarming bacteria, *P. mirabilis* exists as small swimmer cells in liquid media. When placed on a solid surface, *P. mirabilis* undergoes a biochemical and physical change, differentiating into elongated, aseptate, polyploid, hyper-flagellated swarmer cells. These cells also up-regulate virulence factors as well as a plethora of other genes. Unlike most other swarming bacteria, *Proteus* is able to swarm on high concentrations of agar (>2.0%). *P. mirabilis* forms a circular swarming pattern that resembles a 'bull's eye' because of its unique ability to consolidate, or de-differentiate, back into swimmer cells. This process of differentiation and consolidation is repeated many times to produce the 'bull's eye'.

The first step of swarming is the recognition of solid surfaces. Wild-type cells only differentiate after sensing they are on a surface. The formation of swarmer cells in liquid by genetic manipulations shows the need for surface sensing can be bypassed. The study of these mutations, or overexpression phenotypes, might provide clues to the mechanism(s) of surface sensing. As discussed earlier, inhibition of flagella is a current model for surface sensing. When *P. mirabilis* is incubated in liquid media with anti-flagellar antibodies, or with a viscous agent, it differentiates into swarmer cells (20). Mutants lacking flagella are unable to differentiate, confirming this idea (15).

Mutations in some flagella components, such as FliG (motor-switch complex) and FliL (unknown), are able to differentiate in liquid (20). It is thought the FliL protein interacts with the motor-switch complex to indicate torque on the

flagella from the surface, and that these mutants mimic this torque in liquid, causing the cells to differentiate even in liquid. It has been proposed FliL signaling also works through the WosA protein, which when overexpressed causes hyper-swarming and elongated cells in liquid culture (63). A mutation in *fliL* causes an increase in *wosA* transcription, suggesting there might be a link between the two and *flhDC* expression (63).

Two-component systems (TCS) are one of the main ways bacteria control gene expression based on information from outside of the cell (167). The canonical model for TCS involves a histidine sensor kinase which upon signal activation dimerizes and autophosphorylates a specific histidine residue, and a response regulator, which receives the phosphate on an aspartate residue. Most response regulators act at a transcriptional level by interacting with DNA directly. *P. mirabilis* has a predicted 16 TCS, although only two have been shown to be involved in swarming (136). The Rpp and Rcs TCS both play a role in swarming by affecting *flhDC* expression. The Rpp system appears to respond to polymyxin B, while the exact signal for Rcs activation is unclear (188).

The Rcs system is more complex than the canonical TCS and consists of multiple parts. In addition to the sensor kinase (RcsC) and response regulator (RcsB), there is also a phospho-transfer protein (RcsD), an outer-membrane activator protein (RcsF) and in some cases an accessory DNA binding protein (RcsA) (for more on how the Rcs system works in *P. mirabilis* see Chapter 4) (105). Unlike the Rpp system, mutations in the Rcs system (*rcsC*, *rcsB*, or *rcsD*) cause the formation of swarmer cells in liquid as well as hyper-swarming (18, 36,

99). It appears that RcsF is dispensable for swarming in wild-type cells, however, there might be a small role for RcsF in the absence of O-antigen (Morgenstein and Rather, in submission). The Rcs system has been extensively studied in *E. coli* and *Salmonella* as well as other swarming organisms and is a known repressor of *flhDC* (28, 29, 35, 48-50, 91, 106, 108, 172, 187). The Rcs system controls swarming motility through the regulation of *flhDC*, although the entire Rcs regulon is not known. Other genes under control of the Rcs system are most likely involved in the control of swarming because mutations that increase *flhDC* expression do not necessarily cause differentiation in liquid (36). It is not known what controls the Rcs system, however, it is thought that stresses in the periplasm or outer membrane can activate the system, such as surface growth, or osmotic and peptidoglycan stresses (49, 59, 91, 156). If the Rcs system senses surfaces to initiate the formation of swarmer cells in *P. mirabilis*, it may explain why mutations in the system remove the requirement for surfaces and allow for differentiation in liquid.

Other than FliL and Rcs mutations, the only other known gene that can be mutated and result in differentiation in liquid is *lon* protease. Why mutations in *lon* hyper-swarm and differentiate in liquid is not entirely understood, however, one of the targets of Lon is *flhD*. In the *lon* mutant, the excess FlhD might account for the increase in swarming, but as stated earlier, mutations that increase *flhDC* expression do not always result in liquid differentiation (36, 37). Another substrate of Lon is RcsA. Although the role of RcsA during swarming is

not known, it is possible RcsA is needed for cell elongation. In a *lon* mutant, RcsA concentrations are higher leading to differentiation in liquid (168).

Recently, Morgenstein et al. showed evidence for another external cell structure to be involved in surface sensing. They showed *P. mirabilis* mutants lacking a full length O-antigen are unable to swarm, or up-regulate flagella on surfaces, but are able to swim, indicating the cells have functioning flagella (122). Unlike in *S. enterica*, swarming was not restored in these *P. mirabilis* mutants upon the addition of surfactin or the use of Eiken agar, indicating a role for O-antigen other than acting as a wetting agent. Swarming could be restored by the overexpression of *flhDC* or by mutations in the Rcs signaling pathway, suggesting that O-antigen has a role in a signaling pathway that affects *flhDC* expression. Through the use of Northern and Western blots, it was shown *flhDC* expression does not increase on surfaces in the O-antigen mutant as it does in wild-type cells (122). How O-antigen signals surface sensing is not known, although, it has been proposed that there are two additional inputs into the Rcs system. These inputs, UmoB (IgaA), an integral membrane protein that in *Salmonella* has been shown to signal to the Rcs system, and UmoD, a proposed periplasmic protein, were originally discovered in a screen for the suppression of a swarming defect in a *flgN* mutant (42). Preliminary data in that study suggest these two proteins work in the same pathway. Our lab has shown that UmoB does indeed work through the Rcs system as it does in *Salmonella* and that UmoD is necessary for swarming in an O-antigen dependent manner (Morgenstein and Rather in submission, Chapter 4). It is still unclear how O-

antigen signals through UmoD and the exact role that UmoD and UmoB play in this signaling.

Other studies have previously shown LPS is important in swarming. During surface growth and differentiation, LPS undergoes biochemical changes leading to swarmer cells with a higher proportion of lipid bilayer in the outer-membrane and more long chain O-antigen units than swimmer cells (7, 9, 57). Mutational analysis has also shown an important role for LPS. Using genetic approaches, mutations in *waaL* (O-antigen ligase), *cld* (*wzz*) (O-antigen chain length determinant), and genes for inner core assembly (*waaD*, *waaC*), were shown to be impaired for swarming (16, 122).

Differentiation

Elongation

Once cells recognize they are on a surface they can differentiate into swarmer cells. Differentiation has two phases: elongation and flagella synthesis. It is possible elongation and hyper-flagellation regulate each other as cells with null alleles in *flhDC* are unable to elongate (33). While much is known about the second phase of differentiation, little is known about cellular elongation. Swarmer cells are elongated ~10-30X the length of swimmer cells, and lack septa. The cause of division inhibition is unknown. Interestingly, swarmer cells continue to replicate the chromosome as they grow leading to a long cell that maintains a DNA::cell size ratio with the chromosomes neatly organized throughout the cell (8, 53). Future studies could analyze FtsZ localization during

swimming and swarming to see if there is a difference. The study of division inhibition during swarming may lead to the discovery of new and novel FtsZ regulators.

flhDC Regulation

The second step of differentiation is the up-regulation of flagella through up-regulation of *flhDC*. During swarming, cells exist in a hyper-flagellated state with over 50X more flagella than their swimming counterparts (34). As discussed earlier, flagella synthesis is tightly controlled through a 3-tiered regulatory system. Tier 1 consists of the master regulator *flhDC*; at which most of the flagella regulation during swarming exists. There are many proteins that play a role in *flhDC* regulation in *P. mirabilis.* Some of these proteins, such as the Rcs system have been discussed previously. However, there are additional proteins that control *flhDC* expression that will be discussed below.

Leucine-responsive regulatory protein (Lrp) and UmoAC are positive regulators of *flhDC*, while Mrp and RsmA (CsrA), are negative regulators (see recent review (123) or Chapter 2) (42, 65, 97, 134). When either *lrp* or *umoAC* are mutated, swarming is altered. Mutations in *lrp* completely abrogate swarming because of an inability to increase flagella number in these cells (65). Lrp responds to amino acids levels and may be a way for the cells to control swarming based on nutrient availability. The UmoA and UmoC proteins have no known function. They were found along with UmoB and UmoD in a screen for suppressors of a *flgN* mutant. All four proteins are able to restore swarming in

this strain through the increase of *flhDC* expression, and when mutated inhibit swaming (57). The degree to which each of the Umo proteins controls *flhDC* expression is different, with UmoB and UmoD having the greatest affect on *flhDC* expression and therefore swarming motility.

The negative regulators of *flhDC* expression both inhibit swarming and cell elongation when overexpressed. *P. mirabilis* produces a variety of fimbriae that are expressed during sessile life. Because the production of flagella is energy consuming, it benefits the cell to inversely express fimbriae and flagella, given that only one can be used at a time. To stop both from being produced, some fimbrial operons contain a *flhDC* repressor. At the end of the MR/P fimbria operon is a gene encoding MrpJ, which is a direct repressor of *flhDC* (95, 134). When MrpJ is overexpressed in wild-type cells, flagella production is inhibited and the percentage of elongated cells is reduced leading to a reduced swarming phenotype. RsmA (CsrA), another inhibitor of *flhDC* expression, is an RNA binding protein that controls the expression of many genes; from stationary-phase genes to genes involved in flagella synthesis (146). In *E. coli*, CsrA positively regulates *flhDC* expression while, in *P. mirabilis*, it acts as a repressor (97, 192). When RsmA is over-produced both *flhDC* expression and cell elongation are reduced, resulting in repression of swarming (97).

Density Dependency

Another aspect of swarmer cell differentiation is the sensing of cell density. The initiation of swarming in *P. mirabilis* is density dependent,

suggesting a possible role for quorum sensing. This signal is most likely *Proteus* specific because a mixture of *E. coli* and *P. mirabilis* initiate swarming at a time correlating to *Proteus* only dense populations (18). Quorum sensing is a means of bacterial communication that normally involves one of a few pathways: acyl-homoserine lactone (LuxIR), AI-1 (LuxMN), or AI-2 (LuxSQ) producing pathways (191). Recent sequencing of the genome revealed *P. mirabilis* did not have homologs of either LuxI or LuxM, and studies of a *luxS* mutant showed no affect on swarming, indicating that none of these three common quorum sensing pathways are used in *P. mirabilis* during swarming (136, 150). This does not mean *P. mirabilis* does not use quorum sensing; only that it uses a different molecule. Putrescine has been proposed as a possible quorum sensing molecule because swarming is delayed or inhibited completely in mutants with variable production of putrescine (170). Mutants lacking putrescine production do not have flagella synthesis problems; indicating the putrescine pathway acts downstream of *flhDC* expression and possibly is involved with elongation. Another possibility is fatty acids act as a quorum sensing molecule. Different fatty acids have opposite effects on swarming when added exogenously to media and some of these affects work through the Rcs TCS (98). However, a physiological role for fatty acids during swarming and a mechanism for cell-cell communication are not known at this time.

Raft Formation/Cellular Movement

The final step of swarming is the formation of swarming rafts and the actual movement of cells outward from the central inoculum. After a period of time, for reasons unknown, the cells consolidate (de-differentiate) back into swimmer cells. The newly formed swimmer cells eventually differentiate back into swarmer cells and move outward again. As this process repeats, a pattern of swarm fronts and terraces is formed, resembling a 'bull's eye' pattern (144). A possible reason for cell density requirements is the need for the formation of these swarming rafts, as individual cells are not motile. Once cells differentiate, they align themselves along their long axis during which the copious amounts of flagella become entangled, most likely keeping the raft together (78). While it appears macroscopically that the swarm front is one continuous layer of cells, it is actually a combination of many smaller rafts, all swarming out from the inoculum (69, 144). The edge of the swarm colony is not smooth but instead jagged (69, 144).

How *P. mirabilis* cells overcome the friction associated with surface movement is not fully understood. There does seem to be a correlation with the amount of flagellation and the agar concentration that cells can swarm on. Both *Vibrio* and *Proteus* can swarm on high concentrations of agar and both produce large amounts of flagella compared to the flagellation of *Pseudomonas* or *E. coli* swarm cells. Hyper-flagellation does not appear to be enough to over come friction alone. In *S. enterica*, a role for O-antigen in surface wetting has been shown and although a purely analogous role in *P. mirabilis* does not appear to be

the case it is still possible that O-antigen has a small role in surface wetting (122, 174). During swarming, a slime layer has been observed trailing swarming rafts, and has been shown to be coordinately regulated with swarmer cell development, but a direct role for this slime in assisting with wettability has not been shown (51, 161). More recently, another molecule has been discovered that is secreted by *P. mirabilis* and is needed for swarming. This colony migration factor (cmf) is a capsule polysaccharide that when absent slows down the velocity of swarming without affecting differentiation or flagella function (58). It is unclear exactly how cmf works, but it has been proposed that it may act as a wetting agent to reduce friction, or even as a matrix to help stabilize swarming rafts (139).

Developmental biology is a large topic that covers many different fields. Bacterial development is probably most well studied in *Bacillus subtilis* and endospore formation, however, other bacteria undergo different developmental programs as well. A common thread in developmental biology is gene regulation. As can be seen, swarming is a complicated form of motility that allows for the study of multiple aspects of bacterial biology. In the beginning of this chapter virulence was discussed, along with the role of swarming, in not only infection, but also virulence gene regulation.

The study of swarming also lends itself to the study of basic biology. How do bacterial cells sense their environment and change their gene expression accordingly? This question has been discussed in terms of surface sensing and swarmer cell differentiation but the ideas can be applied to other topics of

bacteriology, such as biofilm development. Gene regulation is tantamount to controlling swarmer cell development. Studying swarming in genetically tractable organisms allows for new insights into genetic programs such as flagellar regulation or cell division and possibly new roles for characterized molecules, such as O-antigen.

The following chapters offer a detailed examination of the role of O-antigen in surface sensing in *P. mirabilis* and the role of the Rcs TCS. As discussed earlier, O-antigen is proposed to be part of a second surface sensing mechanism (along with flagella inhibition) that signals to *Proteus* cells to differentiate into swarmer cells only on surfaces. The initial discovery of this pathway and a detailed look at the interplay between O-antigen and the Rcs system will be discussed. While more work needs to be done in order to fully understand this interaction, the following work marks a good beginning into the relationship of outer membrane structures and gene regulation.

References

1. **Alamuri, P., K. A. Eaton, S. D. Himpsl, S. N. Smith, and H. L. Mobley.** 2009. Vaccination with Proteus Toxic Agglutinin, a Hemolysin-Independent Cytotoxin *In Vivo*, Protects against *Proteus mirabilis* Urinary Tract Infection. Infection and Immununity **77:**632-641.
2. **Alamuri, P., and H. L. Mobley.** 2008. A Novel Autotransporter of Uropathogenic *Proteus mirabilis* is both a Cytotoxin and an Agglutinin. Molecular Microbiology **68:**997-1017.
3. **Alberti, L., and R. M. Harshey.** 1990. Differentiation of *Serratia marcescens* 274 into Swimmer and Swarmer cells. Journal Bacteriology **172:**4322-4328.
4. **Allison, C., L. Emödy, N. Coleman, and C. Hughes.** 1994. The Role of Swarm Cell Differentiation and Multicellular Migration in the Uropathogenicity of *Proteus mirabilis*. The Journal of Infectious Diseases **169:**1155-1158.
5. **Allison, C., H.-C. Lai, and C. Hughes.** 1992. Co-ordinate Expression of Virulence Genes During Swarm-Cell Differentiaion and Popultion Migration of *Proteus mirabilis*. Molecular Microbiology **6:**1583-1591.
6. **Altman, E., B. A. Harrison, R. K. Latta, K. K. Lee, J. F. Kelly, and P. Thibault.** 2001. Galectin-3-mediated Adherence of *Proteus mirabilis* to

Madin-Darby Canine Kidney Cells. Biochemistry & Cell Biology **79:**783-788.

7. **Armitage, J. P.** 1982. Changes in the Organization of the Outer Membrane of *Proteus mirabilis* During Swarming: Freeze-Fracture Structure and Membrane Fluidity Analysis. Journal of Bacteriology **150:**900-904.

8. **Armitage, J. P., R. J. Rowbury, and D. G. Smith.** 1974. The Effects of Chloramphenicol, Nalidixic Acid and Penicillin on the Growth and Division of Swarming cells of *Proteus mirabilis*. Journal of Medical Microbiology **7:**459-463.

9. **Armitage, J. P., D. G. Smith, and R. J. Rowbury.** 1979. Alterations in the Cell Envelope Composition of *Proteus mirabilis* During the Development of Swarmer Cells. Biochemica et Biophysica Acta **584:**389-397.

10. **Atsumi, T., L. McCartert, and Y. Imae.** 1992. Polar and Lateral Flagellar Motors of Marine *Vibrio* are Driven by Different Ion-Motive Forces. Nature **355:**182-184.

11. **Bahrani, F. K., D. E. Johnson, D. Robbins, and H. L. Mobley.** 1991. *Proteus mirabilis* Flagella and MR/P Fimbriae: Isolation, Purification, N-terminal analysis, and Serum Antibody Response Following Experimental Urinary Tract Infection. Infect. Immun. **59:**3574-3580.

12. **Bahrani, F. K., G. Massad, C. V. Lockatell, D. E. Johnson, R. G. Russell, J. W. Warren, and H. L. Mobley.** 1994. Construction of an MR/P

Fimbrial Mutant of *Proteus mirabilis*: Role in Virulence in a Mouse Model of Ascending Urinary Tract Infection. Infect. Immun. **62:**3363-3371.

13. **Bahrani, F. K., and H. L. Mobley.** 1994. *Proteus mirabilis* MR/P Fimbrial Operon: Genetic Organization, Nucleotide Sequence, and Conditions for Expression. J. Bacteriol. **176:**3412-3419.
14. **Barak, J. D., L. Gorski, A. S. Liang, and K.-E. Narm.** 2009. Previously Uncharacterized *Salmonella enterica* Genes Required for Swarming Play a Role in Seedling Colonization. Microbiology **155:**3701-3709.
15. **Belas, R., D. Erskine, and D. Flaherty.** 1991. *Proteus mirabilis* Mutants Defective in Swarmer Cell Differentiation and Multicellular Behavior. Journal of Bacteriology **173:**6279-6288.
16. **Belas, R., M. Goldman, and K. Ashliman.** 1995. Genetic Analysis of *Proteus mirabilis* Mutants Defective in Swarmer Cell Elongation. Journal of Bacteriology **177:**823-828.
17. **Belas, R., J. Manos, and R. Suvanasuthi.** 2004. *Proteus mirabilis* ZapA Metalloprotease Degrades a Broad Spectrum of Substrates, Including Antimicrobial Peptides. Infection and Immunology **72:**5159-5167.
18. **Belas, R., R. Schneider, and M. Melch.** 1998. Characterization of *Proteus mirabilis* Precocious Swarming Mutants: Identification of *rsb*A, Encoding a Regulator of Swarming Behavior. Journal of Bacteriology **180:**6126-6139.

19. **Belas, R., M. Simon, and M. Silverman.** 1986. Regulation of Lateral Flagella Gene Transcription in *Vibrio parahaemolyticus*. Journal Bacteriology **167:**210-218.

20. **Belas, R., and R. Suvanasuthi.** 2005. The Ability of *Proteus mirabilis* to Sense Surfaces and Regulate Virulence Gene Expression Involves FliL, a Flagellar Basal Body Protein. Journal of Bacteriology **187:**6789-6803.

21. **Berg, H. C.** 2003. The Rotary Motor of Bacterial Flagella. Annual Review of Microbiology **72:**19-54.

22. **Blair, D. F., and H. C. Berg.** 1988. Restoration of Torque in Defective Flagellar Motors. Science **242:**1678-1681.

23. **Borkovich, K. A., L. A. Alex, and M. I. Simon.** 1992. Attenuation of Sensory Receptor Signaling by Covalent Modification. Proceedings of the National Academy of Sciences of the United States of America **89:**6756-6760.

24. **Burall, L. S., J. M. Harro, X. Li, C. V. Lockatell, S. D. Himpsl, J. R. Hebel, D. E. Johnson, and H. L. Mobley.** 2004. *Proteus mirabilis* Genes that Contribute to Pathogenesis of Urinary Tract Infection: Identification of 25 Signature-Tagged Mutants Attenuated at Least 100-Fold. Infect. Immun. **72:**2922-2938.

25. **Burkart, M., A. Toguchi, and R. M. Harshey.** 1998. The Chemotaxis System, but not Chemotaxis, is Essential for Swarming Motility in *Escherichia Ecoli*. Proceedings of the National Academy of Sciences of the United States of America **95:**2568-2573.

26. **Butler, M. T., Q. Wang, and R. M. Harshey.** 2010. Cell Density and Mobility Protect Swarming Bacteria Against Antibiotics. Proceedings of the National Academy of Sciences **107:**3776-3781.

27. **Caiazza, N. C., R. M. Q. Shanks, and G. A. O'Toole.** 2005. Rhamnolipids Modulate Swarming Motility Patterns of *Pseudomonas aeruginosa*. Journal Bacteriology **187:**7351-7361.

28. **Castanie-Cornet, M.-P., K. Cam, and A. Jacq.** 2006. RcsF Is an Outer Membrane Lipoprotein Involved in the RcsCDB Phosphorelay Signaling Pathway in *Escherichia coli*. J. Bacteriol. **188:**4264-4270.

29. **Castelli, M. E., and E. G. Vescovi.** 2010. The Rcs Signal Transduction Pathway Is Triggered by Enterobacterial Common Antigen Structure Alterations in Serratia marcescens. Journal Bacteriology **193:**63-74.

30. **Chen, B. G., L. Turner, and H. C. Berg.** 2007. The Wetting Agent Required for Swarming in *Salmonella enterica* Serovar Typhimurium Is Not a Surfactant. Journal Bacteriology **189:**8750-8753.

31. **Chilcott, G. S., and K. T. Hughes.** 2000. Coupling of Flagellar Gene Expression to Flagellar Assembly in *Salmonella enterica Serovar Typhimurium* and *Escherichia coli*. Microbiol. Mol. Biol. Rev. **64:**694-708.

32. **Chow, A. W., P. R. Taylor, T. T. Yoshikawa, and L. B. Guze.** 1979. A Nosocomial Outbreak of Infections Due to Multiply Resistant *Proteus mirabilis*: Role of Intestinal Colonization as a Major Reservoir. The Journal of Infectious Diseases **139:**621-627.

33. **Claret, L., and C. Hughes.** 2000. Functions of the Subunits in the FlhD2C2 Transcriptional Master Regulator of Bacterial Flagellum Biogenesis and Swarming. Journal of Molecular Biology **303:**467-478.

34. **Claret, L., and C. Hughes.** 2000. Rapid Turnover of FlhD and FlhC, the Flagellar Regulon Transcriptional Activator Proteins, during Proteus Swarming. Journal of Bacteriology **182:**833-836.

35. **Clarke, D. J., S. A. Joyce, C. M. Toutain, A. Jacq, and I. B. Holland.** 2002. Genetic Analysis of the RcsC Sensor Kinase from *Escherichia coli* K-12. Journal of Bacteriology. **184:**1204-1208.

36. **Clemmer, K. M., and P. N. Rather.** 2007. Regulation of *flh*DC Expression in *Proteus mirabilis*. Research in Microbiology**:**295-302.

37. **Clemmer, K. M., and P. N. Rather.** 2008. The Lon Protease Regulates Swarming Motility and virulence Gene Expression in *Proteus mirabilis*. Journal of Medical Microbiology **57:**931-937.

38. **Coker, C., C. A. Poore, X. Li, and H. L. Mobley.** 2000. Pathogenesis of *Proteus mirabilis* Urinary Tract Infection. Microbes and Infection **2:**1497-1505.

39. **Copeland, M. F., S. T. Flickinger, H. H. Tuson, and D. B. Weibel.** 2010. Studying the Dynamics of Flagella in Multicellular Communities of *Escherichia coli* by Using Biarsenical Dyes. Applied Environmental Microbiology **76:**1241-1250.

40. **Darnton, N. C., L. Turner, S. Rojevsky, and H. C. Berg.** 2010. Dynamics of Bacterial Swarming. Biophysical Journal **98:**2082-2090.

41. **Deziel, E., F. Lepine, S. Milot, and R. Villemur.** 2003. *rhlA* is Required for the Production of a Novel Biosurfactant Promoting Swarming Motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the Precursors of Rhamnolipids. Microbiology **149:**2005-2013.

42. **Dufour, A., R. B. Furness, and C. Hughes.** 1998. Novel Genes that Upregulate the *Proteus mirabilis flhDC* Master Operon Controlling Flagellar Biogenesis and Swarming. Molecular Microbiology **29:**741-751.

43. **Dumanski, A. J., H. Hedelin, A. Edin-Liljegren, D. Beauchemin, and R. J. McLean.** 1994. Unique Ability of the *Proteus mirabilis* Capsule to Enhance Mineral Growth in Infectious Urinary Calculi. Infect. Immun. **62:**2998-3003.

44. **Eberl, L., G. Christiansen, S. Molin, and M. Givskov.** 1996. Differentiation of *Serratia liquefaciens* into Swarm Cells is Controlled by the Expression of the *flhD* Master Operon. Journal Bacteriology **178:**554-559.

45. **Eberl, L., S. Molin, and M. Givskov.** 1999. Surface Motility of *Serratia liquefaciens* MG1. Journal Bacteriology **181:**1703-1712.

46. **Ebringer, A., and T. Rashid.** 2009. Rheumatoid Arthritis is Caused by *Proteus*: the Molecular Mimicry Theory and Karl Popper. Frontiers in Bioscience (Elite Ed.) **1:**577-586.

47. **Falkinham, J. O., 3rd, and P. S. Hoffman.** 1984. Unique Developmental Characteristics of the Swarm and Short Cells of *Proteus vulgaris* and *Proteus mirabilis*. Journal Bacteriology **158:**1037-1040.

48. **Farris, C., S. Sanowar, M. W. Bader, R. Pfuetzner, and S. I. Miller.** 2010. Antimicrobial Peptides Activate the Rcs Regulon through the Outer Membrane Lipoprotein RcsF. Journal Bacteriology **192:**4894-4903.

49. **Ferrières, L., and D. J. Clarke.** 2003. The RcsC Sensor Kinase is Required for Normal Biofilm Formation in *Escherichia coli* K-12 and Controls the Expression of a Regulon in Response to Growth on a Solid Surface. Molecular Microbiology **50:**1665-1682.

50. **Francez-Charlot, A., B. Laugel, A. V. Gemert, N. Dubarry, F. Wiorowski, M.-P. Castanié-Cornet, C. Gutierrez, and K. Cam.** 2003. RcsCDB His-Asp Phosphorelay System Negatively Regulates the *flhDC* Operon in *Escherichia coli*. Molecular Microbiology **49:**823-832.

51. **Fuscoe, F. J.** 1973. The Role of Extracellular Slime Secretion in the Swarming of *Proteus*. Medical Laboratory Technology **30:**373-382.

52. **Girgis, H. S., Y. Liu, W. S. Ryu, and S. Tavazoie.** 2007. A Comprehensive Genetic Characterization of Bacterial Motility. PLoS Genet **3:**1644-1660.

53. **Gmeiner, J., E. Sarnow, and K. Milde.** 1985. Cell Cycle Parameters of *Proteus mirabilis*: Interdependence of the Biosynthetic Cell Cycle and the Interdivision Cycle. Journal Bacteriology **164:**741-748.

54. **Gode-Potratz, C. J., D. M. Chodur, and L. L. McCarter.** 2010. Calcium and Iron Regulate Swarming and Type III Secretion in *Vibrio parahaemolyticus*. Journal Bacteriology **192:**6025-6038.

55. **Grahnquist, L., B. Lundberg, and T. K.** 1992. Neonatal *Proteus* Meningoencephalitis. Case Report. Acta Pathologica, Microbiologica et Immunologica Scandinavica **100:**734-736.

56. **Griffith, D. P., D. M. Musher, and C. Itin.** 1976. Urease. The Primary Cause of Infection-Induced Urinary Stones. Investigative urology **13:**346-350.

57. **Gue, M., V. Dupont, A. Dufour, and O. Sire.** 2001. Bacterial Swarming: A Biochemical Time-Resolved FTIR-ATR Study of *Proteus mirabilis* Swarm-Cell Differentiation. Biochemistry **40:**11938-11945.

58. **Gygi, D., M. M. Rahman, H. C. Lai, R. Carlson, J. Guard-Petter, and C. Hughes.** 1995. A Cell-Surface Polysaccharide that Facilitates Rapid Population Migration by Differentiated Swarm Cells of *Proteus mirabilis*. Molecular Microbiology **17:**1167-1175.

59. **Hagiwara, D., M. Sugiura, T. Oshima, H. Mori, H. Aiba, T. Yamashino, and T. Mizuno.** 2003. Genome-Wide Analyses Revealing a Signaling Network of the RcsC-YojN-RcsB Phosphorelay System in *Escherichia coli*. Journal Bacteriology **185:**5735-5746.

60. **Harshey, R. M.** 1994. Bees aren't the Only Ones: Swarming in Gram-Negative Bacteria. Molecular Microbiology **13:**389-394.

61. **Harshey, R. M., and T. Matsuyama.** 1994. Dimorphic Transition in *Escherichia coli* and *Salmonella typhimurium*: Surface-Induced Differentiation into Hyperflagellate Swarmer Cells. Proceedings of the

National Academy of Sciences of the United States of America **91:**8631-8635.

62. **Harvey, P.** 1966. The Oxford Companion to Classical Literature, vol. 3. Oxford University Press, Oxford.

63. **Hatt, J. K., and P. N. Rather.** 2008. Characterization of a Novel Gene, *wosA*, Regulating FlhDC Expression in Proteus mirabilis. J. Bacteriol. **190:**1946-1955.

64. **Hauser, G.** 1885. über Fäulnissbacterien und deren Bezieungen zur Septicämie. Ein Betrag zur Morphologie der Spaltpilze**:**1-94.

65. **Hay, N. A., D. J. Tipper, D. Gygi, and C. Hughes.** 1997. A Nonswarming Mutant of *Proteus mirabilis* Lacks the Lrp Global Transcriptional Regulator. J. Bacteriol. **179:**4741-4746.

66. **Hazelbauer, G. L., J. J. Falke, and J. S. Parkinson.** 2008. Bacterial Chemoreceptors: High-Performance Signaling in Networked Arrays. Trends in Biochemical Sciences **33:**9-19.

67. **Henrichsen, J.** 1972. Bacterial Surface Translocation: a Survey and a Classification. Microbiol. Mol. Biol. Rev. **36:**478-503.

68. **Hess, J. F., K. Oosawa, N. Kaplan, and M. I. Simon.** 1988. Phosphorylation of Three Proteins in the Signaling Pathway of Bacterial Chemotaxis. Cell **53:**79-87.

69. **Hoeniger, J. F.** 1964. Cellular Changes Accompanying the Swarming of *Proteus mirabilis*. I. Observations of Living Cultures. Canadian Journal of Microbiology **10:**1-9.

70. **Hughes, K. T., K. L. Gillen, M. J. Semon, and J. E. Karlinsey.** 1993. Sensing Structural Intermediates in Bacterial Flagellar Assembly by Export of a Negative Regulator. Science **262:**1277-1280.

71. **Inoue, T., R. Shingaki, S. Hirose, K. Waki, H. Mori, and K. Fukui.** 2007. Genome-Wide Screening of Genes Required for Swarming Motility in *Escherichia coli* K-12. Journal Bacteriology **189:**950-957.

72. **Isenstein, D., and E. Honig.** 1990. *Proteus vulgaris* empyema and increased pleural fluid pH. Chest **97:**511.

73. **Jansen, A. M., C. V. Lockatell, D. E. Johnson, and H. L. Mobley.** 2003. Visualization of *Proteus mirabilis* Morphotypes in the Urinary Tract: the Elongated Swarmer Cell Is Rarely Observed in Ascending Urinary Tract Infection. Infect. Immun. **71:**3607-3613.

