INDEPENDENCE AND INTERDEPENDENCE: SIGNAL TRANSDUCTION OF TWO CHEMOSENSORY RECEPTORS IMPORTANT FOR THE REGULATION OF GLIDING MOTILITY IN *MYXOCOCCUS XANTHUS*

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Summary

The *Myxococcus xanthus* Dif and Frz chemosensory pathways play important roles in the regulation of gliding motility. The Dif system regulates the production of exopolysaccheride (EPS), which is essential for social motility and fruiting body formation. The Frz pathway controls reversal frequency, which is fundamental for directed movement by this surface-gliding bacterium. In addition, both pathways are involved in the chemotactic response towards several phosphatidylethanolamine (PE) species such that the Dif pathway is required for excitation while the Frz pathway is essential for adaptation. In this study we addressed three crucial questions regarding the signal processing of these two chemosensory pathways by focusing on DifA and FrzCD, the MCP homologs from their respective pathways.

First, the receptor protein in the Dif pathway, DifA, lacks a perisplasmic domain, the typical signal-sensing structure. To examine whether DifA shares similar transmembrane signaling mechanism with typical transmembrane sensor proteins (MCPs and sensor kinases), we constructed a chimeric protein that is composed of the N-terminus of NarX (nitrate sensor kinase) and the C-terminus of DifA. This NarX-DifA chimera restores the DifA functionality (EPS production, agglutination, S-motility and development) to a Δ*difA* mutant in a nitrate-

dependent manner, suggesting DifA shares a similar transmembrane signaling mechanism with typical MCPs and sensor kinases despite its unorthodox structure.

Second, the *M. xanthus* chemotaxis is still controversial. It has been argued that the taxis-like response in this slowly gliding bacterium could result from physiological effects of certain chemicals. To study motility regulation by the Frz pathway, we constructed two chimeras between the N-terminus of NarX and C-terminus of FrzCD, which is the receptor protein of the Frz pathway. The two chimeras, $NazD_F$ and $NazD_R$, are identical except that $NazD_R$ contains a G51R mutation in the otherwise wild-type NarX sensory module. This G51R mutation was shown to reverse the signaling output of a NarX-Tar chimera to nitrate. We discovered that nitrate specifically decreased the reversal frequency of $NazD_F$-expressing cells and increased that of $NazD_R$-expressing cells. These results show that directional motility in *M. xanthus* can be regulated independently of cellular metabolism and physiology. Surprisingly, the $NazD_R$ strain failed to adapt to nitrate in temporal assays, as did the wild type to known repellents. Therefore, the lack of temporal adaptation to negative stimuli is an intrinsic property in *M. xanthus* motility regulation.

Third, the Dif and Frz pathways are both involved in the chemotactic response towards certain PE molecules such that the Dif pathway is required for excitation and while the Frz system is essential for adaptation. In addition, 12:0 PE, known to be sensed by DifA, results in increased FrzCD methylation. These findings suggested that in the regulation of PE response, two pathways communicate with each other to mediate adaptation. Here we provided evidence to indicate that DifA does not undergo methylation during EPS regulation and PE chemotaxis. On the other hand, using mutants expressing the NarX-DifA chimera, it was found that signal transduction through DifA, DifC (CheW-like) and DifE (CheA-like) modulates FrzCD methylation. Surprisingly, the attractant 12:0 PE can modulate FrzCD methylation in two ways distinguishable by the dependency on DifA, DifC and DifE. The

DifACE-independent mechanism, which may result from specific sensing of 12:0 PE by FrzCD, increases FrzCD methylation as expected. Unexpectedly, 12:0 PE decreases FrzCD methylation with the DifACE-dependent mechanism. This “opposite” FrzCD methylation by DifACE-dependent signaling was supported by results from NafA-expressing mutants because nitrate, which acts as a repellent, increases FrzCD methylation. Based on these findings, we proposed a model for chemotaxis toward 12:0 PE (and 16:1 PE). In this model, DifA and FrzCD both sense the same signal and activate the pathways of excitation (Dif) and adaptation (Frz) independently. The two pathways communicate with each other via methylation crosstalk between DifACE and FrzCD in such a way that processes of excitation and adaptation can be coordinated.

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CHAPTER 1

Introduction and Literature Review

1. The general features of *Myxococcus xanthus*

1-1. Life cycle

Myxococcus xanthus is the model species of the Gram-negative myxobacteria that are characterized by "social behaviors" (Dworkin, 1996; Shimkets, 1999). In its vegetative stage, *M. xanthus* utilizes decaying organic matter or hunts other bacterial species (Shimkets, 1990; Reichenbach, 1999) to obtain amino acids and peptides but not carbohydrates (Dworkin, 1962; Watson and Dworkin, 1968; Bretscher and Kaiser, 1978). To search for food, *M. xanthus* cells typically organize into coordinated swarms so that numerous individuals collectively produce antibiotics and digestive enzymes to facilitate efficient feeding (Rosenberg *et al.*, 1977). During hunting, contact with prey cells elicits a distinct rippling response by numerous *M. xanthus* cells (Reichenbach, 1999; Berleman *et al.*, 2006). It was proposed that rippling, occurring underneath the prey cells, effectively "grinds" the prey cell layers above so that nutrients are effectively released and utilized (Berleman *et al.*, 2006). The intensive intercellular coordination is perhaps one of the important reasons that *M. xanthus* and other myxobacteria are successful colonizers, as exemplified by their uqibitous existence from the South Pole to the tropics as well as their high levels of biomass in cultivated land where there are millions of myxobacterial cells per gram of soil (Reichenbach, 1999).

The wide distribution of myxobacteria also correlates with their environmental adaptability. Most known myxobacteria isolated from soil grow well at 30 ˚C (Reichenbach, 1999), yet some strains are able to grow at 4-6 ˚C (Zhukova, 1963). In the field, myxobacteria have been isolated from the northern tip of the North American continent (Brockman and Boyd, 1963). Although all known myxobacteria are strictly aerobic, they can pass through the mammalian digestive tract, which may help their distribution (Reichenbach, 1999). Finally,

myxobacteria favor neutral to slightly alkaline conditions, but they can be found in highly acidic environments (Reichenbach, 1999).

M. xanthus and other myxobacteria have also evolved a "social" mechanism to cope with the lack of food, which is a perpetual challenge in natural environments. When nutrients are insufficient, up to 10^5 cells aggregate toward a center and eventually build a multicellular dome-structured fruiting body that is visible to the naked eye. Fruiting body formation is a delicately controlled process that requires several days to complete (Shimkets, 1990; Dworkin, 1996). Within fruiting bodies, only 10-20% of the original population of cells differentiate into metabolically quiescent and stress-resistant myxospores (Wireman and Dworkin, 1975), which can germinate once nutrients are available. Although it has been assumed that fruiting body formation is a response specifically to low nutrient level, a recent study revealed an alternative mechanism in which development is also stimulated by the interactions between *M. xanthus* and its prey as co-inoculation of *M. xanthus* with suitable prey enables fruiting body formation even on rich medium (Berleman and Kirby, 2007). Despite the uncertainties regarding the initiation of development, it is clear that the formation of fruiting bodies and myxospores ensures the survival of myxobacteria under environmental stresses.

1-2. Motility

The display of *M. xanthus* social behaviors requires movement (Ward and Zusman, 1999; Kaplan, 2003). As an unflagellated species, *M. xanthus* is only motile along its long axis and on solid surfaces via a mechanism collectively referred to as gliding. *M. xanthus* glides at a rate of only 2-4 μm per minute, or approximately 500 times slower than the swimming speed of *E. coli* (25 μm per second). During movement, *M. xanthus* cells periodically stop and reverse directions. Although less frequently, they can also resume

gliding in the same direction after a pause. Wild-type *M. xanthus* cells reverse gliding direction every 6-8 min on average. The reversal frequency is affected by environmental stimuli, such as attractants and repellents, as well as by cell density (Dworkin, 1983; McBride *et al.*, 1992; Shi *et al.*, 1996; Kearns and Shimkets, 1998). The regulation of reversal frequency underlies the directionality of *M. xanthus* gliding motility on a two-dimensional surface.

M. xanthus gliding is controlled by two distinct and synergistic systems: the adventurous (A-) and the social (S-) motility systems (Hodgkin and Kaiser, 1979b). A-motility powers the movement of isolated cells and small cell groups, while S-motility propels the translocation of large cell groups. Dual motility systems are also present in some flagellated bacteria to facilitate locomotion under different conditions. For example, *Vibrio*, *Proteus* and *Serratia* use swimming in liquid and swarming on agar surfaces (Shapiro, 1998). Likewise for *M. xanthus*, A-motility functions better on a relatively firm and dry surface (e.g., 1.5 % agar), while the S-engine is more efficient on relatively a soft and wet surface (e.g., 0.4 % agar) (Shi and Zusman, 1993). As such, the dual motility systems enable *M. xanthus* to move effectively over a wide range of surfaces.

Three types of cell surface structures have been implicated in *M. xanthus* S-motility, type IV pili (TFP) (Kaiser, 1979; Wu and Kaiser, 1995), exopolysaccharide (EPS) (Shimkets, 1986; Yang *et al.*, 2000; Bellenger *et al.*, 2002; Black and Yang, 2004) and lipopolysaccharide (LPS) (Bowden and Kaplan, 1998). TFP, mainly protein filaments composed of pilin or PilA, are typically 5–7 nm in diameter and several micrometers in length. This polarly localized structure has been directly observed to power the twitching motility of *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa*, presumably by cycles of TFP extension, binding on the substratum and retraction (Merz *et al.*, 2000; Skerker and Berg, 2001). Pilus retraction is likely mediated by the PilT protein (Kaiser, 2000). The TFP of *M.*

xanthus appears to function in a similar fashion (Sun *et al.*, 2000), but requires EPS (Behmlander and Dworkin, 1994a) on the surface of a neighboring cell (Li *et al.*, 2003). This explains why S-motility requires cell-cell contact and is active only when cells are in close proximity. Currently, the involvement of LPS in S-motility of *M. xanthus* is not fully understood.

In comparison, the mechanism underlying A-motility is less certain and even controversial. Myxobacteria and other gliding bacteria such as Cyanobacteria, *Cytophaga* and *Flexibacter* leave trails of slime on solid surfaces (Spormann, 1999). The release of a slime trail was hypothesized to propel myxobacteria a long time ago (Kuhlwein, 1953). However, there has not been any direct evidence to link slime secretion to motility until a few years ago. It was found that a nozzle-like structure in cyanobacteria releases slime in a rate matching the speed of bacterial movement (Hoiczyk and Baumeister, 1998; Hoiczyk, 2000). Similar nozzle structures were also found at the cell poles of *M. xanthus*, implying a similar mechanism for A-motility (Wolgemuth *et al.*, 2002). A later study showed that some A-motility mutants apparently secreted slime from both cell poles, suggesting that the stalemate of A-motility resulted from counteracting forces (Yu and Kaiser, 2007). However, if slime secretion indeed generates propulsion, the competing forces at both cell poles would enable mutant cells to exhibit somewhat jerky motion unless the force generated at both cell poles is absolutely equal at any moment (Mignot, 2007). Alternatively, because *M. xanthus* cells frequently bend and make turns during their movement, propulsion at both cell poles should increase bending of cell bodies. Yet, the mutants that secreted slime from both cell poles did not appear to exhibit such consequence (Yu and Kaiser, 2007). In addition, the slime secretion model would place the motor at the cell's posterior. Correspondingly, the speed of A-motility would be reduced if cell length increases. Nevertheless, the velocity of A-motility was not significantly affected when cells were artificially elongated up to ten times with the antibiotic

Cephalexin (Sun *et al.*, 1999). This suggested that the A-engine is evenly distributed along the cell body rather than localized at cell poles. Such a hypothesis of A-engine distribution was directly demonstrated by a later study (Sliusarenko *et al.*, 2007). Explorations of the distribution of motors suggested that slime secretion might not be the A-motility engine. Rather, it is more plausible that the secretion of those hydrated polymers could play a passive role in motility, such as a lubricant to enhance cell gliding (Mignot, 2007).

Instead, an alternative model proposes that A-motility is powered by an intracellular motor complex that pushes against the substratum to move the cell body forward (Mignot *et al.*, 2007). A crucial component of this complex is possibly AglZ, which is essential for A-motility. This protein has a N-terminal receiver domain and the C-terminal heptad repeats of a coiled-coil structure (Yang *et al.*, 2004). With the aid of a fluorescence fusion protein, AglZ was found to be distributed at several positions along the cell body (Mignot *et al.*, 2007). As the cell moves, AglZ-YFP clusters stay at fixed positions with respect to the substrate, instead of with the moving cell body. In addition, AglZ-YFP clusters assemble at the new leading parts of cell body and dissemble at the lagging end in a rhythm matching cell reversal (Mignot *et al.*, 2007). These observations supported that the A-engine might share functional similarities with the focal adhesion–based motility complexes found in eukaryotic organisms (Wozniak *et al.*, 2004; Baum *et al.*, 2006).

Analysis of individual cell behavior and colony expansion suggested that the propulsion by A- and S-engines is generated in the same direction (Kaiser and Crosby, 1983; Spormann, 1999). As cells reverse, the directionality of the two engines switches synchronously (Kaiser and Crosby, 1983; Blackhart and Zusman, 1985). How is the coordination of two distinct motility systems achieved? It has been shown as the S-engine reverses, FrzS, which is a required component for S-motility and has a receiver domain in the N-terminus, oscillates between cell poles accordingly (Mignot *et al.*, 2005). Similarly, as the A-engine switches

direction, RomR, a response regulator that is essential for A-motility, assembles to the new lagging pole in a rhythm matching cell reversal (Leonardy *et al.*, 2007). In addition, MglA, a 22 kDa protein that shares sequence similarity with certain eukaryotic GTPases (Hartzell, 1997), is required for both A- and S-motility systems (Hodgkin and Kaiser, 1977; Stephens *et al.*, 1989). Interestingly, MglA interacts with AglZ (Yang *et al.*, 2004) and is required for dynamic localization of RomR (Leonardy *et al.*, 2007), suggesting that MglA is part of a master regulatory system to coordinate the directionality of A- and S-engines.

1-3. Developmental signaling

The multicellular development of *M. xanthus* requires the delicate control of cell motility as well as cell differentiation over the course of several days (Shimkets, 1990; Dworkin and Kaiser, 1993; Curtis *et al.*, 2007). Not surprisingly, intensive signaling is involved in orchestrating the behavior of developmental cells (Shimkets, 1999; Kaiser, 2004). Guanosine-5'-(tri)di-3'-diphosphate [(p)ppGpp], a common intracellular signal for stringent or starvation conditions among bacteria (Cashel and Rudd, 1987; Chatterji and Kumar Ojha, 2001), is likely the signal for initiating *M. xanthus* development. This is evident by the correlation of (p)ppGpp accumulation and developmental induction, as well as by its ability to initiate developmental gene expression (Manoil and Kaiser, 1980a, b; Singer and Kaiser, 1995; Harris *et al.*, 1998; Diodati *et al.*, 2006; Ossa *et al.*, 2007). The level of ppGpp is influenced by several proteins including RelA, SocE, CsgA and Nla4 (Singer and Kaiser, 1995; Crawford and Shimkets, 2000a, b; Ossa *et al.*, 2007). RelA is the putative pppGpp synthetase I enzyme (Singer and Kaiser, 1995), while SocE, CsgA and Nla4 appear to regulate the (p)ppGpp levels. Recent studies suggest that SocE inhibits (p)ppGpp production when nutrients are plentiful. Conversely, CsgA and Nla4 are required for maintaining relatively high levels of (p)ppGpp under starvation conditions. SocE is highly basic and

possibly a soluble protein (Crawford and Shimkets, 2000b). CsgA is homologous to short-chain alcohol dehydrogenases (Lee and Shimkets, 1994). Nla4 belongs to a family of enhancer binding proteins that are often components in signal transduction pathways (Xu and Hoover, 2001). It is clear that the (p)ppGpp levels must be elaborately controlled by multiple factors and systems so that the development can properly progress.

As development starts, *M. xanthus* cells produce several sets of intercellular signals. Five groups of extracellular developmental signals, controlled by the loci of *asg*, *bsg*, *csg*, *dsg* and *esg* (Downard *et al.*, 1993; Ellehauge *et al.*, 1998), were identified using extracellular complementation on different mutant groups (e.g., a mutant forms fruiting bodies only when mixed with cells of wild type or different mutant groups) (McVittie *et al.*, 1962; Hagen *et al.*, 1978). Obviously, these mutants are only defective in producing certain signals but are still capable of responding to them.

A-signal is composed of small peptides and amino acids that result from proteolysis (Kuspa *et al.*, 1992a; Plamann *et al.*, 1992). A-signal appears to function at a concentration between 10 μM and 10 mM (Kaplan and Plamann, 1996). The most potent A-effectors are tyrosine, proline, tryptophan, phenylalanine, leucine and isoleucine. A-signaling possibly serves for quorum-sensing to initiate early developmental gene expression when the cell density reaches a sufficient level (Kuspa *et al.*, 1992b; Kaplan and Plamann, 1996; Kaplan, 2003). The generation of A-signal requires AsgA and AsgD, both containing a response regulator domain and a histidine kinase domain (Plamann *et al.*, 1995; Cho and Zusman, 1999). In addition, a DNA-binding protein AsgB (Plamann *et al.*, 1994) and a sigma factor RpoD (Davis *et al.*, 1995) are also involved. The Asg signaling pathway likely integrates the signals of starvation and cell density and results in production of certain proteinases, which degrade surface proteins of *M. xanthus* cells to constituent amino acids and peptides (Kaplan and Plamann, 1996).