74. **Johnson, D. E., R. G. Russell, C. V. Lockatell, J. C. Zulty, J. W. Warren, and H. L. Mobley.** 1993. Contribution of *Proteus mirabilis* Urease to Persistence, Urolithiasis, and Acute Pyelonephritis in a Mouse Model of Ascending Urinary Tract Infection. Infect. Immun. **61:**2748-2754.

75. **Jones, B. D., C. V. Lockatell, D. E. Johnson, J. W. Warren, and H. L. Mobley.** 1990. Construction of a Urease-Negative Mutant of *Proteus mirabili*s: Analysis of Virulence in a Mouse Model of Ascending Urinary Tract Infection. Infect. Immun. **58:**1120-1123.

76. **Jones, B. D., and H. L. Mobley.** 1988. *Proteus mirabilis* Urease: Genetic Organization, Regulation, and Expression of Structural Genes. J. Bacteriol. **170:**3342-3349.

77. **Jones, B. V., E. Mahenthiralingam, N. A. Sabbuba, and D. J. Stickler.** 2005. Role of Swarming in the Formation of Crystalline *Proteus mirabilis* Biofilms on Urinary Catheters. J Med Microbiol **54:**807-813.

78. **Jones, B. V., R. Young, E. Mahenthiralingam, and D. J. Stickler.** 2004. Ultrastructure of *Proteus mirabilis* Swarmer Cell Rafts and Role of Swarming in Catheter-Associated Urinary Tract Infection. Infect. Immun. **72:**3941-3950.

79. **Kawagishi, I., M. Imagawa, Y. Imae, L. McCarter, and M. Homma.** 1996. The Sodium-Driven Polar Flagellar Motor of Marine *Vibrio* as the Mechanosensor that Regulates Lateral Flagellar Expression. Molecular Microbiology **20:**693-699.

80. **Kehry, M. R., and F. W. Dahlquist.** 1982. Adaptation in Bacterial Chemotaxis: CheB-dependent Modification Permits Additional Methylations of Sensory Transducer Proteins. Cell **29:**761-772.

81. **Kim, B.-N., N. J. Kim, M.-N. Kim, Y. S. Kim, J.-H. Woo, and J. Ryu.** 2003. Bacteraemia Due to Tribe *Proteeae*: A Review of 132 Cases During a Decade (1991--2000). Scandinavian Journal of Infectious Diseases **35:**98.

82. **Kim, K. K., H. Yokota, and S.-H. Kim.** 1999. Four-helical-bundle Structure of the Cytoplasmic Domain of a Serine Chemotaxis Receptor. Nature **400:**787.

83. **Kim, S.-H., W. Wang, and K. K. Kim.** 2002. Dynamic and Clustering Model of Bacterial Chemotaxis Receptors: Structural Basis for Signaling

and High Sensitivity. Proceedings of the National Academy of Sciences of the United States of America **99:**11611-11615.

84. **Kim, W., T. Killam, V. Sood, and M. G. Surette.** 2003. Swarm-Cell Differentiation in *Salmonella enterica* Serovar Typhimurium Results in Elevated Resistance to Multiple Antibiotics. Journal Bacteriology **185:**3111-3117.

85. **Kim, W., and M. G. Surette.** 2004. Metabolic Differentiation in Actively Swarming *Salmonella*. Molecular Microbiology **54:**702-714.

86. **Kim, W., and M. G. Surette.** 2003. Swarming Populations of *Salmonella* Represent a Unique Physiological State Coupled to Multiple Mechanisms of Antibiotic Resistance. Biological Proceedings Online **5:**189-196.

87. **Kohler, T., L. K. Curty, F. Barja, C. van Delden, and J.-C. Pechere.** 2000. Swarming of *Pseudomonas aeruginosa* is Dependent on Cell-to-Cell Signaling and Requires Flagella and Pili. Journal Bacteriology **182:**5990-5996.

88. **Kornfeld, S. J., and A. G. Plaut.** 1981. Secretory Immunity and the Bacterial IgA Proteases. Reviews of Infectious Diseases **3:**521-534.

89. **Kutsukake, K.** 1994. Excretion of the Anti-Sigma Factor Through a Flagellar Substructure Couples Flagellar Gene Expression with Flagellar Assembly in *Salmonella typhimurium*. Molecular and General Genetics **243:**605-612.

90. **Lai, S., J. Tremblay, and E. Déziel.** 2009. Swarming Motility: a Multicellular Behaviour Conferring Antimicrobial Resistance. Environmental Microbiology **11:**126-136.

91. **Laubacher, M. E., and S. E. Ades.** 2008. The Rcs Phosphorelay Is a Cell Envelope Stress Response Activated by Peptidoglycan Stress and Contributes to Intrinsic Antibiotic Resistance. J. Bacteriol. **190:**2065-2074.

92. **Lee, K. K., B. A. Harrison, R. Latta, and E. Altman.** 2000. The Binding of *Proteus mirabilis* Nonagglutinating Fimbriae to Ganglio-series Asialoglycolipids and Lactosyl Ceramide. Canadian Journal of Microbiology **46:**961-966.

93. **Li, J., R. V. Swanson, M. I. Simon, and R. M. Weis.** 1995. Response Regulators CheB and CheY Exhibit Competitive Binding to the Kinase CheA. Biochemistry **34:**14626-14636.

94. **Li, X., C. V. Lockatell, D. E. Johnson, and H. L. Mobley.** 2002. Identification of MrpI as the Sole Recombinase that Regulates the Phase Variation of MR/P Fimbria, a Bladder Colonization Factor of Uropathogenic *Proteus mirabilis*. Molecular Microbiology **45:**865-874.

95. **Li, X., D. A. Rasko, C. V. Lockatell, D. E. Johnson, and H. L. Mobley.** 2001. Repression of Bacterial Motility by a Novel Fimbrial Gene Product. EMBO J **20:**4854-4862.

96. **Li, X., H. Zhao, C. V. Lockatell, C. B. Drachenberg, D. E. Johnson, and H. L. Mobley.** 2002. Visualization of *Proteus mirabilis* within the Matrix of

Urease-Induced Bladder Stones during Experimental Urinary Tract Infection. Infect. Immun. **70:**389-394.

97. **Liaw, S.-J., H.-C. Lai, S.-W. Ho, K.-T. Luh, and W.-B. Wang.** 2003. Role of RsmA in the Regulation of Swarming Motility and Virulence Factor Expression in *Proteus mirabilis*. Journal of Medical Microbiology **52:**19-28.

98. **Liaw, S.-J., H.-C. Lai, and W.-B. Wang.** 2004. Modulation of Swarming and Virulence by Fatty Acids through the RsbA Protein in *Proteus mirabilis*. Infection and Immununity **72:**6836-6845.

99. **Liaw, S.-J., H. C. Lai, S. W. Ho, K. T. Luh, and W. B. Wang.** 2001. Characterisation of p-nitrophenylglycerol-resistant *Proteus mirabilis* Super-Swarming Mutants. Journal of Medical Microbiology. **50:**1039-1048.

100. **Lindum, P. W., U. Anthoni, C. Christophersen, L. Eberl, S. Molin, and M. Givskov.** 1998. N-Acyl-L-Homoserine Lactone Autoinducers Control Production of an Extracellular Lipopeptide Biosurfactant Required for Swarming Motility of *Serratia liquefaciens* MG1. Journal Bacteriology **180:**6384-6388.

101. **Liu, J.-H., M.-J. Lai, S. Ang, J.-C. Shu, P.-C. Soo, Y.-T. Horng, W.-C. Yi, H.-C. Lai, K.-T. Luh, S.-W. Ho, and S. Swift.** 2000. Role of f*lhDC* in the Expression of the Nuclease Gene *nucA,* Cell Division and Flagellar Synthesis in *Serratia marcescens*. Journal of Biomedical Science **7:**475-483.

102. **Lux, R., and W. Shi.** 2004. Chemotaxis-guided Movements in Bacteria. Critical Reviews in Oral Biology & Medicine **15:**207-220.

103. **Macnab, R. M.** 2003. How Bacteria Assemble Flagella. Annual Review of Microbiology **57:**77-100.

104. **Maddock, J. R., and L. Shapiro.** 1993. Polar Location of the Chemoreceptor Complex in the Escherichia coli Cell. Science **259:**1717-1723.

105. **Majdalani, N., and S. Gottesman.** 2005. The Rcs Phosphorelay: A Complex Signal Transduction System. Annual Review of Microbiology **59:**379-405.

106. **Majdalani, N., M. Heck, V. Stout, and S. Gottesman.** 2005. Role of RcsF in Signaling to the Rcs Phosphorelay Pathway in *Escherichia coli*. Journal Bacteriology **187:**6770-6778.

107. **Mariconda, S., Q. Wang, and R. M. Harshey.** 2006. A Mechanical Role for the Chemotaxis System in Swarming Motility. Molecular Microbiology **60:**1590-1602.

108. **Mariscotti, J. F., and F. Garcia-del Portillo.** 2008. Instability of the *Salmonella* RcsCDB Signalling System in the Absence of the Attenuator IgaA. Microbiology **154:**1372-1383.

109. **Marx, A. C., M. F. Hartshorne, M. A. Stull, and C. L. Truwit.** 1988. Case Report 496: Intraosseous gas in *Proteus mirabilis* Osteomyelitis Complicating Bone Infarcts in Sickle Cell Disease. Skeletal Radiation **17:**510-513.

110. **Massad, G., C. V. Lockatell, D. E. Johnson, and H. L. Mobley.** 1994. *Proteus mirabilis* Fimbriae: Construction of an Isogenic *pmfA* Mutant and

Analysis of Virulence in a CBA Mouse Model of Ascending Urinary Tract Infection. Infect. Immun. **62:**536-542.

111. **Matsuyama, T., K. Kaneda, Y. Nakagawa, K. Isa, H. Hara-Hotta, and I. Yano.** 1992. A Novel Extracellular Cyclic Lipopeptide which Promotes Flagellum-Dependent and -Independent Spreading Growth of *Serratia marcescens*. Journal Bacteriology **174:**1769-1776.

112. **Mattick, J. S.** 2002. Type IV Pili and Twitching Motility. Annual Review of Microbiology **56:**289-314.

113. **McCarter, L., M. Hilmen, and M. Silverman.** 1988. Flagellar Dynamometer Controls Swarmer Cell Differentiation of *V. parahaemolyticus*. Cell **54:**345-351.

114. **McCarter, L., and M. Silverman.** 1989. Iron Regulation of Swarmer Cell Differentiation of *Vibrio parahaemolyticus*. Journal Bacteriology **171:**731-736.

115. **McCarter, L., and M. Silverman.** 1990. Surface-Induced Swarmer Cell Differentiation of *Vibrio parahaemoiyticus*. Molecular Microbiology **4:**1057-1062.

116. **McEvoy, M. M., A. Bren, M. Eisenbach, and F. W. Dahlquist.** 1999. Identification of the Binding Interfaces on CheY for Two of its Targets the Phosphatase CheZ and the Flagellar Switch Protein FliM. Journal of Molecular Biology **289:**1423-1433.

117. **McLean, R. J. C., J. C. Nickel, K.-J. Cheng, J. W. Costerton, and J. G. Banwell.** 1988. The Ecology and Pathogenicity of Urease-Producing Bacteria in the Urinary Tract. Critical Reviews in Microbiology **16:**37-79.

118. **Mignot, T., J. W. Shaevitz, P. L. Hartzell, and D. R. Zusman.** 2007. Evidence That Focal Adhesion Complexes Power Bacterial Gliding Motility. Science **315:**853-856.

119. **Milligan, D. L., and D. E. Koshland.** 1988. Site-directed Cross-linking. Establishing the Dimeric Structure of the Aspartate Receptor of Bacterial Chemotaxis. Journal of Biological Chemistry **263:**6268-6275.

120. **Mobley, H. L., M. D. Island, and R. P. Hausinger.** 1995. Molecular Biology of Microbial Ureases. Microbiological reviews **59:**451-480.

121. **Mobley, H. L., and J. W. Warren.** 1987. Urease-positive Bacteriuria and Obstruction of Long-Term Urinary Catheters. J. Clin. Microbiol. **25:**2216-2217.

122. **Morgenstein, R. M., K. M. Clemmer, and P. N. Rather.** 2010. Loss of the WaaL O-Antigen Ligase Prevents Surface Activation of the Flagellar Gene Cascade in *Proteus mirabilis*. Journal of Bacteriology **192:**3213-3221.

123. **Morgenstein, R. M., B. Szostek, and P. N. Rather.** 2010. Regulation of Gene Expression During Swarmer Cell Differentiation in *Proteus mirabilis*. FEMS Microbiology Reviews **34:**753-763.

124. **Mulks, M. H., R. J. Shoberg, and P. M. B. Virginia L. Clark.** 1994. Bacterial Immunoglobulin A1 Proteases, p. 543-554, Methods in Enzymology, vol. 235. Academic Press.

125. **Nicholson, E. B., E. A. Concaugh, and H. L. Mobley.** 1991. *Proteus mirabilis* Urease: Use of a *ureA-lacZ* Fusion Demonstrates that Induction is Highly Specific for Urea. Infect. Immun. **59:**3360-3365.

126. **Nielubowicz, G. R., and H. L. Mobley.** 2010. Host-Pathogen Interactions in Urinary Tract Infection. Nature Reviews. Urology. **7:**430-441.

127. **Nielubowicz, G. R., S. N. Smith, and H. L. Mobley.** 2008. Outer Membrane Antigens of the Uropathogen *Proteus mirabilis* Recognized by the Humoral Response during Experimental Murine Urinary Tract Infection. Infect. Immun. **76:**4222-4231.

128. **O'Hara, C. M., F. W. Brenner, and J. M. Miller.** 2000. Classification, Identification, and Clinical Significance of *Proteus*, *Providencia*, and *Morganella*. Clin. Microbiol. Rev. **13:**534-546.

129. **O'Rear, J., L. Alberti, and R. M. Harshey.** 1992. Mutations that Impair Swarming Motility in *Serratia marcescens* 274 Include but are not Limited to Those Affecting Chemotaxis or Flagellar Function. Journal Bacteriology **174:**6125-6137.

130. **Ohnishi, K., K. Kutsukake, H. Suzuki, and T. Iino.** 1992. Gene *fliA* Encodes an Alternative Sigma Factor Specific for Flagellar Operons in *Salmonella typhimurium*. Molecular and General Genetics **221:**139-147.

131. **Ohnishi, K., K. Kutsukake, H. Suzuki, and T. Lino.** 1992. A Novel Transcriptional Regulation Mechanism in the Flagellar Regulon of *Salmonella typhimurium*: an Anti-Sigma Factor Inhibits the Activity of the

Flagellum-Specific Sigma Factor, Sigma F. Molecular Microbiology **6:**3149-3157.

132. **Overhage, J., S. Lewenza, A. K. Marr, and R. E. W. Hancock.** 2007. Identification of Genes Involved in Swarming Motility Using a *Pseudomonas aeruginosa* PAO1 Mini-Tn5-lux Mutant Library. Journal Bacteriology **189:**2164-2169.

133. **Pacheco, G., and V. M. Dias.** 1955. Contribution to the Normal Fecal Flora of the Hamster: *Proteus mirabilis* in Normal Feces of Hamster. Memorias do Instituto Oswaldo Cruz **53:**41-42.

134. **Pearson, M. M., and H. L. Mobley.** 2008. Repression of Motility During Fimbrial Expression: Identification of 14 *mrpJ* Gene Paralogues in *Proteus mirabilis*. Molecular Microbiology **69:**548-558.

135. **Pearson, M. M., D. A. Rasko, S. N. Smith, and H. L. Mobley.** 2010. Transcriptome of Swarming *Proteus mirabilis*. Infect. Immun. **78:**2834-2845.

136. **Pearson, M. M., M. Sebaihia, C. Churcher, M. A. Quail, A. S. Seshasayee, N. M. Luscombe, Z. Abdellah, C. Arrosmith, B. Atkin, T. Chillingworth, H. Hauser, K. Jagels, S. Moule, K. Mungall, H. Norbertczak, E. Rabbinowitsch, D. Walker, S. Whithead, N. R. Thomson, P. N. Rather, J. Parkhill, and H. L. Mobley.** 2008. Complete Genome Sequence of Uropathogenic *Proteus mirabilis*, a Master of both Adherence and Motility. J. Bacteriol. **190:**4027-4037.

137. **Peerbooms, P. G., A. M. Verweij, and D. M. MacLaren.** 1983. Investigation of the Haemolytic Activity of *Proteus mirabilis* strains. Antonie Van Leeuwenhoek **49:**1-11.

138. **Peerbooms, P. G., A. M. Verweij, and D. M. MacLaren.** 1984. Vero Cell Invasiveness of *Proteus mirabilis*. Infect. Immun. **43:**1068-1071.

139. **Rahman, M. M., J. Guard-Petter, K. Asokan, C. Hughes, and R. W. Carlson.** 1999. The Structure of the Colony Migration Factor from Pathogenic *Proteus mirabilis*. A Capsular Polysaccharide that Facilitates Swarming. J. Biol. Chem. **274:**22993-22998.

140. **Rashid, M. H., and A. Kornberg.** 2000. Inorganic Polyphosphate is Needed for Swimming, Swarming, and Twitching Motilities of *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences of the United States of America **97:**4885-4890.

141. **Rashid, T., and A. Ebringer.** 2008. Rheumatoid Arthritis in Smokers Could be Linked to *Proteus* Urinary Tract Infections. Medical Hypotheses **70:**975-980.

142. **Rashid, T., K. S. Jayakumar, A. Binder, S. Ellis, P. Cunningham, and A. Ebringer.** 2007. Rheumatoid Arthritis Patients have Elevated Antibodies to Cross-Reactive and Non Cross-Reactive Antigens from *Proteus* Microbes. Clinical and Experimenal Rheumatology **25:**259-267.

143. **Rather, P. N.** 2005. Swarmer Cell Differentiation in *Proteus mirabilis*. Environmental Microbiology **7:**1065-1073.

144. **Rauprich, O., M. Matsushita, C. J. Weijer, F. Siegert, S. E. Esipov, and J. A. Shapiro.** 1996. Periodic Phenomena in *Proteus mirabilis* Swarm Colony Development. J. Bacteriol. **178:**6525-6538.

145. **Roberts, J. A., M. Bernice Kaack, and E. N. Fussell.** 1993. Adherence to Urethral Catheters by Bacteria Causing Nosocomial Infections. Urology **41:**338-340, 341-342.

146. **Romeo, T., M. Gong, M. Y. Liu, and A. M. Brun-Zinkernagel.** 1993. Identification and Molecular Characterization of *csrA*, a Pleiotropic Gene from *Escherichia coli* that Affects Glycogen Biosynthesis, Gluconeogenesis, Cell Size, and Surface Properties. Journal Bacteriology **175:**4744-4755.

147. **Rózalski, A., H. Długońska, and K. Kotełko.** 1986. Cell invasiveness of Proteus mirabilis and Proteus vulgaris strains. Archivum Immunologiea et Therapiea Experimentalis **34:**505-512.

148. **Rozalski, A., Z. Sidorczyk, and K. Kotelko.** 1997. Potential Virulence Factors of *Proteus* Bacilli. Microbiol. Mol. Biol. Rev. **61:**65-89.

149. **Sar, N., L. McCarter, M. Simon, and M. Silverman.** 1990. Chemotactic Control of the Two Flagellar Systems of *Vibrio parahaemolyticus*. J. Bacteriol. **172:**334-341.

150. **Schneider, R., C. V. Lockatell, D. Johnson, and R. Belas.** 2002. Detection and Mutation of a *luxS*-Encoded Autoinducer in *Proteus mirabilis*. Microbiology **148:**773-782.

151. **Senior, B. W., L. M. Loomes, and M. A. Kerr.** 1991. Microbial IgA Proteases and Virulence. Reviews in Medical Microbiolgy **43:**176-184.

152. **Senior, B. W., L. M. Loomes, and M. A. Kerr.** 1991. The Production and Activity *in vivo* of *Proteus mirabilis* IgA Protease in Infections of the Urinary Tract. J Med Microbiol **35:**203-207.

153. **Shinoda, S., and K. Okamoto.** 1977. Formation and Function of *Vibrio parahaemolyticus* lateral flagella. Journal Bacteriology **129:**1266-1271.

154. **Silverblatt, F. J., and I. Ofek.** 1978. Influence of Pili on the Virulence of *Proteus mirabilis* in Experimental Hematogenous Pyelonephritis. The Journal of Infectious Diseases **138:**664-667.

155. **Sivick, K. E., and H. L. Mobley.** 2010. Waging War against Uropathogenic *Escherichia coli*: Winning Back the Urinary Tract. Infect. Immun. **78:**568-585.

156. **Sledjeski, D., and S. Gottesman.** 1996. Osmotic Shock Induction of Capsule Synthesis in *Escherichia coli K-12*. J. Bacteriol. **178:**1204-1206.

157. **Soo, P.-C., Y.-T. Horng, J.-R. Wei, J.-C. Shu, C.-C. Lu, and H.-C. Lai.** 2008. Regulation of Swarming Motility and *flhDC*(*Sm)* Expression by RssAB Signaling in *Serratia marcescens*. Journal Bacteriology **190:**2496-2504.

158. **Sosa, V., G. Schlapp, and P. Zunino.** 2006. *Proteus mirabilis* Isolates of Different Origins do not Show Correlation with Virulence Attributes and can Colonize the Urinary Tract of Mice. Microbiology **152:**2149-2157.

159. **Spahiu, L., and V. Hasbatha.** 2010. Most Frequent Causes of Urinary Tract Infections in Children. Medicinski Arhiv **64:**88-90.

160. **Springer, W. R., and D. E. Koshland.** 1977. Identification of a Protein Methyltransferase as the *cheR* Gene Product in the Bacterial Sensing System. Proceedings of the National Academy of Sciences of the United States of America **74:**533-537.

161. **Stahl, S. J., K. R. Stewart, and F. D. Williams.** 1983. Extracellular Slime Associated with *Proteus mirabilis* During Swarming. J. Bacteriol. **154:**930-937.

162. **Stahl, S. J., and F. D. Williams.** 1981. Immunofluorescent Evidence of *Proteus mirabilis* Swarm Cell Formation on Sterilized Rat Feces. Applied Environmental Microbiology **41:**801-806.

163. **Stamm, W. E.** 1999. Urinary tract Infections, p. 649-656. *In* R. K. Root (ed.), Clinical Infectious Diseases: a Practical Approach. Oxford University Press Inc., New York.

164. **Stickler, D., L. Ganderton, J. King, J. Nettleton, and C. Winters.** 1993. *Proteus mirabilis*t; Biofilms and the Encrustation of Urethral Catheters. Urological Research **21:**407-411.

165. **Stickler, D., R. Young, G. Jones, N. Sabbuba, and N. Morris.** 2003. Why are Foley Catheters so Vulnerable to Encrustation and Blockage by Crystalline Bacterial Biofilm? Urological Research **31:**306-311.

166. **Stickler, D. J., and J. Zimakoff.** 1994. Complications of Urinary Tract Infections Associated with Devices Used for Long-Term Bladder Management. Journal of Hospital Infection **28:**177-194.

167. **Stock, A. M., V. L. Robinson, and P. N. Goudreau.** 2000. Two-Component Signal Transduction. Annual Review of Biochemistry **69:**183-215.

168. **Stout, V., A. Torres-Cabassa, M. R. Maurizi, D. Gutnick, and S. Gottesman.** 1991. RcsA, an Unstable Positive Regulator of Capsular Polysaccharide Synthesis. Journal Bacteriology **173:**1738-1747.

169. **Studdert, C. A., and J. S. Parkinson.** 2004. Crosslinking Snapshots of Bacterial Chemoreceptor Squads. Proceedings of the National Academy of Sciences of the United States of America **101:**2117-2122.

170. **Sturgill, G., and P. N. Rather.** 2004. Evidence that Putrescine Acts as an Extracellular Signal Required for Swarming in *Proteus mirabilis*. Molecular Microbiology **51:**437-446.

171. **Swihart, K. G., and R. A. Welch.** 1990. Cytotoxic Activity of the *Proteus* Hemolysin HpmA. Infect. Immun. **58:**1861-1869.

172. **Takeda, S.-i., Y. Fujisawa, M. Matsubara, H. Aiba, and T. Mizuno.** 2001. A Novel Feature of the Multistep Phosphorelay in *Escherichia coli*: A Revised Model of the RcsC;YojN;RcsB Signalling Pathway Implicated in Capsular Synthesis and Swarming Behaviour. Molecular Microbiology **40:**440-450.

173. **Thormann, K. M., and A. Paulick.** 2010. Tuning the Flagellar Motor. Microbiology **156:**1275-1283.

174. **Toguchi, A., M. Siano, M. Burkart, and R. M. Harshey.** 2000. Genetics of Swarming Motility in *Salmonella enterica Serovar Typhimurium*: Critical Role for Lipopolysaccharide. J. Bacteriol. **182:**6308-6321.

175. **Tolker-Nielsen, T., A. B. Christensen, K. Holmstrom, L. Eberl, T. B. Rasmussen, C. Sternberg, A. Heydorn, S. Molin, and M. Givskov.** 2000. Assessment of *flhDC* mRNA Levels in *Serratia liquefaciens* Swarm Cells. Journal Bacteriology **182:**2680-2686.

176. **Tremblay, J., A.-P. Richardson, F. Lépine, and E. Déziel.** 2007. Self-Produced Extracellular Stimuli Modulate the *Pseudomonas aeruginosa* Swarming Motility Behaviour. Environmental Microbiology **9:**2622-2630.

177. **Turner, L., W. S. Ryu, and H. C. Berg.** 2000. Real-Time Imaging of Fluorescent Flagellar Filaments. Journal of Bacteriology **182:**2793-2801.

178. **Turner, L., R. Zhang, N. C. Darnton, and H. C. Berg.** 2010. Visualization of Flagella During Bacterial Swarming. Journal Bacteriology **192:**3259-3267.

179. **Ulitzur, S.** 1974. Induction of Swarming in *Vibrio parahaemolyticus*. Archives of Microbiology **101:**357-363.

180. **Uphoff, T. S., and R. A. Welch.** 1990. Nucleotide Sequencing of the *Proteus mirabilis* Calcium-Independent Hemolysin Genes (*hpmA* and *hpmB)* Reveals Sequence Similarity with the *Serratia marcescens* Hemolysin Genes (*shlA* and *shlB*). J. Bacteriol. **172:**1206-1216.

181. **Vladimirov, N., and V. Sourjik.** 2009. Chemotaxis: How Bacteria use Memory. Biological Chemistry **390:**1097-1104.

182. **Wadhams, G. H., and J. P. Armitage.** 2004. Making Sense of it all: Bacterial Chemotaxis. Nature Reviews Molecular Cell Biology **5:**1024-1037.

183. **Walker, K. E., S. Moghaddame-Jafari, C. V. Lockatell, D. Johnson, and R. Belas.** 1999. ZapA, the IgA-degrading Metalloprotease of *Proteus mirabilis*, is a Virulence Factor Expressed Specifically in Swarmer Cells. Molecular Microbiology **32:**825-836.

184. **Wang, Q., J. G. Frye, M. McClelland, and R. M. Harshey.** 2004. Gene Expression Patterns During Swarming in *Salmonella typhimurium*: Genes Specific to Surface Growth and Putative New Motility and Pathogenicity Genes. Molecular Microbiology **52:**169-187.

185. **Wang, Q., S. Mariconda, A. Suzuki, M. McClelland, and R. M. Harshey.** 2006. Uncovering a Large Set of Genes That Affect Surface Motility in *Salmonella enterica* Serovar Typhimurium. Journal Bacteriology **188:**7981-7984.

186. **Wang, Q., A. Suzuki, S. Mariconda, S. Porwollik, and R. M. Harshey.** 2005. Sensing Wetness: A New Role for the Bacterial Flagellum. EMBO Journal **24:**2034-2042.

187. **Wang, Q., Y. Zhao, M. McClelland, and R. M. Harshey.** 2007. The RcsCDB Signaling System and Swarming Motility in *Salmonella enterica*

Serovar Typhimurium: Dual Regulation of Flagellar and SPI-2 Virulence Genes. Journal Bacteriology **189:**8447-8457.

188. **Wang, W.-B., I.-C. Chen, S.-S. Jiang, H.-R. Chen, C.-Y. Hsu, P.-R. Hsueh, W.-B. Hsu, and S.-J. Liaw.** 2008. Role of RppA in the Regulation of Polymyxin B Susceptibility, Swarming, and Virulence Factor Expression in *Proteus mirabilis*. Infectection and Immununity **76:**2051-2062.

189. **Warren, J. W., J. H. Tenney, J. M. Hoopes, H. L. Muncie, and W. C. Anthony.** 1982. A Prospective Microbiologic Study of Bacteriuria in Patients with Chronic Indwelling Urethral Catheters. The Journal of Infectious Diseases **146:**719-723.

190. **Wassif, C., D. Cheek, and R. Belas.** 1995. Molecular Analysis of a Metalloprotease from *Proteus mirabilis*. J. Bacteriol. **177:**5790-5798.

191. **Waters, C. M., and B. L. Bassler.** 2005. Quorum Sensing: Cell-to-Cell Communication in Bacteria. Annual Review of Cell and Developmental Biology **21:**319-346.

192. **Wei, B. L., A.-M. Brun-Zinkernagel, J. W. Simecka, B. M. Prüß, P. Babitzke, and T. Romeo.** 2001. Positive Regulation of Motility and *flhDC* Expression by the RNA-Binding Protein CsrA of *Escherichia coli*. Molecular Microbiology **40:**245-256.

193. **Welch, M., K. Oosawa, S. Aizawa, and M. Eisenbach.** 1993. Phosphorylation-dependent Binding of a Signal Molecule to the Flagellar Switch of Bacteria. Proceedings of the National Academy of Sciences of the United States of America **90:**8787-8791.

194. **Welch, R. A.** 1987. Identification of Two Different Hemolysin Determinants in Uropathogenic *Proteus* Isolates. Infect. Immun. **55:**2183-2190.

195. **Williams, F. D., D. M. Anderson, P. S. Hoffman, R. H. Schwarzhoff, and S. Leonard.** 1976. Evidence Against the Involvement of Chemotaxis in Swarming of *Proteus mirabilis*. Journal of Bacteriology **127:**237-248.

196. **Wolgemuth, C., E. Hoiczyk, D. Kaiser, and G. Oster.** 2002. How *Myxobacteria* Glide. Current Biology **12:**369-377.

197. **Wozniak, C. E., F. F. V. Chevance, and K. T. Hughes.** 2010. Multiple Promoters Contribute to Swarming and the Coordination of Transcription with Flagellar Assembly in *Salmonella*. J. Bacteriol. **192:**4752-4762.

198. **Wray, S. K., S. I. Hull, R. G. Cook, J. Barrish, and R. A. Hull.** 1986. Identification and Characterization of a Uroepithelial Cell Adhesin from a Uropathogenic Isolate of *Proteus mirabilis*. Infect. Immun. **54:**43-49.

199. **Zhao, H., X. Li, D. E. Johnson, I. Blomfield, and H. L. Mobley.** 1997. *In vivo* Phase Variation of MR/P Fimbrial Gene Expression in *Proteus mirabilis* Infecting the Urinary Tract. Molecular Microbiology **23:**1009-1019.

200. **Zhao, H., R. B. Thompson, V. Lockatell, D. E. Johnson, and H. L. Mobley.** 1998. Use of Green Fluorescent Protein To Assess Urease Gene Expression by Uropathogenic *Proteus mirabilis* during Experimental Ascending Urinary Tract Infection. Infect. Immun. **66:**330-335.

201. **Zunino, P., C. Piccini, and C. Legnani-Fajardo.** 1994. Flagellate and Non-flagellate *Proteus mirabilis* in the Development of Experimental Urinary Tract Infection. Microbial Pathogenesis **16:**379-385.