C-signaling is mainly mediated by CsgA (Shimkets, 1999; Kaiser, 2004), which is also a major modulator of (p)ppGpp levels as stated earlier. CsgA is a 17 kDa protein maturated from its 25 kDa precursor after an enzymatic cleavage (Kim and Kaiser, 1990a; Lobedanz and Sogaard-Andersen, 2003). C-signaling requires cell-cell contacts (Kim and Kaiser, 1990b) and functions in a concentration-dependent manner (Shimkets, 1999). At low concentrations, CsgA modulates cell reversal frequency to regulate directional movement and developmental rippling (Sager and Kaiser, 1994). High concentrations of CsgA induce the expression of late developmental genes and the differentiation of vegetative cells into dormant myxospores (Kim and Kaiser, 1990a; Li *et al.*, 1992). C-signaling presumably regulates several downstream components (Bonner and Shimkets, 2001), including FruA (Ogawa *et al.*, 1996), DevRS (Thony-Meyer and Kaiser, 1993) and the Frz chemosensory pathway (Blackhart and Zusman, 1985). FruA is a response regulator (Ogawa *et al.*, 1996; Ellehauge *et al.*, 1998) which, upon activation by C-signal, modulates the Frz pathway (Sogaard-Andersen and Kaiser, 1996) and the transcription of the sporulation locus *devRS* (Ellehauge *et al.*, 1998).

The *esg* locus encodes two subunits of the branched-chain keto acid dehydrogenase (Toal *et al.*, 1995), an enzyme crucial for the synthesis of branched-chain fatty acids. The *esg*-encoded enzyme is likely involved in the synthesis of certain lipids, which might serve as intercellular signals for development (Toal *et al.*, 1995). This hypothesis is in agreement with the findings that several phosphatidylethanolamine (PE) species elicit chemotactic responses from *M. xanthus*, and that the responses to 12:0 PE and 16:1 PE are the most pronounced under starvation conditions (Kearns and Shimkets, 2001; Bonner and Shimkets, 2006).

The remaining two signals, defined by *bsgA* and *dsgA* mutants, are still poorly understood. BsgA is homologous to the *E. coli* Lon protein, an ATP-dependent protease (Gill *et al.*, 1993), while *dsgA* encodes the translation initiation factor IF3 (Cheng *et al.*, 1994). It is unclear how these two signals function in developmental signaling.

1-4. Genomic features of *M. xanthus* and other myxobacteria

According to the sequences of 16S ribosomal RNA, myxobacteria constitute a coherent group within the δ subgroup of proteobacteria, which also includes the sulfur- and sulfate-reducing bacteria and the bdellovibrios (Shimkets and Woese, 1992). The genomes of two myxobacteria, *M. xanthus* and *Sorangium cellulosum*, were recently sequenced (Goldman *et al.*, 2006; Schneiker *et al.*, 2007). The genomic comparison between these two organisms reveals some interesting facts. Although these two myxobacteria share many morphological and behavorial similarities, *M. xanthus* and *S. cellulosum* are quite different genomically. *M. xanthus* has a genome of 9.14 Mb, which is similar to that of some other myxobacteria, e.g., *Stigmatella aurantica* and *S. erecta* (Shimkets, 1993). Surprisingly, the *S. cellulosum* genome is 13.03 Mb, considerably larger than that of *M. xanthus*. In fact, *S. cellulosum* has the largest genome among sequenced bacteria (Schneiker *et al.*, 2007). In addition, only 30.5% of the predicted protein coding sequences of *S. cellulosum* are homologous to their *M. xanthus* counterparts. More importantly, the gene arrangement of these two myxobacteria shares no apparent global similarity, indicating a high level of evolutionary divergence between the two species. These differences or divergence probably partially reflect the metabolic characteristics in their vegetative stages: *M. xanthus* actively hunts other bacteria, while *S. cellulosum* mainly relies on degrading complex plant materials. In addition, whereas *M. xanthus* cannot make branched-chain amino acids, *S. cellulosum* can grow on minimal medium, indicating its capacity to synthesize all amino acids and vitamins (Gerth *et al.*, 2003). Despite the high level of global difference between the genomes of these two myxobacteria, the overall distribution of genes dedicated to specific functions is similar between *M. xanthus* and *S. cellulosum*, suggesting that certain groups of genes are conserved among myxobacteria (Schneiker *et al.*, 2007).

Interestingly, myxobacteria exhibit several genetic features of eukaryotes, in particular, the eukaryotic-like serine/threonine/tyrosine protein kinases (ELK) (Kim and Kaiser, 1991; Goldman *et al.*, 2006). For example, *M. xanthus* has 102 ELKs (http://tigrblast.tigr.org) while *S. cellulosum* has 317 (Schneiker *et al.*, 2007). These eukaryotic genetic features have increasingly been demonstrated to play important roles in regulating the complex social life of *M. xanthus* (Nariya and Inouye, 2002; Nariya and Inouye, 2005; Nariya and Inouye, 2006).

2. Classical chemotaxis pathways and homologous systems in *M. xanthus*

2-1. Chemotactic behavioral response

Chemotaxis, the biased movement toward attractants or away from repellents, is the best-understood mechanism of motility regulation in prokaryotic species (Falke *et al.*, 1997; Szurmant and Ordal, 2004). In a homogenous liquid environment, bacterial cells alternatively display two types of swimming behaviors: straight run and tumble. A run facilitates a cell to move a certain distance whereas a tumble results in no net translocation and a new direction for the following run. Tumble frequency is influenced by chemoeffectors through chemosensory signaling. An attractant suppresses tumbling so it extends the average run of swimming cells. Conversely, repellent increases tumble frequency so that cells are more likely to change directions. The initial change of tumble frequency, or excitation, is followed by adaptation, which restores tumble frequency to the pre-stimulus or pre-excitation level. This adaptation process provides a "memory" that helps the cell to determine whether higher or lower concentrations of a stimulus are being reached. The sequential excution of exitation and adaptation enables swimming bacteria to respond to a chemical gradient and to chemotax towards favorable environments.

2-2. *E. coli* chemotaxis pathway

A chemotaxis pathway functions as a two-component system, which provides the predominant signal transduction mechanism in prokaryotes (Stock *et al.*, 2000). The classical two-component system is composed of a transmembrane sensor kinase and a cytoplasmic response regulator. The sensing of an environmental signal by the sensor kinase at the periplasm results in autophosphorylation on a conserved histidine residue. The phosphate is then transferred to an aspartate in the response regulator, which in turn generates a response. Typically, such a signaling event results in the up- or down-regulation of specific genes (Stock *et al.*, 2000). Chemotaxis pathways have undergone considerable changes in order to direct motility in response to chemical gradients. Nevertheless, some basic signaling features of the two-component prototype, such as transmembrane signaling (Utsumi *et al.*, 1989; Ward *et al.*, 2002) and a phospho-relay (Falke *et al.*, 1997), are still conserved in chemotaxis regulation.

Chemotaxis has been well studied in *E. coli*, whose chemosensory pathway contains methyl-accepting chemotaxis proteins (MCPs), CheW, CheA, CheY, CheB, CheY and CheZ (Fig. 1-1) (Falke *et al.*, 1997). MCPs are typically transmembrane sensors and are composed of highly conserved signaling domains and distinct periplasmic domains that are responsible for sensing signals. A chemosensory pathway typically contains multiple MCPs, which enable the system to integrate different signals to make a general directional decision (Falke *et al.*, 1997; Szurmant and Ordal, 2004). The enteric system contains six MCPs including Tar, Tsr, Trg, Tap, Tcp and Aer, each of which is responsible for sensing several specific stimuli. The signal sensing typically occurs at the periplasmic domain and leads to conformational changes that are transmitted across the cell membrane to the cytoplasmic histidine kinase CheA, which is anchored to MCPs via the scaffold protein CheW (Fig. 1-1). The phosphate group is subsequently relayed to the response regulator CheY to increase its binding affinity

toward the motor complex. Motor binding by CheY increases the probability of clockwise motor rotation, which leads to tumbling. In general, chemotactic bacteria require fast responses (on a timescale of seconds) to cope with ever changing environments (Szurmant and Ordal, 2004). The phosphatase CheZ accelerates the low hydrolysis rate of CheY-P and helps to maintain the chemotactic robustness of the system. As a consequence of this signaling process, an attractant suppresses the phosphorylation of CheA and CheY and decreases the likelihood of tumbling, while a repellent does the opposite.

Adaptation typically occurs seconds after excitation and is mainly achieved through MCP methylation that occurs on specific glutamate residues in the methylation domains (Falke *et al.*, 1997; Szurmant and Ordal, 2004). Such covalent modification presumably causes a conformational change in the MCP signaling domain that attenuates the stimulus-induced changes in CheA kinase activity (Levit *et al.*, 1998). MCP methylation, which stimulates CheA kinase, is catalyzed by the methyltransferase CheR. The methylesterase CheB removes methyl groups from MCPs. CheB is also a response regulator that is phosphorylated by CheA, thus providing a feedback control of this adaptation system. For example, repellent binding by MCP stimulates synthesis of CheA-P, which correspondingly increases CheB-P levels. Increased CheB-P leads to increased methylesterase activity and MCP demethylation to suppress CheA autophosphorylation.

2-3. Chemosensory diversity in prokaryotes

Chemosensory pathways have been found in many species of eubacteria and archea (Szurmant and Ordal, 2004; Zusman *et al.*, 2007), but variations exist among these systems. In general, the signaling in the excitation circuit (MCP, CheW, CheA and CheY) is quite similar among various prokaryotes, while there are enormous differences in other aspects such as signal removal and adaptation (Szurmant and Ordal, 2004). For example, CheZ

appears to only be present in proteobacterial species including *E. coli*; CheV and CheC are not found in *E. coli* and have been demonstrated to mediate methylation-independent adaptation in the Gram-positive bacterium *Bacillus subtilis* (Szurmant and Ordal, 2004). CheV contains a CheW domain fused with a response regulator domain and can function similarly as CheW to connect MCPs with CheA. The phosphorylated CheV appears to re-adjust the interaction of MCP with CheA to bring about adaptation (Karatan *et al.*, 2001). In *Helicobacter pylori*, CheV is perhaps the only adaptation mechanism since this bacterium lacks CheR and CheB (Pittman *et al.*, 2001). Like CheB, CheV is the adaptation modulator accepting phosphate from CheA. Interestingly, the phosphatase CheC appears to be modulated by its substrate CheY-P to generate adaptation by influencing the CheD-MCP interaction that is essential for CheA activation in *B. subtilis* (Muff and Ordal, 2007). These differences in signal removal and adaptation underscore the chemosensory diversity among swimming bacteria.

In addition to directing movement, chemosensory pathways have also been adapted to regulate non-tactic functions, such as pilus production (Bhaya *et al.*, 2001), flagellum biosynthesis (Berleman and Bauer, 2005a), cyst cell development (Berleman and Bauer, 2005b) and swarming cell differentiation (Burkart *et al.*, 1998).

Chemosensory systems have also been discovered in unflagellated gliding bacteria including cyanobacteria and *M. xanthus* (Zusman *et al.*, 2007). Three chemosensory pathways are all required for the TFP-dependent phototactic response in cyanobacteria *Synechocystis* sp. strain PCC6803 (Bhaya, 2004). Interestingly, *M. xanthus* possesses eight chemosensory pathways (Zusman *et al.*, 2007). This is perhaps consistent with the hypothesis that this bacterium has experienced extensive gene duplication (Goldman *et al.*, 2006). These *M. xanthus* chemosensory pathways have been shown to regulate diverse processes including reversal frequency of gliding motility (Frz), EPS production (Dif), lipid chemotaxis (Dif and

Frz), developmental gene expression (Che3), coordination of reversal frequency and velocity (Che4), type IV pilus assembly (Che6) and fatty acid composition (Che7). Currently, the Dif and Frz pathways are two of the best-studied chemosensory systems in *M. xanthus* (Zusman *et al.*, 2007).

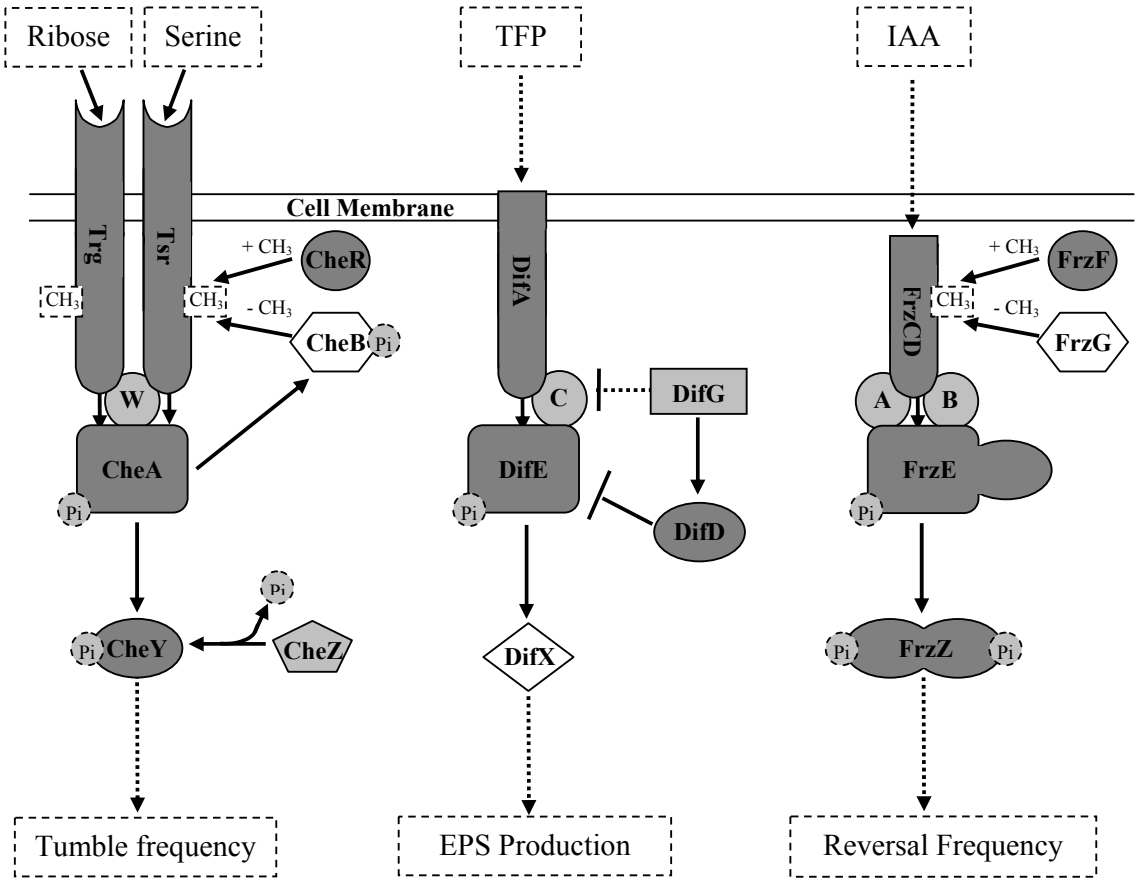

Figure 1-1. A comparison of the *E. coli* chemotaxis pathway with the *M. xanthus* Dif and Frz chemosensory systems. For simplicity, only two of the *E. coli* MCPs, Trg and Tsr, are shown in the diagram. Likewise, only one signal is shown for each MCP although it may sense other signals. DifA and FrzCD are homologs of MCP but with unconventional structures. Otherwise, the proteins of identical shapes and colors indicate homology. For example, DifD is a CheY homolog whereas DifC, FrzA and FrzB are all homologous to CheW. FrzE is a CheA-CheY fusion protein while FrzZ is composed of two CheY domains. Arrows and bars represent stimulatory and inhibitory effects, respectively. DifX is a hypothetical protein that is downstream of DifE in the regulation of EPS production. Solid lines illustrate direct interactions while dashed lines indicate indirect or hypothetical interactions. The Dif and Frz pathways are both involved in PE chemotaxis, but how they interact with each other remains unkown. The phosphorylation of DifE is hypothetical. TFP: type IV pilus. IAA: isoamyl alcohol.

3. *M. xanthus* Dif and Frz pathways

3-1. EPS regulation by the Dif pathway

The *dif* locus encodes six proteins, DifA, DifB, DifC, DifD, DifE and DifG. All except DifB are homologous to known chemosensory proteins (Fig. 1-1) (Yang *et al.*, 1998b). The Dif pathway regulates the production of EPS (Yang *et al.*, 1998b; Bellenger *et al.*, 2002; Black and Yang, 2004), a surface component essential for S-motility and development. DifA, DifC and DifE, homologs of MCP, CheW and CheA, are essential for EPS production (Yang *et al.*, 1998b; Bellenger *et al.*, 2002; Black and Yang, 2004). They likely form a transmembrane ternary complex similar to that of MCP, CheW and CheA in the *E. coli* chemotaxis pathway (Fig. 1-1) (Yang and Li, 2005). Type IV pili (TFP), the motor for S-motility (Wall and Kaiser, 1999; Mattick, 2002), appears to mediate the signal into the DifACE complex for activating EPS production (Black *et al.*, 2006). The signal perception by DifA presumably modulates the autophorylation of DifE. However, DifD, the CheY homolog, is not downstream of DifE in EPS regulation (Black and Yang, 2004). A hypothetical protein DifX was proposed to function downstream of DifE to mediate EPS output (Fig. 1-1) (Black and Yang, 2004). DifD may instead function as a phosphate sink to divert the phosphate flow from the central pathway and suppress the signaling output (Black and Yang, 2004), as does CheY1 of the *Sinorhizobium meliloti* chemotaxis system (Schmitt, 2002). DifG, a CheC homolog, is another negative regulator of EPS production. DifG likely interacts with DifD to influence the output (Yang and Li, 2005), and the possibility of DifG directly interacting with the DifACE complex has not been ruled out (Black and Yang, unpublished). An unusual feature of the Dif pathway is the lack of CheB and CheR homologs (Fig. 1-1), which are almost universal in chemosensory pathways (Zusman *et al.*, 2007). This might correlate with the fact that EPS production is not a tactic response, therefore adaptation might not be needed in its regulation.