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 and Suvanasuthi, 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, 2004; Belas and Suvanasuthi, 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 et al, 1979, Armitage, 1982; Belas et al, 1995; Gue et al, 2001), 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 σ^{28}(Claret, L. and C. Hughes 2000). The expression of σ^{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*, et al.*, 1993; Romeo, 1998) by affecting the stability of mRNA (Liu*, et al.*, 1995; Liu*, et al.*, 1998). In several *Enterobacteriaceae,* a counterpart of RsmA, RsmB, has been identified. RsmB is an untranslated regulatory RNA that binds and neutralizes RsmA (Liu*, et al.*, 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 (Cui*, et al.*, 1995; Mukherjee*, et al.*, 1996;

Liu*, et al.*, 1998; Wei*, et al.*, 2001; Liaw*, et al.*, 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*, et al.*, 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*, et al.*, 2001), while in the *Erwinia* species RsmA represses swarming and virulence factor expression (Cui*, et al.*, 1995, Mukherjee*, et al.*, 1996; Liu*, et al.*, 1998). 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*, et al.*, 2003). Finally, over-expressing *rsmA* in a strain

mutant for *rsbA (rcsD)*, a protein required for RcsB phosphorylation and subsequent repression of *flhDC* (Liaw*, et al.*, 2001), suppresses the hyperswarming phenotype of the *rsbA* mutant (Liaw*, et al.*, 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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References

Alavi M & Belas R (2001) Surface sensing, swarmer cell differentiation, and biofilm development. Methods Enzym 336:29-40

Allison C, Lai HC & Hughes C (1992) Co-ordinate expression of virulence genes during swarm-cell differentiation and population migration of *Proteus mirabilis*. Mol. Microbiol. 6:1583-1591.

Allison C, Lai HC, Gygi D & Hughes C (1993) Cell differentiation of *Proteus mirabilis* is initiated by glutamine, a specific chemoattractant for swarming cells. Mol. Microbiol. 8:53-60

Allison C, Emody L, Coleman N & Hughes C (1994) The role of swarm cell differentiation and multicellular migration in the uropathogenicity of *Proteus mirabilis.* J. Infect. Dis. 169:1155-1158.

Armitage JP, Smith DG, Rowbury RJ (1979) Alterations in the cell envelope composition of *Proteus mirabilis* during the development of swarmer cells. Biochim Biophys Acta 584:389-397.

Armitage JP (1982) Changes in the organization of the outer membrane of *Proteus mirabilis* during swarming: freeze-fracture structure and membrane fluidity analysis. J. Bacteriol. 150:900-904.

Arouh S (2001) Analytical model for ring formation by bacterial swarmers. Physical Review E 63, 031908 1-14

Bahrani FK & Mobley HL (1994) *Proteus mirabilis* MR/P fimbrial operon: genetic organization, nucleotide sequence, and conditions for expression. J. Bacteriol. 176:3412-3419.

Belas, R (1994) Expression of multiple flagellin-encoding genes of *Proteus mirabilis*. J. Bacteriol. 176:7169-7181.

Belas R, Goldman M & Ashliman K (1995) Genetic analysis of *Proteus mirabilis* mutants defective in swarmer cell elongation. J. Bacteriol. 177:823-828.

Belas R, Schneider R & Melch, M (1998) Characterization of *Proteus mirabilis* precocious swarming mutants: identification of *rsb*A, encoding a regulator of swarming behavior. J Bacteriol 180:6126-6139.

Belas R & Suvanasuthi R (2005) The ability of *Proteus mirabilis* to sense surfaces and regulate virulence gene expression involves FliL, a flagellar basal body protein. J. Bacteriol 187:6789-6803.

Calvo JM & Matthews RG (1994) The leucine-responsive regulatory protein, a global regulator of metabolism in *Escherichia coli.* Microbiol Rev. 58: 466-490.

Cano DA, Dominguez-Bernal G, Tierrez A, Garcia-Del Portillo F & Casadesus J (2002) Regulation of capsule synthesis and cell motility in *Salmonella enterica* by the essential gene *igaA*. Genetics 162:1513-1523.

Cano DA, Martinez-Moya M, Pucciarelli MG, Groisman EA, Casadesus J & Garcia-Del Portillo F (2001) *Salmonella enterica Serovar Typhimurium* response involved in attenuation of pathogen intracellular proliferation. Infect. Immun. 69:6463-6474.

Castanie-Cornet MP, Cam K & Jacq A (2006) RcsF is an outer membrane lipoprotein involved in the RcsCDB phosphorelay signaling pathway in *Escherichia coli*. J. Bacteriol. 188:4264-4270.

Chilcott GS & Hughes KT (2000) Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica* Serovar Typhimurium and *Escherichia coli.* Microbiol Mol Biol Rev 64:694-708

Claret L & Hughes C (2000) Functions of the subunits of the $FlhD_2C_2$ transcriptional master regulator of bacterial flagellum biogenesis and swarming. J. Mol. Biol. 303:467-478.

Clemmer KM & Rather PN (2008) The Lon protease regulates swarming motility and virulence gene expression in *Proteus mirabilis*. J Med Microbiol 57:931-937.

Clemmer KM & Rather PN (2007) Regulation of *flh*DC Expression in *Proteus mirabilis*. Res Microbiol 158:295-302.

Cui Y, Chatterjee A, Liu Y, Dumenyo CK & Chatterjee AK (1995) Identification of a global repressor gene, rsmA, of Erwinia carotovora subsp. carotovora that controls extracellular enzymes, N-(3-oxohexanoyl)-L-homoserine lactone, and pathogenicity in soft-rotting Erwinia spp. J Bacteriol 177: 5108-5115.

Daniels R, Reynaert S, Hoekstra H, Verreth C, Janssens J, Braeken K, Fauvart M, Beullens S, Heusdens C, Lambrichts I, De Vos DE, Vanderleyden J, Vermant J & Michiels J (2006) Quorum signal molecules as biosurfactants affecting swarming in *Rhizobium etli*. Proc. Natl. Acad. Sci. U S A. 2006 103:14965-14970.

Dufour A, Furness RB & Hughes C (1998) Novel genes that upregulate the *Proteus mirabilis flhDC* master operon controlling flagellar biogenesis and swarming. Mol Microbiol 29:741-751.

Esipov SE & Shapiro JA (1998) Kinetic model of *Proteus mirabilis* swarm colony development. J. Math Biol 36:249-268

Ferrières L & Clarke DJ (2003) The RcsC sensor kinase is required for normal biofilm formation in *Escherichia coli* K-12 and controls the expression of a regulon in response to growth on a solid surface. Mol Microbiol 50:1665-1682.

Fraser GM, Claret L, Furness R, Gupta S & Hughes C (2002) Swarming-coupled expression of the *Proteus mirabilis hpmBA* haemolysin operon. Microbiology. 148:2191-2201.

Friedberg D, Platko JV, Tyler B & Calvo JM (1995) The amino acid sequence of Lrp is highly conserved in four enteric microorganisms. J Bacteriol 177: 1624-1626.

Gue M, Dupont V, Dufour A & Sire O (2001) Bacterial swarming: a biochemical time-resolved FTIR-ATR study of *Proteus mirabilis* swarm-cell differentiation. Biochemistry 40:11938-11945.

Gygi, D, Rahman, MM, Lai, HC, Carlson, R, Guard-Petter, J & Hughes, C. (1995) A cell-surface polysaccharide that facilitates rapid population migration by differentiated swarm cells of *Proteus mirabilis.* Mol Microbiol. 17:1167-1175.

Hagiwara D, Sugiura M, Oshima T, Mori H, Aiba H, Yamashino T & Mizuno T (2003) Genome-wide analyses revealing a signaling network of the RcsC-YojN-RcsB phosphorelay system in *Escherichia coli*. J. Bacteriol. 185:5735-5746.

Harshey RM (2003) Bacterial motility on a surface: many ways to a common goal. Annu. Rev. Microbiol. 57:249-273.

Hatt JK & Rather PN (2008) Characterization of a novel gene, *wosA,* regulating FlhDC expression in *Proteus mirabilis*. J Bacteriol 190:1946-1955.

Hay NA, Tipper DJ, Gygi D & Hughes C (1997) A nonswarming mutant of *Proteus mirabilis* lacks the Lrp global transcriptional regulator. J Bacteriol 179: 4741-4746.

Jansen AM, Lockatell CV, Johnson DE & Mobley HL (2003) Visualization of *Proteus mirabilis* morphotypes in the urinary tract: the elongated swarmer cell is rarely observed in ascending urinary tract infection. Infect. Immunity. 71:3607-3613

Jones BV, Young R, Mahenthiralingam, E & Stickler, DJ (2004) Ultrastructure of *Proteus mirabilis* swarmer cell rafts and role of swarming in catheter associated urinary tract infection. Infect. Immun. 72:3941-3950.

Laubacher ME & Ades SE (2008) The Rcs phosphorelay is a cell envelope stress response activated by peptidoglycan stress and contributes to intrinsic antibiotic resistance. J. Bacteriol. 190:2065-2074.

Legnani-Fajardo C, Zunino P, Piccini C, Allen A & Maskell D (1996) Defined mutants of *Proteus mirabilis* lacking flagella cause ascending urinary tract infection in mice. Microb. Pathog. 21:395-405

Lahaye E, Aubry T, Fleury T & Sire O (2007) Does water activity rule *P. mirabilis* periodic swarming? II. Viscoelasticity and water balance during swarming Biomacromolecules 8:1228-1235

Li X, Rasko DA, Lockatell CV, Johnson DE & Mobley HL (2001) Repression of bacterial motility by a novel fimbrial gene product. EMBO J 20:4854-4862.

Liaw SJ, Lai HC, Ho SW, Luh KT & Wang WB (2001) Characterisation of p-nitrophenylglycerol-resistant *Proteus mirabilis* super-swarming mutants. J. Med. Microbiol. 50:1039-1048.

Liaw SJ, Lai HC, Ho SW, Luh KT & Wang WB (2003) Role of RsmA in the regulation of swarming motility and virulence factor expression in *Proteus mirabilis.* J Med Microbiol 52: 19-28.

Lindum PW, Anthoni U, Christophersen C, Eberl L, Molin S & Givskov M (1998) N-Acyl-L-homoserine lactone autoinducers control production of an extracellular lipopeptide biosurfactant required for swarming motility of *Serratia liquefaciens* MG1. J. Bacteriol. 180:6384-6388.

Lintner RE, Mishra PK, Srivastava P, Martinez-Vaz BM, Khodursky, AB & Blumenthal RM (2008) Limited functional conservation of a global regulator among related bacterial genera: Lrp in *Escherichia, Proteus* and *Vibrio.* BMC Microbiol 8:60

Liu MY, Yang H & Romeo T (1995) The product of the pleiotropic *Escherichia coli* gene *csrA* modulates glycogen biosynthesis via effects on mRNA stability. J Bacteriol 177: 2663-2672.

Liu Y, Cui Y, Mukherjee A & Chatterjee AK (1998) Characterization of a novel RNA regulator of Erwinia carotovora ssp. carotovora that controls production of extracellular enzymes and secondary metabolites. Mol Microbiol 29:219-234.

Majdalani N, Heck M, Stout V & Gottesman S (2005) Role of RcsF in signaling to the Rcs phosphorelay pathway in *Escherichia coli*. J. Bacteriol. 187:6770-6778.

Matsuyama T, Takagi Y, Nakagawa, Y, Itoh H, Wakita J & Matsushita M (2000) Dynamic aspects of the structured cell population in a swarming colony of *Proteus mirabilis.* J Bacteriol 182:385-393

McCarter L & Silverman M (1990) Surface-induced swarmer cell differentiation of *Vibrio parahaemolyticus.* Mol Micro 4:1057-1062

Medvedev GS, Kopell N & Kaper TJ (2000) A reaction-diffusion system with periodic front dynamics. J Appl Math 60:1601-1638.

Mobley HL & Belas R (1995) Swarming and pathogenicity of *Proteus mirabilis* in the urinary tract. Trends Microbiol. 3:280-284.

Mobley HL, Belas R, Lockatell V, Chippendale G, Trifillis AL, Johnson DE & Warren JW (1996) Construction of a flagellum negative mutant of *Proteus mirabilis:* effect on internalization by human renal epithelial cells and virulence in a mouse model of ascending urinary tract infection. Infect. Immun. 64:5332-5340.

Morgenstein RM, Clemmer KM and Rather PN (2010) Loss of the WaaL O-antigen ligase prevents surface activation of the flagellar gene cascade in *Proteus mirabilis.* J. Bacteriol, in press

Mukherjee A, Cui Y, Liu Y, Dumenyo CK & Chatterjee AK (1996) Global regulation in Erwinia species by *Erwinia carotovora rsmA,* a homologue of *Escherichia coli csrA*: repression of secondary metabolites, pathogenicity and hypersensitive reaction. Microbiology 142 (Pt 2): 427-434.

Newman EB & Lin R (1995) Leucine-responsive regulatory protein: a global regulator of gene expression in *Escherichia coli.* Annu Rev Microbiol 49:747-775.

Pearson MM & Mobley HL (2008) Repression of motility during fimbrial expression: identification of 14 *mrpJ* gene paralogues in *Proteus mirabilis*. Mol Microbiol 69:548-558.

Pearson MM, Sebaihia M, Churcher C, Quail MA, Seshasayee AS, Luscombe NM, Abdellah Z, Arrosmith C, Atkin B, Chillingworth T, Hauser H, Jagels K, Moule S, Mungall K, Norbertczak H, Rabbinowitsch E, Walker D, Whithead S, Thomson NR, Rather PN, Parkhill J & Mobley HL (2008) Complete genome sequence of uropathogenic *Proteus mirabilis*, a master of both adherence and motility. J Bacteriol. 190:4027-4037.

Rather PN (2005) Swarmer cell differentiation in *Proteus mirabilis.* Environ Microbiol 8:1065-1073

Rauprich O, Matsushita M, Weijer CJ, Siegert F, Esipov SE & Shapiro JA (1996) Periodic phenomena in *Proteus mirabilis* swarm colony development. J Bacteriol. 178:6525-6538.

Romeo T, Gong M, Liu MY & Brun-Zinkernagel AM (1993) Identification and molecular characterization of *csrA,* a pleiotropic gene from *Escherichia coli* that affects glycogen biosynthesis, gluconeogenesis, cell size, and surface properties. J Bacteriol 175: 4744-4755.

Rozalski A, Sidorczyk Z & Kotelko, K (1997) Potential virulence factors of *Proteus* bacilli. Micro. Mol. Biol. Reviews. 61:65-89.

Schneider R, Lockatell CV, Johnson D & Belas R (2002) Detection and mutation of a *luxS*-encoded autoinducer in *Proteus mirabilis.* Microbiology. 148:773-782.

Siomi H, Choi M, Siomi MC, Nussbaum RL & Dreyfuss G (1994) Essential role for KH domains in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. Cell 77: 33-39.

Sledjeski DD & Gottesman S (1996) Osmotic shock induction of capsule synthesis in *Escherichia coli* K-12. J Bacteriol. 178:1204-1206.

Stevenson LG & Rather PN (2006) A novel gene involved in regulating the flagellar gene cascade in Proteus mirabilis. J Bacteriol 188: 7830-7839.

Stock AM, Robinson VL & Goudreau PN (2000) Two-component signal transduction. Ann Rev Biochem 69:183-215.

Sturgill G & Rather PN (2004) Evidence that putrescine acts as an extracellular signal required for swarming in *Proteus mirabilis.* Mol. Microbiol. 51:437-446.

Toguchi A, Siano M, Burkart M & Harshey RM (2000) Genetics of swarming motility in Salmonella enterica serovar typhimurium: critical role for lipopolysaccharide. J Bacteriol. 182:6308-6321.

Tsilibaris V, Maenhaut-Michel G & VanMelderen L (2006) Biological roles of the Lon ATP-dependent protease. Res. Microbiol. 157:701-713.

Walker KE, Moghaddame-Jafari S, Lockatell CV, Johnson D & Belas R (1999) ZapA, the IgA-degrading metalloprotease of *Proteus mirabilis,* is a virulence factor expressed specifically in swarmer cells. Mol Microbiol 32: 825-836.

Wang Q, Zhao Y, McClelland M & Harshey RM (2007) The RcsCDB signaling system and swarming motility in *Salmonella enterica* serovar typhimurium: dual regulation of flagellar and SPI-2 virulence genes. J Bacteriol. 189:8447-8457.

Wang WB, Chen IC, Jiang SS, Chen HR, Hsu CY, Hsueh PR, Hsu WB, & Liaw SJ (2008) Role of RppA in the regulation of polymyxin B susceptibility, swarming, and virulence factor expression in *Proteus mirabilis*. Infect Immun. 76:2051-2062.

Waters CM & Bassler BL (2005) Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol. 21:319-346

Wei BL, Brun-Zinkernagel AM, Simecka JW, Pruss BM, Babitzke P & Romeo T (2001) Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli.* Mol Microbiol 40:245-256.

Zunino P, Piccini C & Legnani-Fajardo C (1994) Flagellate and non-flagellate *Proteus mirabilis* in the development of experimental urinary tract infection. Microb. Pathog. 16:379-385.

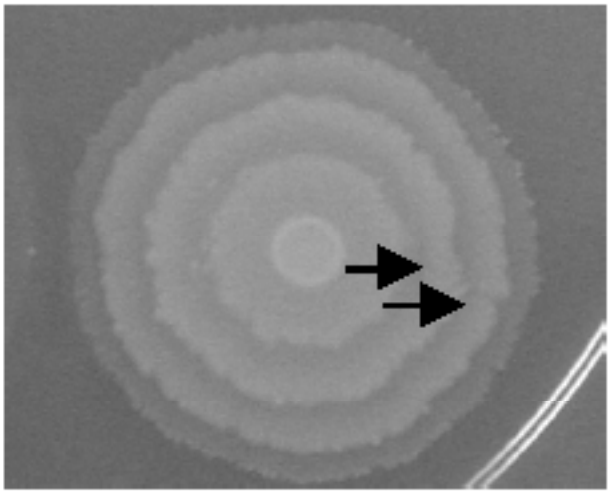
Figure legends.

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.

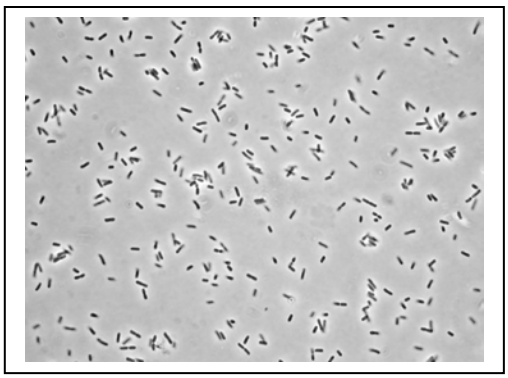
Figure 2. Key regulators of gene expression during swarming. A summary of the genes that are known to regulate gene expression during swarming in *P. mirabilis* is shown. The predicted location of each gene product in the outer membrane (OM), inner cytoplasmic membrane (IM) or cytoplasm is shown.

Figure 1.

A.



B.



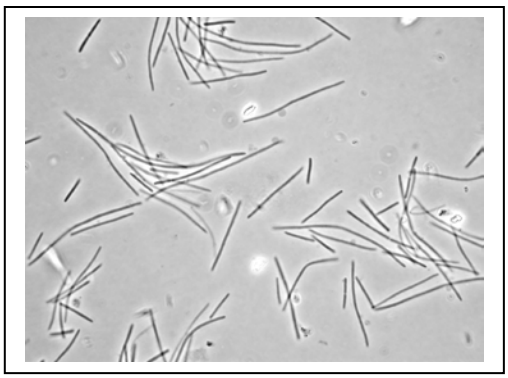
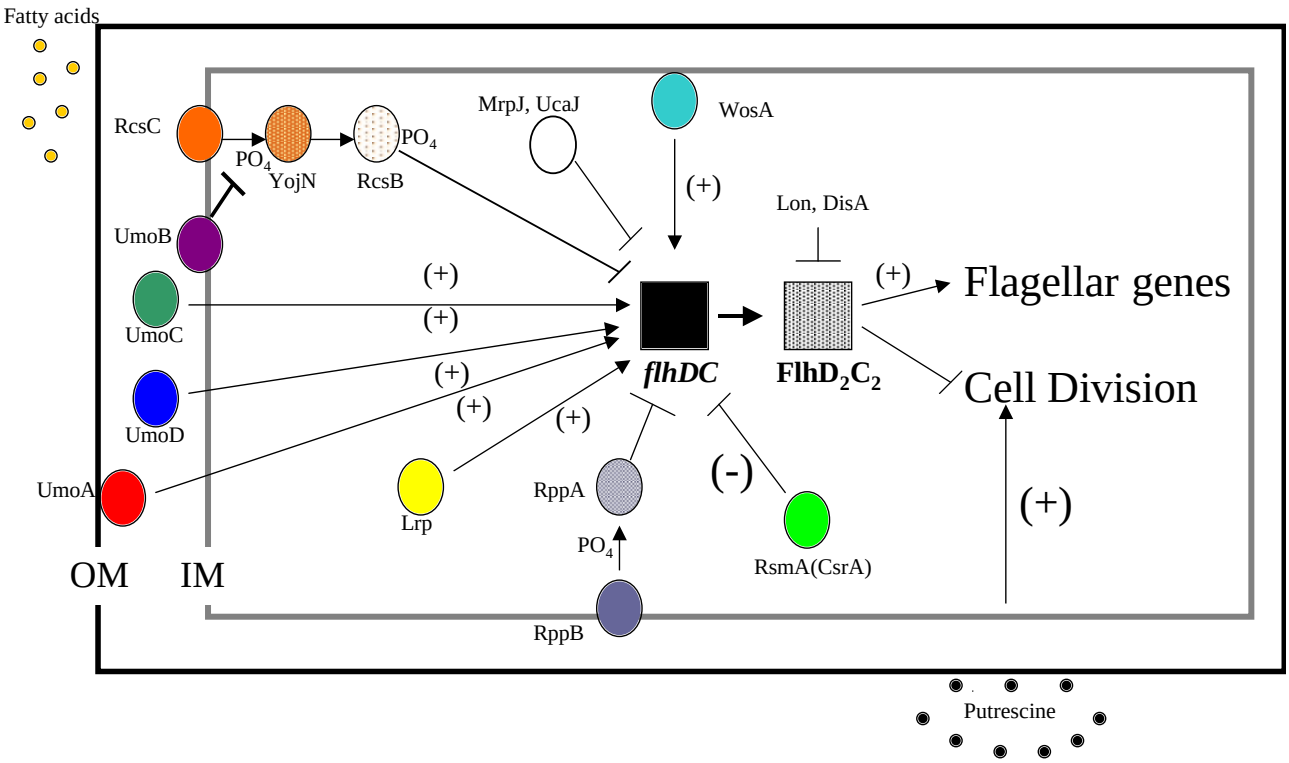


Figure 2.

Key regulatory mechanisms involved in swarming.



Chapter 3: Loss of the WaaL O-antigen ligase prevents surface activation of the flagellar gene cascade in *Proteus mirabilis*

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Abstract

Proteus mirabilis is a Gram-negative bacterium that undergoes a physical and biochemical change from a vegetative swimmer cell (a typical Gram-negative rod) to an elongated swarmer cell when grown on a solid surface. In this study, we report that a transposon insertion in the *waaL* gene, encoding O-antigen ligase, blocked swarming motility on solid surfaces, but had little effect on swimming motility in soft agar. The *waaL* mutant was unable to differentiate into a swarmer cell. Differentiation was also prevented by a mutation in *wzz,* encoding a chain length determinant for O-antigen, but not by a mutation in *wzyE,* encoding an enzyme that polymerizes Enterobacterial Common Antigen (ECA), a different surface polysaccharide to the lipidA::core. In wild-type *P. mirabilis,* increased expression of the *flhDC* operon occurs after growth on solid surfaces and is required for the high-level expression of flagellin that is characteristic of swarmer cells. However, in both the *waaL* and *wzz* mutants, the *flhDC* operon was not activated during growth on agar. A loss of function mutation in the *rcsB* response regulator or overexpression of *flhDC* restored swarming to the *waaL* mutant, despite the absence of O-antigen. Therefore, although O-antigen may serve a role in swarming by promoting wettability, the loss of O-antigen blocks a regulatory pathway that links surface contact with the upregulation of *flhDC* expression.

Introduction

Proteus mirabilis is a Gram-negative, rod-shaped bacterium that causes urinary tract infections in patients with catheters or abnormal urethras (50). It has been well studied for its ability to swarm, a flagella-based, solid-surface associated, social movement. In liquid broth *P. mirabilis* are peritrichously flagellated swimmer cells with a few flagella. Three to four hours after contact with a solid medium, the cells begin to differentiate into elongated rods that are 20-40 fold longer than their liquid counterparts, and have a >50 fold increase in flagella. These cells are also multi-nucleated and aseptate (reviewed in references (21, 41)). The swarmer cells join together to form a swarming raft and as a group swarm out from a central inoculum until an unknown signal is sensed and the cells consolidate, or de-differentiate, back into swimmer cells (24). This process repeats itself to form a characteristic bull's eye pattern on an agar plate (42). At present, several known signals for differentiation have been identified and include; the inhibition of flagellar rotation and the accumulation of putrescine. However, it is thought that there are additional unknown signals involved (4, 53).

Flagella play an important role in swarming as surface sensors and for propulsion; therefore their expression is tightly regulated. The regulation of flagella in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium has been well studied and is complex; involving a three-tiered regulatory cascade (reviewed in (11)). The master regulator for flagellar synthesis is $FlhD_2C_2$ encoded by the *flhDC* operon. These genes are considered class I genes and control the expression of the class II genes, which are involved in the hook and

basal body construction. An alternative sigma factor, sigma 28, also a class II gene, controls the expression of class III genes such as *flaA,* the flagellin structural gene, and is only activated upon release of an anti-sigma factor, FlgM, which is exported through the completed hook/basal body structure (11). $FlhD_2C_2$ is necessary for swarming in *P. mirabilis* (12-14, 16, 52). During swarmer cell differentiation, the transcript levels of *flhDC* rise almost 50-fold, therefore mutations in genes that regulate *flhDC* levels can have dramatic effects on swarming. For example, mutations in the leucine-responsive regulatory protein (a positive regulator of *flhDC*) block swarming, while mutations in components of the RcsBCD phosphorelay (a negative regulator of *flhDC*) result in hyper-swarming, and even elongation in liquid, a condition normally non-permissive to cell differentiation (4, 22, 29).

The outer membrane of Gram-negative bacteria, especially the O-antigen, is highly immunogenic, acts as a phage receptor and participates in development. *P. mirabilis* has a typical Gram-negative outer membrane in which there is a phospholipid monolayer on the periplasmic side and lipopolysaccharide (LPS) as the outermost leaflet of the membrane (48). The LPS contains a lipid A region, core region, and O-antigen region (43). Enteric bacteria such as *E. coli*, *S. enterica,* or *P. mirabilis* can have either the O-antigen or another polysaccharide termed the Enterobacterial Common Antigen (ECA) attached to the lipid A::core moiety (46, 47). These two antigens are synthesized via different pathways, but they are both attached to the lipid A::core by the same protein; *waaL,* or O-antigen ligase (27).

LPS and ECA have been implicated to play roles in bacterial developmental signaling and motility. Bowden and Kaplan demonstrated a role for O-antigen in *Myxococcus xanthus* social motility and fruiting-body development where O-antigen mutants were defective in S-motility and exhibited aggregation defects during development (6). Toguchi et al. demonstrated a role for LPS in *S. enterica* swarming motility (54). The O-antigen mutant failed to show a reduction in flagellar synthesis, leading the authors to hypothesize that the O-antigen was part of an extracellular milieu that acted as a wettability agent to reduce surface friction (54). ECA has also been shown to be important in bacterial swarming. In *Serratia marcescens*, completed ECA was needed for the upregulation of the master regulator *flhDC*. Without functional ECA, swarming motility was abolished while swimming motility was only decreased (9). Finally, recent studies in *E. coli* indicate that functional LPS is required for both swimming and swarming motility (18). In that study, it was demonstrated that LPS-defective mutants failed to activate the *flhDC* operon, encoding the class I master activator. Interestingly, swarming could be restored by mutations that disrupt the Rcs pathway, suggesting that functional LPS was involved in relieving the RcsB mediated repression during swarming on solid surfaces. In addition, that study revealed that truncations into the inner core of LPS did not prevent swarming when *flhDC* expression was maintained (18).

Additional surface components may also play a role in swarming in *P. mirabilis* (17, 51, 55). A slime layer behind a swarming raft was first observed by Fuscoe in 1973 (17). The production of slime was later found to be coordinated

with swarmer cell development, but a direct link between this slime and swarming was never confirmed (51). More recently, a capsular polysaccharide (colony migration factor (20)) was shown to be needed for swarming and was proposed to reduce surface friction while possibly acting as a matrix for swarming raft formation (40). Along with Cmf, the O-antigen has been implicated to have a role during swarming in *P. mirabilis*. A transposon library was screened for mutants that could not elongate, and insertions were found in the *cld* gene encoding an O-antigen chain length determinant and in the *waaD* and *waaC* (formerly *rfaD/rfaC*) genes required for inner core LPS synthesis (3). Finally, using Fourier transform infared spectroscopy, it was shown that different LPS forms are present on the cell surface during various stages of the swarm cycle, and that the fatty acid composition of the membrane also changes during swarming (19). This indicates *P. mirabilis* carefully controls its membranes during a swarming cycle. These data show that LPS is required for normal swarming, but the basis for this requirement is not understood.

We have found that a transposon insertion in the *P. mirabilis rfaL* (*waaL*) gene, encoding O-antigen ligase, blocks swarmer cell differentiation by preventing the increase in *flhDC* expression that occurs when vegetative cells are grown on solid surfaces. We show the swarming defect in *P. mirabilis* was specific to the loss of O-antigen and not to ECA. Swarmer cell differentiation and swarming defects could be overcome in the *waaL* mutant by overexpressing *flhDC* in *trans.* In addition, swarming was restored by loss of function mutations in the *rcsB* gene, encoding a response regulator in the RscBCD system that

regulates *flhDC* and additional genes in response to growth on solid surfaces and cell envelope stress (28, 30). Finally, our data demonstrates that swarming in *P. mirabilis,* like *E. coli,* can occur in the absence of O-antigen.

Materials and Methods

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. Both *E. coli* and *P. mirabilis* were grown in modified Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) at 37^0C, or LB plates kept at 37^0C. For swim and swarm assays, the agar concentration was 0.3% and 1.5% respectively. Antibiotics were used for selection at concentrations of 25 μg/mL for both chloramphenicol and streptomycin for *E. coli.* Antibiotic concentrations for the selection of *P. mirabilis* were 100μg/mL for chloramphenicol, 35μg/mL for streptomycin, 20μg/mL for kanamycin, and 15μg/mL for tetracycline.

Transposon mutagenesis. PM7002 was mated with SM10 λpir with pUT::mini-Tn*5lacZ1* and the exconjugants with transposon insertions were selected on LB plates supplemented with kanamycin and tetracycline. After overnight incubation at 37^0C, the cells were patched onto 2% LB to screen for the ability to swarm. To identify the insertion site of the transposon in non-swarming mutants, chromosomal DNA was digested with *Bam*HI, ligated to pACYC184, and transformed into *E. coli* XLI. Kanamycin resistant clones were sequenced using a transposon specific primer that read outward from one end into the flanking chromosomal DNA.

Southern blot analysis. To map the transposon insertion in PM942, chromosomal DNA was prepared, digested with *Bam*HI, transferred to a

nitrocellulose membrane, and probed with a digoxigenin labeled probe to the kanamycin cassette of mini-Tn*5lacZ1.* To confirm the matings resulted in the appropriate gene disruptions, chromosomal DNA from the *wzz*, *wzyE*, and *rscB* mutants were extracted, and separately digested with *Bgl*II, *Sal*I, and *Eco*RI before being transferred to a nitrocellulose membrane, and probed with a gene specific digoxigenin labeled probe.

Motility assays. To examine the ability of colonies to swim, cultures were grown overnight in LB media with appropriate antibiotics. All samples were normalized to the same $O.D._{600}$. 5μL droplets were placed on a 0.3% LB plate, with chloramphenicol and incubated for 8 hrs at 37^{o}C. Swarm assays were done identically except the inoculum was spotted onto a 1.5% agar plate.

Mutant construction. Internal fragments of the *wzz, wzyE,* and *rcsB* genes were generated by PCR using the primer set intWzz.for/intWzz.rev, intWzyE.for/intWzyE.rev, and RcsB.for/RcsB.rev respectively (Table 2). Products were digested with *Xba*I and *Sal*I and ligated to pKNG101 cut with the same enzymes. Plasmids were initially electroporated into CC118 □*pir* and then electroporated into SM10λpir for conjugal mating with PM7002 (*wzz* and *wzyE* mutations) and PM942 (*rcsB* mutation) on LB plates. Exconjugants representing Campbell-type integration events that disrupted each gene were selected on LB plates with tetracycline and streptomycin and mutations were confirmed by Southern blot analysis (see above).