The specific signal(s) that activates EPS production has not been explicitly proven. Several studies indicated that at low cell density, starvation and intercellular proximity might stimulate EPS production (Shimkets, 1986; Behmlander and Dworkin, 1991). It is perhaps plausible that *M. xanthus* cells do not synthesize the motility component EPS if there are abundant nutrients because cells do not need to move to search for food, as supported by suppression of *M. xanthus* motility on agar containing a rich medium (Shi *et al.*, 1993). However, lack of nutrients may change the sedentary vegetative state of *M. xanthus* cells. For example, starvation triggers fruiting body formation while insufficient nutrients may force cells to find a better food source. EPS is apparently essential for development and beneficial for movement since the S-engine requires this surface component. In addition, only cells at a high enough density can undergo development or perform S-motility, which explains the necessity of neighboring cells for the production of EPS. Nevertheless, how these signals (starvation and intercellular proximity) are processed remains unkown.

3-2. The regulation of reversal frequency by the Frz pathway

The Frz pathway controls reversal frequency (Blackhart and Zusman, 1985), which enables the surface-gliding *M. xanthus* to control direction. The *frz* null mutants barely reverse and form "frizzy" filaments under developmental starvation. The Frz pathway is composed of seven proteins, FrzCD (MCP), FrzA (CheW), FrzB (CheW), FrzE (CheA-CheY fusion), FrzF (CheR), FrzG (CheB) and FrzZ (CheY-CheY fusion) (Fig. 1-1). Like MCP proteins in swimming bacteria (Falke *et al.*, 1997; Szurmant and Ordal, 2004), FrzCD exhibits methylation changes upon stimulation (McBride *et al.*, 1992; Shi *et al.*, 1993). Small alcohols, chloroform and dimethyl sulfoxide (DMSO) cause demethylation of FrzCD, while complex media ingredients such as yeast extract and casitone increase FrzCD methylation (McBride *et al.*, 1992; Shi *et al.*, 1993). In addition, *M. xanthus* colonies have been shown to

migrate towards nutrients and away from isoamyl alcohol (IAA) or DMSO (Shi *et al.*, 1993). The tactic behavior of *M. xanthus*, mediated by the Frz pathway, correlates with the change of reversal frequency since reversal is stimulated by repellents (IAA and DMSO) and suppressed by attractants (nutrients) (McBride *et al.*, 1992; Shi *et al.*, 1993). Thus, the functionality of the Frz pathway is very similar to the generic chemotaxis systems found in swimming bacteria (Falke *et al.*, 1997; Szurmant and Ordal, 2004).

Based on the phenotypes of deletion mutants in vegetative swarming, development and the repellent response, FrzCD, FrzA and the CheA domain of FrzE likely form a core signaling complex (Bustamante *et al.*, 2004). Strikingly, FrzCD, a soluble transducer that lacks transmembrane and perisplasmic domains (Fig. 1-1), senses a signal using its C-terminal signaling domains (Bustamante *et al.*, 2004). As expected, the CheR homolog FrzF methylates FrzCD (McBride *et al.*, 1992) and is required for fruiting body formation and normal vegetative swarming (McBride *et al.*, 1992). Although FrzG may demethylate FrzCD, *frzG* mutants display a similar pattern of FrzCD methylation as the wild type (McBride *et al.*, 1992), and exhibit an intermediate phenotype in vegetative swarming (Bustamante *et al.*, 2004). This suggested that unlike *E. coli* CheB, FrzG does not form a negative feedback loop with the core Frz components. The signaling output of the Frz pathway is mediated by FrzZ (Inclan *et al.*, 2007). The conserved aspartate residues (D52 and D220) in both CheY-like domains of FrzZ accept phosphate groups from the CheA domain of FrzE (Fig. 1-1). When both D52 and D220 were changed to glutamate, FrzZ phosphorylation was abolished and the double mutant exhibited the *frz* null phenotype. Thus, the signaling of the Frz chemosensory pathway has apparent differences in comparison with that of chemotaxis pathways in swimming bacteria (Falke *et al.*, 1997; Szurmant and Ordal, 2004).

3-3. Co-regulation of PE chemotaxis by the Dif and Frz pathways

It has been argued that with a velocity slower than the diffusion rate of small chemicals, *M. xanthus* is unlikely chemotactic (Dworkin, 1983; Dworkin and Eide, 1983). In the backdrop of such debate, the identification of several species of water-insoluble phosphatidylethanolamine (PE) [dilauroyl (12:0) PE, 16:1 PE and dioleoyl (18:1) PE] as *M. xanthus* chemoattractants (Kearns and Shimkets, 1998; Kearns *et al.*, 2000; Bonner *et al.*, 2005) provides great insight into the motility regulation of this gliding bacterium. 16:1 PE, a characteristic constituent of the *M. xanthus* cell membrane (Curtis *et al.*, 2006), is proposed to serve as a self-recognition signal. 18:1 PE, a common membrane component of other soil-dwelling bacteria, but not *M. xanthus*, could be a signal for hunting (Kearns *et al.*, 2000). *M. xanthus* cells exhibit biased movement toward PE, which correlates with temporal cell responses: excitation (suppression of reversal frequency) followed by adaptation (restoration of basal reversal frequency) (Kearns and Shimkets, 1998).

Surprisingly, the regulation of PE chemotaxis requires both Dif and Frz chemosensory pathways: Dif is essential for excitation and Frz for adaptation (Kearns and Shimkets, 1998). The signaling of 12:0 PE and 16:1 PE also requires extracellular fibrils (Kearns *et al.*, 2000) that contain equal amount of EPS and protein (Behmlander and Dworkin, 1994a, b). This might explain why the cellular responses to 12:0 PE and 16:1 PE are the most apparent under starvation conditions (Kearns and Shimkets, 1998; Kearns *et al.*, 2001), which up-regulates EPS production (Shimkets, 1986; Behmlander and Dworkin, 1991). 12:0 PE and 16:1 PE are proposed to be first perceived by fibrils, which then deliver the signal to the Dif pathway (Kearns *et al.*, 2000). Signaling of 18:1 PE, on the other hand, requires only DifE, but not DifA or DifC (Bonner *et al.*, 2005), implying another transducer for 18:1 PE chemotaxis. The responses of 12:0 PE, 16:1 PE and 18:1 PE all require the CheY homolog DifD, which is not required for EPS production. Furthermore, both DifB and DifG are not essential for

excitation, but are involved in adaptation to 16:1 PE (likely 12:0 PE as well) (Bonner *et al.*, 2005). Currently, no study has been undertaken to elucidate how the Frz pathway mediates adaptation in PE response, although 12:0 PE was shown to increase FrzCD methylation (McBride *et al.*, 1992).

4. Questions regarding the Dif and Frz chemosensory pathways

We are interested in examining the following questions regarding the signaling of the two chemosensory pathways that are important for the regulation of gliding motility in *M. xanthus*.

First, DifA, the MCP homolog in the Dif pathway, may be responsible for sensing signals (Fig. 1-1). Indeed, DifA contains most of the features of sensory transducers, including two transmembrane helices and a HAMP linker (Yang *et al.*, 1998b). However, it lacks a prominent periplasmic domain. Therefore, does DifA shares similar transmembrane signaling mechanisms that are conserved among classical MCPs and sensor kinases (Ward *et al.*, 2002)?

Second, *M. xanthus* chemotaxis is still controversial. In addition to the problem that *M. xanthus* velocity is slower than the diffusion rate of small molecules, the known chemoeffectors including isoamyl alcohol, chloroform, DMSO, complex media ingredients and PE could affect *M. xanthus* motility through physiological/metabolic changes instead of chemotaxis. Thus, we want to ask: does *M. xanthus* possess motility regulation independent of cell physiology and metabolism?

Lastly, because the Dif and Frz pathways are involved in excitation and adaptation of PE chemotaxis respectively (Kearns and Shimkets, 1998; Kearns *et al.*, 2000; Bonner *et al.*, 2005), the two pathways likely interact with each other during the PE response. The third

question is, where does the putative Dif-Frz crosstalk occur and how does the Frz pathway regulate chemotactic adaptation?

CHAPTER 2

Nitrate-dependent activation of the Dif signaling pathway of *Myxococcus xanthus* mediated by a NarX-DifA interspecies chimera

Xu, Q., Black, W. P., Ward, S. M., and Yang, Z. (2005) Nitrate-dependent activation of the Dif signaling pathway of *Myxococcus xanthus* mediated by a NarX-DifA interspecies chimera. J. Bacteriol. 187: 6410-6418.

Summary

Myxococcus xanthus fibril exopolysaccharide (EPS), essential for the social gliding motility and development of this bacterium, is regulated by the Dif chemotaxis-like pathway. DifA, an MCP homolog, is proposed to mediate signal input to the Dif pathway. However, DifA lacks a prominent periplasmic domain, which in classical chemoreceptors is responsible for signal perception and for initiating transmembrane signaling. To investigate the signaling properties of DifA, we constructed a NarX-DifA (NafA) chimera from the sensory module of *Escherichia coli* NarX and the signaling module of *M. xanthus* DifA. We report here the first functional chimeric signal transducer constructed using genes from organisms in two different phylogenetic subdivisions. When expressed in *M. xanthus*, NafA restored fruiting body formation, EPS production, and S-motility to *difA* mutants in the presence of nitrate. Studies with various double mutants indicate that NafA requires the downstream Dif proteins to function. We propose that signal inputs to the Dif pathway and transmembrane signaling by DifA are essential for the regulation of EPS production in *M. xanthus*. Despite the apparent structural differences, DifA appears to share similar transmembrane signaling mechanisms with enteric sensor kinases and chemoreceptors.

Introduction

Myxococcus xanthus is a Gram-negative bacterium with a multicellular developmental process and distinct motility systems (Dworkin, 1996; Shimkets, 1999). Under nutrient limitation, tens of thousands of *M. xanthus* cells aggregate to form fruiting bodies on solid substrata. Vegetative cells within fruiting bodies eventually differentiate into dormant and stress-resistant myxospores. These spores can germinate and reenter the vegetative cell cycle when conditions become favorable for growth. *M. xanthus* cells move on surfaces by gliding during both developmental aggregation and vegetative growth. The gliding motility of *M. xanthus* is controlled by the adventurous (A) and the social (S) motility systems. A-motility enables the movement of well-isolated cells, and S-motility refers to the movement of large cell groups. S-motility appears more important for *M. xanthus* development, because all known S-motility mutants are defective in fruiting body formation to various extents (Hodgkin and Kaiser, 1979b; MacNeil *et al.*, 1994).

Fibril exopolysaccharides (EPS) (Shimkets, 1986; Weimer *et al.*, 1998; Yang *et al.*, 2000; Bellenger *et al.*, 2002; Black and Yang, 2004) have been demonstrated to be crucial for S-motility. It was proposed that fibril EPS may mediate the retraction of type IV pili (Li *et al.*, 2003), the likely motor for S-motility (Wall and Kaiser, 1999). The regulation of fibril EPS clearly requires multiple genetic loci, including *tgl* (Dana and Shimkets, 1993), *stk* (Dana and Shimkets, 1993; Kim *et al.*, 1999), *sglK* (Weimer *et al.*, 1998; Yang *et al.*, 1998a), *eps* and *eas* (Lu *et al.*, 2005), *nla24* (Lancero *et al.*, 2004) and *dif* (Yang *et al.*, 2000; Bellenger *et al.*, 2002; Black and Yang, 2004). The *dif* locus encodes proteins with extensive homology to bacterial chemotaxis proteins. DifA is homologous to MCP, DifC to CheW, DifD to CheY, DifE to CheA, and DifG to CheC (Yang *et al.*, 1998b; Black and Yang, 2004). Deletion of most *dif* genes results in perturbation of EPS production as well as defects in S-motility and fruiting body formation (Yang *et al.*, 1998b; Yang *et al.*, 2000; Bellenger *et al.*, 2002; Black

and Yang, 2004). The homology suggests that the Dif pathway may function similarly to the bacterial chemotaxis pathways, in which signal perception mediated by the periplasmic domains of classical MCPs modulates the strength of downstream responses (Szurmant and Ordal, 2004; Armitage *et al.*, 2005). It is proposed that in the regulation of EPS production, DifA perceives signals and activates the downstream DifE kinase through the coupling protein DifC (Bellenger *et al.*, 2002; Black and Yang, 2004; Yang and Li, 2005). A recent study shows that DifC can indeed mediate interactions between DifA and DifE to form a ternary signaling complex (Yang and Li, 2005). On the other hand, although DifA is an MCP homolog with two putative transmembrane domains, it lacks an apparent periplasmic domain and is therefore unlikely to be capable of direct ligand-binding as do classical bacterial chemoreceptors (Yang *et al.*, 1998b; Yang and Li, 2005).

In the present study, we used a chimera to investigate the signaling properties of DifA and the Dif pathway. Functional chimeras were constructed previously between different chemoreceptors, between different sensor kinases, and between chemoreceptors and sensor kinases (Krikos *et al.*, 1985; Utsumi *et al.*, 1989; Baumgartner *et al.*, 1994; Feng *et al.*, 1997; Ward *et al.*, 2002; Appleman *et al.*, 2003; Bibikov *et al.*, 2004). The structural basis behind the functionality of these chimeras is that transmembrane signaling mechanisms are well conserved among bacterial MCPs and sensor kinases (Ward *et al.*, 2002). We chose the sensory module of NarX for the construction of a chimera with DifA mainly because nitrate, one of the signals for NarX, had no obvious effect on growth and development of wild-type *M. xanthus* at concentrations up to 1 mM (data not shown), which is sufficient for maximum NarX activation (Lee *et al.*, 1999). We show here that the NarX-DifA (NafA) chimera, despite being a cross-species hybrid protein, is able to activate the *M. xanthus* Dif pathway. When expressed at levels comparable to DifA expression in wild type, NafA restored fruiting body formation, EPS production and S-motility to *difA* mutants in the presence of nitrate;

without nitrate, NafA failed to complement *difA* deletions. Examination of *nafA* in Δ*difA* Δ*difC*, Δ*difA* Δ*difD*, Δ*difA* Δ*difE* and Δ*difA* Δ*difG* double deletion backgrounds indicates that the NafA chimera signals through the Dif pathway in response to nitrate. The results suggest that N-terminus of DifA apparently mediates signal perception, and the C-terminus is sufficient for interactions with the downstream components of the pathway in EPS regulation. This is in contrast with FrzCD whose N-terminus is not required for signal perception in chemotactic responses (Bustamante *et al.*, 2004). The functionality of NafA in *M. xanthus* also implies that DifA likely shares similar transmembrane signaling mechanisms with other bacterial chemoreceptors and sensor kinases (Draheim *et al.*, 2005).

Table 2-1. *M. xanthus* strains and plasmids used in this study.

Designation	Genotype or description	Source or reference
Strains		
DK1622	Wild type	(Kaiser, 1979)
SW403	Δ*difC*	(Bellenger *et al.*, 2002)
YZ601	Δ*difA*	This study
YZ603	Δ*difE*	(Black and Yang, 2004)
YZ604	Δ*difG*	(Black and Yang, 2004)
YZ613	Δ*difD*	(Black and Yang, 2004)
YZ720	Δ*difA* Δ*difC*	This study
YZ653	Δ*difA* Δ*difD*	This study
YZ719	Δ*difA* Δ*difE*	This study
YZ654	Δ*difA* Δ*difG*	This study
YZ716	Δ*difA*/P*tar-nafA*; Kan^r	This study
YZ724	Δ*difA*/P*dif-nafA*; Kan^r	This study
YZ722	Δ*difA* Δ*difC*/P*tar-nafA*; Kan^r	This study
YZ659	Δ*difA* Δ*dif*D/P*tar-nafA*; Kan^r	This study
YZ721	Δ*difA* Δ*difE*/P*tar-nafA*; Kan^r	This study
YZ660	Δ*difA* Δ*difG*/P*tar-nafA*; Kan^r	This study
YZ730	Δ*difA* Δ*difC*/P*dif-nafA*; Kan^r	This study
YZ731	Δ*difA* Δ*dif*D/P*dif-nafA*; Kan^r	This study
YZ732	Δ*difA* Δ*difE*/P*dif-nafA*; Kan^r	This study
YZ733	Δ*difA* Δ*difG*/P*dif-nafA*; Kan^r	This study
YZ735	*aglU*; Tet^r	This study
YZ736	Δ*difA aglU*; Tet^r	This study
YZ738	Δ*difA aglU*/P*dif-nafA*; Kan^r; Tet^r	This study
Plasmids		
pZErO-2	Cloning vector with *ccdB* for lethal selection; Kan^r	Invitrogen
pYC274	Mx8 phage ATT site; *lacZα* for screening; Kan^r	(Guo *et al.*, 1996)
pWB200	Mx8 phage ATT site; *ccdB* for lethal selection; Kan^r	This study
pACYC184	Tet^r; Cm^r	(Rose, 1988)
pXQ703	pACYC184 with *Hind*III and *Xba*I sites removed	This study
pBluescript II SK (+)	Cloning vector with *lacZα* for screening; Amp^r	Stratagene
pXQ723	*lacZα* for screening; Tet^r	This study
pBJ113	Gene replacement vector with KG cassette; Kan^r	(Julien *et al.*, 2000)
pWB116	*difA* in-frame deletion in pBJ113	This study
pAD56	P*tar-nart*; Amp^r	(Ward *et al.*, 2002)
pXQ706	DNA with the DifA C-terminus in pWB200	This study
pXQ713	P*tar-nafA* in pWB200; Kan^r	This study
pXQ719	P*dif-nafA* in pWB200; Kan^r	This study
pXQ730	700 bp *aglU* internal fragment in pXQ723; Tet^r	This study

Results

Construction and expression of NarX-DifA (NafA) chimera in *M. xanthus*

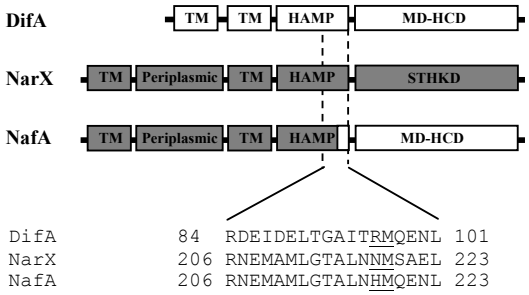

Figure 2-1. Construction of NarX-DifA (NafA) chimera. TM: transmembrane domain; HAMP: HAMP linker region; STHKD: signal transduction histidine kinase domain; MD-HCD: methylation and highly conserved (signaling) domains. The diagram is not drawn to scale. The amino acid sequences at the bottom are from the indicated region of HAMP linkers of NarX, DifA and NafA and the underlined residues indicate the junction of the NarX-DifA fusion.