Northern blot analysis. Cells were grown overnight in LB. All samples were normalized to the same $O.D._{600}$. 150μL droplets were spread onto 2% LB plates in parallel to produce cultures that were synchronously differentiating. The cells were collected from each plate at the indicated time points with LB media and normalized to an $O.D._{600}$ 0.7. One milliliter of the cells were centrifuged at 12,000 RPM. Total RNA was isolated using the Masterpure RNA purification kit (Epicentre, Madison WI). Equal amounts of RNA were run on a 1.2% formaldehyde agarose gel, and transferred to a nitrocellulose membrane. A DNA probe specific to *flhDC* was labeled with digoxigenin and used to examine transcript levels by chemiluminescence using the CDP-Star substrate (Roche Applied Science).

Western blot analysis. Cells were grown overnight in LB. All samples were normalized to the same $O.D._{600}$. 150μL droplets were spread onto 2% LB plates in parallel to produce colonies that were synchronously differentiating. The cells were collected from each plate at the indicated time points with LB media and normalized to an $O.D._{600}$ 1.000. One milliliter of cells were centrifuged at 12,000 RPM for one minute. The pellet was resuspended in Laemmli Sample Buffer (Bio-Rad) with beta-mercaptoethanol. Protein levels were normalized, run on a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane. The primary antibody used was a rabbit anti-FlaA antibody. The secondary antibody was a donkey anti-rabbit antibody conjugated with peroxidase.

LPS purification and visualization. LPS was isolated from cells using a modified version of the method of Marolda et al. (33). Briefly, cells were grown overnight in appropriate antibiotics. Equal amounts of cells were inoculated into fresh LB with antibiotics and allowed to grow to exponential phase. 100µL droplets were spread onto a 1.5% LB plate and collected after 4 hours of incubation at 37^{o}C with PBS (pH 7.2). After lysing, the cells were treated with proteinase K overnight. In the morning fresh proteinase K was added for 4 hours and the lysates were exposed to hot phenol. Ethyl ether was used to remove any phenol. Once the ether was removed, the LPS was suspended in loading buffer and run on a 12% SDS-PAGE gel. The LPS was visualized using the method of Kittelberger and Hilbink (26).

Complementation of *waaL* and *wzz* mutations. To ensure that the phenotypes seen in the *waaL* and *wzz* mutant backgrounds were due to the specific mutations and not polar effects or second site mutations, the full length version of each gene, including the native ribosome binding site, was generated by PCR. The *waaL* gene was cloned into pACYC184 at the *Bam*HI and *Sal*I sites using primers 942.for and 942.rev, while *wzz* was cloned into pACYC184 at the *Eco*RV and *Bam*HI sites using Wzz.for and Wzz.rev (see Table 2 for primer sequences).

Results

A mini-Tn*5Cm* insertion in the *waaL* gene blocks swarming, but not swimming motility. Swarming and swimming in *P. mirabilis* both require proper flagellar function and a functional chemotaxis system (7, 21, 41). Swimming occurs in liquid and in soft motility agar (0.2-0.4%), while swarming only occurs on a solid surface, such as 1.5% agar plates. In addition, swarming is characterized by the differentiation of vegetative cells to elongated swarmer cells, which then interact to form swarming rafts (24, 41). In order to further elucidate the pathway(s) required for surface recognition and swarmer cell differentiation in *P. mirabilis,* mini-Tn*5lacZ1* transposon mutagenesis was performed on PM7002, a wild type strain of *P. mirabilis,* and a mutant, PM942, that was unable to swarm, but maintained the ability to swim was isolated.

The site of the mini-Tn*5lacZ* transposon insertion in PM942 was determined as described in the Materials and Methods. The insertion was at a position corresponding to amino acid 183 within an open reading frame encoding a 422 amino acid protein. A BLAST search of the deduced protein from PM7002 exhibited 100% identity over the sequenced region to PMI3163 from the genome of HI4320 (36). Additional BLAST searches revealed homology to putative WaaL (RfaL) orthologs from *Photorhabdus luminescens* (50% amino acid identity) and *Salmonella enterica* serovar Heidelberg, Agona, and Schwarzengrund (39% identity). The WaaL protein functions as an O-antigen ligase that links undecaprenol bound O-antigen subunits to the outer core of lipopolysaccharide (LPS) (34). The Kyte-Doolittle hydropathy profile of the putative *P. mirabilis*

WaaL ortholog was highly similar to WaaL proteins from *Salmonella enterica* serovar typhi, *Escherichia coli* O157:H7 and *Pseudomonas aeruginosa* PAO1 (Fig. 1). The similarity in hydropathy profiles between various WaaL proteins from gram-negative bacteria has been noted previously and used to identify potential *waaL* encoding genes, despite the large differences in amino acid similarity between the WaaL proteins (1)**.** Based on the above data and the analysis of O-antigen presented below, the PMI3163 gene was hereafter designated *waaL* (44)*.*

PM942 *waaL*::mini-Tn*5lacZ1* exhibited essentially wild-type levels of swimming when assayed on 0.3% agar, indicating it possessed functional flagellar and chemotactic systems, but was unable to swarm (Fig. 2). To determine if the observed swarming defect in PM942 was due to loss of *waaL* function and not from a polar effect or a secondary unlinked mutation, the *waaL* gene was amplified from the chromosome of PM7002 and cloned into pACYC184 resulting in plasmid pRM5. In PM942 containing pRM5, swarming motility was restored to wild-type levels (Fig. 2).

O-antigen profile of the PM942 mutant. To test if the *waaL* gene product functioned in a manner consistent with an O-antigen ligase and was a WaaL (RfaL) ortholog*,* LPS was isolated from the wild-type PM7002, PM942 *waaL*::mini-Tn*5lacZ1*/pACYC184), and PM942/pRM5 (pACYC184 + *waaL*) and analyzed by SDS-PAGE. Figure 3 shows that the wild type *P. mirabilis* PM7002 strain exhibited the core and core +1 bands. In addition, there was also a ladder

of bands with a broad distribution of varying lengths that represented the O-antigen of varying lengths. In PM942 *waaL*::mini-Tn*5lacZ1,* the core +1 band was absent and there was no ladder of O-antigen in the low to mid-size length. However, there was a faint banding pattern of material that extended much higher in the gel than the material from wild-type cells. In PM942/pRM5, the LPS profile was restored back to that of wild-type PM7002 (Fig. 3).

The presence of the high molecular weight material in PM942 varied in individual LPS preps and we hypothesized that this material might represent O-antigen subunits that remained linked to undecaprenol–PP due to the absence of WaaL activity. Alternatively, this material could represent an undefined polymer whose production is induced in the absence of O-antigen. To test whether this material was O-antigen, a mutation was made in the gene (*wzz*) encoding the O-antigen chain length determinant in PM942. This mutation is predicted to reduce the length of O-antigen whether it is linked to undecaprenol or the LPS core. The *waaL/wzz* double mutant exhibited an LPS profile that only consisted of core, indicating the material was likely composed of O-antigen linked to undecaprenol-PP (data not shown). The above data, taken together with the concomitant loss of core +1 and the short to intermediate O-antigen chains is consistent with loss of O-antigen ligase activity in PM942.

Loss of O-antigen and not ECA is responsible for the swarming defect of a *waaL* mutant. In enteric bacteria, another outer membrane component exists called the Enterobacterial Common Antigen (ECA) (27, 47). This can exist on

the outer surface in two forms; linked to diacylglycerol or linked to the lipid A::core. In *E. coli,* WaaL can link either O-antigen or ECA subunits to the lipid A::core (27, 45). In *S. marcescens* ECA completion acts as a checkpoint for *flhDC* activation (9). To test whether the lack of ECA or O-antigen due to the *waaL* mutation was causing the swarming defect, a mutation in *wzyE* (PMI3326), encoding the ECA polymerase was made in PM7002. The *wzyE* mutant exhibited a significant growth defect, but was still able to swarm after overnight growth indicating that ECA is not needed for swarming (data not shown).

Loss of full length O-antigen in a *wzz* mutant confers a swarming defect. To independently confirm the requirement of full length O-antigen in swarming, a mutation was made in the O-antigen chain length determinant (*cld*), *wzz* (PMI2182) by insertion of the suicide plasmid pKNG101, hereafter designated (*wzz::Sm*^{R}) (25, 39). This mutation should result in O-antigen subunits with reduced chain length being connected to the lipid A::core (39). The mutation in *wzz* abolished swarming in RM16 (*wzz::Sm*^{R}) and could be complemented by the cloned *wzz* gene in pACYC184 (pRM19) (Fig. 4). Analysis of LPS profiles by SDS-PAGE gel demonstrated that in the *wzz* mutant (*wzz::Sm*^{R}/pACYC184), O-antigen of very short chain length was present and in the *wzz* complemented strain (*wzz::Sm*^{R}/pACYC184 + *wzz*), the wild-type pattern of O-antigen distribution was restored (Fig. 3). These data provide additional evidence that full length O-antigen is needed for swarming.

***waaL* and *wzz* mutants fail to activate the flagellar gene cascade.** The basis for the decreased swarming in *waaL* and *wzz* mutants was investigated. The most obvious explanation was that loss of surface O-antigen prevented swarming because it normally acted as a lubricant or increased "wettability" by extracting water from the agar. A similar role for O-antigen has been proposed in *S. typhimurium* (54). However, the addition of a surfactant, such as surfactin from *Bacillus subtilis* or purified LPS from wild-type *P. mirabilis* to the agar did not restore swarming to PM942 (data not shown). When *waaL* or *wzz* mutant cells were examined microscopically at the outside edge of growth, there were no swarmer cells observed (data not shown). To examine the basis for this lack of differentiation, we examined the expression of flagellin, encoded by the *flaA* gene, which is a hallmark of the differentiation process. Analysis of FlaA (flagellin) levels at various times during swarmer cell differentiation in the wild-type and *waaL* mutant (PM942) indicated the characteristic rise in flagellin expression beginning at 3 hours post inoculation on LB plates (T_3) in the wild-type strain was not observed in PM942 even after 6 hours of growth on agar plates (T_6) (Fig. 5A). However, liquid grown cells exhibited similar amounts of flagellin (Fig. 5A, T_0 sample).

To determine if the failure of PM942 to activate FlaA (flagellin) during swarmer cell differentiation was due to insufficient levels of the class 1 activator, $FlhD_2C_2$, Northern blots were used to examine *flhDC* mRNA accumulation in wild-type and PM942 cells at hourly time points representing various stages of swarmer cell differentiation. In PM942, there was no detectable activation of

flhDC expression at any point during swarmer cell differentiation (T_2-T_6) as seen in Fig. 5B.

The above data indicated that the *waaL* mutant was defective in activating the *flhDC* operon when grown on solid surfaces. To further investigate if this was a consequence of altered surface O-antigen, we examined the ability of the *wzz::Sm*^{R} mutant, exhibiting O-antigen of reduced chain length, to activate flagellin expression on solid surfaces. Like the *waaL* mutation, the *wzz* mutation also resulted in the failure to activate both *flhDC* and flagellin expression after growth on solid surfaces (Fig. 5C).

Restoring *flhDC* expression in the *waaL* mutant rescues swarming. To test whether the failure of the *waaL* mutant to activate *flhDC* on solid surfaces was primarily, if not exclusively, responsible for the inability of PM942 to swarm, *flhDC* was expressed from a constitutive promoter (*E. coli lac*$_p$) on the plasmid pFDCH1 (12). In PM942/pFDCH1, swarming motility was restored and was actually increased over wild-type (Fig. 6). This was likely due to the increased expression of FlhDC. The LPS profile of PM942/pFDCH1 was identical to PM942 indicating that overexpression of *flhDC* did not restore O-antigen synthesis (data not shown). Taken together, the above data indicated there was not an intrinsic inability of the *waaL* mutant to swarm, but that a signaling defect resulting from the *waaL* mutation prevented *flhDC* activation, which is critical for activation of the *flaA* flagellin gene and additional genes involved in differentiation.

Inactivation of the RcsB response regulator restores swarmer cell differentiation to the *waaL* mutant. The above data suggested that surface O-antigen is required to relay a signal that leads to *flhDC* activation. It was hypothesized this signal may be mediated by surface contact and involve the RcsBCD phosphorelay, since this system has previously been implicated in sensing solid surfaces and membrane stress. In addition, mutations in this pathway result in hyperswarming in *P. mirabilis* due, in part, to overexpression of *flhDC* (4, 13, 29). To test a possible role of RscB in relaying a signal from the O-antigen, a *waaL/rcsB* double mutant, designated RM7, was constructed and demonstrated that swarming was restored when *rcsB* was inactivated in PM942 *waaL*::mini-Tn*5lacZ1* (Fig. 6). The *waaL/rcsB* double mutant, like the *flhDC* overexpressing strain, did not restore O-antigen addition to LPS (data not shown).

Discussion

Previous studies have implicated a role for the outer surface of *P. mirabilis* in swarming, where both a slime layer and a capsular polysaccharide, designated colony migration factor (Cmf), are required (17, 20, 40, 51). In this study, an additional role for the cell surface in swarming was identified. A transposon insertion in a putative *waaL* ortholog (PMI3163), involved in both O-antigen and ECA synthesis, blocked the ability of *P. mirabilis* to swarm. Our study is not the first to report a role for *waaL* in swarming. Studies by Toguchi et al in *Salmonella enterica* Serovar Typhimurium demonstrated that *waaL* was required for

swarming motility (54) However, in contrast to *P. mirabilis,* the loss of *waaL* in *S. enterica* was not associated with a failure to upregulate flagellin expression during swarming (54).

The LPS profile of the *P. mirabilis waaL* mutant in Fig. 3 is consistent with loss of O-antigen ligase activity, with a concomitant loss of the O-antigen +1 band and the ladder of O-antigen repeats. Although the *P. mirabilis* WaaL protein shared limited sequence homology with other WaaL proteins, this is a common feature among WaaL proteins from different bacteria and even among WaaL proteins from different serotypes of the same species (1, 23, 37, 38, 49) For example, WaaL proteins between *E. coli* K-12 and R3 share limited sequence homology and those between different serotypes of *Vibrio cholerae* O1 and V194 share only 24% identity and are not capable of cross-complementing the respective mutations (23, 49)**.** The *P. mirabilis* WaaL protein displayed a high degree of similarity in Kyte-Doolittle hydropathy profiles to WaaL proteins from other gram-negative bacteria (Fig. 1).

Since the *waaL* mutation alters both O-antigen and ECA addition to the lipid A core, individual mutations were made in the *wzyE* and *wzz* genes, encoding ECA polymerase and an O-antigen chain length determinant, respectively (27, 39, 44). Swarming was abolished in the *wzz* mutant, where the O-antigen is synthesized in a truncated form and ECA is unaffected, but the *wzyE* mutant, defective in ECA synthesis, was still able to swarm. Therefore, O-antigen, but not ECA, is required for swarming. This contrasts to the proposed role of ECA in *Serratia marcescens*, where it was necessary for swarming and

flhDC activation and was suggested to act as a checkpoint for *flhDC* activation (9). An analogous role for O-antigen in *P. mirabilis* seems unlikely, since liquid grown at cultures at early stationary phase do not upregulate *flhDC*, but have complete O-antigen (data not shown).

In the *waaL* mutant, mRNA for *flhDC* and the flagellin protein (FlaA) failed to increase during growth on solid surfaces (Fig. 5). However, swarming in the *waaL* mutant was restored by artificially raising the levels of *flhDC* or by a loss of function mutation in the RcsB response regulator (Fig. 6) and neither condition restored O-antigen synthesis (data not shown). This indicated that the failure of *waaL* mutants to differentiate and swarm on solid surfaces was not due to an intrinsic structural or physical defect resulting from the absence of O-antigen, but may have resulted from the interruption of a surface signaling pathway that increased expression of the master regulator *flhDC.* This does not preclude a second role for O-antigen in facilitating movement by promoting wettatability in a manner similar to that proposed for O-antigen in *Salmonella enterica* serovar typhimurium (54).

At the present time, the mechanism by which a *waaL* mutation results in a failure to activate *flhDC* on solid surfaces is unclear, but several possibilities exist. In *E. coli,* mutations that truncate the inner core of LPS activate the Rcs system, resulting in greater repression of *flhDC* and inhibition of swimming and swarming (18). Based on this, the loss of O-antigen ligase activity in *P. mirabilis* may lead to cell envelope stress via the accumulation of unligated O-antigen intermediates in the periplasm. In turn, this may activate the RcsBCD system

resulting in greater repression of *flhDC.* However, this seems unlikely based on the following: (i) the levels of *flhDC*-dependent FlaA expression are similar in liquid grown wild-type or PM942 cells (T_O sample in Fig 5A), (ii) both strains swim at equal efficiency (Fig. 2) and (iii) a *wzz* mutant, which contains short O-antigen length without the accumulation of unligated O-antigen precursors in the periplasm, is also unable to activate *flhDC* and *flaA* on surfaces (Fig. 5C).

A second possibility is that O-antigen is acting as a sensor to monitor a solid surface. If O-antigen functions in surface sensing, it may require the RcsBCD phosophorelay, composed of the RcsC sensor kinase, RcsD (RsbA, YojN) and the RcsB response regulator, which has a role in surface sensing in other bacteria, such as *E. coli* (15). This model is also based on previous studies, where mutations in the *P. mirabilis rcsB, rcsC* or *rcsD(rsbA)* genes result in overexpression of *flhDC,* differentiation under non-permissive conditions (liquid), and hyper-swarming on agar surfaces (4, 13, 29). Upon contact of O-antigen with solid surfaces, it is hypothesized that a change in the outer membrane occurs that decreases the RcsC kinase and/or increases phosphatase activity resulting in lower levels of phosphorylated RcsB and de-repression of *flhDC.* A mediator of this signal between the outer membrane and RcsC may be an outer membrane protein such as RcsF and/or the putative inner membrane protein UmoB (IgaA), both of which regulate RcsC activity (8, 14, 18, 31).

This study adds to the complexity of how *P. mirabilis* senses a solid surface and regulates differentiation. One mechanism has been proposed by

Belas and Suvanasuthi, and involves a role for flagella in sensing solid surfaces (2, 5). In these studies, when *P. mirabilis* was grown in liquid culture, conditions that inhibited flagellar rotation such as the addition of anti-FlaA antibodies, or a thickening agent, resulted in the formation of swarmer cells (5). Although it is possible that the loss of O-antigen alters flagellar synthesis or function, this seems unlikely because the *waaL* mutant exhibited swimming motility that was similar to wild-type (Fig. 2). Therefore, flagellar inhibition should be relayed in a similar manner for both wild-type and *waaL* mutant strains upon placement on solid media. It also seems unlikely that the *waaL* mutation specifically decreases flagellar function on solid surfaces because then the *waaL* mutant would be predicted to differentiate more efficiently than wild-type, since inhibition of flagellar rotation is a signal for differentiation. Based on this information, is seems likely that two distinct sensing mechanisms operate in *P. mirabilis* to regulate the ability to swarm, one involving the inhibition of flagellar rotation and a second mechanism that requires O-antigen. We propose that the inhibition of flagellar rotation activates an undefined pathway that regulates genes required for swarmer cell elongation. In a separate pathway, O-antigen contact with solid surfaces activates the flagellar gene cascade needed for the copious amounts of flagella required for movement of a swarmer cell. In support of the two pathways for swarmer cell differentiation, unpublished studies from our lab have shown that a *P. mirabilis motA* mutant, unable to rotate its flagella, fails to differentiate but correctly activates *flhDC* expression upon contact with a solid surface. Future

work will investigate which genes are controlled by flagellar inhibition and which are controlled by the O-antigen mediated surface signaling pathway.

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References

1. **Abeyrathne, P. D., C. Daniels, K. K. Poon, M. J. Matewish, and J. S. Lam.** 2005. Functional characterization of WaaL, a ligase associated with linking O-antigen polysaccharide to the core of *Pseudomonas aeruginosa* lipopolysaccharide. J. Bacteriol. **187:**3002-3012.
2. **Alavi, M., and R. Belas.** 2001. Surface sensing, swarmer cell differentiation, and biofilm Development, p. 29-40, Methods in Enzymology, vol. Volume 336. Academic Press.
3. **Belas, R., M. Goldman, and K. Ashliman.** 1995. Genetic analysis of *Proteus mirabilis* mutants defective in swarmer cell elongation. J. Bacteriol. **177:**823-828.
4. **Belas, R., R. Schneider, and M. Melch.** 1998. Characterization of *Proteus mirabilis* Precocious swarming mutants: identification of *rsb*A, encoding a regulator of swarming behavior. J. Bacteriol. **180:**6126-6139.
5. **Belas, R., and R. Suvanasuthi.** 2005. The ability of *Proteus mirabilis* to sense surfaces and regulate virulence gene expression involves FliL, a flagellar basal body protein. J. Bacteriol. **187:**6789-6803.
6. **Bowden, M. G., and H. B. Kaplan.** 1998. The *Myxococcus xanthus* lipopolysaccharide O-antigen is required for social motility and multicellular development. Mol. Microbiol. **30:**275-284.
7. **Burall, L. S., J. M. Harro, X. Li, C. V. Lockatell, S. D. Himpsl, J. R. Hebel, D. E. Johnson, and H. L. Mobley.** 2004. *Proteus mirabilis* genes that contribute to pathogenesis of urinary tract infection: identification of 25

signature-tagged mutants attenuated at least 100-fold. Infect. Immun. **72:**2922-2938.

8. **Castanie-Cornet, M. P., K. Cam, and A. Jacq.** 2006. RcsF is an outer membrane lipoprotein involved in the RcsCDB phosphorelay signaling pathway in *Escherichia coli*. J. Bacteriol. **188:**4264-4270.

9. **Castelli, M. E., G. V. Fedrigo, A. L. Clementin, M. V. Ielmini, M. F. Feldman, and E. Garcia Vescovi.** 2008. Enterobacterial common antigen integrity is a checkpoint for flagellar biogenesis in *Serratia marcescens*. J. Bacteriol. **190:**213-220.

10. **Chang, A. C., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:**1141-1156.

11. **Chilcott, G. S., and K. T. Hughes.** 2000. Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica Serovar Typhimurium* and *Escherichia coli*. Microbiol. Mol. Biol. Rev. **64:**694-708.

12. **Clemmer, K. M., and P. N. Rather.** 2008. The Lon protease regulates swarming motility and virulence gene expression in *Proteus mirabilis*. J. Med. Microbiol. **57:**931-937.

13. **Clemmer, K. M., and P. N. Rather.** 2007. Regulation of *flhDC* expression in *Proteus mirabilis*. Res. Microbiol. 158**:**295-302.

14. **Dufour, A., R. B. Furness, and C. Hughes.** 1998. Novel genes that upregulate the *Proteus mirabilis flhDC* master operon controlling flagellar biogenesis and swarming. Mol. Microbiol. **29:**741-751.

15. **Ferrières, L., and D. J. Clarke.** 2003. The RcsC sensor kinase is required for normal biofilm formation in *Escherichia coli* K-12 and controls the expression of a regulon in response to growth on a solid surface. Mol. Microbiol. **50:**1665-1682.

16. **Furness, R. B., G. M. Fraser, N. A. Hay, and C. Hughes.** 1997. Negative feedback from a *Proteus* class II flagellum export defect to the *flhDC* master operon controlling cell division and flagellum assembly. J. Bacteriol. **179:**5585-5588.

17. **Fuscoe, F. J.** 1973. The role of extracellular slime secretion in the swarming of *Proteus*. Medical Lab. Tech. **30:**373-382.

18. **Girgis, H. S., Y. Liu, W. S. Ryu, and S. Tavazoie.** 2007. A comprehensive genetic characterization of bacterial motility. PLoS Genet **3:**1644-1660.

19. **Gue, M., V. Dupont, A. Dufour, and O. Sire.** 2001. Bacterial swarming: a biochemical time-resolved FTIR-ATR study of *Proteus mirabilis* swarm-cell differentiation. Biochemistry **40:**11938-11945.

20. **Gygi, D., M. M. Rahman, H. C. Lai, R. Carlson, J. Guard-Petter, and C. Hughes.** 1995. A cell-surface polysaccharide that facilitates rapid population migration by differentiated swarm cells of *Proteus mirabilis*. Mol. Microbiol. **17:**1167-1175.

21. **Harshey, R. M.** 2003. Bacterial motility on a surface: many ways to a common goal. Ann. Rev. Microbiol. **57:**249-273.

22. **Hay, N. A., D. J. Tipper, D. Gygi, and C. Hughes.** 1997. A nonswarming mutant of *Proteus mirabilis* lacks the Lrp global transcriptional regulator. J. Bacteriol. **179:**4741-4746.

23. **Heinrichs, D. E., M. A. Monteiro, M. B. Perry, and C. Whitfield.** 1998. The assembly system for the lipopolysaccharide R2 Core-type of *Escherichia coli* is a hybrid of those found in *Escherichia coli* K-12 and *Salmonella enterica:* Structure and function of the R2 WaaK and WaaL homologs. J. Biol. Chem. **273:**8849-8859.

24. **Jones, B. V., R. Young, E. Mahenthiralingam, and D. J. Stickler.** 2004. Ultrastructure of *Proteus mirabilis* swarmer cell rafts and role of swarming in catheter-associated urinary tract infection. Infect. Immun. **72:**3941-3950.

25. **Kaniga, K., I. Delor, and G. R. Cornelis.** 1991. A wide-host-range suicide vector for improving reverse genetics in gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. Gene **109:**137-141.

26. **Kittelberger, R., and F. Hilbink.** 1993. Sensitive silver-staining detection of bacterial lipopolysaccharides in polyacrylamide gels. Journal of Biochemical and Biophysical Methods **26:**81-86.

27. **Kuhn, H. M., U. Meier-Dieter, and H. Mayer.** 1988. ECA, the Enterobacterial common antigen. FEMS Microbiology Review **4:**195-222.

28. **Laubacher, M. E., and S. E. Ades.** 2008. The Rcs phosphorelay is a cell envelope stress response activated by peptidoglycan stress and contributes to intrinsic antibiotic resistance. J. Bacteriol. **190:**2065-2074.

29. **Liaw, S.-J., H. C. Lai, S. W. Ho, K. T. Luh, and W. B. Wang.** 2001. Characterisation of p-nitrophenylglycerol-resistant *Proteus mirabilis* super-swarming Mutants. J. Med. Microbiol. **50:**1039-1048.

30. **Majdalani, N., and S. Gottesman.** 2007. Genetic dissection of signaling through the Rcs phosphorelay. Methods in Enzymology **423:**349-362.

31. **Majdalani, N., M. Heck, V. Stout, and S. Gottesman.** 2005. Role of RcsF in signaling to the Rcs phosphorelay pathway in *Escherichia coli*. J. Bacteriol. **187:**6770-6778.

32. **Manoil, C., and J. Beckwith.** 1985. TnphoA: a transposon probe for protein export signals. Proceedings of the National Academy of Sciences of the United States of America **82:**8129-8133.

33. **Marolda, C. L., P. Lahiry, E. Vines, S. Saldias, and M. A. Valvano.** 2006. Micromethods for characterization of lipid A-core and O-antigen lipopolysaccharide. Methods in Molecular Biology: Glycobiology Protocols **347:**237-252.

34. **McGrath, B. C., and M. J. Osborn.** 1991. Localization of the terminal steps of O-antigen synthesis in *Salmonella typhimurium*. J. Bacteriol. **173:**649-654.

35. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. J. Bacteriol. **170:**2575-2583.

36. **Pearson, M. M., M. Sebaihia, C. Churcher, M. A. Quail, A. S. Seshasayee, N. M. Luscombe, Z. Abdellah, C. Arrosmith, B. Atkin, T. Chillingworth, H. Hauser, K. Jagels, S. Moule, K. Mungall, H. Norbertczak, E. Rabbinowitsch, D. Walker, S. Whithead, N. R. Thomson, P. N. Rather, J. Parkhill, and H. L. Mobley.** 2008. Complete Genome Sequence of Uropathogenic *Proteus mirabilis*, a Master of both Adherence and Motility. J. Bacteriol. **190:**4027-4037.

37. **Pérez, J. M., M. A. McGarry, C. L. Marolda, and M. A. Valvano.** 2008. Functional analysis of the large periplasmic loop of the *Escherichia coli* K-12 WaaL O-antigen ligase. Molecular Microbiology **70:**1424-1440.

38. **Raetz, C. R., C. M. Reynolds, M. S. Trent, and R. E. Bishop.** 2007. Lipid A modification systems in gram-negative bacteria. Annual Review of Biochemistry **76:**295-329.

39. **Raetz, C. R., and C. Whitfield.** 2002. Lipopolysaccharide endotoxins. Annual Review of Biochemistry **71:**635-700.

40. **Rahman, M. M., J. Guard-Petter, K. Asokan, C. Hughes, and R. W. Carlson.** 1999. The Structure of the colony migration factor from pathogenic *Proteus mirabilis*. a capsular polysaccharide that facilitates swarming. J. Biol. Chem. **274:**22993-22998.

41. **Rather, P. N.** 2005. Swarmer cell differentiation in *Proteus mirabilis*. Environmental Microbiology **7:**1065-1073.

42. **Rauprich, O., M. Matsushita, C. J. Weijer, F. Siegert, S. E. Esipov, and J. A. Shapiro.** 1996. Periodic phenomena in *Proteus mirabilis* swarm colony development. J. Bacteriol. **178:**6525-6538.

43. **Reeves, P. P., and L. Wang.** 2002. Genomic organization of LPS-specific loci. Current Topics in Microbiology and Immunology **264:**109-135.

44. **Reeves, P. R., M. Hobbs, M. A. Valvano, M. Skurnik, C. Whitfield, D. Coplin, N. Kido, J. Klena, D. Maskell, C. R. Raetz, and P. D. Rick.** 1996. Bacterial polysaccharide synthesis and gene nomenclature. Trends in Microbiology **4:**495-503.

45. **Rick, P. D., H. Mayer, B. A. Neumeyer, S. Wolski, and D. Bitter-Suermann.** 1985. Biosynthesis of enterobacterial common antigen. J. Bacteriol. **162:**494-503.

46. **Rinno, J., J. Gmeiner, J. R. Golecki, and H. Mayer.** 1980. Localization of enterobacterial common antigen: *Proteus mirabilis* and its various L-forms. J. Bacteriol. **141:**822-827.

47. **Rinno, J., J. R. Golecki, and H. Mayer.** 1980. Localization of enterobacterial common antigen: immunogenic and nonimmunogenic enterobacterial common antigen-containing *Escherichia coli*. J. Bacteriol. **141:**814-821.

48. **Rozalski, A., L. Brade, P. Kosma, B. J. Appelmelk, C. Krogmann, and H. Brade.** 1989. Epitope specificities of murine monoclonal and rabbit polyclonal antibodies against enterobacterial lipopolysaccharides of the Re chemotype. Infect. Immun. **57:**2645-2652.

49. **Schild, S., A.-K. Lamprecht, and J. Reidl.** 2005. Molecular and functional characterization of O-antigen transfer in *Vibrio cholerae*. J. Biol. Chem. **280:**25936-25947.

50. **Senior, B. W.** 1983. *Proteus morgani* is less frequently associated with urinary tract infections than *Proteus mirabilis*--an explanation. J. Med. Microbiol. **16:**317-22.

51. **Stahl, S. J., K. R. Stewart, and F. D. Williams.** 1983. Extracellular slime associated with *Proteus mirabilis* during swarming. J. Bacteriol. **154:**930-937.

52. **Stevenson, L. G., and P. N. Rather.** 2006. A novel gene involved in regulating the flagellar gene cascade in *Proteus mirabilis*. J. Bacteriol. **188:**7830-7839.

53. **Sturgill, G., and P. N. Rather.** 2004. Evidence that putrescine acts as an extracellular signal required for swarming in *Proteus mirabilis*. Mol. Microbiol. **51:**437-446.

54. **Toguchi, A., M. Siano, M. Burkart, and R. M. Harshey.** 2000. Genetics of swarming motility in *Salmonella enterica Serovar Typhimurium*: critical role for lipopolysaccharide. J. Bacteriol. **182:**6308-6321.

55. **VanderMolen, G. E., and F. D. Williams.** 1977. Observation of the swarming of *Proteus mirabilis* with scanning electron microscopy. Canadian Journal of Microbiology **23:**107-112.

Figure legends.

Figure 1. Kyte-Doolittle hydropathy profiles of WaaL proteins. The Kyte-Doolittle hydropathy profile of the PMI3163 open reading from *P. mirabilis* was compared to WaaL proteins from other gram-negative bacteria. The hydropathy profiles were generated using the Biology Workbench program (http://seqtool.sdsc.edu/CGI/BW.cgi).