Except for the lack of a prominent periplasmic domain, DifA possesses all the primary structural features of classical MCPs: two transmembrane domains, a HAMP linker region, and methylation and signaling domains (Fig. 2-1; Yang *et al.*, 1998b). It is unclear whether DifA mediates signal inputs to the Dif pathway and if so, how it may transmit the signal to the proteins downstream. In order to better understand the signaling properties of DifA and the Dif pathway, NafA, a chimera with the N-terminus of NarX and the C-terminus of DifA, was constructed (Fig. 2-1). An *Nde*I site was introduced into *narX* by substituting alanine 218 with a histidine (Ward *et al.*, 2002), which joins to methionine 97 of DifA in NafA (Fig. 1). Two plasmids containing the chimeric *nafA* gene were generated: pXQ713, referred to as the P*tar* construct hereafter because it has *nafA* controlled by the *E. coli tar* promoter, and pXQ719, referred to as the P*dif* construct because it has *nafA* controlled by the *M. xanthus dif* promoter (Table 2-1). These two constructs were transformed into YZ601, which contains a new and more complete deletion of *difA*. The resulting strains YZ716 (Δ*difA*/P*tar*) and YZ724 (Δ*difA*/P*dif*) (Table 2-1) were used throughout this study. The expression of NafA was examined by immunoblotting using polyclonal antibodies against the C-terminus of DifA (Yang Z., unpublished data) under vegetative conditions with or without

nitrate. The results indicated that YZ724 expressed NafA at a similar level as DifA in DK1622 but YZ716 had no detectable levels of NafA by immunoblotting (data not shown). The results also showed that the presence of 5, 35 and 100 μM nitrate does not have an apparent effect on NafA expression (data not shown); any effects by nitrate on *M. xanthus* strains containing *nafA* should not be attributed to any regulation of NafA expression by nitrate.

DifA N-terminus is likely essential for its function

M. xanthus difA mutants are known to be defective in formation of fruiting bodies (Yang *et al.*, 1998b; Yang *et al.*, 2000). The newly constructed Δ*difA* mutant (YZ601) showed no development under starvation conditions that prompted normal development of the wild type (DK1622) (Fig. 2-1 A, first row). YZ716 and YZ724 also exhibited no development under these conditions (Fig. 2-1 A, first row), indicating that the introduction of the *nafA* chimeric gene *per se* did not restore development. Since YZ724 expresses NafA to similar levels as DifA (data not shown) and NafA contains the C-terminus but not the N-terminus of DifA (Fig. 2-1), these observations imply that the N-terminus of DifA is required for DifA function in development. This is in contrast to FrzCD, whose N-terminus can be removed without causing apparent defects in the Frz signaling pathway (Bustamante *et al.*, 2004). We propose that the N-terminus of DifA is responsible for mediating signal input to the Dif pathway, and that its C-terminus interacts with downstream components.

NafA restores fruiting body formation to *difA* mutants in response to nitrate

If the N-terminus of DifA is involved in signal perception, nitrate might activate the Dif pathway through NafA by providing the signal as is the case with Nart, the Nar-Tar chimera (Ward *et al.*, 2002). Although nitrite and nitrate both are sensed by NarX and Nart (Lee *et al.*,

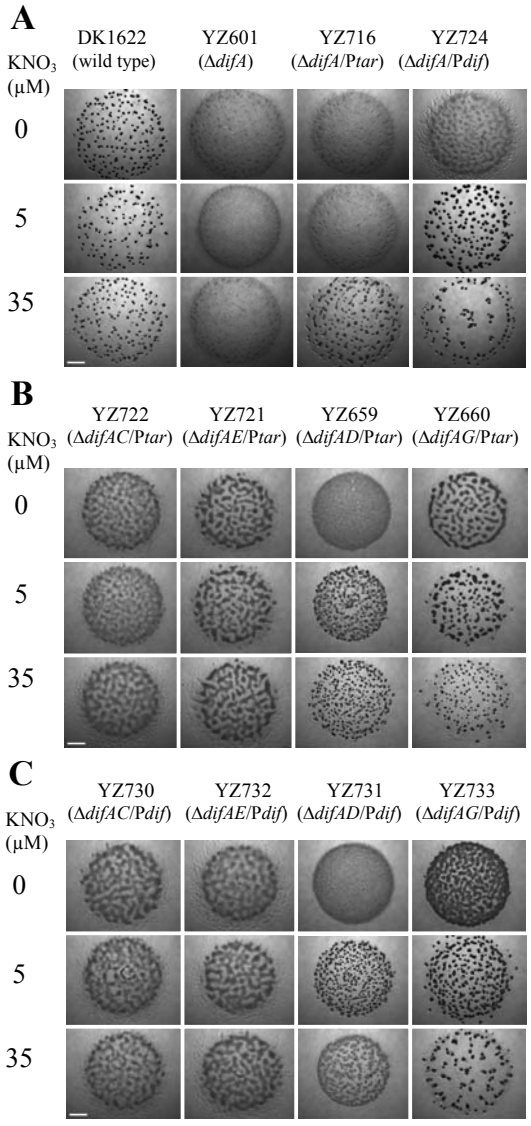

Figure 2-2. Fruiting body formation on CF plates supplemented with KNO_3. A. Wild type, Δ*difA* mutant, and two *nafA*-carrying strains in Δ*difA* background. B. Mutants with the P*tar* construct (pXQ713) in double deletion backgrounds of Δ*difA* Δ*difC*, Δ*difA* Δ*difE*, Δ*difA* Δ*difD* and Δ*difA* Δ*difG*. C. Mutants with the P*dif* construct (pXQ719) in double deletion backgrounds of Δ*difA* Δ*difC*, Δ*difA* Δ*difE*, Δ*difA* Δ*difD* and Δ*difA* Δ*difG*. Five microliters of cell suspension (approximately 5×10^9 cells/ml) in MOPS buffer was spotted onto CF plates with KNO_3 at indicated concentrations. Pictures were taken after incubation at 32ºC for 3 days. The scale bar at the lower left represents 1 mm. P*tar* and P*dif* are the abbreviations of the two *nafA* constructs under the controls of *E. coli tar* promoter and *M. xanthus dif* promoter, respectively.

1999; Ward *et al.*, 2002), only nitrate was used in this study because 1 mM nitrite severely inhibited *M. xanthus* growth (data not shown), whereas full activation of NarX requires 3.5 mM nitrite (Lee *et al.*, 1999).

Nitrate clearly influenced the development of the two strains harboring the *nafA* constructs (Fig. 2-2 A). Both YZ716 (Δ*difA*/P*tar*) and YZ724 (Δ*difA*/P*dif*) formed fruiting bodies in the presence of 35 μM nitrate. YZ724, but not YZ716, also did so with 5 μM nitrate. In the absence of nitrate, neither strain developed. The fruiting bodies formed by these strains in the presence of adequate nitrate all contained refractile and spherical myxospores (data not shown). The development of YZ716 was surprising since this strain did not produce enough NafA to be detected by immunoblotting (data not shown). This indicates that *nafA* was expressed from the *E. coli tar* promoter in *M. xanthus* at a sufficient level to initiate fruiting body formation. Fruiting bodies of YZ716 produced under these conditions, however, showed apparent defects when

compared to the wild type. They appeared to be less compact and irregular in shape, with more cells remaining outside of the aggregates. These defects were perhaps caused by insufficient EPS production. The overall conclusion, however, is that NafA can restore development to *difA* mutants in the presence of nitrate.

The results in Figure 2-2 A indicate that nitrate concentration affects fruiting body morphology formed by strain YZ724. At 5 μM nitrate they were comparable to those formed by the wild type. At 35 μM nitrate, however, the YZ724 fruiting bodies were variable in size and not evenly distributed. These defects were even more severe at higher concentrations of nitrate (data not shown), suggesting that the overstimulation of the Dif pathway leads to developmental defects. We suggest that unregulated production of EPS resulting from excessive stimulation of the Dif pathway (Fig. 2-4) is responsible for the observed abnormalities of YZ724 in development. This is consistent with the similarly observed developmental defects of *difD*, *difG* and *stk* mutants, all of which overproduce EPS (Dana and Shimkets, 1993; Black and Yang, 2004).

NafA signals through the Dif pathway

It is highly likely that DifC and DifE, two central components of the Dif pathway, function downstream of DifA (Yang *et al.*, 1998b; Yang *et al.*, 2000; Bellenger *et al.*, 2002; Black and Yang, 2004; Yang and Li, 2005). DifD and DifG, which are negative regulators of the Dif pathway (Black and Yang, 2004), may or may not be required for the activation of EPS production. It could be argued that NafA, a chimera with mixed components from two different subdivisions of proteobacteria, might bypass the Dif pathway and stimulate *M. xanthus* development through other mechanisms. If NafA restores the development by interacting with the downstream Dif components, the DifC and DifE proteins should be

involved. To test this point, the *nafA* plasmids were introduced into Δ*difA* Δ*difC*, Δ*difA* Δ*difD*, Δ*difA* Δ*difE* and Δ*difA* Δ*difG* double deletion strains.

As shown in Figures 2-2 B and 2-2 C, nitrate did not restore development in the *difA difC* or *difA difE* strains in the presence of *nafA*. In contrast, in the presence of nitrate both P*tar* and P*dif* constructs supported developmental aggregation of Δ*difA* Δ*difD* and Δ*difA* Δ*difG* mutants. The results agree with Black and Yang (Black and Yang, 2004) in showing that deletion of *difD* or *difG* does not eliminate development but rather alters the appearance of fruiting bodies, perhaps because of elevated EPS production. More importantly, the observations here demonstrated that NafA requires both *difC* and *difE* to restore fruiting development and that the NafA chimera likely signals through the downstream elements of the Dif pathway in response to nitrate stimulation. This is consistent with a model in which the C-terminus of activated DifA interacts with downstream Dif components to activate EPS production (Black and Yang, 2004; Yang and Li, 2005). In addition, the results here suggest that neither DifD nor DifG functions downstream of DifA because the Δ*difA* Δ*difD* and the Δ*difA* Δ*difG* double mutants showed similar developmental phenotypes as the Δ*difA* single mutant under all conditions (Fig. 2-2 B and 2-2 C).

NafA rescues cellular cohesion and EPS production to *difA* mutants in response to nitrate

Previous studies suggested that Dif proteins control development by regulating EPS production and S-motility (Yang *et al.*, 1998b; Yang *et al.*, 2000; Bellenger *et al.*, 2002; Black and Yang, 2004). An apparent explanation for the nitrate-induced fruiting body formation by *nafA* containing strains was the restoration of EPS production. Since *M. xanthus* cellular cohesion requires EPS (Shimkets, 1986; Yang *et al.*, 2000; Black and Yang, 2004), agglutination assays were performed to examine whether nitrate could induce cellular

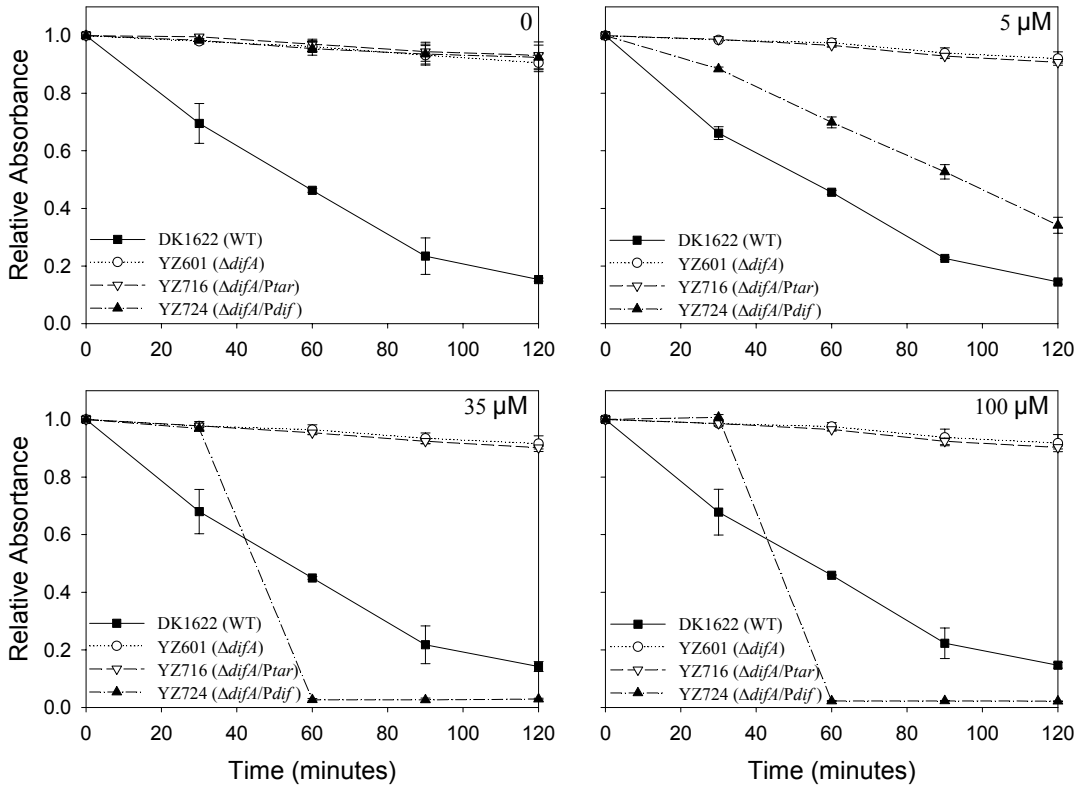

Figure 2-3. Agglutination assay. Cells grown overnight in CTT were washed and re-suspended to approximately 2.5×10^8 cells/ml in agglutination buffer with KNO_3 at concentrations indicated at the upper right of each panel. OD was measured every 30 min for 2 hours. Relative absorbance was obtained by dividing the OD at each time point by the initial OD value.

cohesion (Fig. 2-3). Cells of strain DK1622 agglutinated similarly under all conditions tested as indicated by decreasing optical density at 600 nm (OD_{600}) over time. In contrast, the OD_{600} of YZ601 remained stable at nitrate concentrations of 0, 5, 35 and 100 μM. Nitrate by itself therefore had little effect on the agglutination of the wild type and the *difA* mutant strains. On the other hand, although strain YZ724 (Δ*difA*/P*dif*) showed agglutination patterns similar to those of strain YZ601 in the absence of nitrate, it agglutinated at all three nitrate concentrations examined (5, 35 and 100 μM, Fig. 2-3). These results provide further support for the conclusion that nitrate stimulates the Dif pathway through NafA to activate EPS production. For comparison, strain YZ716 (Δ*difA*/P*tar*) did not agglutinate at all nitrate

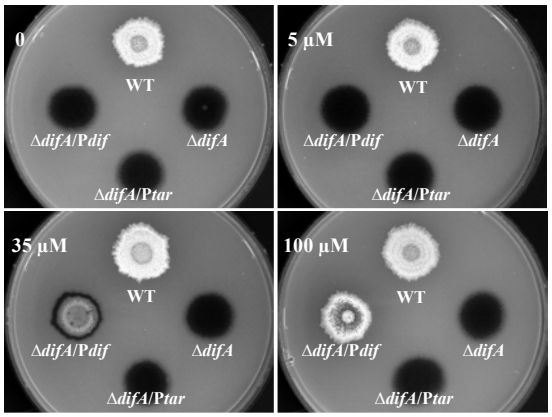

Figure 2-4. EPS production under different nitrate concentrations. Five microliters of cells at ~ 5×10^7 cells/ml in MOPS buffer were spotted onto CTT plates containing 50 μg of calcofluor white/ml and KNO_3 at different concentrations as indicated in the upper left of each picture. After incubation at 32ºC for 7 days, the plates were photographed under the illumination of UV light (365 nm). The diameter of the plates shown is 9 cm. WT (DK1622); Δ*difA* (YZ601); Δ*difA*/P*tar* (YZ716); Δ*difA*/P*dif* (YZ724).

concentrations of 5, 35 and 100 μM, suggesting that its EPS production was insufficient to support significant cell adhesion.

The results of fruiting body formation (Fig. 2-2) and agglutination (Fig. 2-3) strongly suggest that nitrate could activate the Dif pathway and lead to EPS production. The fluorescent dye calcofluor white was used to analyze EPS production more directly as described in **Experimental procedures** after seven days of incubation (Fig. 2-4). Wild-type strain DK1622 showed the same level of EPS as illustrated by the consistent fluorescence intensity under all conditions. Strains YZ601 and YZ716, produced no detectable level of EPS with or without nitrate. In contrast, YZ724 gave a clear fluorescence signal at 35 and 100 μM nitrate although not at 5 μM. At 100 μM, its fluorescence intensity was close to that of strain DK1622. These results clearly demonstrate that YZ724 produces EPS in response to nitrate.

NafA restores S-motility to *difA* mutants

If NafA turns on the Dif pathway in response to nitrate, it should lead to the restoration of S-motility to *difA* mutants expressing *nafA*. S-motility was first examined using swarming assays on soft CTT plates (0.4% agar). As shown in Fig. 2-5 A, the DK1622 strain (wild type) spread considerably and produced rough flares at the colony edge in the presence and absence of nitrate. YZ601 (Δ*difA*) and YZ716 (Δ*difA*/P*tar*) colonies expanded insignificantly and had

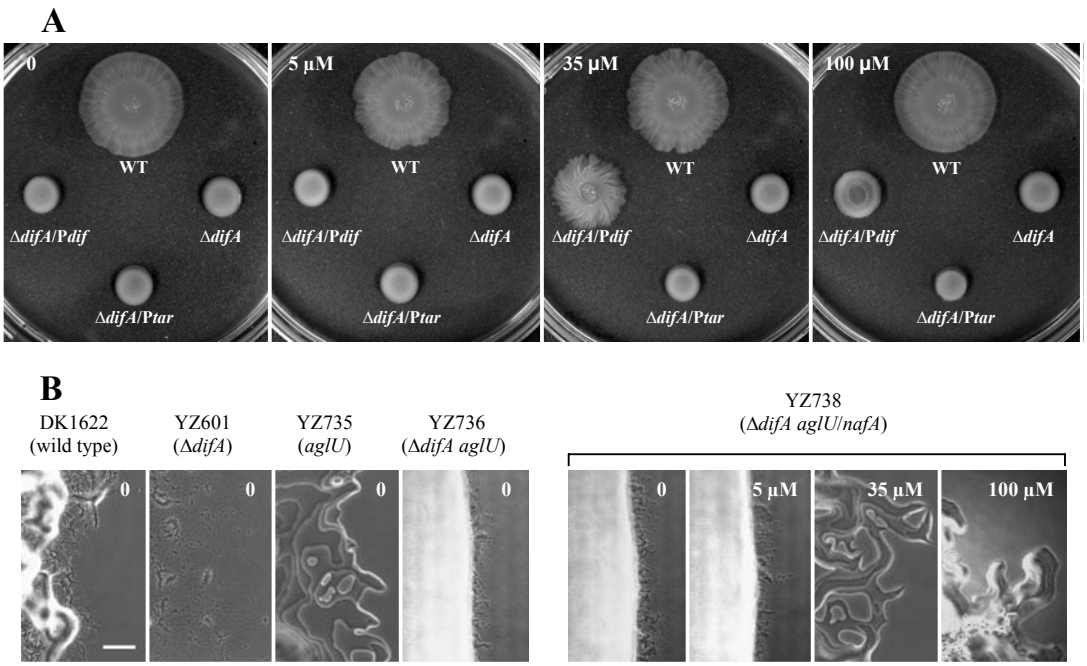

Figure 2-5. Examination of S-motility using soft (0.4% agar) (A) and hard (1.5% agar) (B) CTT plates. A. Five microliters of cells at ~1×10^{10} cells/ml were spotted onto soft CTT plates containing KNO_3 at concentrations as indicated at the upper left of each picture. Plates were photographed after incubation at 32ºC for 3 days. WT (DK1622); Δ*difA* (YZ601); Δ*difA*/P*tar* (YZ716); Δ*difA*/P*dif* (YZ724). The diameter of the plates shown is 9 cm. B. Five microliters of cells (approximately 5×10^9 cells/ml) were spotted onto CTT plates with or without KNO_3. After 2 days of incubation at 32ºC, the colony edges were photographed under phase contrast microscope. The bar, 100 μm.

a glossy appearance and smooth edges under all conditions examined. At 0 and 5 μM nitrate, YZ724 (Δ*difA*/P*dif*) showed a similar smooth colony morphology. However, at 35 μM nitrate, the YZ724 colonies expanded significantly and exhibited rougher colony edges. This demonstrates that appropriate amounts of nitrate supplementation can restore S-motility to *difA* mutants expressing the NafA chimera.

Surprisingly, although developmental aggregation, agglutination, and EPS production were all restored to YZ724 by 100 μM, its colony showed little expansion at 100 μM nitrate on soft CTT plates (Fig. 2-5 A), indicating defects in S-motility. This motility defect could either be qualitative or quantitative, that is, either S-motility was not restored to YZ724 or S-motility was restored but somehow inhibited or reduced at 100 μM nitrate. To examine S-motility qualitatively (Hodgkin and Kaiser, 1979a, b), an A-motility mutation *aglU* (Youderian *et al.*, 2003) was introduced into strain YZ724 (see **Experimental procedures**)

and the motility of the resultant strain was compared with that of parental strains using hard CTT plates (1.5% agar). Figure 2-5 B shows that DK1622 (wild type) moved both as large cell groups (S-motility) and well-isolated cells (A-motility). Strain YZ601 (Δ*difA*) moved primarily as isolated cells and in small cell groups. As expected, strain YZ735 (*aglU*) showed the typical A^- S^+ colony edges. Strain YZ736 (Δ*difA aglU*), which lacks genes essential for both motility systems, displayed only residual motility as reported previously (Wu *et al.*, 1997; Bellenger *et al.*, 2002). The colony edge morphology of these strains (DK1622, YZ601, YZ735, and YZ736) was not affected by nitrate (data not shown) as expected. In contrast, when the P*dif-nafA* plasmid was introduced into the *difA aglU* strain, the resulting transformant (YZ738) exhibited nitrate-induced movement of large cell groups at both 35 and 100 μM nitrate (Fig. 2-5 B). YZ738 had S-motile flares at both 35 and100 μM nitrate, although the flares at 100 μM appeared larger, fewer, and blunter. There was no induced S-motility flare at 5 μM nitrate. These observations confirmed that NafA restores S-motility to *difA* mutants qualitatively in a nitrate-dependent manner and that the defects in S-motility at 100 μM (Fig. 2-5 A) are quantitative in nature.

Continuous and overproduction of EPS may inhibit *M. xanthus* S-motility

The quantitative defects in S-motility of the NafA-expressing strain (YZ724) at 100 μM nitrate (Fig. 2-5 A) could be the result of continuously high levels of EPS production in the presence of 100 μM nitrate. To examine this possibility, EPS production of YZ724 at 100 μM nitrate was examined by calcofluor white binding every 12 h for 7 days. The results at 12, 36, 60, and 132 h are shown in Figure 2-6. As indicated by emitted fluorescence, EPS production by the NafA-expressing YZ724 was very substantial and readily detectable at 12 h, whereas the wild type produced little EPS until the third day (60 h). Even at the sixth day (132 h), the intensity of fluorescence from the wild type was less than that from the NafA-expressing

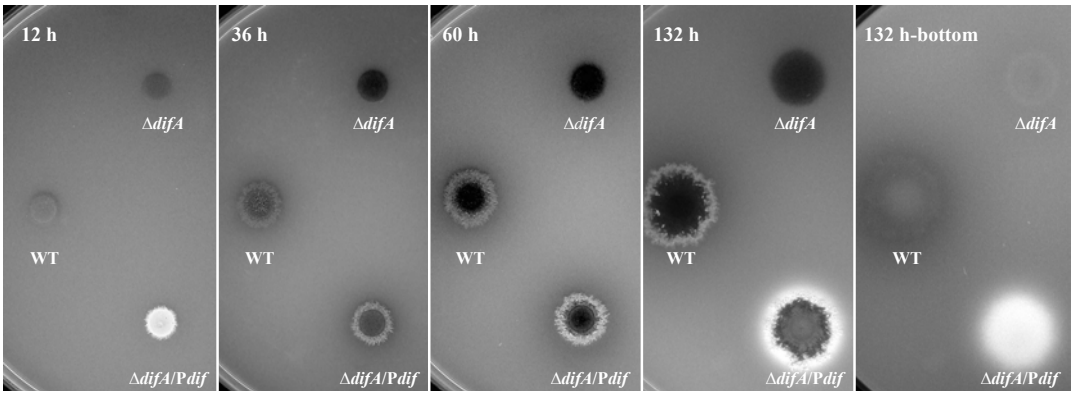

Figure 2-6. EPS production at different time points upon exposure to 100 µM nitrate. Cells grown without nitrate were washed and resuspended at ~1 x 10^{10} cells/ml in MOPS buffer. Five-microliter aliquots of the cell suspension were spotted onto CTT plates containing 50 µg/ml of calcofluor white and 100 µM KNO_3. After incubation at 32°C for the indicated times (hours), the plates were photographed as in Figure 2-4 except for the last picture, which was photographed upside down from the bottom through the agar. The diameter of the plates used is 9 cm. WT, DK1622; Δ*difA*, YZ601; Δ*difA*/P*dif*, YZ724.

strain under these assay conditions. The differences in fluorescence intensity were even more dramatic when the plates were viewed from the bottom. The last photograph in Figure 2-6 shows the fluorescence at the sixth day (132 h, bottom) when the plate was viewed upside down with UV illumination. It clearly showed that the NafA-expressing strain produces significantly more EPS than the wild type. The nonfluorescent center of YZ724 colonies when viewed from the top was possibly because the cells closer to the agar surface bound all the available dye and no calcofluor white could diffuse through to the cells in the top layers. The results here indicate that continuously high levels of EPS production coincide with the swarming defects of YZ724 at 100 µM nitrate on soft agar (Fig. 2-5 A), possibly suggesting a cause-and-effect relationship. It should be noted that there are differences between the experiments in Figures 2-4 and 2-6: cell suspension at ~ 5 x 10^7 cells/ml was used as inoculum for Figure 2-4, and ~ 1 x 10^{10} cells/ml was used for Figure 2-6, because sufficient numbers of cells had to be present for the detection of fluorescence for the experiments in Figure 2-6 at the early time points.

Discussion

NafA, a chimera between the NarX sensory module and the DifA methylation and signaling domains, restores fruiting body formation, EPS production, and S-motility to *difA* mutants in a nitrate-dependent manner. We propose that DifA, despite its lack of a prominent periplasmic domain, has similar modular structures as the classical bacterial MCPs (Szurmant and Ordal, 2004). That is, the N-terminus of DifA appears to receive signal input, and the C-terminus is responsible for interactions with downstream components in the regulation of EPS production. The functioning of NafA in *M. xanthus* suggests that DifA shares similar transmembrane signaling mechanisms with classical bacterial MCPs and sensor kinases. The responses mediated by the NafA chimera also suggest a correlation between stimulus strength and the level of EPS production, as expected if the control of the Dif pathway mimics physiologically relevant events. In addition, the Δ*difA* Δ*difD* double mutant was complemented by the *nafA* plasmids similarly as the *difA* mutant (Fig. 2-2 B and C), suggesting that DifD, the CheY homolog, is not necessarily downstream of DifA in the regulation of EPS production. Since DifA, DifC and DifE form a signaling complex (Yang and Li, 2005) as MCPs, CheW and CheA in bacterial chemotaxis systems, DifD is therefore unlikely downstream of DifE in the regulation of EPS in *M. xanthus* (Black and Yang, 2004).

Most prokaryotic chemoreceptors have the general transmembrane topology as the classical *E. coli* MCPs (Tar, Tsr, Trg and Tap) (Zhulin, 2001). Nevertheless, some MCP homologs with structural features like DifA have been shown to be functional signal transducers. For example, *E. coli* Aer and *Halobacterium salinarium* HtrI, possess two transmembrane domains with no apparent perisplasmic domain (Hoff *et al.*, 1997; Bibikov *et al.*, 2004). The *E. coli* aerotaxis receptor Aer detects the redox state of the cell through a flavin adenine dinucleotide (FAD) that binds to the N-terminus of the transducer (Bibikov *et*

al., 2000; Bibikov *et al.*, 2004). *H. salinarium* HtrI senses light through interactions of its N-terminal transmembrane domain with its cognate sensory rhodopsin SRI (Hoff *et al.*, 1997). Although the signals sensed by DifA to stimulate EPS production remain unknown, our results with NafA suggest that DifA may detect signals in a manner similar to Aer and HtrI.

Our results with NafA also lead to the conclusion that *M. xanthus* EPS production is elaborately regulated under both vegetative and developmental conditions. The NafA-expressing strain YZ724 is sensitive to nitrate concentration during both development on starvation medium and vegetative swarming on soft agar. Although strain YZ724 forms fruiting bodies similar to those of the wild type at 5 μM nitrate, its fruiting bodies at 35 μM (Fig. 2-2 A) and higher nitrate concentrations (data not shown) displayed obvious defects. Similarly, S-motility during vegetative swarming by this strain on soft agar is very sensitive to nitrate concentrations (Fig. 2-5 A). The expansion of YZ724 colonies at 35 μM is similar to that of wild-type colonies, but at either 5 μM or 100 μM, swarming is severely impaired. The decrease in colony expansion on soft agar at 100 μM nitrate could be due to growth defects brought about by unregulated EPS production (Fig. 2-6). We argue that growth defects are unlikely because the expansion of YZ724 colonies on hard agar, which depends more heavily on A-motility (Shi and Zusman, 1993), is not appreciably diminished by 100 μM nitrate (Fig. 2-4 and data not shown). The defects at 100 μM nitrate are instead reminiscent of motility and developmental phenotypes of *difD* and *stk* mutants which overproduce EPS (Dana and Shimkets, 1993; Black and Yang, 2004). These results indicate that EPS production must be controlled precisely during both the vegetative and developmental cycles of *M. xanthus*. It is not clear how continuous and/or overproduction of EPS affects *M. xanthus* S-motility.

A related observation is that the restoration of vegetative swarming to a NafA-expression strain requires higher concentrations of nitrate than the restoration of development.

Development can be partially restored to strain YZ716 (Δ*difA*/P*tar*) at 35 μM nitrate (Fig. 2-2A), but S-motility and detectable EPS production could not be restored even at 100 μM for this strain (Fig. 2-4 and 2-5 A). Similarly, YZ724 (Δ*difA*/P*dif*) requires 35 μM nitrate to restore detectable EPS production and S-motility (Fig. 2-4 and 2-5 A) but only 5 μM to restore development (Fig. 2-2 A). One possible explanation is that S-motility requires higher level of EPS production than development. Alternatively, because EPS production was measured only under vegetative conditions in this study (Fig. 2-4), it is possible that development and vegetative swarming have similar requirements for EPS production but developmental conditions allow more EPS production than vegetative conditions even with the same signal strength to the Dif pathway. Although we have no convincing evidence to favor or exclude either of these two possibilities at the present, these observations suggest that elaborate regulation of EPS production is important for the vegetative and the developmental life cycles of *M. xanthus*.

Experimental procedures

Strains, plasmids and growth conditions

The plasmids and *M. xanthus* strains used in this study are listed in Table 2-1. *M. xanthus* was grown on Casitone-Tris (CTT) plates or in CTT liquid medium (Kaiser, 1979) at 32ºC on a rotary shaker at 300 rotations per min (rpm). The exception was that Casitone-yeast extract (CYE) (Campos and Zusman, 1975) plates were used for strain maintenance. CF plates were used as the development-inducing medium for *M. xanthus* (Hagen *et al.*, 1978). The E. coli strain XL1-Blue (Stratagene), used for routine cloning and plasmid construction, was grown on Luria-Bertani (LB) plates or LB liquid (Miller, 1972) at 37ºC. Plates contained 1.5% agar unless noted otherwise. When necessary, kanamycin and tetracycline were added to media at 100 μg/ml and 15 μg/ml, respectively.