Figure 2. WaaL is necessary for swarming, but not for swimming. The swimming (left panel) and swarming (right panel) phenotypes of the following strains are shown; PM7002 wild-type/pACYC184, PM942 *waaL*::mini-Tn*5lacZ1*/pACYC184 and PM942 *waaL*::mini-Tn*5lacZ1*/pRM5 (pACYC184 + *waaL*). For both swarming and swimming assays, strains were grown overnight in the appropriate antibiotics, followed by adjusting each culture to the same optical density and 5µL drops were placed on each plate. Swimming assays were done in LB with 0.3% agar and swarming was done on LB plates with 1.5% agar that were pre-dried for 30 minutes at 37^{o}C. Plates were photographed after 8 hours incubation at 37^{o}C.

Figure 3. SDS-PAGE analysis of O-antigen production. O-antigen length was determined by SDS-PAGE analysis of LPS preparations followed by silver staining. The designated strains are WT: PM7002/pACYC184, *waaL*$^{-}$: PM942 *waaL*::mini-Tn*5lacZ1*/pACYC184, *waaL*$^{-}$/*waaL*$^{+}$: PM942 *waaL*::mini-

Tn*5lacZ1*/pACYC184 + *waaL*, *wzz*$^{-}$: RM16 *wzz::Sm*^{R}/pACYC184 vector only, and *wzz*$^{-}$*/wzz*$^{+}$: RM16 *wzz::Sm*^{R}/pACYC184 + *wzz*.

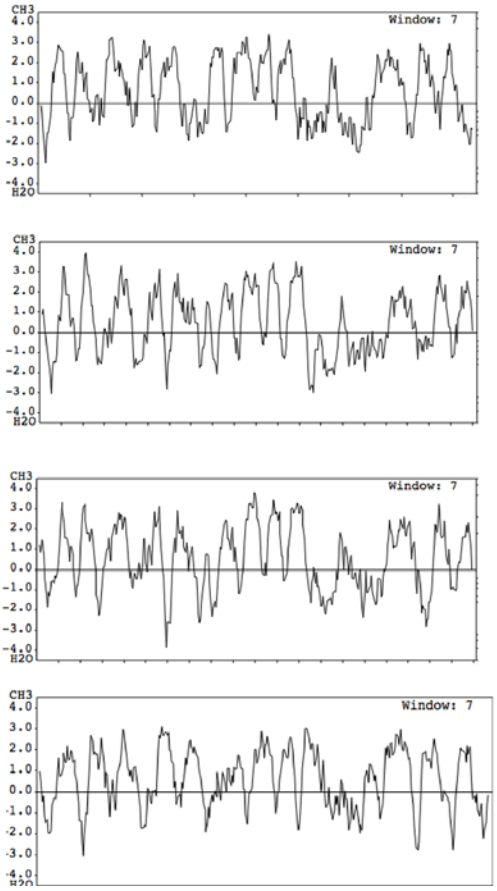
Figure 4. A *wzz* mutation prevents swarming. The swarming phenotypes of the indicated strains were determined by growing cells up overnight in LB broth with the appropriate antibiotics and adjusting cultures to the same optical density. For the swarming assays, three individual 5µL drops for each strain were placed on the same LB plate and migration distances were measured at 30 minute intervals. The starting diameter of each spot was 5 mm. The average of three measurements is shown. PM7002 wild-type, RM16 *wzz::Sm*^{R}/pACYC184 vector and RM16 *wzz::Sm*^{R}/pRM19 (pACYC184 + *wzz*).

Figure 5. Analysis of FlaA and *flhDC* expression in wild-type and *waaL* mutant strains. Cells were grown up overnight to an O.D.$_{600}$ of 1.8 and 200µL of the culture was spread onto separate 2% agar plates for hourly collection up to six hours. The overnight culture was diluted to an O.D.$_{600}$ of 1.0 for the T_0 sample. Cells were collected off plates as described previously (19) and duplicate samples were lysed in Laemmli buffer or used for RNA isolation. The amounts of protein and RNA were standardized and equal amounts were loaded for each sample. (A) Western blot analysis of FlaA protein levels. The T_4-T_6 samples were run on a separate gel and processed at the same time as the T_0-T_3 samples (B) Northern blot analysis of *flhDC* mRNA accumulation of RNA collected from the same cell sample as in panel A. Ethidium bromide staining of

the RNA used in Panel B is shown. Panel C: Northern blot analysis of *flhDC* mRNA accumulation and Western blot analysis of FlaA protein expression in cells harvested at T_4 during swarmer cell differentiation. The same cell pellets from each strain were split and used for each analysis.

Figure 6. Suppression of the swarming defect in a *waaL* mutant. In panel A, the swarming phenotype of wild-type PM7002/pACYC184, PM942 *waaL*::mini-Tn*5lacZ1*/pACYC184, PM942 *waaL*::mini-Tn*5lacZ1*/pFDCH1, and RM7 (*waaL*::mini-Tn*5lacZ1, rcsB::Sm*^{R}) and is shown on a 1.5% agar plates after growth at 37^{o}C for 8 hours. In panel B, the swarming distance of the strains used in panel A is shown as a function of time.

Figure 1



P. mirabilis HI4320

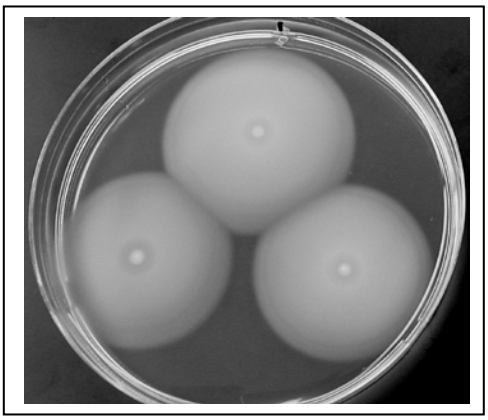
S. enterica serovar typhi

E. coli O157

P. aeruginosa PAO1

Figure 2

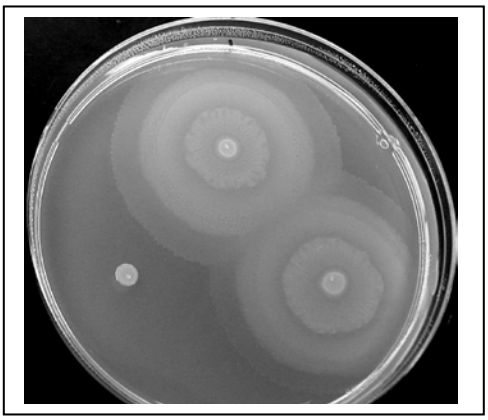
PM7002/pACYC184



waaL::mini-Tn*5* pACYC184

waaL::mini-Tn*5* pRM5 (184 + *waaL*)

PM7002/pACYC184



waaL::mini-Tn*5* pACYC184

waaL::mini-Tn*5* pRM5 (184 + *waaL*)

Figure 3

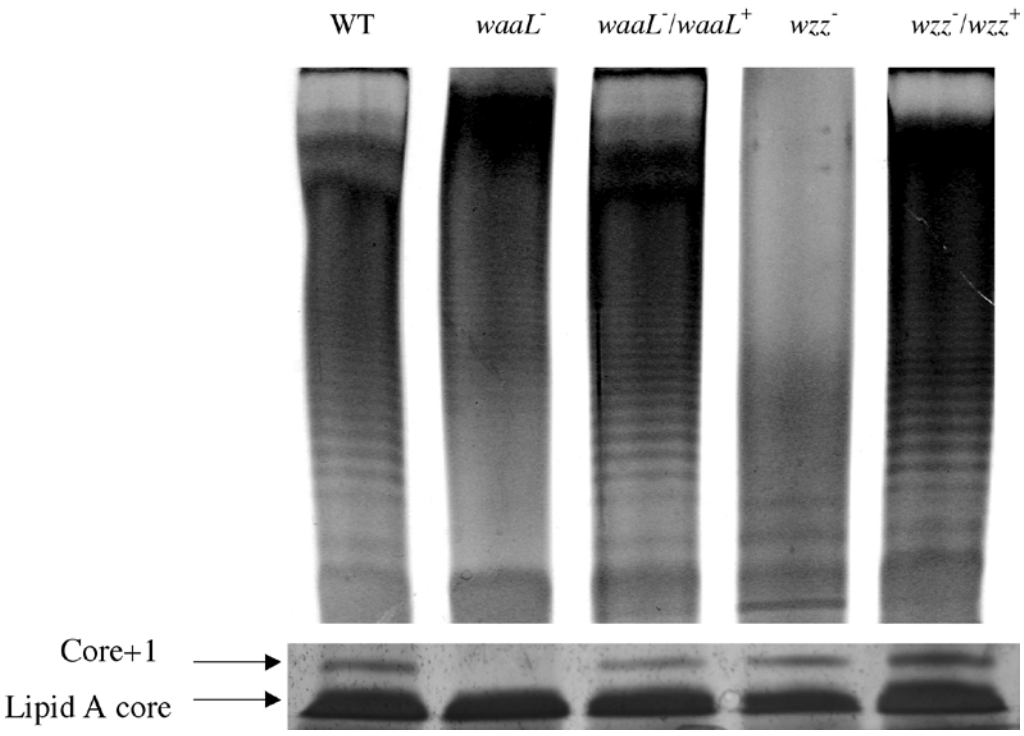


Figure 4

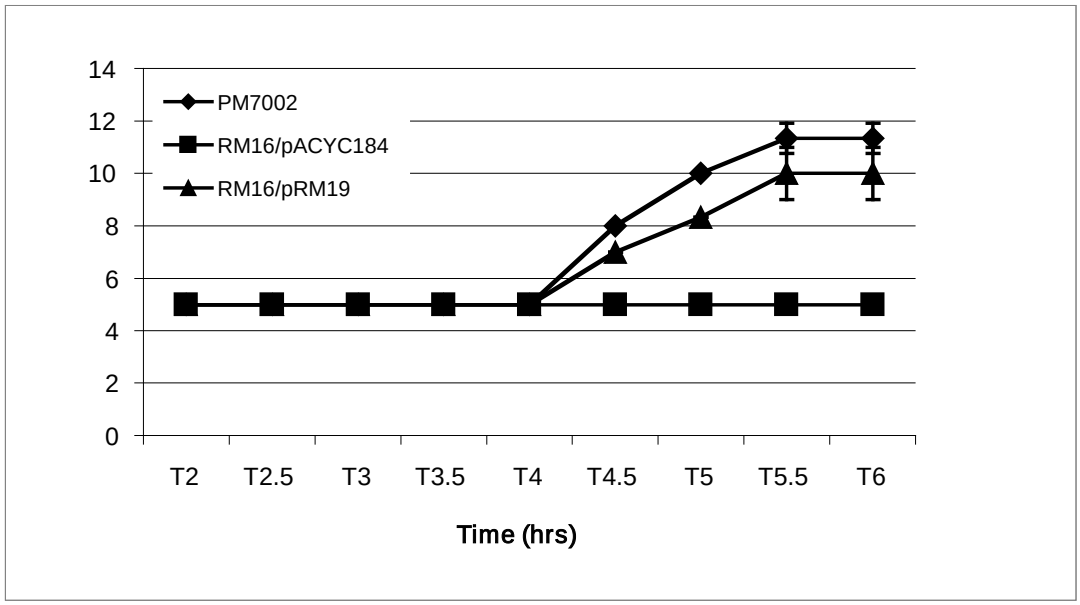

14
12
10
8
6
4
2
0
PM7002
RM16/pACYC184
RM16/pRM19
T2
T2.5
T3
T3.5
T4
T4.5
T5
T5.5
T6
Time (hrs)

Figure 5

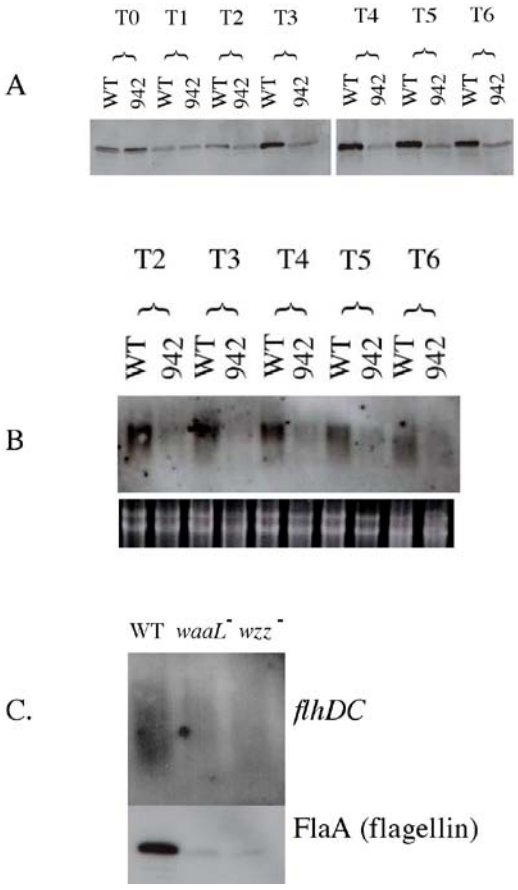
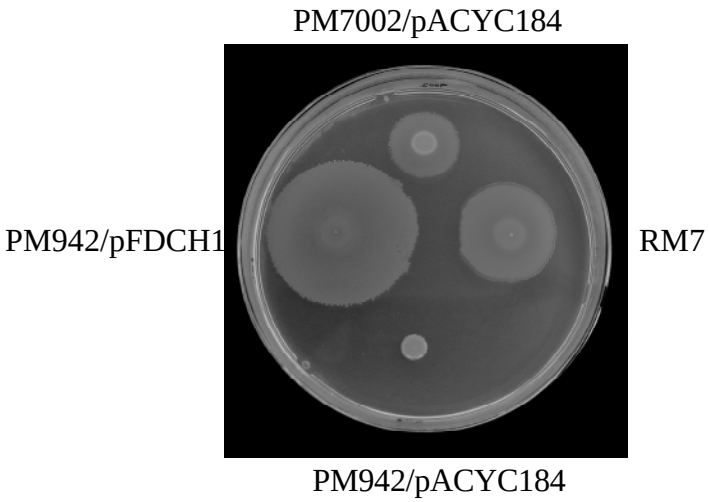

A
T0 T1 T2 T3 T4 T5 T6
WT 942 WT 942 WT 942 WT 942 WT 942 WT 942 WT 942
B
T2 T3 T4 T5 T6
WT 942 WT 942 WT 942 WT 942 WT 942
C.
WT waaL- wzz-
flhDC
FlaA (flagellin)

Figure 6

A.


PM7002/pACYC184
PM942/pFDCH1
RM7
PM942/pACYC184

B.

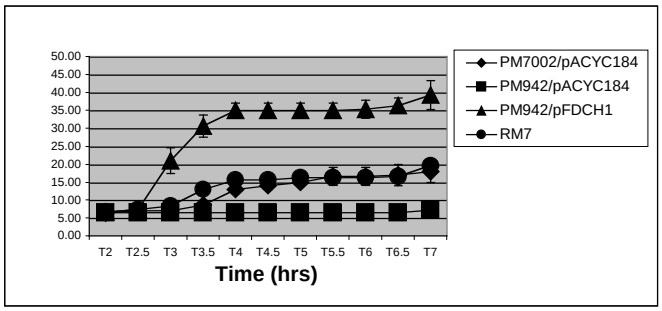

50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
T2
T2.5
T3
T3.5
T4
T4.5
T5
T5.5
T6
T6.5
T7
Time (hrs)
PM7002/pACYC184
PM942/pACYC184
PM942/pFDCH1
RM7

Table 1: Strains and Plasmids

Strain or plasmid	Description/genotype	Source or Reference
E. coli strains		
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
XL1	*endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44* F'f *hsdR17(*r_K^- m_K^+*)*	Laboratory stock
CC118 λ*pir*	*araD139* Δ *(ara leu)7697* Δ*lacZ74 phoA*Δ*20 galE galK thi rpsE rpoB argE(Am) recA1*	(32)
SM10 λ*pir*	*thi thr leu tonA supE recA* RP4-2Tc::Mu Km^r λ*pir*	(35)
P. mirabilis strains		
PM7002	Wild type; Tc^r	ATCC
PM942	*waaL::*mini-Tn5*lacZ1*-Kan^r	This study
RM5	PM942/pACYC184	This study
	PM942/pFDCH1	This study

RM6	PM942 *rcsB::Sm*^{R}	This study
RM7	PM942/pACYC184 + *waaL*	This study
RM9	PM7002 *wzyE::Sm*^{R}	This study
RM14	PM7002 *wzz::Sm*^{R}	This study
RM16		
Plasmids		
pACYC184	Low copy Cm^R	(10)
pBC	High copy Cm^R	Stratagene
pKNG101	R6K-derived suicide vector; Sm^R	(25)
pRM5	pACYC184 +*waaL*	This study
pRM14	pKNG101::*wzy* (internal fragment)	This study
pRM17	pKNG101::*wzz* (internal fragment)	This study
pFDCH1	pACYC184 + *flhDC*	(12)
pRM18	pKNG101::*rcsB* (internal fragment) in CC118	(13)
pRM19	pACYC184 + *wzz*	This study

Table 2: Primers

Primer name	**Primer Sequence**	**Purpose**
942.for	ATCGAGGATCCTTATTGTATGATGAGCCATTC	Complementation
942.rev	ATGACATGTCGACTTAGCTAACGGATGTATCTTC	Complementation
intWzyE.for	ATGACATGTCGACAGCCTCTAGCGAGCCTTCTAGG	Deletion mutant
intWzyE.rev	ACAGTCTAGAACGACATCTGGTCTTTGTGGC	Deletion mutant
intWzz.for	ATGACTAGTCGACATGTTGATACTGGCGTAAATG	Deletion mutant
intWzz.rev	ACAGTCTAGATTCAGAACTGACCGTTGTAGG	Deletion mutant
Wzz.for	AATACTGATATCACGATTATCGGATTAGG	Complementation
Wzz.rev	ATCGTGGATCCACCTACTTTTATTTGTGG	Complementation
RcsB.for	GTACAGTCGACTCACCGACCTATCTATGCCT	Deletion mutant
RcsB.rev	GTACAGTCGACTCACCGACCTATCTATGCCT	Deletion mutant

Chapter 4: Genetic Dissection of the Rcs Signaling Pathway and its Role in Swarming Motility in *Proteus mirabilis*

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This manuscript was written by R.M. with editorial changes made by P.R.

Abstract

Proteus mirabilis is a Gram-negative bacterium that exists as a short rod when grown in liquid media, but during growth on surfaces undergoes a distinct physical and biochemical change that culminates in the formation of a swarmer cell. How *P. mirabilis* senses a surface is not fully understood; however, the inhibition of flagella rotation and accumulation of putrescine have been proposed to be sensory mechanisms. Our lab isolated a transposon insertion in *waaL*, encoding O-antigen ligase (PM942*waaL::km*^{r}), which results in loss of swarming, but not swimming motility. The swarming defect in the *waaL* mutant results from a failure to activate *flhDC,* the class 1 activator of the flagellar gene cascade, when grown on solid surfaces, and was restored by overexpression of *flhDC* in trans or by making a mutation in the response regulator *rcsB*. Mutations were made in *rcsC*, *rcsB*, *rcsF*, *umoB* (*igaA*), and *umoD* in wild-type and *waaL* backgrounds to test the hypothesis that O-antigen was needed for surface sensing by acting through the Rcs phosphorelay. Comparison of the swarming phenotypes of the single and double mutants, as well as overexpression strains, showed that there is a differential effect of RcsF and UmoB on swarming in wild-type and *waaL* backgrounds and we show that RcsF inhibits UmoB activity, but not UmoD activity in a wild-type background. The data demonstrates that along with RcsF, UmoD is another input acting on the Rcs system, and that this second input is activated by O-antigen contact with solid surfaces.

Introduction

Proteus mirabilis is a Gram-negative bacillus that exhibits a cooperative form of motility termed swarming. In liquid culture, *P. mirabilis* exists as peritrichously flagellated swimmer cells that are only a few microns in length. Once placed on solid surfaces, the cells undergo physical and biochemical changes to form swarmer cells. One change is the up-regulation of *flhDC*, the flagellar master regulator, resulting in swarmer cells that are elongated (20-50 times longer than swimmer cells) and hyper-flagellated (50-100 fold more flagella), while being multi-nucleated and aseptate (reviewed in (24, 39, 41)). These elongated cells align parallel to each other, entangling their flagella to from a swarming raft (26). As a group, this raft radiates out from the central inoculum to form a ring of swarming. When an unknown signal is sensed, the cells consolidate, or de-differentiate, back into swimmer cells. This process repeats to form a characteristic bull's eye pattern on an agar plate (42). How bacteria in general, and *Proteus* more specifically, recognize they are on a surface and change their gene expression profile accordingly is just beginning to be understood. One hypothesis is that inhibition of flagella rotation along with putrescine accumulation are signals leading to the up-regulation of *flhDC* and other swarming associated genes in *P. mirabilis* (1, 44). More recently, Morgenstein et al. proposed a role for the O-antigen in sensing surfaces (38). They hypothesize O-antigen is needed to sense solid surfaces and to relay this signal to the cell through the Rcs two-component system.

Two-component regulatory systems (TCS) are one of the most common ways bacteria control gene expression in response to external signals. The canonical TCS consists of an inner membrane bound sensor kinase, which dimerizes and autophosphorylates itself on a specific histidine, and a cytoplasmic response regulator, which receives this phosphate. The phosphate group from the sensor kinase is passed to an aspartate on the response regulator, activating it, thus allowing gene expression to be controlled through direct binding of the promoter region of the controlled genes (although not all response regulators control gene expression (22)) (43). While TCS are important in bacterial physiology, their prevalence in individual bacteria may vary, from 0 TCS in *Mycoplasma genitalium*, to 80 in *Synechocystis* sp. (36, 37). *P. mirabilis* is predicted to have 16 TCS, although only the Rcs and Rpp systems have been directly shown to influence motility (2, 11, 29, 40, 48).

The Rcs TCS has been well studied in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (10, 12, 15, 17, 18, 23, 28, 30, 45, 47). The Rcs system is more complicated than the canonical TCS; along with the response regulator (RcsB) and sensor kinase (RcsC), it also uses an outer membrane activator protein (RcsF) and a phosphotransfer protein (RcsD) (6, 20, 30, 32). A stimulus can be sensed through one of two pathways depending on the origin of the stimulus. If the signal originates externally, it can go through the outer membrane and RcsF, which relays the signal to RcsC. However, if the signal originates in the periplasm or cytoplasmic membrane it proceeds directly to RcsC, which upon autophosphorylation of its own His and Asp residues transfers

the phosphate to a His residue on RcsD. In turn, RcsD then transfers the phosphate to the Asp on RcsB (28, 31, 32). The phosphorylated RcsB can bind DNA and act as either a repressor or an activator (31). More recently, another input, UmoB (IgaA), has been implicated in controlling the Rcs system (4, 5, 13, 14, 19, 33, 34, 46). The Rcs system has been shown to respond to various stresses, such as those caused by perturbations in the cell envelope and peptidoglycan, or by osmotic stress (28, 49).

The Rcs TCS is important for motility in a variety of organisms. As a repressor of *flhDC* the Rcs system controls motility at the level of flagella synthesis. During swarming the Rcs system is presumably de-activated in order to allow for the copious amounts of flagella seen during swarming. In support of this idea, mutations in the Rcs pathway lead to a hyper-swarming phenotype in *P. mirabilis* (2, 11, 29, 48). Work in both *P. mirabilis* and *Serratia marcescens* have indicated that outer-membrane structures are required for swarming (7, 8, 38). Recently the Rcs system has been shown to be regulated by Enterobacterial Common Antigen in *S. marcescens* and suggested to be regulated by O-antigen sensing in *P. mirabilis* (8, 38).

UmoB was discovered along with three other genes (UmoA, UmoC, UmoD) in a search for suppressors of a swarming defect in a *flgN* mutant using an overexpression library. It was shown that suppression by all four loci was due to up-regulation of *flhDC*, and loss of function mutations in these genes caused a decrease in *flhDC* expression as well as a concomitant lack of swarming to varying degrees (14). UmoB and UmoD exhibited the most severe phenotypes

in terms of swarming and *flhDC* regulation (14). A few years later, while looking for *Salmonella* mutants that could grow in fibroblast cells, Cano et al. discovered an UmoB homolog, which also regulates *flhDC* expression, that they termed IgaA (4, 5). A major difference between the proteins in *Salmonella* and *Proteus* is that a loss of function of IgaA is lethal, while loss of UmoB function is not (4). The lethality of a true IgaA loss of function mutant has necessitated the use of leaky alleles such as *igaA1*, that retain some activity, and has enabled suppressor mutations to be found that all map to the Rcs system (4, 5, 34).

The direct role IgaA has on the Rcs system in not known, however there is evidence that suggests a possible role involving direct interaction with a protein in the Rcs system. Using the *igaA1* mutant and tagged Rcs components, an *igaA1* mutation was shown to not have an effect on Rcs protein levels, indicating a post-translational role for IgaA function (13). The *igaA1* mutation, in conjunction with an *rcsB* mutation, showed an RcsB activated promoter was more active in the *igaA1* background than wild-type. As presumed an *igaA1rcsB* double mutant has no increase in activity, indicating that the Rcs system is activated in the absence of IgaA. However, an *rcsB* mutant has less expression than wild-type, indicating IgaA might allow a basal level of activity of the Rcs TCS (13). To further link IgaA and the Rcs system, microarray data were used to link gene expression in *igaA1, igaA1rcsB* and *rcsB* mutants, during high osmolarity, a condition that activates the Rcs system (33, 49). The authors also looked at the expression of an Rcs controlled virulence factor *spvA* in the different backgrounds and saw a decrease of expression in the *igaA1* strain and the *rcsB*

mutant. This lead to a hypothesis that *spvA* is both positively and negatively regulated by RcsB and IgaA is needed to control the levels of phosphorylated RcsB by either acting on RcsC or RcsD (the inner membrane components) (33). Where IgaA actually enters the Rcs system is still not known.

Our previous study indicated a role for O-antigen in surface sensing and a role for the Rcs TCS in relaying the surface signal, to transcriptional regulation (38). Here, we genetically dissect the Rcs system and show that signaling upon surface contact is different in wild-type and O-antigen minus cells. We show that like IgaA, UmoB works through the Rcs system, and that O-antigen is needed for this. We also propose a role for UmoD in this signaling pathway through interactions with UmoB.

Materials and Methods

Strains and media. For cloning purposes *E. coli* strain XL1 was used. For conjugal matings *E. coli* strain SM10λpir (35) was used as the donor strain and either PM7002 (wild-type) or PM942 (waaL::miniTn5) *P. mirabilis* strains were used as the recipients. *E. coli* and *P. mirabilis* were both grown in modified Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) shaking at 37^0C, or on LB plates kept at 37^0C. Swarm assays were performed on 1.5% agar plates with appropriate antibiotics. Antibiotics were used for selection at concentrations of 25 μg/mL for both chloramphenicol and streptomycin, 20μg/mL of kanamycin, and 100μg/mL for ampicillin for *E. coli*. Antibiotic concentrations for the selection of *P. mirabilis* were 100μg/mL for chloramphenicol, 35μg/mL for streptomycin, 20μg/mL for kanamycin, 15μg/mL for tetracycline, and 300μg/mL for ampicillin.

Cloning. *rcsF* and *umoD* were cloned into pACYC184 (9) and pBC (Stratagene) and expressed from the *tet* and *lac* promoters respectively using *Bam*HI and *Sal*I sites added to the genes through PCR. Each gene has its own ribosome-binding site. *umoB* and *umoD* were cloned into the multi-cloning site of pTrc99A and expressed from the *lac* promoter using *Bam*HI and *Sal*I sites added to the genes through PCR. IPTG was not added for expression of these genes expressed from pTrc99A, as the promoter was leaky and produced a hyper-swarming phenotype. When both plasmids were expressed in a cell at the same time,

double antibiotic selection with chloramphenical and ampicillin at the concentrations listed above was used. See table 1 for a list of primers used.

Construction of mutations. For allelic replacement *rcsF* and *umoB* were cloned into pBC and digested with *Bgl*II and *Hind*III respectively. The kanamycin kixx cassette was ligated into the gene at either the *Bgl*II or *Hind*III site. These constructs were sub-cloned into pKNG101 (27) and maintained in *E. coli* SM10λpir . The strains were mated with either PM7002 or PM942*waaL::km*^{r} and selected on tetracyline and streptomycin to select for a Campbell type insertion. The transconjugants were then grown without selection, to cause a double crossover event, which results in excision of the vector and either restoration of the wild-type allele or leaving only the mutated allele at the wild-type locus. Serial dilutions were plated on LB or LB strep^{35} plates to select for any colonies that retained the vector. Kanamycin was used to select for the colonies that have lost the vector and have the mutated allele. Allelic replacement was confirmed by Southern blot (see below). See table 1 for primers used.

Campbell insertions were used to make mutations in *rcsB* and *rcsC.* An internal gene fragment was cloned into pKNG101 using restriction ends made by PCR at either the *Xba*I/*BamH*I or *BamH*I/*Sal*I sites (see table 1 for primers used). The pKNG101 clones were electroporated into *E. coli* SM10λpir, and then mated with either PM7002 or PM942*waaL::km*^{r}. Exconjugants representing Campbell-type integration events that disrupted each gene were selected on LB plates with tetracycline and streptomycin and mutations were confirmed by Southern blot.

Southern blot analysis. To confirm the matings resulted in the appropriate gene disruptions, chromosomal DNA from the *rcsB*, *rcsC*, *rscF*, and *umoB* mutants were extracted, and separately digested with *Eco*RI, *Sal*I, *Hind*III, and *Eco*RI before being transferred to a nitrocellulose membrane, and probed with a gene specific digoxigenin labeled probe.

Swarm assays. To examine the swarming phenotype of the strains used in this study, the strains were grown overnight in LB media with appropriate antibiotics at 37^0C shaking. Fresh LB was used to equilibrate the $O.D._{600}$ of the cultures. 2μL drops of each culture were spotted on an LB plate in triplicate. Measurements of the swarming diameter were taken every 30 minutes starting at 2.5 hours post inoculation.

Northern blot analysis. Cells were grown overnight in LB. All samples were normalized to the same $O.D._{600}$ with fresh LB. 150μL drops were spread onto 2% LB plates in parallel to produce cultures that were synchronously differentiating. The cells were collected from each plate 4 hours after inoculation with LB media and spun for 1 minute at 12,000rpm. Total RNA was isolated using the Masterpure RNA purification kit (Epicentre, Madison WI). Equal amounts of RNA were run on a 1.2% formaldehyde agarose gel, and transferred to a nitrocellulose membrane. A DNA probe specific to each gene was labeled with digoxigenin and used to examine transcript levels by chemiluminescence using the CDP-Star substrate (Roche Applied Science).

Results

Differential effects of *rcs* mutations in wild-type and *waaL* backgrounds. *Proteus* strain PM942*waaL::km*^{r} has a mutation in *waaL*, resulting in a lack of O-antigen and swarming due to mis-regulation of *flhDC* upon contact with solid surfaces, which could be compensated for by a mutation in *rcsB* (38). If O-antigen signals to the Rcs system it was though that the outer membrane associated protein RcsF would be involved in relaying a signal through the Rcs TCS. To test this hypothesis, allelic replacement was performed on wild-type and PM942*waaL::km*^{r} cells to create a null allele of *rcsF::km*^{r}. The swarming phenotype of the *rcsF::km*^{r} mutant was compared to *rcsC::sm*^{r} and *rcsB::sm*^{r} loss of function mutations. As seen in Fig. 1, the *rcsF::km*^{r} mutation has no effect on swarming in wild-type cells; however there was a increase in swarming when *rcsF::km*^{r} was present in the *waaL* mutant background. These results suggest that RcsF is not needed in wild-type cells for swarming, and there is another input into the Rcs system that is O-antigen dependent. In wild-type cells, this O-antigen dependent pathway is active, thereby masking the effects of an *rcsF* mutation. However, in PM942*waaL::km*^{r}, the lack of O-antigen keeps this pathway deactivated allowing the loss of *rcsF* to partially suppress the swarming defect caused by the lack of O-antigen.