Plasmid constructions

Two new cloning vectors, pWB200 and pXQ723, were constructed in this study. To construct pWB200, an 1.8-kb fragment containing the Mx8 phage attachment (ATT) site was amplified from pYC274 (Guo *et al.*, 1996) and cloned into pZErO-2 (Invitrogen), which had been digested with *Bgl*I and *Dra*III and filled in with T4 DNA polymerase (New England BioLab). pWB200 contains *ccdB* for lethal selection in *E. coli* and the Mx8 ATT for integration in *M. xanthus*. To construct pXQ723, the *Hind*III and the *Xba*I sites of pACYC184 (Rose, 1988) were first removed by digestion with these enzymes, filled in with T4 DNA polymerase and then re-ligated to generate pXQ703. A 0.9 kb *Afl*III-*Dra*III fragment from pBluescript II SK(+) (Stratagene) was cloned into *Ava*I and *Sac*II digested pXQ703 after both were treated with T4 DNA polymerase. The resulting pXQ723 contains

the tetracycline-resistance gene and the replication origin from pACYC184 and the *lacZα* for blue-white screening from pBluescript II SK (+).

pWB116 and pXQ730 were used to construct *M. xanthus difA* and *aglU* mutants, respectively. To construct pWB116, the *difA* deletion plasmid, a DNA fragment with *difA* in-frame deletion was generated using a two-step, overlap polymerase chain reaction (PCR) (Sambrook and Russell, 2001) and cloned into *Sma*I of pBJ113 (Julien *et al.*, 2000). This deletion construct removed the complete *difA* open reading frame (Yang *et al.*, 1998b) except the last codon. To construct pXQ730, the *aglU* insertion plasmid, a 700 bp internal fragment of *aglU* was amplified from *M. xanthus* genomic DNA using oligonucleotides (5'-GGAATTCTGATGGCCTCGCTGGTGATG-3' and 5'-GGAATTCACCTTCATGGGCGGCGCGTC-3'), digested with *Eco*RI and cloned into the same site of pXQ723.

To construct pXQ713, a 1.1 kb of *difA* C-terminal fragment was PCR amplified and codon 96 (CGC) of *difA* was changed to CAT in this fragment to create an *Nde*I site (CATATG). This *difA* C-terminal fragment was cloned into the *Eco*RV site of pWB200 in the same orientation as the *E. coli lac* promoter to first generate pXQ706. A 2.0 kb *Eco*RI-*Nde*I fragment encoding the NarX N-terminus and the upstream *tar* promoter from pAD56 (Ward *et al.*, 2002) was cloned into the same sites of pXQ706 to create pXQ713. pXQ713 was digested with *BamH*I, filled in with T4 DNA polymerase and then digested with *Hind*III; a 0.5 kb PCR fragment containing the *dif* promoter was digested with *Hind*III and ligated into the treated pXQ713 as described above to create pXQ719.

Construction of *M. xanthus* strains.

Mutants with in-frame deletions in *dif* genes were constructed by using the positive-negative kanamycin/galactose (KG) method (Ueki *et al.*, 1996). To construct *difA* deletion

mutants, pWB116 was electroporated (Kashefi and Hartzell, 1995) into DK1622 (wild type), SW403 (Δ*difC*) (Bellenger *et al.*, 2002), YZ603 (Δ*difE*), YZ613 (Δ*difD*) and YZ604 (Δ*difG*) (Black and Yang, 2004) and selected by kanamycin resistance. Mutants of Δ*difA* (YZ601), Δ*difA* Δ*difC* (YZ720), Δ*difA* Δ*difE* (YZ719), Δ*difA* Δ*difD* (YZ653) and Δ*difA* Δ*difG* (YZ654) were subsequently identified by their resistance to galactose and sensitivity to kanamycin and further confirmed by PCR. These *dif* mutants were transformed with pXQ713 or pXQ719 by electroporation (Kashefi and Hartzell, 1995) to produce *nafA*-carrying strains (Table 2-1). To construct *M. xanthus aglU* insertion mutants, pXQ730 was used to transform DK1622 (wild type), YZ601 (Δ*difA*) and YZ724 (Δ*difA*/P*dif*) to generate YZ735 (*aglU*), YZ736 (Δ*difA aglU*) and YZ738 (Δ*difA aglU*/P*dif*).

Examination of NafA expression

M. xanthus was cultured in CTT liquid overnight with or without KNO_3 to approximately 1.0-1.5×10^8 cells/ml. About 2.5 x 10^8 cells were harvested, washed with cold 50 mM Tris-HCl (pH 7.4) and resuspended in 50 μl of loading buffer (2% SDS, 5% mercaptoethanol, 8.5% glycerol) (Sambrook and Russell, 2001). Ten microliters of these samples, after being boiled for 5 min, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide gel. Immunoblotting was performed as described (Sambrook and Russell, 2001) using antibody against cytoplasmic domains of DifA (Lancero *et al.*, 2004; Yang Z., unpublished data).

Phenotypic analysis of *M. xanthus* strains

M. xanthus first grown in CTT liquid to approximately 1.0 x 10^8 to 2.0 x 10^8 cells/ml for all phenotypic analyses. For examination of fruiting body formation, cells were harvested, washed and resuspended in MOPS (morpholinepropanesulfonic acid) buffer (10 mM MOPS,

2 mM $MgSO_4$, pH 7.6) at approximately 5 x 10^9 cells/ml. Five microliters of these cell suspensions were spotted onto the surface of CF plates supplemented with 0, 5 or 35 μM KNO_3. Fruiting body formation was examined and documented after 3 days of incubation at 32ºC.

For assessment of cellular cohesion, cells from liquid culture were washed with agglutination buffer (10 mM MOPS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 6.8) (Dana and Shimkets, 1993), and resuspended to approximately 2.5 x 10^8 cells/ml in agglutination buffer with 0, 5, 35 or 100 μM nitrate. Optical density (OD) at 600 nm was recorded every 30 min for 2 hours and was normalized against the initial OD reading. Calcofluor white binding (Dana and Shimkets, 1993; Black and Yang, 2004) was used to evaluate EPS production. Cells from liquid culture were washed and resuspended in MOPS buffer at appropriate cell densities. Five microliters of these cell suspensions were spotted onto the surface of CTT plates supplemented with calcofluor white (50 μg/ml) and KNO_3 (0, 5, 35 or 100 μM). The plates were incubated at 32ºC for appropriate time before they were examined and documented under the illumination of UV light (365 nm).

For analysis of S-motility on soft agar, cells from liquid culture were washed and resuspended in MOPS buffer at approximately 1 x 10^{10} cells/ml. Five microliters of these cell suspensions were spotted onto the surface of soft CTT plates (0.4% agar) supplemented with 0, 5, 35 or 100 μM KNO_3. Colony expansion was examined and photographed after 3 days of incubation at 32ºC. For analysis of motility on hard agar, 5 μl of a cell suspension at approximately 5×10^9 cells/ml was spotted onto CTT plates (1.5% agar) with or without 35 μM KNO_3. After 2 days of incubation at 32ºC, colony edges were photographed using phase-contrast microscopy.

CHAPTER 3

Chemotaxis mediated by NarX-FrzCD chimeras and non-adapting repellent responses in *Myxococcus xanthus*

Xu, Q., Black, W. P., Mauriello, E. M. F., Zusman, D. R., and Yang, Z. (2007) Chemotaxis mediated by NarX-FrzCD chimeras and non-adapting repellent responses in *Myxococcus xanthus*. Mol. Microbiol. 66: 1370-1381.

Summary

Myxococcus xanthus requires gliding motility for swarming and fruiting body formation. It uses the Frz chemosensory pathway to regulate cell reversals. FrzCD is a cytoplasmic chemoreceptor required for sensing effectors for this pathway. NarX is a transmembrane sensor for nitrate from *Escherichia coli*. In this study, two NarX-FrzCD chimeras were constructed to investigate *M. xanthus* chemotaxis: $NazD_F$ contains the N-terminal sensory module of NarX fused to the C-terminal signaling domain of FrzCD; $NazD_R$ is similar except that it contains a G51R mutation in the NarX domain known to reverse the signaling output of a NarX-Tar chimera to nitrate. We report that while nitrate had no effect on the wild type, it decreased the reversal frequency of *M. xanthus* expressing $NazD_F$ and increased that of *M. xanthus* expressing $NazD_R$. These results show that directional motility in *M. xanthus* can be regulated independently of cellular metabolism and physiology. Surprisingly, the $NazD_R$ strain failed to adapt to nitrate in temporal assays as did the wild type to known repellents. Therefore, the lack of temporal adaptation to negative stimuli is a general feature in *M. xanthus*. Thus, the appearance of biased movements by *M. xanthus* in repellent gradients is likely due to the inhibition of net translocation by repellents.

Introduction

Although flagella mediated motility is the leading model for the study of bacterial chemotaxis (Falke *et al.*, 1997; Szurmant and Ordal, 2004), many bacteria display movement without the aid of flagella (McBride, 2001). Translocation along the long axis of a cell on a solid surface without the aid of flagella is called 'gliding motility' (McBride, 2001), and is found in many branches of the bacterial phylogenetic tree. These bacteria, including *Myxococcus xanthus*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa* and cyanobacteria, show directed cell movements (Sandstrom *et al.*, 1983; Ward and Zusman, 1997; Chung *et al.*, 2001; Bhaya, 2004; Bonner and Shimkets, 2006). However, the mechanism of chemotaxis and its regulation in the gliding bacteria are not as well understood as in swimming bacteria (Falke *et al.*, 1997; Szurmant and Ordal, 2004).

The Gram-negative bacterium *M. xanthus* requires gliding motility for its complex life cycle, including vegetative swarming and fruiting body formation (Shimkets, 1990; Ward and Zusman, 1999). *M. xanthus* contains two different motility systems for gliding: S-motility, which is powered by type IV pili (Merz *et al.*, 2000; Sun *et al.*, 2000; Skerker and Berg, 2001), and A-motility, which involves adhesion complexes and unidentified motors (Mignot *et al.*, 2007). *M. xanthus* cells move only on solid surfaces, and the wild type reverses its gliding direction about every 6-8 minutes in the absence of tactic stimulation. Phosphatidylethanolamine (PE) was shown to suppress cellular reversals whereas isoamyl alcohol (IAA) and dimethyl sulfoxide (DMSO) were found to stimulate cellular reversals (McBride *et al.*, 1992; Kearns and Shimkets, 2001). Furthermore, *M. xanthus* swarms (colonies) have been shown to migrate preferentially into areas with nutrients or PE and avoid those with IAA or DMSO (Shi *et al.*, 1993; Kearns and Shimkets, 1998). These studies

identified nutrients and PE as chemoattractants and DMSO along with IAA and other small alcohols as repellents for *M. xanthus*.

Cell reversals in *M. xanthus* are controlled by the Frz chemosensory system (Ward and Zusman, 1997), and mutations in *frz* genes lead to alteration of reversal frequency (Blackhart and Zusman, 1985; McCleary *et al.*, 1990; McBride *et al.*, 1992). The components of the Frz system are similar in many ways to those of the enteric chemotaxis pathways. For example, FrzCD, a cytoplasmic receptor, is homologous to the methyl-accepting chemotaxis proteins (MCP) (Ward and Zusman, 1997). Additionally, methylation of FrzCD is increased in the presence of suppressors of cellular reversals (attractants) and decreased in the presence of chemicals that stimulated reversals (repellents) (McBride *et al.*, 1992). These observations are consistent with the hypothesis that *M. xanthus* cells exhibit chemotaxis and that the Frz pathway plays a crucial role in this process.

It should be noted that nutrients, PE, IAA and DMSO likely affect the physiology of *M. xanthus* and may alter cell movement independently of chemotactic regulation. For example, rich media inhibits *M. xanthus* motility (Dworkin and Eide, 1983; McBride *et al.*, 1992; Shi and Zusman, 1994; Ward *et al.*, 1998). PE, a major constituent of *M. xanthus* membranes, could affect gliding directly by modulating the membranes or acting as nutrients (McBride *et al.*, 1992; Avadhani *et al.*, 2006; Bonner and Shimkets, 2006; Curtis *et al.*, 2006; Moraleda-Munoz and Shimkets, 2007). In addition, PE can be toxic to *M. xanthus* and lead to the loss of motility (McBride *et al.*, 1992; Xu and Yang, unpublished data). It is known that ethanol, which interferes with cell membrane function, inhibits agglutination of *M. xanthus* at 9.5% (Shimkets, 1986). Likewise, the small alcohol IAA inhibited agglutination of *M. xanthus* at 0.03% (Xu and Yang, unpublished data). DMSO at 2% or higher causes *M. xanthus* to sporulate (Shi *et al.*, 1994). We were concerned that the physiological and

metabolic effects of these chemicals and nutrients could be responsible for the observed behavioral changes in *M. xanthus* independently of chemotaxis regulation.

To further investigate *M. xanthus* tactic responses, we constructed two chimeras, $NazD_F$ and $NazD_R$. $NazD_F$ contains the sensory module of NarX from *Escherichia coli* (Stewart and Berg, 1988; Kalman and Gunsalus, 1990) fused to the signaling module of FrzCD; $NazD_R$ is essentially identical to $NazD_F$, except that it contains a G51R point mutation that reverses the chemotactic output of a NarX-Tar chimera toward nitrate stimulation in *E. coli* (Ward *et al.*, 2006). We demonstrate here that *M. xanthus* cells expressing these chimeras are able to initiate tactic responses to nitrate independent of direct physiological effects from other chemicals. Specifically, nitrate suppressed the reversal frequency of Δ*frzCD*/*nazD*$_F$ cells and elevated those of the Δ*frzCD*/*nazD*$_R$ mutant. Surprisingly, Δ*frzCD*/*nazD*$_R$ cells failed to adapt to nitrate as a repellent in temporal assays. Nitrate also inhibited swarm expansion and development of the $NazD_R$-expressing strain. The lack of adaptation to negative stimulation was found to be common in *M. xanthus* taxis, since wild-type cells failed to adapt to the presence of known repellents in various assays. Mechanisms without temporal adaptation for biased movement in a gradient of negative stimuli are discussed.

Table 3-1. Strains and plasmids.

Designation	Genotype or description	Source or reference
Strains		
DZ2	Wild type	(Campos and Zusman, 1975)
DZ4480	Δ*frzCD*	(Bustamante *et al.*, 2004)
YZ1701	Δ*frzCD*/*narX-frzCD*	This study
YZ1702	Δ*frzCD*/*narX-frzCD*(G51R)	This study
Plasmids		
pWB200	Mx8 ATT site; *ccdB* lethal selection; Kan^r	(Xu *et al.*, 2005)
pUC18	pBlue screening; Amp^r	(Norrander *et al.*, 1983)
pXQ701	DNA encoding NarX N-terminus in pUC18	This study
pXQ703	*narX-frzCD* chimeric gene in pUC18	This study
pXQ724	*dif* promoter in pWB200	This study
pXQ726	P*dif-narX-frzCD* in pWB200, $NazD_F$	This study
pAD56(G51R)	*narX-tar*(G51R) gene	(Ward *et al.*, 2006)
pXQ728	P*dif-narX-frzCD*(G51R) in pWB200, $NazD_R$	This study

Results

Construction of NarX-FrzCD chimeras

To examine the responses of *M. xanthus* to the same chemical as both positive and negative chemoeffectors, two NarX-FrzCD chimeric receptors were constructed based on sequence similarities within the receptor domains (Fig. 3-1). Hybrid receptors generally fuse a sensor domain from one receptor to a signaling domain from another within the HAMP domain (Utsumi *et al.*, 1989; Baumgartner *et al.*, 1994; Aravind and Ponting, 1999; Ward *et al.*, 2002; Appleman *et al.*, 2003; Xu *et al.*, 2005; Ward *et al.*, 2006). However, FrzCD, a cytoplasmic MCP, lacks an obvious HAMP domain (Bustamante *et al.*, 2004). Nevertheless, the amino acids from Ala 88 to Val 138 of FrzCD do share similarities with the HAMP domains of several transmembrane sensors, including *M. xanthus* DifA, *Bacillus subtilis* TlpB as well as *E. coli* Tar and NarX (Fig. 3-1 A). Two NarX-FrzCD chimeras, $NazD_F$ and $NazD_R$, were constructed as described in **Experimental procedures** (Table 3-1). The cloning

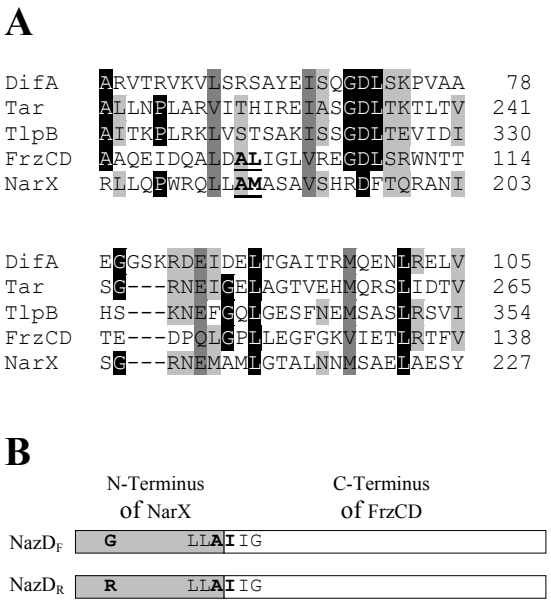

Figure 3-1. Construction of NarX-FrzCD chimeras. A. Sequence alignment of A88-V138 of FrzCD with HAMP linkers of DifA, Tar (*E. coli*), TlpB (*B. subtilis*) and NarX (*E. coli*). Similar and identical residues are shaded in gray and black respectively. The underlined residues in FrzCD and NarX indicate the junction of NarX-FrzCD chimeras. B. The two NarX-FrzCD chimeras, $NazD_F$ and $NazD_R$, consist of the N-terminus of NarX and the C-terminus of FrzCD. L99 of FrzCD (underlined in A), after being mutated to isoleucine, joins A186 of NarX (underlined in A) to create the $NazD_F$. The G51R mutation was introduced into $NazD_F$ to generate $NazD_R$.

process introduced into FrzCD an L99I substitution (Fig. 3-1 A) that joined Ala 186 of NarX for the construction of the chimeras (Fig. 3-1). While $NazD_F$ contains a wild-type NarX segment, $NazD_R$ contains a G51R mutation which altered the activity of NarX and reversed the signaling output of a NarX-Tar chimera in *E. coli* (Williams and Stewart, 1997; Ward *et al.*, 2006). Both $NazD_F$ and $NazD_R$ are under the control of a *difA* promoter in the plasmid constructs, which also contain the MX8 phage attachment site (Magrini *et al.*, 1999) to facilitate subsequent integration into *M. xanthus* chromosome.