UmoB and UmoD effect swarming to varying degrees. The Rcs system has been shown to repress the *flhDC* operon *(11)*. The UmoA-D proteins have been shown to increase *flhDC* expression when expressed in high copy (14). One of

these genes, *umoB,* has a known homolog in *S. enterica*, *igaA,* which is known to play a role in the regulation of the Rcs system (13, 14, 33, 46). While the function of UmoD is not known, it has been shown that when both UmoB and UmoD are mutated, loss of swarming occurs because of a block in *flhDC* activation. UmoB is able to suppress the swarming defect in a *umoD* mutant, but not vice versa, indicating there is potential for these proteins to be acting in the same pathway [(14) and data not shown]. Because of their roles in *flhDC* expression and possible connection to the Rcs system UmoB and UmoD were further explored for their roles in swarming and Rcs activation.

UmoB and UmoD were overexpressed in wild-type and PM942*waaL::km*^{r} cells to assess their ability to effect swarming. As previously reported, UmoB and UmoD overexpression leads to hyper-swarming in wild-type cells (Fig. 2) (14). The level of hyper-swarming was compared to an *rcsB::sm*^{r} strain and Fig. 2A shows cells overexpressing UmoD swarm to a comparable level to cells with the *rcsB::sm*^{r} mutation. Wild-type cells overexpressing UmoB exhibited enhanced swarming, but not to the degree conferred by the overexpression of UmoD. Interestingly, in PM942*waaL::km*^{r}, UmoB overexpression had little effect on swarming (Fig. 2B). However, UmoD overexpression was able to restore swarming in PM942*waaL::km*^{r} to a much greater degree, though still not to the levels of the *waaL::km*^{r}*;rcsB::sm*^{r} double mutant. It appears from the data that both UmoB and UmoD activities are decreased in PM942*waaL::km*^{r}.

UmoB works through the Rcs TCS. In order to genetically determine if UmoB is an input into the Rcs TCS, the swarming phenotype of an *umoB::km*^{r} strain made by allelic replacement, was compared to the phenotype of an *umoB::km*^{r}*;rcsB::sm*^{r} double mutant. The *umoB::km*^{r} mutant does not swarm (Fig 3A), while a *rcsB::sm*^{r} mutant hyper-swarms (Fig 1A and 3B) (2, 11, 14). As seen in Fig. 3A the swarming phenotype of the *umoB::km*^{r}*;rcsB::sm*^{r} double mutant is the same as an *rcsB::sm*^{r} single mutant suggesting they act in the same pathway. Also, an UmoB overexpressing strain was compared to an *rcsB::sm*^{r} strain overexpressing UmoB. If the two proteins work in the same pathway there should not be an additive effect on swarming. When a *rcsB::sm*^{r} mutant overexpresses UmoB it does not exhibit an additive effect on swarming (Fig. 3B). These data show that UmoB does indeed work through the Rcs system as its homolog IgaA does in *S. enterica* (4, 33, 46).

Next it was determined if UmoB worked through the same mechanism in PM942*waaL::km*^{r} to ascertain if and how O-antigen was needed for UmoB activity. In Fig. 3C it can be seen that as in wild-type cells, UmoB works through the Rcs system in PM942*waaL::km*^{r}. These data suggest that the UmoB present in PM942*waaL::km*^{r} works similarly to the UmoB in wild-type cells, and there must be something disrupting its functional state, stopping it from restoring swarming in PM942*waaL::km*^{r}.

RcsF overexpression counters the effect of UmoB overexpression, but not UmoD. The Rcs system contains a predicted outer membrane associated

protein RcsF that is needed for signaling to RcsC when the signal originates outside the periplasm (20, 23, 30, 32). It is not known which protein RcsF interacts with, or even if it directly interacts with a known Rcs component.

To test if RcsF activates the Rcs system through UmoB, both RcsF and UmoB were expressed in wild-type cells on medium copy number plasmids. UmoB was expressed from pTrc99A from the *lac* promoter, however IPTG was not added. RcsF was expressed on the compatible pACYC184 from the constitutively active *tet* promoter. Both genes have their own ribosome binding sites. When RcsF is overexpressed by itself, there is only a slight negative effect on swarming, in stark contrast to overexpression of UmoB, which leads to a hyper-swarming phenotype (Fig 4A). However, when both genes are overexpressed together, RcsF inhibits the hyper-swarming phenotype caused by UmoB overexpression, restoring swarming to wild-type levels (Fig. 4A). There was no appreciable defect in growth in any of the strains used that would account for the swarming effects (data not shown). This suggests that either, RcsF is interacting with UmoB and blocking its activity, or RcsF is interacting downstream of UmoB to block UmoB function. It is also possible that RcsF is acting upstream on an activator of UmoB.

Previous data suggests that UmoB acts downstream of UmoD (14). If RcsF interacts with a downstream partner of UmoB, or with its upstream partner, UmoD, one would expect the overexpression of RcsF and UmoD to not hyper-swarm. This was not the case as wild-type cells overexpressing both UmoD and RcsF still hyper-swarm (Fig. 4B). *umoD* was expressed from pTrc99A without

the addition of IPTG, and there were no growth defects (data not shown). These data indicate that RcsF does not interact with UmoD.

To further elucidate the role of RcsF and its interplay with UmoB, both proteins were overexpressed in PM942*waaL::km*^{r}. Because PM942*waaL::km*^{r} does not swarm, any repressive effect of RcsF overexpression was not evident. When RcsF is overexpressed at the same time as UmoB or UmoD in PM942*waaL::km*^{r}, RcsF inhibits swarming in both cases (Fig. 4CD). When overexpressed at the same time as UmoD, RcsF also inhibits swarming in PM942*waaL::km*^{r}, in contrast to wild-type cells where there was no effect. These data suggest that either UmoD, RcsF, or both exist in different states in wild-type and PM942*waaL::km*^{r} cells. In wild-type, during swarming UmoD is in an active state that can inhibit the repressive effects that RcsF has on UmoB activity, or RcsF function is turned off in an O-antigen dependent manner. Conversely, in PM942*waaL::km*^{r}, UmoD is not activated and exists primarily in the off state. Because UmoD is not active in PM942*waaL::km*^{r}, overexpression of RcsF can block the swarming caused by UmoD overexpression, as opposed to wild-type cells where the activated UmoD appears to be dominant to RcsF activity.

RcsF is not needed for UmoB or UmoD function. The above data demonstrate that RscF is constitutively active on solid surfaces and inhibiting the activity of UmoB in PM942*waaL::km*^{r}. In order to see if RcsF is needed for UmoB or UmoD activity, either gene was overexpressed in wild-type and PM942*waaL::km*^{r} strains lacking *rcsF*. As stated above, in wild-type cells, UmoD

is active and dominant to RcsF activity, therefore, it stands to reason an *rcsF* mutation would not have an effect on swarming in UmoB or UmoD overexpressing strains. As seen in Fig. 5A-B, overexpression of UmoB or UmoD in an *rcsF* mutant has no additional effect. However, in PM942*waaL::km*^{r}, where the UmoD is not active, an *rcsF* mutation does have a considerable effect on swarming in both the UmoB and UmoD overexpressing strains (Fig. 5C-D). This is likely because in PM942*waaL::km*^{r} RcsF activity is constitutively inhibiting UmoB. Removing the repressive effects of RcsF on UmoB frees UmoB to effect Rcs phosphorylation.

***rcsF, umoB,* and *umoD* transcription is unchanged in PM942*waaL::km*^{r}.** Northern blots were performed on total cellular RNA from wild-type cells and PM942*waaL::km*^{r} cells collected four hours post surface contact to identify if the differential effects on swarming were due to differences in transcription of *rcsF, umoB* or *umoD*. Previous authors had been unable to see *umoB* transcripts by Northern blot indicating there might be little transcription of this gene (14). We were clearly able to see transcripts of all genes looked at. There were no differences in mRNA levels of any of the genes checked between wild-type and PM942*waaL::km*^{r} (Fig. 6C). To ensure cells were differentiating properly *flhDC* levels were examined (Fig. 6D). As previously reported, wild-type *flhDC* levels reach a max around T4, but PM942*waaL::km*^{r} *flhDC* levels do not rise (38). These data indicate any differences in swarming seen between wild-type and PM942*waaL::km*^{r} are not due to transcription of *rcsF, umoB* or *umoD*.

UmoB and UmoD work together in PM942*waaL::km^r*. The above data indicate UmoB and UmoD work together. To test this hypothesis further, *umoB* and *umoD* were overexpressed from pTrc99A and pACYC184 respectively in PM942*waaL::km^r*. UmoD overexpression leads to the same level of swarming in both pTRC99A and pACYC184 (data not shown). The hypothesis tested is that UmoD interacts with UmoB and in PM942*waaL::km^r* this interaction does not happen because UmoD is not activated. This will hold if the overexpression of both proteins increases swarming over overexpression of the individual proteins (Fig. 2B). This datum shows overexpression of both proteins does increase swarming (Fig. 7), most likely because UmoD, even inactive, has a greater chance of interacting with an UmoB protein when there are more of both proteins around. Once this interaction occurs, the UmoB is activated and can repress the Rcs system allowing *flhDC* to be expressed.

Discussion

How does a bacterial cell sense its environment? This is a crucial question in microbiology. *Proteus mirabilis* is a useful model for studying the way bacteria sense solid surfaces because of a lifestyle change that occurs on solid surfaces compared to liquid cultures. Upon contact with solid surfaces, *P. mirabilis* begins to differentiate into swarmer cells. To initiate swarming it was first purposed that inhibition of flagella rotation on solid surfaces was sensed by the cells and caused a change in gene expression (1, 3). More recently, work has been done showing a requirement for O-antigen in surface sensing (38). In this study, we genetically manipulate the Rcs system in both wild-type and O-antigen minus cells (PM942*waaL::km*^{r}) in order to establish a link between the two. We show that RcsF is not the only input into the Rcs TCS in *P. mirabilis* but that there are two new inputs. First, we showed UmoB (IgaA) works through the Rcs system (Fig. 3) and secondly, UmoD is an input into the Rcs system through possible interactions with UmoB (Fig. 4).

A detailed breakdown of the Rcs TCS in *P. mirabilis* was needed because only a few of the components have been looked at and only *rcsB* has been examined in an O-antigen minus strain (2, 11, 29, 38). As previously determined, *rcsB* and *rcsC* mutations in wild-type cells result in a hyper-swarming phenotype. It was hypothesized that RcsF would play a large role in signal transduction. However, an *rcsF* mutation had no effect on swarming, indicating the possible existence of other inputs (Fig. 1A). In PM942*waaL::km*^{r}, both the *rcsB* and *rcsC* mutations restore swarming near wild-type levels; however the *rcsF* mutation

only has a small effect on swarming (Fig. 1B). This indicates that any other input is likely to be regulated in an O-antigen dependent manner, masking a phenotype of the *rcsF* mutant in wild-type cells because of a functional O-antigen. This result is similar to *E. coli* where LPS mutants that were deficient in swarming could have this phenotype suppressed by an RcsF mutation (21). In *P. mirabilis* an *rcsF* mutation does not result in full suppression of the swarming defect seen in cells lacking an O-antigen.

The lack of a major phenotype of the *rcsF* mutant suggested there were other inputs. A candidate for this input was UmoB because IgaA (a homolog) has been shown in *S. enterica* to work through the Rcs TCS (13, 33, 34, 46). Using genetic approaches Fig. 3 shows that UmoB does indeed work through the Rcs TCS in both wild-type and *waaL* cells, indicating there is no functional difference in how the system works in these two strains, only a difference in activation of the system. The original work characterizing UmoB, as does our unpublished results, show evidence that UmoD might be in the same pathway as UmoB (14). UmoD was therefore examined along with UmoB in future experiments.

In wild-type cells, a *rcsF* mutation has no effect, however in PM942*waaL::km*^{r}, there is a small effect when *rcsF* is mutated (Fig. 1). To further tease out a role for RcsF in swarming, it was overexpressed in strains, with either a wild-type or O-antigen minus background, overexpressing either UmoB or UmoD. In both backgrounds, overexpression of RcsF was able to inhibit the hyper-swarming caused by UmoB overexpression (Fig. 4A,C).

However, unlike with UmoB, the concomitant overexpression of RcsF and UmoD only had an effect in PM942*waaL::km*^{r} (Fig. 4B,D). These data show the activity of UmoD is different in wild-type and PM942*waaL::km*^{r} cells. It appears that UmoD is activated in an O-antigen dependent manner to be dominant over the activity RcsF has on UmoB.

We also show that RcsF is not needed for signaling in wild-type cells and is in fact inhibited on surfaces. *rcsF* can be mutated without having an effect on swarming in either wild-type or wild-type overexpressing UmoB or UmoD cells (Fig. 1A, 5AB). In PM942*waaL::km*^{r} there is an effect of mutating *rcsF* in all cases. Swarming is increased when UmoB or UmoD are overexpressed indicating that unlike in wild-type cells, RcsF activity in PM942*waaL::km*^{r} cannot be turned off (Fig. 5C-D). By removing the inhibitory effect of RcsF the swarming effect of UmoB or UmoD overexpression can more easily be seen.

It is possible that there are transcriptional differences between *rcsF*, *umoB*, or *umoD* in wild-type or PM942*waaL::km*^{r} cells that accounts for the observed swarming phenotypes. Northern blots were performed to measure mRNA levels during swarming (Fig. 6). There were no differences in mRNA level in either strain indicating a post-transcriptional mechanism for the observed swarming differences.

If UmoD functions as we suggest, overexpression of UmoD and UmoB in PM942*waaL::km*^{r} would cause a swarming phenotype greater than either gene by itself. This is because the increased amounts of UmoD would have a greater chance of interacting with UmoB if there are more of both around. An increase in

swarming was seen when both proteins were over expressed at the same time (Fig, 7). The model also suggests that a mutation in *umoD* would behave in a manner similar to that seen in PM942*waaL::km*^{r}. While an *umoD* mutant does display a similar swarming phenotype to PM942*waaL::km*^{r}, this phenotype is suppressed to a higher level when UmoB is overexpressed. This difference appears to be due to an increase in FlaA levels. Why the *umoD* mutant has FlaA levels increased to a greater degree than PM942*waaL::km*^{r} when UmoB is overexpressed is not known. We suggest in PM942*waaL::km*^{r} UmoD is locked in a dominant form, inhibiting UmoB and swarming. It is possible that UmoD has an unknown role in its "inactive state", allowing for a different phenotype when UmoD is removed completely versus having UmoD inactive as in PM942*waaL::km*^{r}. There is also a possible role for O-antigen in lubricating the swarming surface as well as signaling to RcsF. This wetting effect could allow for an increase of swarming in the *umoD* mutant that is now seen in PM942*waaL::km*^{r}. In confirmation that *umoD::km*^{r} acts like PM942*waaL::km*^{r}, a *umoD::km*^{r}*;rcsB::sm*^{r} double mutant was made. This strain is able to hyper-swarm similar to an *rcsB* mutation in wild-type cells, confirming a role for UmoD in the Rcs system during swarming.

Using the above data, we have formulated a model for how *P. mirabilis* senses surfaces using O-antigen and how Rcs signaling is used to control *flhDC* expression (Fig. 8). As cells begin to swarm (T_4), O-antigen signals to UmoD to switch activities. This newly activated UmoD activates UmoB. UmoB inhibits the Rcs system, most likely through RcsC or RcsD, which relieves the repression of

flhDC, allowing for the activation of the flagellar gene cascade. O-antigen interactions with the surface also signal to turn off RcsF function. RcsF appears to work in an opposite role of UmoD to inhibit UmoB function.

A recent study by Farris et al. proposed a model for Rcs activation by antimicrobial peptides (16). They suggest insertion of the peptide in the outer membrane pinches the membrane bringing RcsF into contact with an inner membrane protein. It is possible that surface contacts cause a similar membrane pinch in an O-antigen dependant manner, which allows UmoD and UmoB to interact and stops RcsF . UmoB is predicted to have a large periplasmic loop we propose is the site for RcsF and UmoD interactions (13). Future experiments will examine the physical interactions between these proteins and the role of O-antigen in regulating their activity.

In other organisms outer membrane structures have been tied to the Rcs system as well. In *E. coli*, mutations in LPS, like in *P. mirabilis*, disrupt swarming motility (21, 25). These mutations can be suppressed through mutations in the Rcs pathway. Unlike in *P. mirabilis, rcsF* mutations are able to fully restore swarming in *E. coli* (21). In *S. marcescens*, the completion of intact enterobacterial common antigen (ECA) has been suggested to be a checkpoint for flagellar synthesis (7). Recently, it was shown that ECA signals through the Rcs pathway to signal for flagellar synthesis. Interestingly, mutations in Rcs genes do not effect motility (either swimming or swarming) unless there is also an ECA mutation (8). This is in contrast to *P. mirabilis* where mutations in the Rcs system lead to hyper-motility during both swimming and swarming (2, 11, 29). It

is difficult to imagine how Rcs mutations would only have an effect in ECA mutant cells but not wild-type cells. Perhaps the mechanism for Rcs activation is different in *S. marcescens* and *P. mirabilis*.

References

1. **Alavi, M., and R. Belas.** 2001. Surface Sensing, Swarmer Cell Differentiation, and Biofilm Development, p. 29-40, Methods in Enzymology, vol. Volume 336. Academic Press.
2. **Belas, R., R. Schneider, and M. Melch.** 1998. Characterization of *Proteus mirabilis* Precocious Swarming Mutants: Identification of *rsb*A, Encoding a Regulator of Swarming Behavior. Journal of Bacteriology **180:**6126-6139.
3. **Belas, R., and R. Suvanasuthi.** 2005. The Ability of *Proteus mirabilis* to Sense Surfaces and Regulate Virulence Gene Expression Involves FliL, a Flagellar Basal Body Protein. Journal of Bacteriology **187:**6789-6803.
4. **Cano, D. A., G. Dominguez-Bernal, A. Tierrez, F. G.-D. Portillo, and J. Casadesus.** 2002. Regulation of Capsule Synthesis and Cell Motility in *Salmonella enterica* by the Essential Gene *igaA*. Genetics **162:**1513-1523.
5. **Cano, D. A., M. Martinez-Moya, M. G. Pucciarelli, E. A. Groisman, J. Casadesus, and F. Garcia-Del Portillo.** 2001. *Salmonella enterica Serovar Typhimurium* Response Involved in Attenuation of Pathogen Intracellular Proliferation. Infect. Immun. **69:**6463-6474.
6. **Castanie-Cornet, M.-P., K. Cam, and A. Jacq.** 2006. RcsF Is an Outer Membrane Lipoprotein Involved in the RcsCDB Phosphorelay Signaling Pathway in *Escherichia coli*. J. Bacteriol. **188:**4264-4270.

7. **Castelli, M. E., G. V. Fedrigo, A. L. Clementin, M. V. Ielmini, M. F. Feldman, and E. G. Vescovi.** 2008. Enterobacterial Common Antigen Integrity is a Checkpoint for Flagellar Biogenesis in *Serratia marcescens*. Journal Bacteriology **190:**213-220.

8. **Castelli, M. E., and E. G. Vescovi.** 2010. The Rcs Signal Transduction Pathway Is Triggered by Enterobacterial Common Antigen Structure Alterations in Serratia marcescens. Journal Bacteriology **193:**63-74.

9. **Chang, A. C., and S. N. Cohen.** 1978. Construction and Characterization of Amplifiable Multicopy DNA Cloning Vehicles Derived from the P15A Cryptic Miniplasmid. J. Bacteriol. **134:**1141-1156.

10. **Clarke, D. J., S. A. Joyce, C. M. Toutain, A. Jacq, and I. B. Holland.** 2002. Genetic Analysis of the RcsC Sensor Kinase from *Escherichia coli* K-12. Journal of Bacteriology. **184:**1204-1208.

11. **Clemmer, K. M., and P. N. Rather.** 2007. Regulation of *flh*DC Expression in *Proteus mirabilis*. Research in Microbiology**:**295-302.

12. **Delgado, M. A., C. Mouslim, and E. A. Groisman.** 2006. The PmrA/PmrB and RcsC/YojN/RcsB Systems Control Expression of the *Salmonella* O-antigen Chain Length Determinant. Molecular Microbiology **60:**39-50.

13. **Dominguez-Bernal, G., M. G. Pucciarelli, F. Ramos-Morales, M. Garcìa-Quintanilla, D. A. Cano, J. Casades˙s, and F. G.-d. Portillo.** 2004. Repression of the RcsC-YojN-RcsB Phosphorelay by the IgaA

Protein is a Requisite for *Salmonella* Virulence. Molecular Microbiology **53:**1437-1449.

14. **Dufour, A., R. B. Furness, and C. Hughes.** 1998. Novel Genes that Upregulate the *Proteus mirabilis flhDC* Master Operon Controlling Flagellar Biogenesis and Swarming. Molecular Microbiology **29:**741-751.
15. **Fabrice Carballes, C. B., Jean-Pierre Bouche, Kaymeuang Cam.** 1999. Regulation of *Escherichia coli* cell division genes *ftsA* and *ftsZ* by the two-component system *rcsC-rcsB*. Molecular Microbiology **34:**442-450.
16. **Farris, C., S. Sanowar, M. W. Bader, R. Pfuetzner, and S. I. Miller.** 2010. Antimicrobial Peptides Activate the Rcs Regulon through the Outer Membrane Lipoprotein RcsF. Journal Bacteriology **192:**4894-4903.
17. **Ferrières, L., and D. J. Clarke.** 2003. The RcsC Sensor Kinase is Required for Normal Biofilm Formation in *Escherichia coli* K-12 and Controls the Expression of a Regulon in Response to Growth on a Solid Surface. Molecular Microbiology **50:**1665-1682.
18. **Francez-Charlot, A., B. Laugel, A. V. Gemert, N. Dubarry, F. Wiorowski, M.-P. Castanié-Cornet, C. Gutierrez, and K. Cam.** 2003. RcsCDB His-Asp Phosphorelay System Negatively Regulates the *flhDC* Operon in *Escherichia coli*. Molecular Microbiology **49:**823-832.
19. **Garcia-Calderon, C. B., J. Casadesus, and F. Ramos-Morales.** 2009. Regulation of *igaA* and the Rcs System by the MviA Response Regulator in *Salmonella enterica*. J. Bacteriol. **191:**2743-2752.

20. **Gervais, F. G., and G. R. Drapeau.** 1992. Identification, Cloning, and Characterization of *rcsF*, a New Regulator Gene for Exopolysaccharide Synthesis that Suppresses the Division Mutation *ftsZ84* in *Escherichia coli* K-12. J. Bacteriol. **174:**8016-8022.

21. **Girgis, H. S., Y. Liu, W. S. Ryu, and S. Tavazoie.** 2007. A Comprehensive Genetic Characterization of Bacterial Motility. PLoS Genet **3:**1644-1660.

22. **Grebe, T. W., and J. B. Stock.** 1999. The Histidine Protein Kinase Superfamily. Advances in Microbial Physiology **41:**139-227.

23. **Hagiwara, D., M. Sugiura, T. Oshima, H. Mori, H. Aiba, T. Yamashino, and T. Mizuno.** 2003. Genome-Wide Analyses Revealing a Signaling Network of the RcsC-YojN-RcsB Phosphorelay System in *Escherichia coli*. Journal Bacteriology **185:**5735-5746.

24. **Harshey, R. M.** 2003. Bacterial Motilitiy on a Surface: Many Ways to a Common Goal. Annual Review of Microbiology **57:**249-273.

25. **Inoue, T., R. Shingaki, S. Hirose, K. Waki, H. Mori, and K. Fukui.** 2007. Genome-Wide Screening of Genes Required for Swarming Motility in *Escherichia coli* K-12. Journal Bacteriology **189:**950-957.

26. **Jones, B. V., R. Young, E. Mahenthiralingam, and D. J. Stickler.** 2004. Ultrastructure of *Proteus mirabilis* Swarmer Cell Rafts and Role of Swarming in Catheter-Associated Urinary Tract Infection. Infect. Immun. **72:**3941-3950.

27. **Kaniga, K., I. Delor, and G. R. Cornelis.** 1991. A Wide-Host-Range Suicide Vector for Improving Reverse Genetics in Gram-negative Bacteria: Inactivation of the *blaA* Gene of *Yersinia enterocolitica*. Gene **109:**137-141.

28. **Laubacher, M. E., and S. E. Ades.** 2008. The Rcs Phosphorelay Is a Cell Envelope Stress Response Activated by Peptidoglycan Stress and Contributes to Intrinsic Antibiotic Resistance. J. Bacteriol. **190:**2065-2074.

29. **Liaw, S.-J., H. C. Lai, S. W. Ho, K. T. Luh, and W. B. Wang.** 2001. Characterisation of p-nitrophenylglycerol-resistant *Proteus mirabilis* Super-Swarming Mutants. Journal of Medical Microbiology. **50:**1039-1048.

30. **Majdalani, N., and S. Gottesman.** 2007. Genetic Dissection of Signaling Through the Rcs Phosphorelay. Methods in Enzymology **423:**349-362.

31. **Majdalani, N., and S. Gottesman.** 2005. The Rcs Phosphorelay: A Complex Signal Transduction System. Annual Review of Microbiology **59:**379-405.

32. **Majdalani, N., M. Heck, V. Stout, and S. Gottesman.** 2005. Role of RcsF in Signaling to the Rcs Phosphorelay Pathway in *Escherichia coli*. Journal Bacteriology **187:**6770-6778.

33. **Mariscotti, J. F., and F. Garcia-del Portillo.** 2009. Genome Expression Analyses Revealing the Modulation of the *Salmonella* Rcs Regulon by the Attenuator IgaA. J. Bacteriol. **191:**1855-1867.

34. **Mariscotti, J. F., and F. Garcia-del Portillo.** 2008. Instability of the *Salmonella* RcsCDB Signalling System in the Absence of the Attenuator IgaA. Microbiology **154:**1372-1383.

35. **Miller, V. L., and J. J. Mekalanos.** 1988. A Novel Suicide Vector and its use in Construction of Insertion Mutations: Osmoregulation of Outer Membrane Proteins and Virulence Determinants in *Vibrio cholerae* Requires *toxR*. J. Bacteriol. **170:**2575-2583.

36. **Mizuno, T.** 1998. His-Asp Phosphotransfer Signal Transduction. Journal of Biochemistry **123:**555-563.

37. **Mizuno, T., T. Kaneko, and S. Tabata.** 1996. Compilation of all Genes Encoding Bacterial Two-Component Signal Transducers in the Genome of the Cyanobacterium, *Synechocystis* sp. Strain PCC 6803. DNA Res **3:**407-414.

38. **Morgenstein, R. M., K. M. Clemmer, and P. N. Rather.** 2010. Loss of the WaaL O-Antigen Ligase Prevents Surface Activation of the Flagellar Gene Cascade in *Proteus mirabilis*. Journal of Bacteriology **192:**3213-3221.

39. **Morgenstein, R. M., B. Szostek, and P. N. Rather.** Regulation of Gene Expression During Swarmer Cell Differentiation in *Proteus mirabilis*. FEMS Microbiology Reviews **E. pub**.

40. **Pearson, M. M., M. Sebaihia, C. Churcher, M. A. Quail, A. S. Seshasayee, N. M. Luscombe, Z. Abdellah, C. Arrosmith, B. Atkin, T. Chillingworth, H. Hauser, K. Jagels, S. Moule, K. Mungall, H. Norbertczak, E. Rabbinowitsch, D. Walker, S. Whithead, N. R.**

Thomson, P. N. Rather, J. Parkhill, and H. L. Mobley. 2008. Complete Genome Sequence of Uropathogenic *Proteus mirabilis*, a Master of both Adherence and Motility. J. Bacteriol. **190:**4027-4037.

41. **Rather, P. N.** 2005. Swarmer Cell Differentiation in *Proteus mirabilis*. Environmental Microbiology **7:**1065-1073.

42. **Rauprich, O., M. Matsushita, C. J. Weijer, F. Siegert, S. E. Esipov, and J. A. Shapiro.** 1996. Periodic Phenomena in *Proteus mirabilis* Swarm Colony Development. J. Bacteriol. **178:**6525-6538.

43. **Stock, A. M., V. L. Robinson, and P. N. Goudreau.** 2000. Two-Component Signal Transduction. Annual Review of Biochemistry **69:**183-215.

44. **Sturgill, G., and P. N. Rather.** 2004. Evidence that Putrescine Acts as an Extracellular Signal Required for Swarming in *Proteus mirabilis*. Molecular Microbiology **51:**437-446.

45. **Takeda, S.-i., Y. Fujisawa, M. Matsubara, H. Aiba, and T. Mizuno.** 2001. A Novel Feature of the Multistep Phosphorelay in *Escherichia coli*: A Revised Model of the RcsC;YojN;RcsB Signalling Pathway Implicated in Capsular Synthesis and Swarming Behaviour. Molecular Microbiology **40:**440-450.

46. **Tierrez, A., and F. Garcia-del Portillo.** 2004. The *Salmonella* Membrane Protein IgaA Modulates the Activity of the RcsC-YojN-RcsB and PhoP-PhoQ Regulons. J. Bacteriol. **186:**7481-7489.

47. **Wang, Q., Y. Zhao, M. McClelland, and R. M. Harshey.** 2007. The RcsCDB Signaling System and Swarming Motility in *Salmonella enterica Serovar Typhimurium*: Dual Regulation of Flagellar and SPI-2 Virulence Genes. Journal Bacteriology **189:**8447-8457.

48. **Wang, W.-B., I.-C. Chen, S.-S. Jiang, H.-R. Chen, C.-Y. Hsu, P.-R. Hsueh, W.-B. Hsu, and S.-J. Liaw.** 2008. Role of RppA in the Regulation of Polymyxin B Susceptibility, Swarming, and Virulence Factor Expression in *Proteus mirabilis*. Infectection and Immununity **76:**2051-2062.

49. **Zhou, L., X.-H. Lei, B. R. Bochner, and B. L. Wanner.** 2003. Phenotype MicroArray Analysis of *Escherichia coli* K-12 Mutants with Deletions of All Two-Component Systems. J. Bacteriol. **185:**4956-4972.

Figure legends.

Figure 1. Differential effects of Rcs mutations on swarming in wild-type and PM942*waaL::km*^{r} cells. The swarming phenotypes of the indicated strains were determined by growing cells up overnight in LB broth with the appropriate antibiotics and adjusting cultures to the same optical density with fresh LB. Panel A shows the swarming phenotype of wild-type *Proteus* and the corresponding Rcs mutations. Panel B shows PM942*waaL::km*^{r} and the corresponding Rcs mutations. Three individual 2µL drops for each strain were placed on the same LB plate and swarming distances were measured at 30 minute intervals. The starting diameter of each spot was 4 mm. The average of the three measurements is shown.

Figure 2. UmoB and UmoD effect swarming to varying degrees. The swarming phenotypes of the indicated strains were determined by growing cells up overnight in LB broth with the appropriate antibiotics and adjusting cultures to the same optical density with fresh LB. Panel A shows the swarming phenotype of *umoB* and *umoD* mutants in wild-type *Proteus* in comparison to an *rcsB* mutant. Panel B shows cells with *umoB* and *umoD* mutations in PM942*waaL::km*^{r} in comparison to a *waaL;rcsB* double mutation. Three individual 2µL drops for each strain were placed on the same LB plate and swarming distances were measured at 30 minute intervals. The starting diameter of each spot was 4 mm. The average of the three measurements is shown.

Figure 3. UmoB is an input into the Rcs TCS. The swarming phenotypes of the indicated strains were determined by growing cells up overnight in LB broth with the appropriate antibiotics and adjusting cultures to the same optical density with fresh LB. Panel A shows the swarming phenotype of wild-type *Proteus* compared to an *umoB* mutant with or without an *rcsB* mutation and an *rcsB* mutant alone. Panel B shows cells over expressing *umoB* in a wild-type or *rcsB* background. Panel C shows PM942*waaL::km*^{r} compared to an *umoB* double mutant or an *umoB;rcsB* triple mutant. Three individual 2µL drops for each strain were placed on the same LB plate and swarming distances were measured at 30 minute intervals. The starting diameter of each spot was 4 mm. The average of the three measurements is shown.

Figure 4. RcsF overexpression counters the phenotype of UmoB overexpression, but not UmoD. The swarming phenotypes of the indicated strains were determined by growing cells up overnight in LB broth with the appropriate antibiotics and adjusting cultures to the same optical density with fresh LB. Panel A shows the swarming phenotype of wild-type cells over expressing *umoB, rcsF,* or both. Panel B shows the swarming phenotype of wild-type cells over expressing *umoD, rcsF,* or both. Panel C shows the swarming phenotype of PM942*waaL::km*^{r} over expressing *umoB, rcsF,* or both. Panel D shows the swarming phenotype of PM942*waaL::km*^{r} over expressing *umoD, rcsF,* or both. Three individual 2µL drops for each strain were placed on the

same LB plate and swarming distances were measured at 30 minute intervals. The starting diameter of each spot was 4 mm. The average of the three measurements is shown.