$NazD_F$ and $NazD_R$ chimeras are expressed and functional in *M. xanthus*

The two plasmids encoding $NazD_F$ (pXQ726) and $NazD_R$ (pXQ728) were electroporated into the Δ*frzCD* mutant DZ4480 to generate YZ1701 (Δ*frzCD*/*nazD*$_F$) and YZ1702 (Δ*frzCD*/*nazD*$_R$), respectively (Table 3-1). Immunoblotting using polyclonal anti-FrzCD antibodies (McCleary *et al.*, 1990) and whole cell lysates showed that the chimeras in YZ1701 and YZ1702 were expressed and that the level of expression was slightly higher than FrzCD in DZ2, the wild-type strain (data not shown). Figure 3-2 shows that the two chimeras, which contain the transmembrane regions of NarX, are localized in the membrane fraction as expected, in contrast to FrzCD, which is cytoplasmic (McBride *et al.*, 1992).

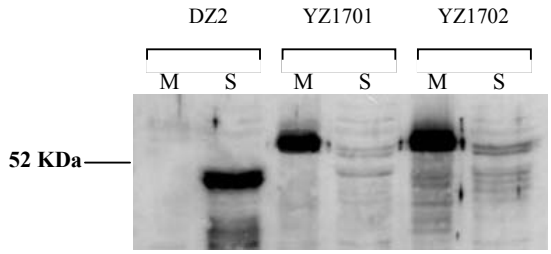

Figure 3-2. Localization of NarX-FrzCD chimeras to the membrane fraction. Membrane (M) and soluble (S) fractions from DZ2 (WT), YZ1701 (Δ*frzCD*/*nazD*$_F$) and YZ1702 (Δ*frzCD*/*nazD*$_R$) were prepared, separated by SDS-PAGE and probed using immunoblotting by anti-FrzCD polyclonal antibodies (See **Experimental procedures**).

We were interested in determining if the NazD$_F$ and NazD$_R$ chimeras were functional, especially since their cellular localization was now in the membrane. Previous work showed that 0.3% isoamyl alcohol (IAA) greatly inhibits swarm expansion of *M. xanthus* (Bustamante *et al.*, 2004). This inhibition was Frz pathway dependent, but only required the signaling domain (C-terminal domain) of FrzCD (Bustamante *et al.*, 2004). Thus, if the NazD$_F$ and NazD$_R$ chimeras are functional, both YZ1701 and YZ102 should respond to IAA since the signaling domain of FrzCD is present in both chimeras. As shown in Fig. 3-3, the parental Δ*frzCD* mutant, DZ4480, was not affected by IAA addition: it showed comparable swarm expansion in the presence or absence of IAA (Bustamante *et al.*, 2004). In contrast, the YZ1701 and YZ102 strains, as well as the wild-type strain DZ2, showed reduced swarming in the presence of 0.3% IAA. These results show that both chimeras are expressed and functional in signaling through the Frz pathway, at least with respect to the repellent response, despite the transformation of the MCP from a cytoplasmic to a transmembrane receptor. It should be noted that both YZ1701 and YZ102 showed diminished swarming on nutrient plates with 1.5% agar (Fig. 3-3).

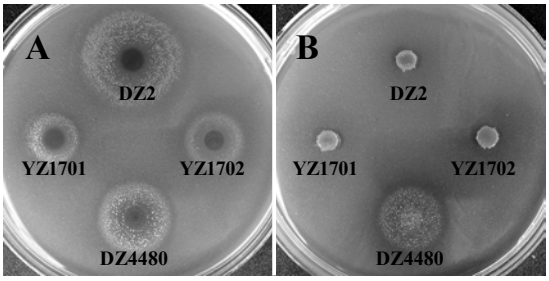

Figure 3-3. Colony expansion on CTT agar with or without IAA. Five microliters of cells in TPM buffer at 5 x 10^9 cells/ml were spotted onto CTT plates (1.5% agar) with (B) or without (A) 0.3% IAA (v/v). The diameter of the plates shown is 9 cm. Strains used are DZ2 (WT), DZ4480 (Δ*frzCD*), YZ1701 (Δ*frzCD*/*nazD*$_F$) and YZ1702 (Δ*frzCD*/*nazD*$_R$).

Nitrate elicits opposite behavioral responses in $NazD_F$- and $NazD_R$-expressing cells

To determine whether $NazD_F$- and $NazD_R$-expressing cells can mediate nitrate-dependent regulation of motility in *M. xanthus*, the behavioral response of cells to nitrate was followed under conditions where cells were isolated from one another on the agar surface. As described in **Experimental procedures**, cells spotted onto TPM plates (0.7% agar) were monitored by time-lapse video microscopy for 30 minutes immediately after exposure to nitrate at 0, 15, 35, 100 and 350 μM. The data in Fig. 3-4 show that, regardless of the presence or absence of nitrate, wild-type (DZ2) cells reversed about once every 6 minutes or 0.16 reversals per minute (rev/min), the Δ*frzCD* mutant (DZ4480) showed essentially zero reversals during the 30 minutes of observation (data not shown). Our observations also indicated that nitrate did not appreciably affect the percentage of motile cells (data not shown). These results indicate that nitrate had no intrinsic effect on the motility behavior of wild-type *M. xanthus* or the Δ*frzCD* mutant. In contrast, nitrate clearly affected the reversal frequency of YZ1701 and YZ1702 (Fig. 3-4). In the absence of nitrate, YZ1701 ($NazD_F$) had a reversal frequency similar to the wild type, but its reversal frequency decreased from about 0.15 to 0.018 rev/min as nitrate concentration increased from 0 to 350 μM. On the other hand, the reversal of YZ1702 ($NazD_R$) increased from the basal level of 0.047 rev/min to 0.46 rev/min as nitrate concentration increased. In relative terms, the tested nitrate concentrations (15, 35, 100 and 350 μM) resulted in 2 to 10 fold changes in the reversal frequency of YZ1701 and

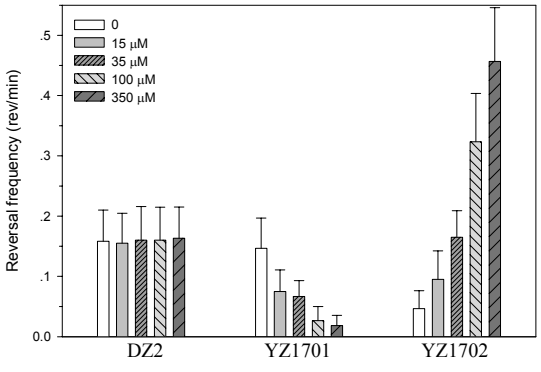

Figure 3-4. Cellular reversal during the first 30 min of exposure to nitrate. Cells at 5.0 x 10^7 cells/ml were transferred to TPM plates (0.7% agar) with different concentrations of nitrate as indicated. Reversal frequency during the first 30 minutes was calculated from 20 cells for each strain as described in **Experimental procedures**. Strains used are DZ2 (WT), YZ1701 (Δ*frzCD*/*nazD*$_F$) and YZ1702 (Δ*frzCD*/*nazD*$_R$).

YZ1702 in opposing directions. No changes could be detected at concentrations at or below 10 μM (data not shown). These results add additional support to the hypothesis that the Frz chemosensory system is indeed a chemotaxis pathway: if the regulation of cell motility in *M. xanthus* could only be achieved through changes in metabolism or cell physiology, nitrate would have been expected to elicit similar responses in the $NazD_F$ and $NazD_R$ expressing strains.

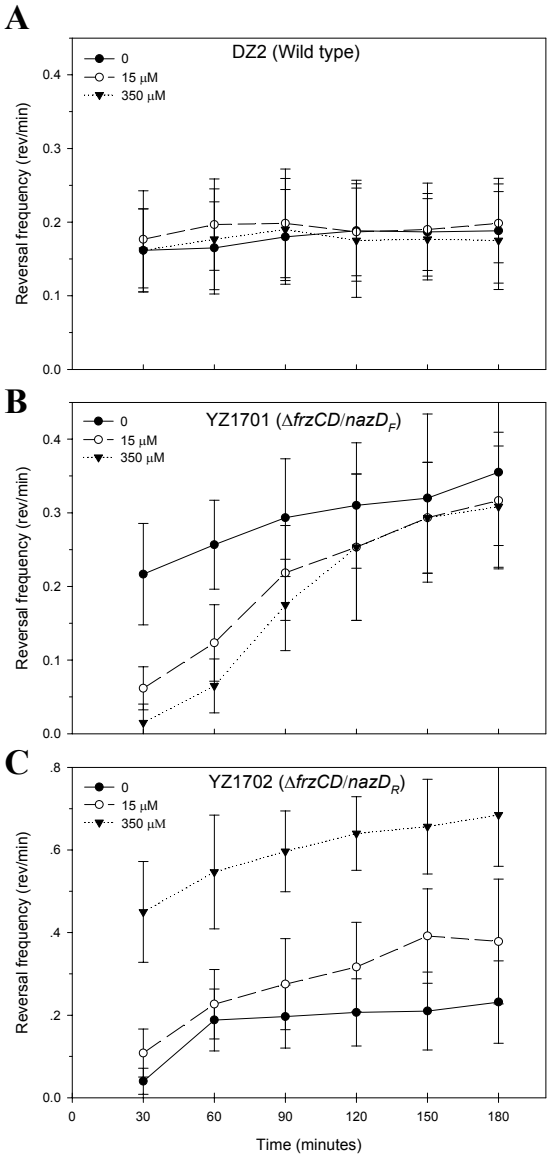

Figure 3-5. Cellular reversal during 3 hours of nitrate exposure. Experiments were performed as described for Fig. 4 for 3 hours. A. DZ2 (WT); B. YZ1701 (Δ*frzCD*/*nazD*$_F$); C. YZ1702 (Δ*frzCD*/*nazD*$_R$).

$NazD_F$- but not $NazD_R$-expressing cells adapt to nitrate exposure

In enteric bacteria, cells adapt to chemotactic stimulation by returning to the unstimulated state after a period of time if the concentration of the chemoeffector stays constant (Macnab and Koshland, 1972; Tsang *et al.*, 1973). To determine whether YZ1701 (Δ*frzCD*/*nazD*$_F$) or YZ1702 (Δ*frzCD*/*nazD*$_R$) adapt to nitrate stimulation, we examined the reversal frequency of cells after exposure to nitrate (0, 15 or 350 μM) for intervals up to three hours. Nitrate exposure had no obvious effect on the reversal frequency of the DZ2 wild-type cells as expected (Fig. 3-5 A). Figures 3-5 B and C show that during the first 30 minutes, in the absence of nitrate,

both $NazD_F$- and $NazD_R$-expressing mutants exhibited reversal frequencies similar to those shown in Figure 3-4; during the next 30 to 60 minutes, the reversal frequency of both strains fluctuated upwards slightly. However, during the first 30 minutes in the presence of nitrate (15 and 350 μM), the reversal frequency of both YZ1701 and YZ1702 changed: nitrate decreased the reversal frequency of YZ1701 and increased that of YZ1702 (Fig. 3-5 B and C). The reversal frequency of YZ1701 in the presence of nitrate eventually approached that in the absence of nitrate (Fig. 3-5 B), indicating that this strain can adapt to the presence of nitrate. On the other hand, the reversal frequency of YZ1702 in the presence of nitrate remained elevated throughout the duration of the experiment. Even at 15 μM, the lowest concentration of nitrate to have detectable effect on *M. xanthus* cell behavior, the reversal frequency was about 1.7 fold of the basal levels at the end of the experiment. These results indicate that the *M. xanthus* strains expressing NarX-FrzCD chimera can adapt to nitrate as an attractant but not as a repellent.

***M. xanthus* cells fail to adapt to known repellents**

The lack of adaptation to nitrate by YZ1702 caused us to investigate the adaptation of wild-type DZ2 to known repellents as isolated cells on agar plates. It should be noted that isolated *M. xanthus* cells only move by A-motility. As shown in Table 3-2, the reversal frequency of wild-type cells increased in the initial exposure to 0.03% IAA (McBride *et al.*, 1992). During the following 2.5 hours, the reversal frequency remained elevated with no evidence of adaptation. Responses to 1% DMSO and 0.03% phenethyl alcohol yielded similar results (Table 3-2). In addition, even at 0.0001% IAA, the lowest concentration to have detectable effect on *M. xanthus* cell behavior, the reversal frequency of the wild-type DZ2 remained elevated after three hours of exposure (data not shown). These results suggest that the lack of adaptation to repellents may be an intrinsic property of the Frz pathway and chemotaxis in *M. xanthus*.

Table 3-2. Cellular reversal of *M. xanthus* wild-type DZ2 in response to known repellents.

Repellents	Reversal Frequency (rev/min)		
	0~30 min	75~105 min	150~180 min
None	0.16 ± 0.05	0.18 ± 0.07	0.19 ± 0.07
0.03% IAA	0.55 ± 0.14	0.55 ± 0.13	0.56 ± 0.14
0.03% Phenethyl alcohol	0.26 ± 0.06	0.28 ± 0.07	0.27 ± 0.06
1% DMSO	0.26 ± 0.05	0.26 ± 0.08	0.27 ± 0.04

Swarm expansion, which involves both A- and S-motility, was also examined following nitrate or repellent stimulation. Figure 3-6 A shows that swarm expansion of DZ2 (wild type), DZ4480 (Δ*frzCD*) and YZ1701 (Δ*frzCD*/*nazD*$_F$) were unaffected by nitrate up to 1000 μM. In contrast, YZ1702 (Δ*frzCD*/*nazD*$_R$) showed reductions in colony expansion with increasing concentrations of nitrate. These results are consistent with our previous data showing that YZ1702 cells failed to adapt to nitrate as a repellent (Fig. 3-5). Moreover, the repellent IAA at 0.1% or higher also caused reductions in swarm expansion (Fig. 3-6 B) for all *M. xanthus* strains tested except the Δ*frzCD* mutant DZ4480, which is non-responsive to such inhibition (Bustamante *et al.*, 2004). IAA had stronger inhibitory effects on YZ1701 and YZ1702 (Fig. 3-6 B) than on the wild type possibly due to higher expression of the chimeras in these mutant strains (Fig. 3-2 and data not shown). In summary, the data here from both

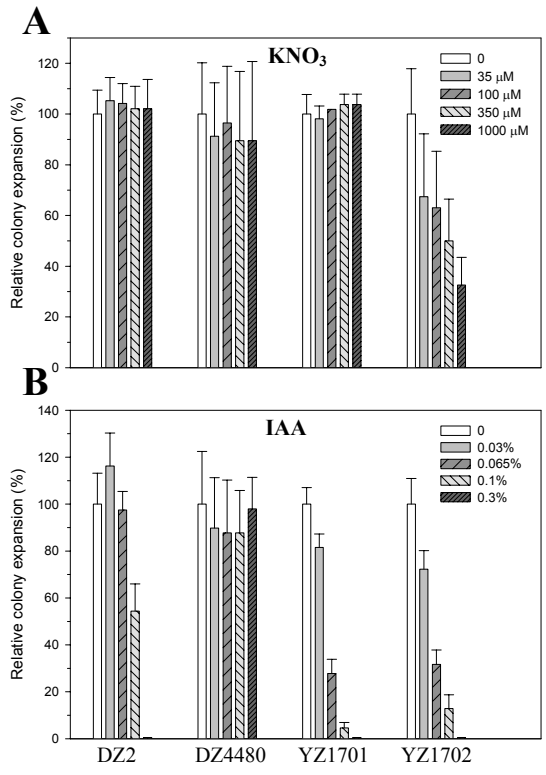

Figure 3-6. Colony expansion with different concentrations of nitrate (A) or IAA (B). Five microliters of cell suspension at 5 x 10^9 cells/ml was spotted onto CTT plates (1.5% agar) with indicated concentration of nitrate or IAA. Relative colony expansion was calculated based on net increases in colony diameter after 72 hours of incubation at 32°C and was normalized to that of the same strain without IAA and nitrate. Strains used are DZ2 (WT), DZ4480 (Δ*frzCD*), YZ1701 (Δ*frzCD*/*nazD*$_F$) and YZ1702 (Δ*frzCD*/*nazD*$_R$).

microscopic and macroscopic assays indicate that *M. xanthus* cells do not adapt to repellent stimulation mediated through the Frz pathway.