Figuire 5. *rcsF* is not needed for UmoB or UmoD activity in PM7002. The swarming phenotypes of the indicated strains were determined by growing cells up overnight in LB broth with the appropriate antibiotics and adjusting cultures to the same optical density with fresh LB. Panel A-B shows the swarming phenotype of *rcsF::kan*^{r} cells in an otherwise wild-type background over expressing *umoB* or *umoD.* Panel C-D shows the swarming phenotype of *waaL::miniTn5;rcsF::kan*^{r} (PM942*waaL::km*^{r};*rcsF*) cells expressing *umoB* or *umoD*. Three individual 2µL drops for each strain were placed on the same LB plate and swarming distances were measured at 30 minute intervals. The starting diameter of each spot was 4 mm. The average of the three measurements is shown.

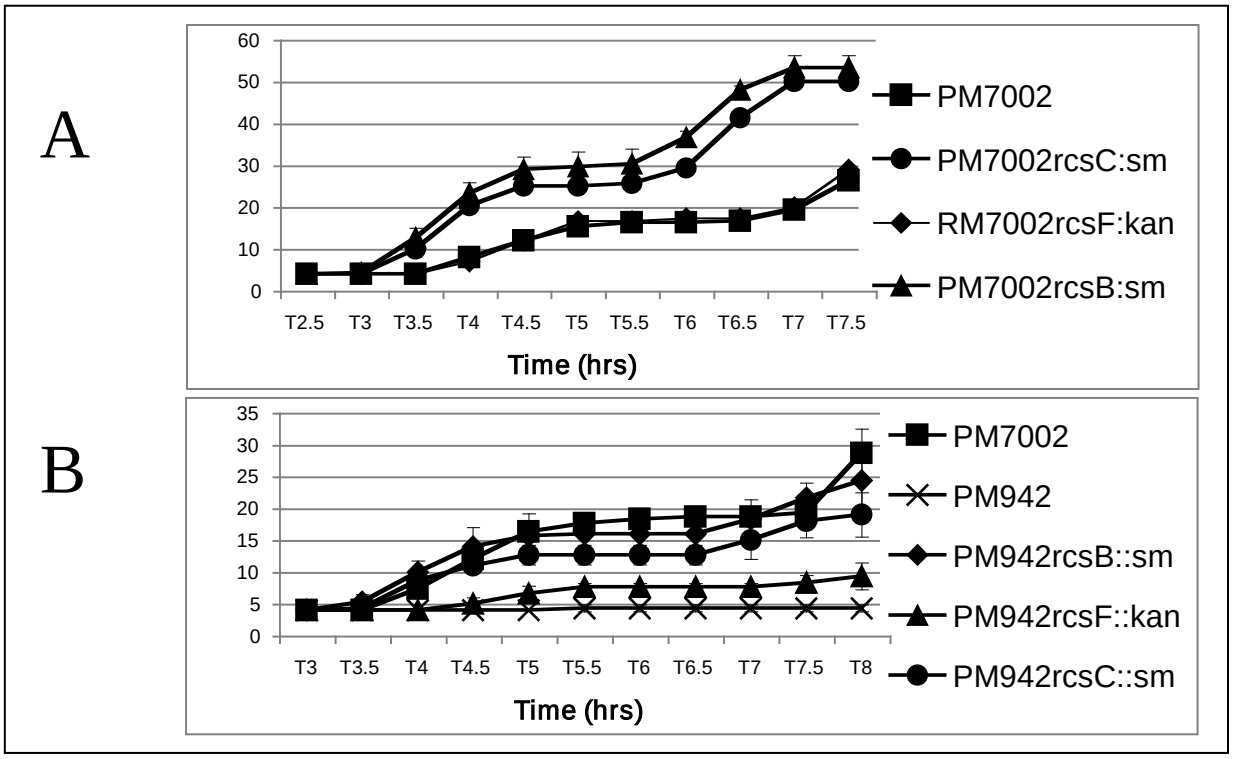
Figure 6. *rcsF*, *umoB*, and *umoD* are transcribed equally in wild-type and PM942*waaL::km*^{r} . Cells were grown up overnight and the $O.D._{600}$ were equilibrated with fresh LB. 150µL of the culture was spread onto separate 2% agar plates to collect after 4 hours. Cells were collected off plates and spun down for RNA isolation. The amounts of RNA were standardized and equal amounts were loaded for each sample (E). (A) Northern blot analysis of *rcsF* transcript levels. (B) Northern blot analysis of *umoB* mRNA accumulation. (C)

Northern blot analysis of *umoD* mRNA levels. (D) Northern blot analysis of *flhDC* mRNA. The same RNA sample was used to perform each Northern.

Figure 7. Overexpression of both *umoB* and *umoD* has an accumulative effect in PM942*waaL::km*^{r}. The swarming phenotypes of the indicated strains were determined by growing cells up overnight in LB broth with the appropriate antibiotics and adjusting cultures to the same optical density with fresh LB. Shown is the swarming phenotype of PM942*waaL::km*^{r} cells over expressing *umoB*, *umoD*, or both. Three individual 2µL drops for each strain were placed on the same LB plate and swarming distances were measured at 30 minute intervals. The starting diameter of each spot was 4 mm. The average of the three measurements is shown.

Figure 8. Model showing Rcs inhibition upon surface contact. On solid surfaces, O-antigen changes UmoD activity to allow it to help transition UmoB to its activated form (indicated by asterisks). RcsF activity is shut down, either through O-antigen independently or though UmoD. Activated UmoB can repress the Rcs system, blocking the repressive effects of RcsB on the *flhDC* promoter, allowing for a large increase in flagella synthesis and swarming.

Figure 1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T2.5
T3
T3.5
T4
T4.5
T5
T5.5
T6
T6.5
T7
T7.5
Time (hrs)
PM7002
PM7002rcsC:sm
RM7002rcsF:kan
PM7002rcsB:sm
B
35
30
25
20
15
10
5
0
T3
T3.5
T4
T4.5
T5
T5.5
T6
T6.5
T7
T7.5
T8
Time (hrs)
PM7002
PM942
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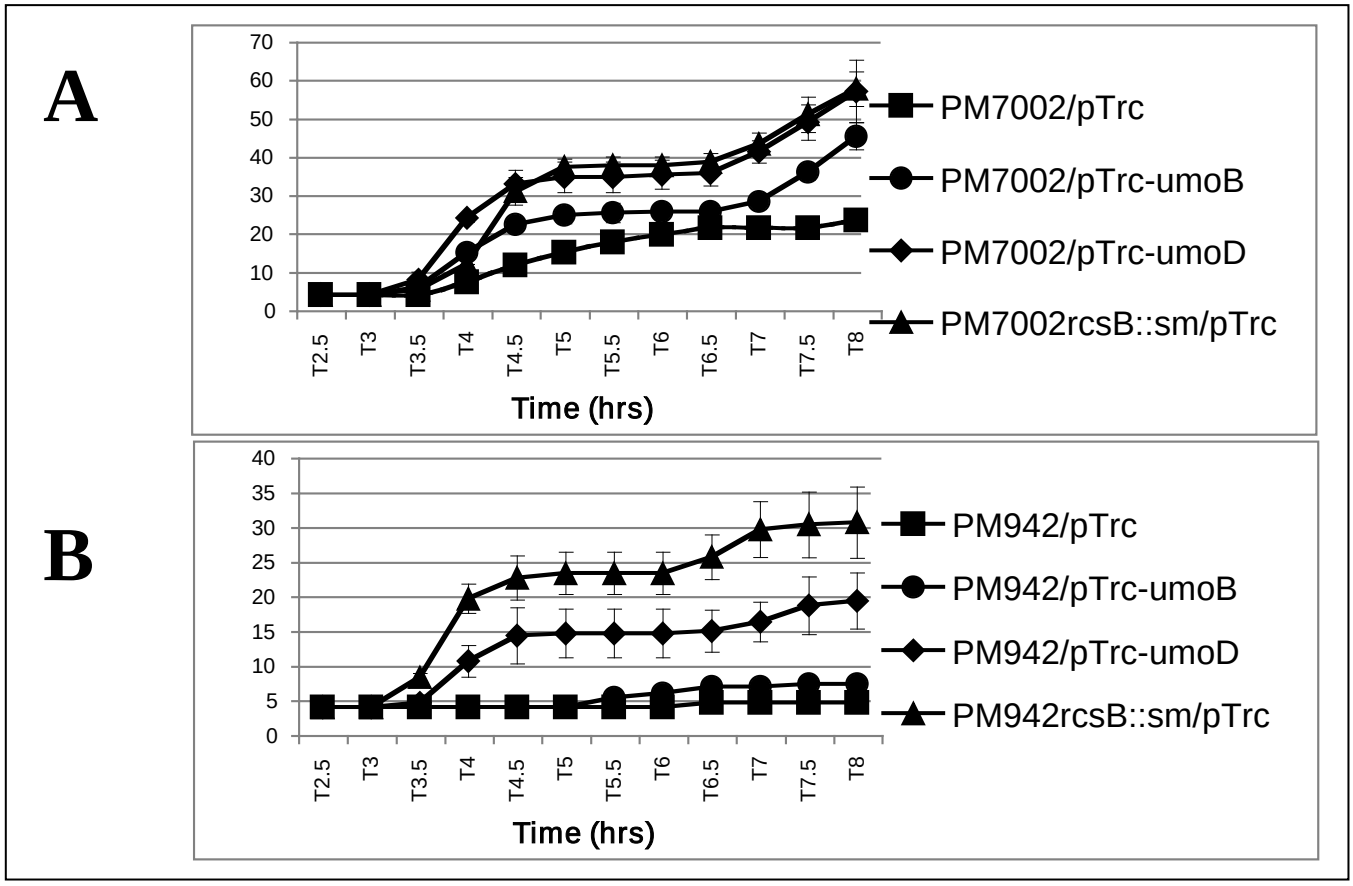
PM942rcsF::kan
PM942rcsC::sm

Figure 2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T3
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T4
T4.5
T5
T5.5
T6
T6.5
T7
T7.5
T8
Time (hrs)
PM7002/pTrc
PM7002/pTrc-umoB
PM7002/pTrc-umoD
PM7002rcsB::sm/pTrc
B
40
35
30
25
20
15
10
5
0
T2.5
T3
T3.5
T4
T4.5
T5
T5.5
T6
T6.5
T7
T7.5
T8
Time (hrs)
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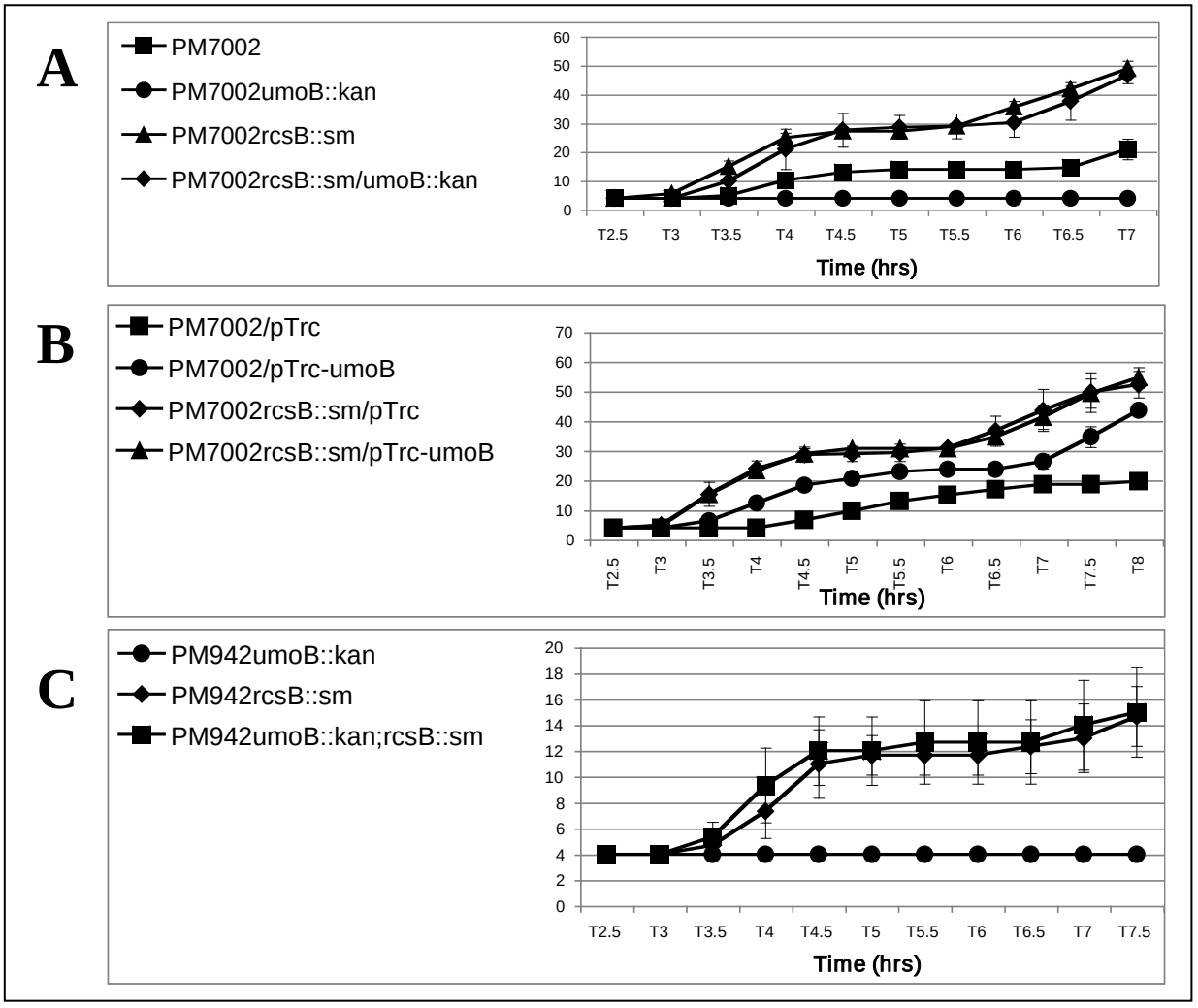
PM942/pTrc-umoB
PM942/pTrc-umoD
PM942rcsB::sm/pTrc

Figure 3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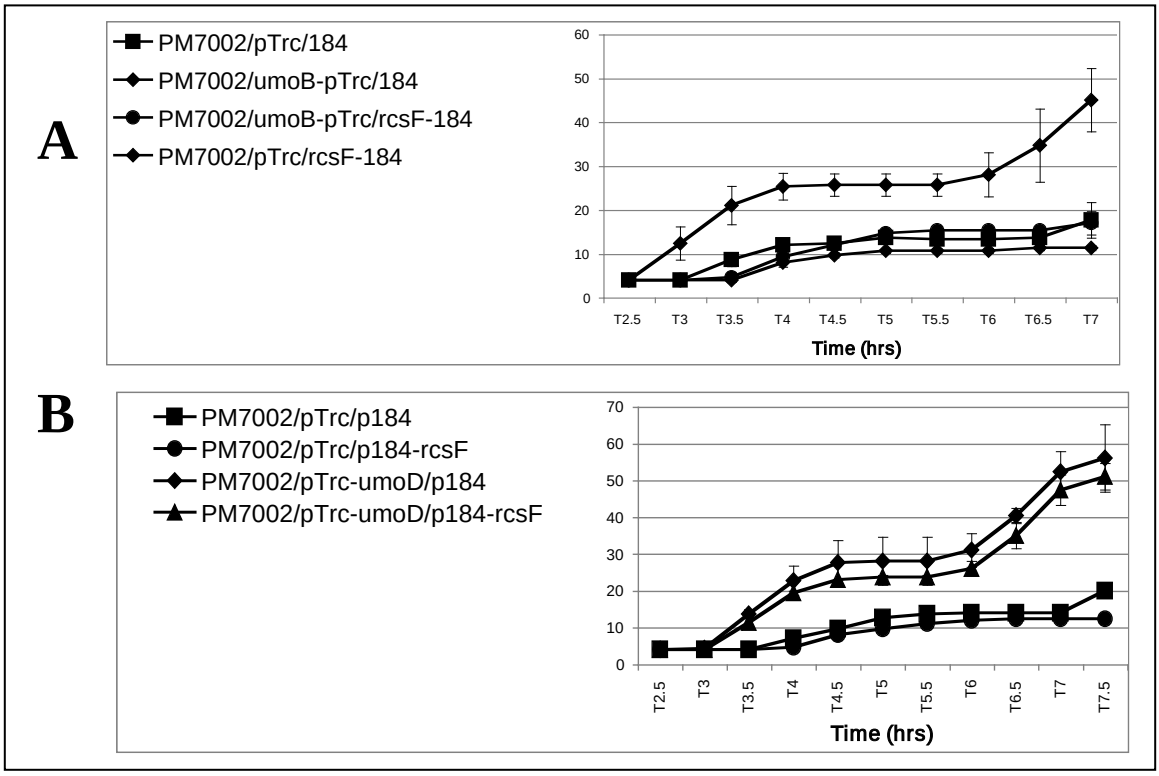
PM7002rcsB::sm/umoB::kan
60
50
40
30
20
10
0
T2.5 T3 T3.5 T4 T4.5 T5 T5.5 T6 T6.5 T7
Time (hrs)
B
PM7002/pTrc
PM7002/pTrc-umoB
PM7002rcsB::sm/pTrc
PM7002rcsB::sm/pTrc-umoB
70
60
50
40
30
20
10
0
T2.5 T3 T3.5 T4 T4.5 T5 T5.5 T6 T6.5 T7 T7.5 T8
Time (hrs)
C
PM942umoB::kan
PM942rcsB::sm
PM942umoB::kan;rcsB::sm
20
18
16
14
12
10
8
6
4
2
0
T2.5 T3 T3.5 T4 T4.5 T5 T5.5 T6 T6.5 T7 T7.5
Time (hrs)

Figure 4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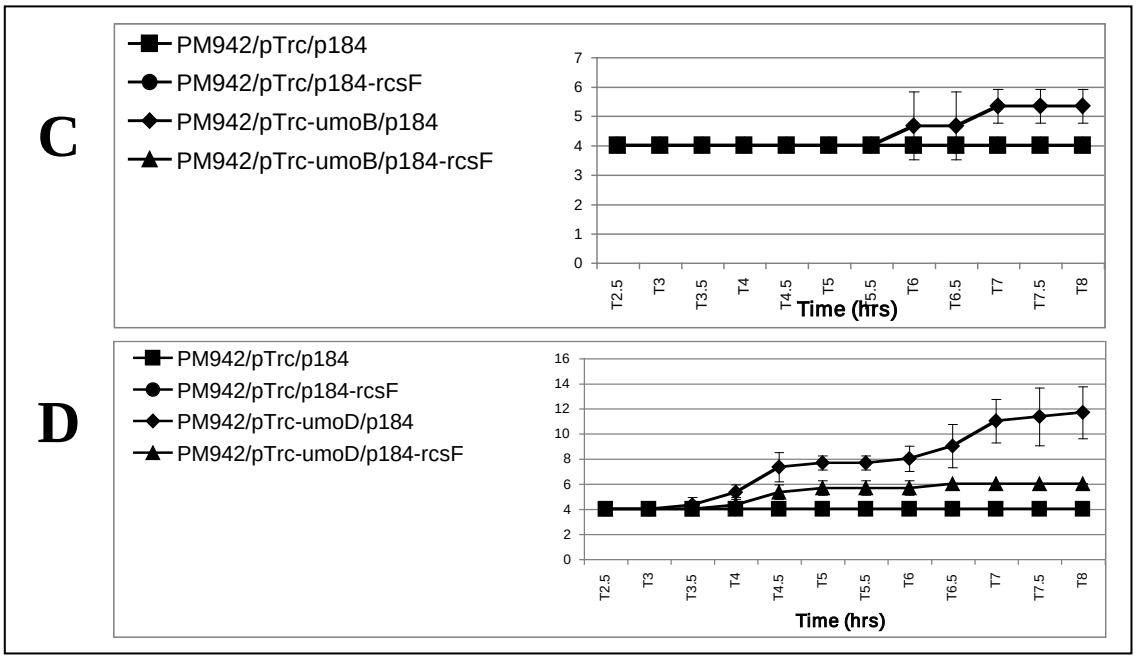
PM7002/umoB-pTrc/rcsF-184
PM7002/pTrc/rcsF-184
60
50
40
30
20
10
0
T2.5 T3 T3.5 T4 T4.5 T5 T5.5 T6 T6.5 T7
Time (hrs)
B
PM7002/pTrc/p184
PM7002/pTrc/p184-rcsF
PM7002/pTrc-umoD/p184
PM7002/pTrc-umoD/p184-rcsF
70
60
50
40
30
20
10
0
T2.5 T3 T3.5 T4 T4.5 T5 T5.5 T6 T6.5 T7 T7.5
Time (hrs)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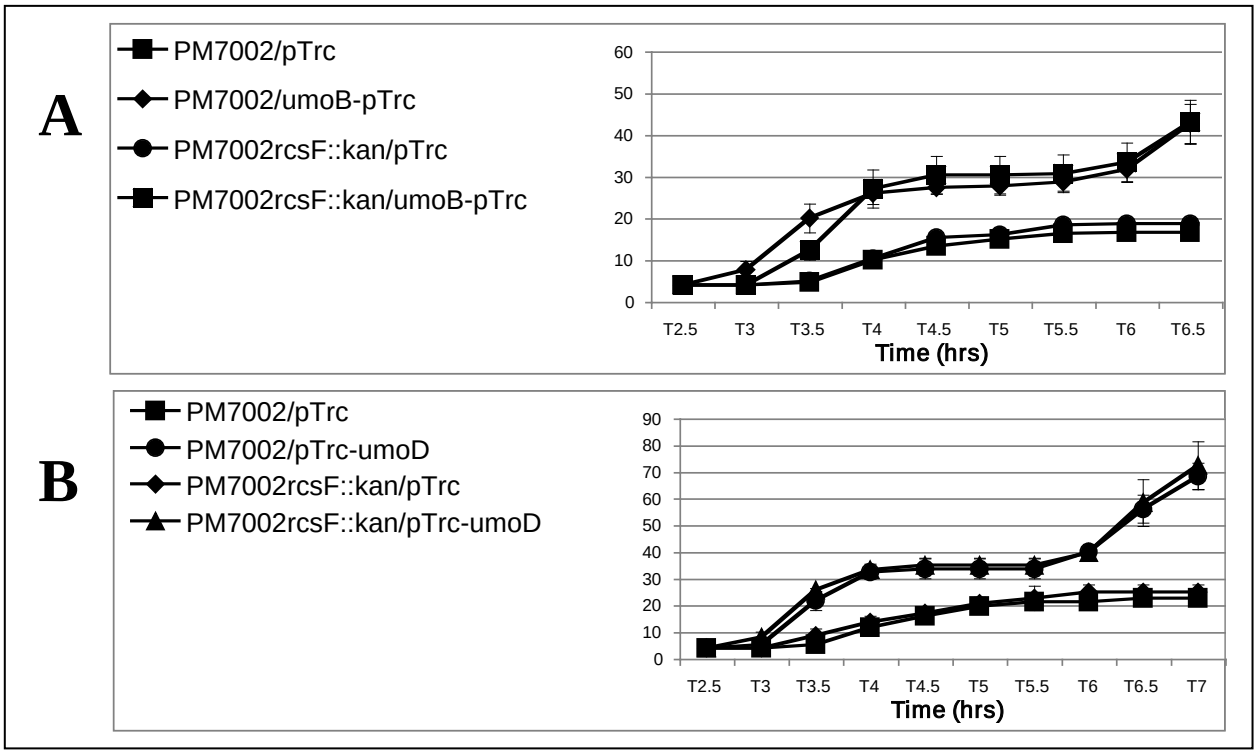
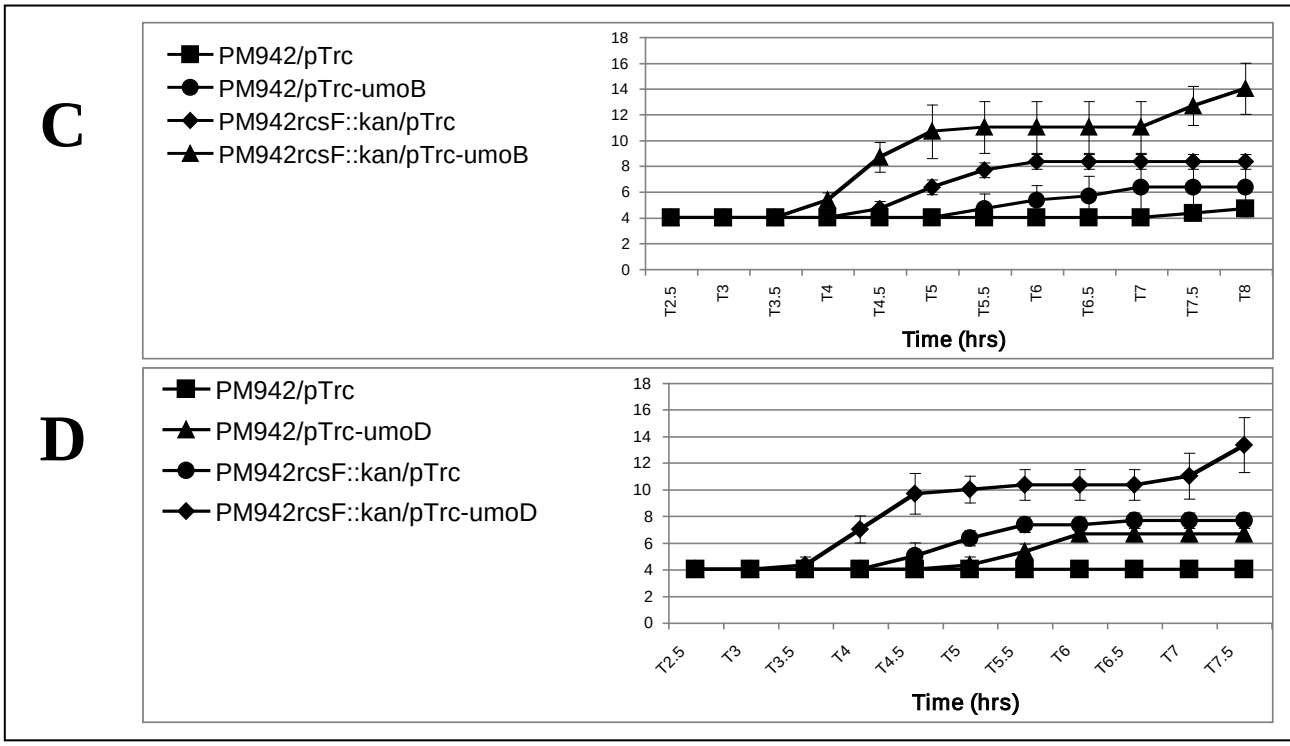
PM942/pTrc/p184-rcsF
PM942/pTrc-umoB/p184
PM942/pTrc-umoB/p184-rcsF
7
6
5
4
3
2
1
0
T2.5 T3 T3.5 T4 T4.5 T5 T5.5 T6 T6.5 T7 T7.5 T8
Time (hrs)
D
PM942/pTrc/p184
PM942/pTrc/p184-rcsF
PM942/pTrc-umoD/p184
PM942/pTrc-umoD/p184-rcsF
16
14
12
10
8
6
4
2
0
T2.5 T3 T3.5 T4 T4.5 T5 T5.5 T6 T6.5 T7 T7.5 T8
Time (hrs)

Figure 5


A
PM7002/pTrc
PM7002/umoB-pTrc
PM7002rcsF::kan/pTrc
PM7002rcsF::kan/umoB-pTrc
60
50
40
30
20
10
0
T2.5 T3 T3.5 T4 T4.5 T5 T5.5 T6 T6.5
Time (hrs)
B
PM7002/pTrc
PM7002/pTrc-umoD
PM7002rcsF::kan/pTrc
PM7002rcsF::kan/pTrc-umoD
90
80
70
60
50
40
30
20
10
0
T2.5 T3 T3.5 T4 T4.5 T5 T5.5 T6 T6.5 T7
Time (hrs)


C
PM942/pTrc
PM942/pTrc-umoB
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PM942rcsF::kan/pTrc-umoB
18
16
14
12
10
8
6
4
2
0
T2.5 T3 T3.5 T4 T4.5 T5 T5.5 T6 T6.5 T7 T7.5 T8
Time (hrs)
D
PM942/pTrc
PM942/pTrc-umoD
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PM942rcsF::kan/pTrc-umoD
18
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Time (hrs)

Figure 6

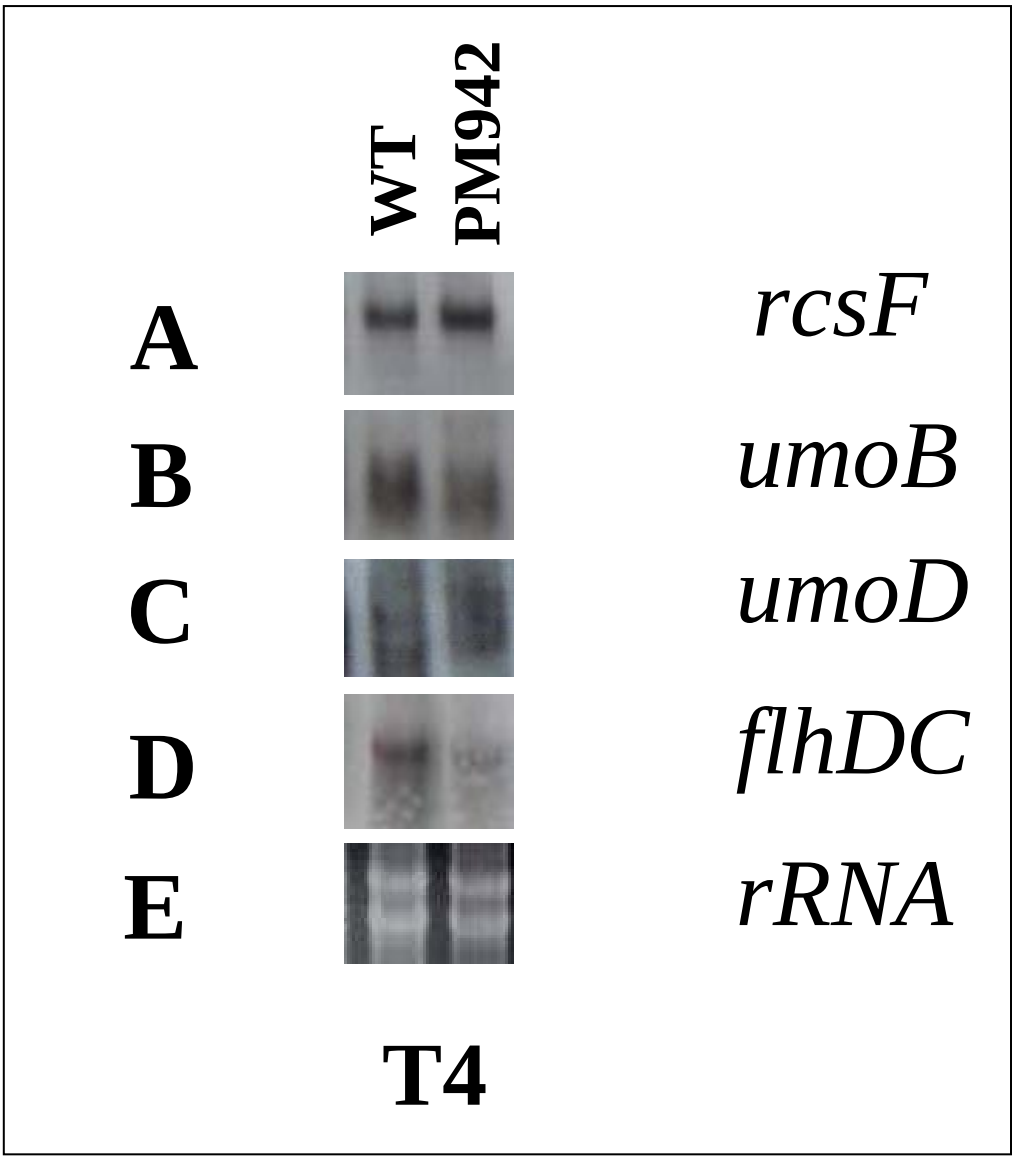
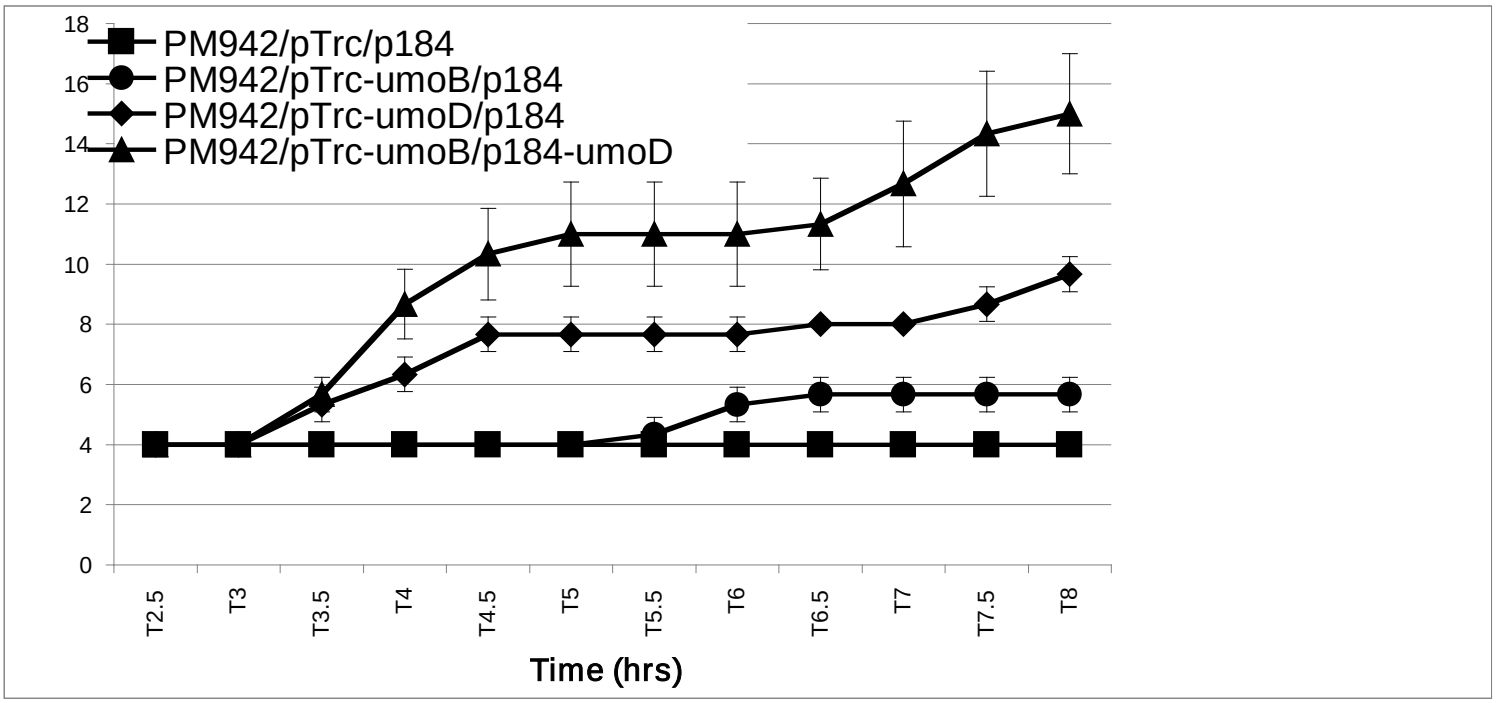


Figure 7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PM942/pTrc-umoB/p184-umoD
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T7
T7.5
T8
Time (hrs)

Figure 8

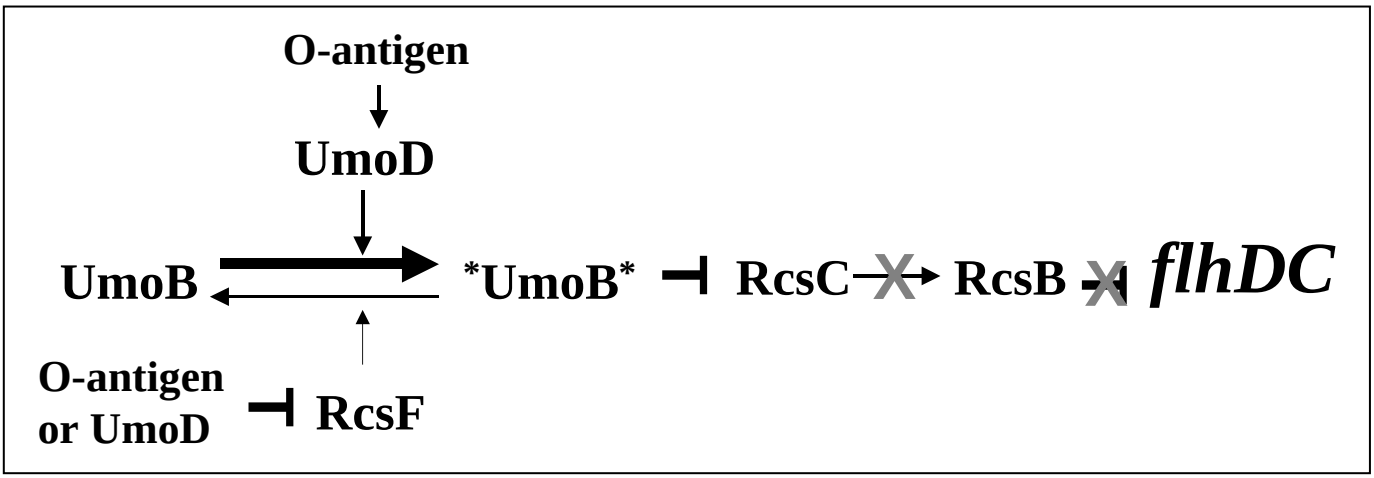
O-antigen
UmoD
UmoB
UmoB
RcsC
RcsB
flhDC
O-antigen
or UmoD
RcsF

Table 1

Primer	Sequence (5'-3')	Purpose
rcsB.for	GTACAGTCGACTCACCGACCTATCTATGCCT	Deletion mutant
rcsB.rev	GTACAGTCGACTCACCGACCTATCTATGCCT	Deletion mutant
rcsCint.for	ATCAAGGATCCAGAGCGTTCCATTTTAACACG	Deletion mutant
rcsCint.rev	ATGCTTAGTCGACATGCTTCACGCTTAGAGGAGC	Deletion mutant
rcsF.for	ATCAGGGATCCATTTGCATTAAATTAGGGC	Mutation/overexpression
rcsF.rev	ATGACATGTCGACATTCATTGAGTAATTAATAGTGC	Mutation/overexpression
umoB.for	ATCAGGGATCCATTGTTACTAAGCAACACC	Mutation/overexpression
umoB.rev	ATGACATGTCGACAGTAAACACATTGCCTTCC	Mutation/overexpression
umoD1A.for	ATCAAGGATCCTGGTGATAAAAGAGTGAAATCC	Mutation/overexpression
UmoD.rev	ATGACATGTCGACTATCAGTTATCAGCGTTAATGC	Mutation/overexpression

Chapter 5: Discussion/Conclusions

P. mirabilis is a Gram-negative bacterium that undergoes a physical and biochemical change during surface growth. When grown on a surface, *P. mirabilis* differentiates from a small, peritrichously flagellated swimmer cell to a hyper-flagellated, elongated, aseptate, multi-nucleated swarmer cell that up-regulates many genes, including virulence factors. These swarmer cells, as a group, are able to move in a coordinated fashion over the surface of an agar plate, performing an act termed swarming. There are many species of bacteria that can swarm, although the type of media required varies among different species (Chapter 1). *P. mirabilis* is able to swarm on media with agar concentrations over 2.0%, while many other swarming bacteria, including *E. coli*, can only swarm on specialized Eiken agar and/or at agar concentrations below 1.0% (25, 44). The amount of hyper-flagellation and elongation exhibited by *P. mirabilis* during swarming makes it unique in its swarming differentiation. Only *Vibrio* hyper-flagellates to such a degree: with both being able to swarm on greater concentrations of agar than other species, suggesting a correlation between the amounts of flagellation and swarming ability.

Most bacteria that swarm form amorphous radial or tendril patterns, however, *P. mirabilis* forms a characteristic ‘bull’s eye’ pattern on agar plates (48). This pattern is formed because *P. mirabilis* cannot maintain itself as a swarmer cell for long periods of time. After only a few hours of swarming, the cells consolidate (de-differentiate) back into swimmer cells. This pattern repeats itself until rings of swarming and consolidation are formed. It is not known why

and how cells consolidate, but it is known *flhDC* expression needs to be reduced in order to attain consolidation (10). Swarming is an energy exhaustive process involving the up-regulation of not only flagella, but many virulence factors as well. There is also a slime layer associated with swarming cells as well as a specialized capsule polysaccharide (18, 24, 41). It is possible that consolidation happens because energy stores are depleted and the cells need to resume normal growth. Another possibility is that as cells swarm out, the slime layer is depleted, and the cells consolidate as they wait for the slime layer to reform.

The ability of cells to elongate is another important unanswered question in the swarming field. When *P. mirabilis* elongates into a swarmer cell it does not stop chromosome replication, nor does it form septa (2, 21). The increase in size and replication of the chromosome should meet the requirements of two cell division checkpoints (46). This means studying swarming could bring insights into new cell division regulators. One hypothesis was that the SOS division inhibitor SulA was induced during swarming and inhibited Z-ring formation (12, 39). In *E. coli*, SulA is a substrate of Lon protease and in *P. mirabilis lon* mutants hyper-swarm and elongate in liquid (11, 37). The reason for this elongation phenotype is not known but was hypothesized that an increase in the levels of SulA protein in this strain resulted in division inhibition. It is also known that *sulA* transcript levels increase during swarming, however a *sulA* mutant does not have a swarming phenotype (unpublished data) indicating SulA is most likely not responsible for elongation during swarming. The mechanism of division inhibition is still unknown. Possible proteins to examine are from the *min* locus, which help

to ensure division occurs at the midcell by inhibiting Z-ring formation at the poles (14). Studying division inhibition in swarmer cells might lead to new mechanisms of division inhibition, which not only could broaden our understanding of cell division, but also produce novel antimicrobials that target these new proteins.

Bacteria encounter many different conditions during their life and must be able to change their genetic programs accordingly. Swarming bacteria change their gene expression upon transfer from liquid to solid media. How bacteria do this is still a mystery. The study of swarming has led to multiple hypotheses concerning the change of gene expression seen from solid surface growth. Work with *V. parahaemolyticus* showed the polar flagellum acts as a surface sensor (27, 33). When grown in liquid media supplemented with either anti-flagellum antibodies or thickening agents cells will induce the expression of swarming specific genes, such as lateral flagella (4, 33). In *P. mirabilis*, it is also assumed that flagella inhibition plays a role in surface sensing (1). Like *V. parahaemolyticus*, when *P. mirabilis* is incubated with anti-flagellar antibodies or a viscous agent, differentiation occurs (5). It is currently not known how flagellar inhibition initiates the differentiation process. It is possible the cell is sensing cell speed or flagella rotational speed, but not actual force (27). In *P. mirabilis*, mutations in *motA*, a flagellar motor protein, produce cells that are unable to rotate their flagella, yet regulate flagella in a similar manner to wild-type cells in response to surfaces (unpublished data). This indicates the flagellar inhibition signal might control elongation rather than *flhDC* expression. It does not help conclude if cell speed or flagellar rotation speed is being measured. These data

also suggest there is another pathway involved in surface sensing which controls *flhDC* expression.

Another possible mechanism for surface sensing involves ion concentration. In *V. parahaemolyticus*, iron and calcium sensing have been shown to be necessary for swarming initiation (22, 34). Even in low iron conditions (which induces lateral gene expression) polar flagellum inhibition is still necessary indicating ion concentration is necessary but not sufficient to induce swarming (34). When agar is added to normally iron rich media it acts as though it were iron-deplete, presumably because diffusion is limited. This indicates that solid surfaces themselves might be limiting nutrient uptake and signaling to the cells to adjust their genetic profile accordingly. However, in *Salmonella*, it was shown that genes induced during low iron conditions were expressed at higher levels on 0.6% swarm agar than on 1.5% agar plates, a condition that should have lower diffusion (49). This indicates that rather than diffusion, there may be a greater need for nutrients in swarmer cells than swimmer cells.

The work presented here (chapters 3-4) discussed the role of O-antigen as another signaling pathway to sense solid surfaces. Mutations in O-antigen result in cells that have fully functional flagella in liquid media, resulting in a wild-type swimming phenotype. However, on surfaces, mutants lacking full length O-antigen do not swarm (38). In *S. enterica*, mutants lacking an O-antigen were unable to swarm due to a loss of lubrication, rather than a signaling problem (47). This mechanism does not appear to be the same in *P. mirabilis* because

Northern blot and Western blot analyses showed flagella induction was impaired only on surfaces, and at the level of *flhDC* expression. When O-antigen is absent, *flhDC* is not induced on surfaces, resulting in cells that do not produce the appropriate levels of flagella to swarm. These cells also have an inability to differentiate, showing a possible connection between increased *flhDC* expression and division inhibition. This shows that O-antigen is needed for signal transduction opposed to wettability as seen in *Salmonella*.

LPS structures such as O-antigen or Enterobacterial Common Antigen (ECA) have been reported to be important for swarming in other organisms. In *E. coli*, mutants in LPS pathways have been identified that are impaired for swarming ability (20, 26). The swarming defects in these mutants could be suppressed through mutations in the Rcs signaling pathway (20). In *S. marcescens*, ECA completion was shown to be a checkpoint for flagellar synthesis (8). *S. marcescens* ECA mutants also have swarming restored when mutations are made in the Rcs system (9). Interestingly, RcsF was fully able to restore swarming in these mutants, but in *P. mirabilis*, RcsF only displays a small role in swarming and does not restore swarming to a wild-type level in a strain lacking O-antigen (Morgenstein and Rather in submission). In *P. mirabilis*, when mutations are made in the Rcs system (other than *rcsF*) not only is there an effect on swarming, but also swimming (3, 10, 29). In both cases, the Rcs mutants exhibit hyper-motility. However, in *S. marcescens*, mutants in the Rcs pathway swarm and swim to the same level as wild-type cells (9). This might indicate there is a difference in Rcs regulation in these species, although it is

hard to understand how these mutations only have an effect in ECA mutants but not in wild-type strains of *S. marcescens*. In both *E. coli and S. marcescens*, swarming was inhibited because *flhDC* was not properly induced on surfaces, similarly to *P. mirabilis*.

The Rcs TCS is a complicated phosphorelay: consisting of more parts than the canonical TCS. First, the sensor kinase (RcsC) is a hybrid kinase that after autophosphorylation transfers the phosphate to its own aspartate, instead of the response regulator's (30). Along with RcsC, there is the response regulator, RcsB. In order for RcsB to be phosphorylated, a phosphotransfer protein is also needed (RcsD), which takes the phosphate onto one of its histidine residues in order to pass it to an aspartate on RcsB. An outer-membrane activator protein (RcsF) and an inner-membrane repressor (UmoB, IgaA) have been identified (6, 7, 16, 19, 31, 32). Our work shows UmoB works as part of the Rcs signaling cascade in *P. mirabilis*, as it does in *Salmonella* (Morgenstein and Rather). We also show that another input into the Rcs system is UmoD, a predicted periplasmic protein (16).

In *P. mirabilis*, O-antigen mutants can swarm properly under the right conditions. When *flhDC* is expressed in trans under a constitutive promoter, or when mutations are made in *rcsC* or *rcsB*, swarming is restored. This swarming can even be greater than wild-type, indicating there is not an intrinsic inability of these cells to swarm, but rather, O-antigen is likely signaling to *flhDC* and in its absence cells do not properly induce *flhDC* on surfaces (38). It is unknown how O-antigen relays information to the cell. Our hypothesis is O-antigen-surface

contacts brings the inner and outer membranes closer together, allowing greater contact between UmoD and UmoB. Upon UmoD-UmoB interactions, UmoB is activated and can inhibit RcsC activity. This turns off RcsC and removes the repressive effects of RcsB on *flhDC* allowing for the large increase of flagella seen during swarming. Also during this time, RcsF, which inhibits UmoB activity, is turned off, increasing the amount of activated UmoB (Morgenstein and Rather).

Two-component systems are one of the most important ways bacteria control gene expression. They are found in almost every bacterial species, although the number in each organism can differ, with *Mycoplasma genitalium* having none and *Synechocystis* sp. having 80 (23, 35, 36). *P. mirabilis* has 16 predicted TCS, although only two have been shown to be explicitly involved in swarming (Rpp, Rcs) (40). Most response regulators bind DNA and act as transcriptional regulators, although some, such as chemotaxis response regulators, bind other proteins. A main interest in the field is how sensor kinases sense and respond to stimuli. For most TCS studied, the exact stimuli is unknown, however almost all of the known stimuli center on ligands binding to the sensor kinase. This is the case during chemotaxis and quorum sensing signaling. This is most likely because it is easier to study ligand-receptor interactions than “abstract” ideas such as stress or temperature change.

While there is much that can be learned from studying canonical TCS, the future of microbiology probably lies in the understanding of how non-ligands are sensed by the cell. Recently, researchers have shown a mechanism for a sensor

kinase to respond to temperature changes in the cell as well as the ability of proteins to localize in the cell based on cell wall curvature (13, 42, 43). The DesK sensor kinase senses temperature by measuring the thickness of the plasma membrane through the use of a “buoy” system, where the amino terminus floats on the membrane at high temperature (thin membrane) and is buried in the membrane at lower temperatures (thick membrane) (13). Another “abstract” concept is the curvature of the membrane. Two different proteins in *B. subtilis* were shown to recognize either positive or negative curvature of the cell membrane (42, 43). It appears that by inserting an amphipathic helix into the membrane these proteins can sense membrane curvature and localize correcly.

The Rcs TCS is a well-studied system, yet no definitive signal has been identified to activate it. It is known peptidoglycan and membrane stress act as at least one signal (28, 45). Growth on surfaces has also been suggested to control Rcs activity (17, 38). We have proposed that O-antigen is needed for Rcs modulation on solid surfaces, however, it is unknown how O-antigen-surface contacts are sensed by the Rcs system. Whatever the signal, it is most likely not a ligand, but an abstract concept, as seen above.

There are many experiments that can be done in the future to solidify the hypotheses expressed in this work. First, it is important to show UmoD modulates the Rcs system and that O-antigen affects this modulation. *In vitro* work can be done with purified proteins to measure the phosphorylation state of RcsB. Using purified UmoB, UmoD, and Rcs components it is possible to determine if the addition of a protein affects RcsB phosporylation. To test the

effects of these proteins and O-antigen *in vivo*, promoter fusions of known Rcs controlled promoters (from *E. coli*) can be fused to *lacZ* to measure activity of these promoters in wild-type and O-antigen backgrounds in conjunction with UmoB, UmoD or Rcs mutations.

After showing the phosphorylation of RcsB is affected in a manner predicted by the model, both biochemistry and genetics can be applied to further characterize the interactions amongst these proteins. Further confirmation of the model would come from showing direct protein-protein interactions between UmoB and UmoD or RcsF. UmoB is predicted to have a large periplasmic loop, which is most likely the site of any interaction with a periplasmic protein (such as UmoD or RscF) (15). Our lab has not been able to purify UmoB due to protein degradation (unpublished results). If it proves impossible to purify the entire UmoB protein, the periplasmic loop could be purified instead. The model purposes that UmoB interacts with both UmoD and RcsF in the periplasm, along with RcsC in the inner-membrane. Alanine-scanning mutagenesis of the periplasmic loop should result in either hyper-functioning or non-functioning protein, which can be assayed through swarming assays. Suppressor mutations of the phenotypes can be found and then mapped. This should supply information regarding protein interactions with UmoB as well as specific residues that are important for function.

References

1. **Alavi, M., and R. Belas.** 2001. Surface Sensing, Swarmer Cell Differentiation, and Biofilm Development, p. 29-40, Methods in Enzymology, vol. Volume 336. Academic Press.
2. **Armitage, J. P., R. J. Rowbury, and D. G. Smith.** 1974. The Effects of Chloramphenicol, Nalidixic Acid and Penicillin on the Growth and Division of Swarming cells of *Proteus mirabilis*. Journal of Medical Microbiology **7:**459-463.
3. **Belas, R., R. Schneider, and M. Melch.** 1998. Characterization of *Proteus mirabilis* Precocious Swarming Mutants: Identification of *rsb*A, Encoding a Regulator of Swarming Behavior. Journal of Bacteriology **180:**6126-6139.
4. **Belas, R., M. Simon, and M. Silverman.** 1986. Regulation of Lateral Flagella Gene Transcription in *Vibrio parahaemolyticus*. Journal Bacteriology **167:**210-218.
5. **Belas, R., and R. Suvanasuthi.** 2005. The Ability of *Proteus mirabilis* to Sense Surfaces and Regulate Virulence Gene Expression Involves FliL, a Flagellar Basal Body Protein. Journal of Bacteriology **187:**6789-6803.
6. **Cano, D. A., G. Dominguez-Bernal, A. Tierrez, F. G.-D. Portillo, and J. Casadesus.** 2002. Regulation of Capsule Synthesis and Cell Motility in *Salmonella enterica* by the Essential Gene *igaA*. Genetics **162:**1513-1523.

7. **Cano, D. A., M. Martinez-Moya, M. G. Pucciarelli, E. A. Groisman, J. Casadesus, and F. Garcia-Del Portillo.** 2001. *Salmonella enterica Serovar Typhimurium* Response Involved in Attenuation of Pathogen Intracellular Proliferation. Infect. Immun. **69:**6463-6474.

8. **Castelli, M. E., G. V. Fedrigo, A. L. Clementin, M. V. Ielmini, M. F. Feldman, and E. G. Vescovi.** 2008. Enterobacterial Common Antigen Integrity is a Checkpoint for Flagellar Biogenesis in *Serratia marcescens*. Journal Bacteriology **190:**213-220.

9. **Castelli, M. E., and E. G. Vescovi.** 2010. The Rcs Signal Transduction Pathway Is Triggered by Enterobacterial Common Antigen Structure Alterations in Serratia marcescens. Journal Bacteriology **193:**63-74.

10. **Clemmer, K. M., and P. N. Rather.** 2007. Regulation of *flh*DC Expression in *Proteus mirabilis*. Research in Microbiology**:**295-302.

11. **Clemmer, K. M., and P. N. Rather.** 2008. The Lon Protease Regulates Swarming Motility and virulence Gene Expression in *Proteus mirabilis*. Journal of Medical Microbiology **57:**931-937.

12. **Cole, S. T.** 1983. Characterisation of the Promoter for the LexA Regulated *sulA* Gene of *Escherichia coli*. MGG. Molecular and general genetics **189:**400-404.

13. **Cybulski, L. E., M. MartÌn, M. C. Mansilla, A. Fernandez, and D. de Mendoza.** 2010. Membrane Thickness Cue for Cold Sensing in a Bacterium. Current Biology **20:**1539-1544.

14. **de Boer, P. A. J., R. E. Crossley, and L. I. Rothfield.** 1989. A Division Inhibitor and a Topological Specificity Factor Coded for by the Minicell Locus Determine Proper Placement of the Division Septum in *E. coli*. Cell **56:**641-649.

15. **Dominguez-Bernal, G., M. G. Pucciarelli, F. Ramos-Morales, M. Garcìa-Quintanilla, D. A. Cano, J. Casades˙s, and F. G.-d. Portillo.** 2004. Repression of the RcsC-YojN-RcsB Phosphorelay by the IgaA Protein is a Requisite for *Salmonella* Virulence. Molecular Microbiology **53:**1437-1449.

16. **Dufour, A., R. B. Furness, and C. Hughes.** 1998. Novel Genes that Upregulate the *Proteus mirabilis flhDC* Master Operon Controlling Flagellar Biogenesis and Swarming. Molecular Microbiology **29:**741-751.

17. **Ferrières, L., and D. J. Clarke.** 2003. The RcsC Sensor Kinase is Required for Normal Biofilm Formation in *Escherichia coli* K-12 and Controls the Expression of a Regulon in Response to Growth on a Solid Surface. Molecular Microbiology **50:**1665-1682.

18. **Fuscoe, F. J.** 1973. The Role of Extracellular Slime Secretion in the Swarming of *Proteus*. Medical Laboratory Technology **30:**373-382.

19. **Gervais, F. G., and G. R. Drapeau.** 1992. Identification, Cloning, and Characterization of *rcsF*, a New Regulator Gene for Exopolysaccharide Synthesis that Suppresses the Division Mutation *ftsZ84* in *Escherichia coli* K-12. J. Bacteriol. **174:**8016-8022.

20. **Girgis, H. S., Y. Liu, W. S. Ryu, and S. Tavazoie.** 2007. A Comprehensive Genetic Characterization of Bacterial Motility. PLoS Genet **3:**1644-1660.

21. **Gmeiner, J., E. Sarnow, and K. Milde.** 1985. Cell Cycle Parameters of *Proteus mirabilis*: Interdependence of the Biosynthetic Cell Cycle and the Interdivision Cycle. Journal Bacteriology **164:**741-748.

22. **Gode-Potratz, C. J., D. M. Chodur, and L. L. McCarter.** 2010. Calcium and Iron Regulate Swarming and Type III Secretion in *Vibrio parahaemolyticus*. Journal Bacteriology **192:**6025-6038.

23. **Grebe, T. W., and J. B. Stock.** 1999. The Histidine Protein Kinase Superfamily. Advances in Microbial Physiology **41:**139-227.

24. **Gygi, D., M. M. Rahman, H. C. Lai, R. Carlson, J. Guard-Petter, and C. Hughes.** 1995. A Cell-Surface Polysaccharide that Facilitates Rapid Population Migration by Differentiated Swarm Cells of *Proteus mirabilis*. Molecular Microbiology **17:**1167-1175.

25. **Harshey, R. M., and T. Matsuyama.** 1994. Dimorphic Transition in *Escherichia coli* and *Salmonella typhimurium*: Surface-Induced Differentiation into Hyperflagellate Swarmer Cells. Proceedings of the National Academy of Sciences of the United States of America **91:**8631-8635.

26. **Inoue, T., R. Shingaki, S. Hirose, K. Waki, H. Mori, and K. Fukui.** 2007. Genome-Wide Screening of Genes Required for Swarming Motility in *Escherichia coli* K-12. Journal Bacteriology **189:**950-957.

27. **Kawagishi, I., M. Imagawa, Y. Imae, L. McCarter, and M. Homma.** 1996. The Sodium-Driven Polar Flagellar Motor of Marine *Vibrio* as the Mechanosensor that Regulates Lateral Flagellar Expression. Molecular Microbiology **20:**693-699.

28. **Laubacher, M. E., and S. E. Ades.** 2008. The Rcs Phosphorelay Is a Cell Envelope Stress Response Activated by Peptidoglycan Stress and Contributes to Intrinsic Antibiotic Resistance. J. Bacteriol. **190:**2065-2074.

29. **Liaw, S.-J., H. C. Lai, S. W. Ho, K. T. Luh, and W. B. Wang.** 2001. Characterisation of p-nitrophenylglycerol-resistant *Proteus mirabilis* Super-Swarming Mutants. Journal of Medical Microbiology. **50:**1039-1048.

30. **Majdalani, N., and S. Gottesman.** 2005. The Rcs Phosphorelay: A Complex Signal Transduction System. Annual Review of Microbiology **59:**379-405.

31. **Majdalani, N., M. Heck, V. Stout, and S. Gottesman.** 2005. Role of RcsF in Signaling to the Rcs Phosphorelay Pathway in *Escherichia coli*. Journal Bacteriology **187:**6770-6778.

32. **Mariscotti, J. F., and F. Garcia-del Portillo.** 2009. Genome Expression Analyses Revealing the Modulation of the *Salmonella* Rcs Regulon by the Attenuator IgaA. J. Bacteriol. **191:**1855-1867.

33. **McCarter, L., M. Hilmen, and M. Silverman.** 1988. Flagellar Dynamometer Controls Swarmer Cell Differentiation of *V. parahaemolyticus*. Cell **54:**345-351.

34. **McCarter, L., and M. Silverman.** 1989. Iron Regulation of Swarmer Cell Differentiation of *Vibrio parahaemolyticus*. Journal Bacteriology **171:**731-736.

35. **Mizuno, T.** 1998. His-Asp Phosphotransfer Signal Transduction. Journal of Biochemistry **123:**555-563.

36. **Mizuno, T., T. Kaneko, and S. Tabata.** 1996. Compilation of all Genes Encoding Bacterial Two-Component Signal Transducers in the Genome of the Cyanobacterium, *Synechocystis* sp. Strain PCC 6803. DNA Res **3:**407-414.

37. **Mizusawa, S., and S. Gottesman.** 1983. Protein Degradation in *Escherichia coli*: the *lon* Gene Controls the Stability of SulA Protein. Proceedings of the National Academy of Sciences of the United States of America **80:**358-362.

38. **Morgenstein, R. M., K. M. Clemmer, and P. N. Rather.** 2010. Loss of the WaaL O-Antigen Ligase Prevents Surface Activation of the Flagellar Gene Cascade in *Proteus mirabilis*. Journal of Bacteriology **192:**3213-3221.

39. **Mukherjee, A., C. Cao, and J. Lutkenhaus.** 1998. Inhibition of FtsZ Polymerization by SulA, an Inhibitor of Septation in *Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America **95:**2885-2890.

40. **Pearson, M. M., M. Sebaihia, C. Churcher, M. A. Quail, A. S. Seshasayee, N. M. Luscombe, Z. Abdellah, C. Arrosmith, B. Atkin, T. Chillingworth, H. Hauser, K. Jagels, S. Moule, K. Mungall, H.**

Norbertczak, E. Rabbinowitsch, D. Walker, S. Whithead, N. R. Thomson, P. N. Rather, J. Parkhill, and H. L. Mobley. 2008. Complete Genome Sequence of Uropathogenic *Proteus mirabilis*, a Master of both Adherence and Motility. J. Bacteriol. **190:**4027-4037.

41. **Rahman, M. M., J. Guard-Petter, K. Asokan, C. Hughes, and R. W. Carlson.** 1999. The Structure of the Colony Migration Factor from Pathogenic *Proteus mirabilis*. A Capsular Polysaccharide that Facilitates Swarming. J. Biol. Chem. **274:**22993-22998.
42. **Ramamurthi, K. S., S. Lecuyer, H. A. Stone, and R. Losick.** 2009. Geometric Cue for Protein Localization in a Bacterium. Science **323:**1354-1357.
43. **Ramamurthi, K. S., and R. Losick.** 2009. Negative Membrane Curvature as a Cue for Subcellular Localization of a Bacterial Protein. Proceedings of the National Academy of Sciences **106:**13541-13545.
44. **Rauprich, O., M. Matsushita, C. J. Weijer, F. Siegert, S. E. Esipov, and J. A. Shapiro.** 1996. Periodic Phenomena in *Proteus mirabilis* Swarm Colony Development. J. Bacteriol. **178:**6525-6538.
45. **Sledjeski, D., and S. Gottesman.** 1996. Osmotic Shock Induction of Capsule Synthesis in *Escherichia coli K-12*. J. Bacteriol. **178:**1204-1206.
46. **Thanbichler, M.** 2010. Synchronization of Chromosome Dynamics and Cell Division in Bacteria. Cold Spring Harbor Perspectives in Biology **2**.

47. **Toguchi, A., M. Siano, M. Burkart, and R. M. Harshey.** 2000. Genetics of Swarming Motility in *Salmonella enterica Serovar Typhimurium*: Critical Role for Lipopolysaccharide. J. Bacteriol. **182:**6308-6321.

48. **Verstraeten, N., K. Braeken, B. Debkumari, M. Fauvart, J. Fransaer, J. Vermant, and J. Michiels.** 2008. Living on a Surface: Swarming and Biofilm Formation. Trends in Microbiology **16:**496-506.

49. **Wang, Q., J. G. Frye, M. McClelland, and R. M. Harshey.** 2004. Gene Expression Patterns During Swarming in *Salmonella typhimurium*: Genes Specific to Surface Growth and Putative New Motility and Pathogenicity Genes. Molecular Microbiology **52:**169-187.