Effect of repellents on *M. xanthus* development

Proper regulation of motility is critical for *M. xanthus* fruiting body formation (Ward and Zusman, 1999). To investigate the impact of repellents on *M. xanthus* fruiting body development, DZ2 (wild type), DZ4480 (Δ*frzCD*), YZ1701 (Δ*frzCD*/*nazD*$_F$) and YZ1702 (Δ*frzCD*/*nazD*$_R$) strains were assayed for fruiting in the presence of nitrate or IAA (Fig. 3-7). Without nitrate and IAA, DZ2 formed normal fruiting bodies and DZ4480 (Δ*frzCD*) formed frizzy filaments. Nitrate supplementation only affected YZ1702; the other strains tested appeared to be unaffected, even at 1000 μM nitrate. Without nitrate, YZ1702 appeared frizzy under developmental conditions. As nitrate concentration increased from 0 to 35 μM (data not shown) and to 100 μM, the morphology of YZ1702 aggregates became less frizzy and more organized (Fig. 3-7). As nitrate concentration increased further, the organization of

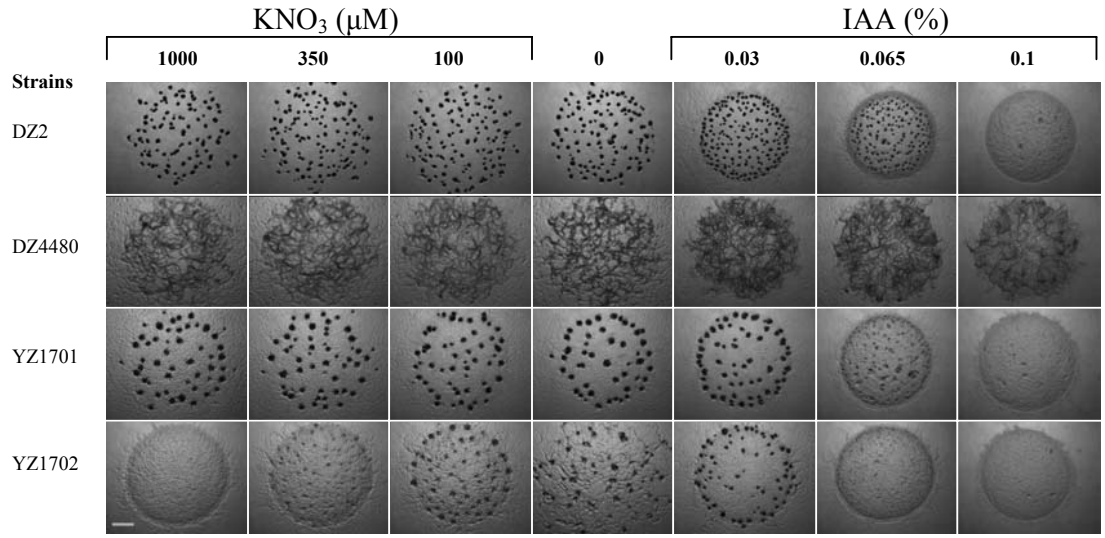

Figure 3-7. Fruiting body formation with or without nitrate or IAA. Five microliters of cell suspension at 5 x 10^9 cells/ml was spotted onto CF agar with indicated concentration of nitrate or IAA. The controls without IAA and nitrate are shown in the middle. Shown on the left are plates containing indicated concentration of nitrate and on the right are those with IAA. Development was documented after 3 days of incubation at 32°C. Bar, 1 mm. Strains used are DZ2 (WT), DZ4480 (Δ*frzCD*), YZ1701 (Δ*frzCD*/*nazD*$_F$) and YZ1702 (Δ*frzCD*/*nazD*$_R$).

YZ1702 aggregates deteriorated. In contrast, IAA affected the development of all strains except the Δ*frzCD* mutant DZ4480 (Fig. 3-7). At 0.065% (data not shown) and 0.1%, (below the concentrations required for complete inhibition of colony expansion (Astling *et al.*, 2006), IAA nearly abolished aggregation of all strains (Fig. 3-7). Thus developing cells, similar to their vegetative counterparts, failed to adapt to the presence of the repellents.

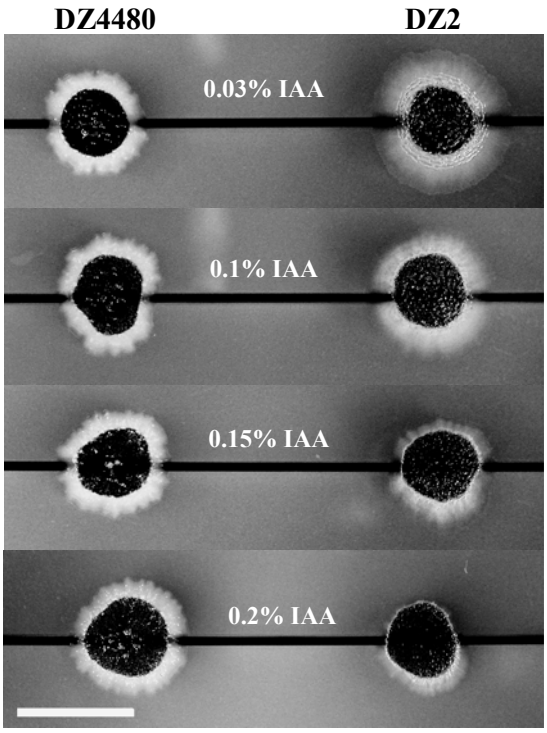

Figure 3-8. Colony swarming in repellent gradients. Ten microliters of cell suspension at 2.5 x 10^{10} cells/ml (is this 9 or 10, inconsistent with methods) with India ink was spotted onto the plates with established IAA gradients. Colony expansion was photographed after 24 hours of incubation at 32°C. The dark center of the colonies indicated by India ink marks the initial placement of the cell suspension. The dark line is the divider that compartmentalized the plate into two sections. The upper compartments in the pictures here contained IAA at the indicated concentrations. Strains used are DZ2 (WT) and DZ4480 (Δ*frzCD*). Bar, 10 mm. More details on the experiments are provided in Experimental procedures.

Biased movement in a repellent gradient

The absence of temporal adaptation to repellents described above caused us to reexamine *M. xanthus* behavior within spatial repellent gradients. For these experiments, we used the assay developed by Shi *et al.* (1993) in which stable and steep gradients of chemicals are established using compartmentalized Petri plates containing various chemicals or nutrients and agar overlays. In these gradients, *M. xanthus* cells appear to exhibit biased movement away from IAA (Shi *et al.*, 1993). Figure 3-8 shows one such experiment where the swarming of the wild-type strain DZ2 is compared to that of the *frzCD* mutant DZ4480 when one of the compartments contains the repellent IAA while the other

compartment does not. The results (Fig. 3-8) show that the *frzCD* mutant is unresponsive to IAA at all concentrations tested, while the wild type shows significant inhibition of swarming by increasing IAA concentrations. That is, the higher the IAA concentration in a gradient, the more the inhibition on the net movement of the wild type (Fig. 3-8). Thus the differential swarming observed in the two compartments in this assay can give the appearance of negative chemotaxis. However, the repellent response here appears to consist of the inhibition of net cell translocation; this agrees with the sustained increase in reversal frequency observed in *M. xanthus* in the presence of repellents (Table 3-2; McBride *et al.*, 1992). This response is in contrast to the one observed for *E. coli* where cells do swim away from repellents using biased random walks (Falke *et al.*, 1997; Szurmant and Ordal, 2004).

Discussion

In the present study, we used two NarX-FrzCD chimeras, $NazD_F$ and $NazD_R$, which differ only by a single residue, to study chemotaxis and signaling through FrzCD in *M. xanthus*. We showed that $NazD_F$ and $NazD_R$ mediate opposite initial responses to nitrate: the reversal of $NazD_F$ cells is suppressed and that of $NazD_R$ is elevated by nitrate (Figs 3-4 and 3-5). To our surprise, nitrate exposure resulted in a sustained elevation of reversal in YZ1702 throughout the three-hour duration of our experiment (Fig. 3-5). It was further discovered that isolated *M. xanthus* cells failed to adapt to the presence of known repellents including IAA, DMSO and phenethyl alcohol after three hours of incubation (Table 3-2). In addition, negative chemostimulation affected *M. xanthus* behavior during fruiting body development as well as vegetative swarming (Fig. 3-6 and 3-7). We also confirmed that *M. xanthus* biases its movement in an IAA gradient (Fig. 3-8) as reported previously (Shi *et al*., 1993). The results here shed light on two questions regarding *M. xanthus* taxis: whether there are signal transduction pathways that directly regulate cell movement independently of cell physiology and whether the mechanism in this gliding myxobacterium conforms to the well-understood chemotaxis regulatory paradigm in flagellated bacteria (Szurmant and Ordal, 2004).

Regulation of gliding motility independent of metabolism and cell physiology

In flagellated bacteria, behavioral changes elicited by chemotactic stimuli can be detected very rapidly, within tens of milliseconds (Sourjik and Berg, 2002; Khan *et al.*, 2004). It has become apparent that their chemotaxis signal transduction regulates motor function independent of changes in cell physiology or metabolism (Adler, 1969; Szurmant and Ordal, 2004). *M. xanthus* moves very slowly and the earliest detectable behavioral changes require many minutes rather than milliseconds (McCleary *et al.*, 1990; McBride *et al.*, 1992; Kearns

and Shimkets, 1998; Bonner *et al.*, 2005). Moreover, as has been discussed earlier, many of the chemicals that are known to alter the motility behavior of *M. xanthus* have the potential to modify cell physiology and affect behavioral changes independently of chemotaxis (see **Introduction**). Consequently, regulation of motility in *M. xanthus* through metabolism or other physiological changes, rather than through chemosensory signal transduction, has remained a viable possibility. Using two engineered MCP receptors that differ by a single amino acid, we showed that *M. xanthus* can respond to nitrate as a negative or a positive stimulus depending on the modification to the chimeric receptor (Fig. 3-4). If changes in motility behavior were due to changes in physiology and not the result of chemotaxis regulation, nitrate would have had the same influence on the movement of *M. xanthus* irrespective of the configuration of the engineered receptor. Although toxicity is always a concern when adding repellents to bacteria, the use of the chimeras and nitrate in our case are not expected to compromise the cells metabolically. These results are consistent with the hypothesis that *M. xanthus* regulates motor function by means of the Frz chemosensory system in much the same way as the flagellated bacteria regulate cell behavior by chemotaxis signal transduction.

Temporal sensing of attractants by *M. xanthus*

In temporal assays, *M. xanthus* cells suppress cellular reversals in response to chemoattractants. These responses are analogous to chemosensory responses of the enteric bacteria to attractants ("tumbles" are suppressed). Initial exposure to nitrate reduces the reversal frequency of YZ1701 (Δ*frzCD*/*nazD*$_F$) (Fig. 3-4) as does PE of the wild-type *M. xanthus* (Kearns and Shimkets, 1998; Kearns *et al.*, 2000; Kearns *et al.*, 2001). Slowly, *M. xanthus* cells adapt to the presence of constant concentrations of attractants by returning to the pre-stimulus reversal frequency (Fig. 3-5; Kearns and Shimkets, 1998; Kearns *et al.*, 2000;

2001b). Adaptation by *M. xanthus* to the attractants took about 1.5 h (Fig. 3-5; Kearns and Shimkets, 1998), rather than the several seconds to minutes as in enteric bacteria (Macnab and Koshland, 1972; Brown and Berg, 1974). The slow rate of adaptation reflects the slow rate of movement of *M. xanthus*. More importantly, *M. xanthus* cells exhibit biased movement toward higher concentration of chemoattractants (Kearns and Shimkets, 1998). Therefore, on both temporal and spatial scales, the behavioral response of *M. xanthus* to chemoattractants is similar to flagellated bacteria. A temporal sensing mechanism in itself is sufficient to explain directed or tactic movement of *M. xanthus* in a spatial gradient of a chemoattractant. Chemokinesis and spatial sensing may exist in *M. xanthus* (Dworkin and Eide, 1983; Ward *et al.*, 1998), but it is not essential to invoke such mechanisms to explain *M. xanthus* taxis in response to positive stimuli.

Behavioral response of *M. xanthus* to negative stimuli

In our experiments, the behavioral responses of individual or isolated *M. xanthus* cells to negative stimulation are quite distinct from those of flagellated bacteria. Although exposure to negative stimuli did result in an initial elevation of reversal frequency as observed by video microscopy (Fig. 3-4 and Table 3-2; McBride *et al.*, 1992), isolated *M. xanthus* cells failed to reset their reversal frequency to the pre-stimulus level (Fig. 3-5, Table 3-2). YZ1702 (Δ*frzCD*/*nazD*$_R$), for example, was unable to adapt to the presence of nitrate after three hours of incubation. Similarly, individual cells of various strains all failed to adapt to the three known repellents IAA, DMSO and phenethyl alcohol (Table 3-2) at non-saturating concentrations as determined previously (McBride *et al.*, 1992; Shi and Zusman, 1994). On a macroscopic scale, these chemicals also affected both vegetative swarming and development (Fig. 3-6 and 3-7). If either the vegetative or the developing cells were able to adapt to these chemicals in tens of minutes or even hours, these chemicals would have had

minimum effects on colony or developmental morphology in the time frame (many days) these experiments were conducted.

The results presented here suggest that behaviorally, *M. xanthus* cells do not adapt to repellents on a single cell basis. This is consistent with a previous report (Shi and Zusman, 1994) indicating that only cells within groups or clumps appeared to adapt to IAA. Movements of cells in groups are driven by both A and S motility, while isolated cells move by A-motility. It is possible that only the S-motility machinery adapted to repellent stimulations. It has been shown previously that colony expansion or swarming of *M. xanthus* on soft agar plate (0.4% agar) depend solely on S-motility (Shi and Zusman, 1993). Yet, colony expansion of the wild-type DZ2 on such agar plates was reduced by 49% and 65% by the addition of 0.065% and 0.1% IAA, respectively (Xu and Yang, unpublished data). In addition, when several A^+S^- and A^-S^+ mutants were examined in the presence of 0, 0.03, 0.1 and 0.3 percent of IAA, the inhibition of colony expansion by IAA showed no particular preference for either A or S motility (Xu and Yang, unpublished data). How might cells in clumps seem to adapt to IAA? One possible explanation is that when a cell moves to the top of a pile/clump, it becomes physically separated from the agar medium that contains IAA and recovers from the transient exposure. This also illustrates one of the difficulties in the studies of chemotaxis in surface motile bacteria and in the interpretation of results from these studies. Based on the above observations along with the results from macroscopic and microscopic analysis (Fig. 3-5, 3-6 and 3-7, Table 3-2), we conclude that *M. xanthus* cells, whether isolated or in groups, do not adapt temporally to repellent stimuli.

Mechanisms for biased movement in a repellent gradient

How might *M. xanthus* achieve tactic or biased movement in a repellent gradient (Fig. 3-8; Shi *et al.*, 1993) without a temporal sensing mechanism? Theoretical calculations indicated that *M. xanthus*, with its size and speed, should be able to use a spatial sensing mechanism for chemotaxis (Berg and Purcell, 1977; Dworkin and Eide, 1983) that does not necessitate temporal adaptation. However, experimental observations make this an unlikely mechanism. For example, if *M. xanthus* showed chemotaxis by spatial sensing, we would have expected net movement down a repellent gradient to exceed or at least equal to that without repellents. The opposite is observed: an effective gradient always reduced rather than promoted the net movement down the gradient or in any other direction (Fig. 3-8). In addition, such a mechanism predicts that cells at the higher end of the repellent gradient would move down the gradient from their initial position. Instead, cells at the edge of a colony at the higher end of an effective gradient either showed little movement or moved up the gradient (Fig. 3-8; Shi *et al.*, 1993). Although a spatial sensing mechanism may not require temporal adaptations, it is not consistent with the observations in *M. xanthus.*

Alternatively, the observed bias in a gradient of IAA can be explained by the inhibition of net movement by repellents through increased reversal without adaptation. It is evident that increases in reversal alone can lead to decreases in net cell movement and colony expansion. Both *frzCD*^{c} and *mglA* mutants display a hyper-reversal phenotype at the cellular level and show no or little colony expansion (Hartzell, 1997; Spormann and Kaiser, 1999; Inclan *et al.*, 2007). Different concentrations of IAA and other negative stimuli essentially phenocopy these two mutants over a wider spectrum. IAA at 0.3% reduced colony expansion to similar degrees as *frzCD*^{c} and *mglA* mutations (Hartzell, 1997; Spormann and Kaiser, 1999; Inclan *et al.*, 2007). The lower the IAA concentration, the less the increase in cell reversal and the less the decrease in colony expansion (Table 3-2, Fig. 3-6 B and data not shown). In a

spatial gradient, cells in a zone with higher IAA concentration would show less net movement than those in a zone with lower IAA concentration. This would give the appearance of biased movement although movements in all direction are reduced. This mechanism for the biased movement of *M. xanthus* in a repellent gradient is consistent with all experimental results without the requirement of adaptation and without invoking spatial sensing. This is also somewhat reminiscent of the photophobic or light-induced avoidance response by many phototrophic microbes (Diehn, 1973; Spudich and Bogomolni, 1984; Sprenger *et al.*, 1993; Lucia *et al.*, 1996; Armitage and Hellingwerf, 2003; Sobierajska *et al.*, 2006).

Signaling by the cytoplasmic receptor FrzCD

A few interesting observations were also made on the signaling of FrzCD with the NazD chimeras. With all the previous chimeras of NarX and MCPs, as long as the wild-type sensory module of NarX is used, nitrate always activated the downstream kinase, as in the case of Nart and NafA (Ward *et al.*, 2002; Xu *et al.*, 2005; Ward *et al.*, 2006). In addition, nitrate stimulates the kinase activity of NarX in its native context in *E. coli* (Stewart and Berg, 1988; Kalman and Gunsalus, 1990). Yet, nitrate perception by $NazD_F$ likely inhibits the cognate kinase since nitrate stimulation decreases the reversal of *M. xanthus* (Fig. 3-4 and 3-5); $NazD_R$, the G51R version, inverted this response. More importantly, despite its cytoplasmic nature, FrzCD contains a HAMP-like linker that connects the N-terminus to the C-terminal signaling domain (Fig. 3-2); conversion of FrzCD to transmembrane chimeras apparently created functional receptors in *M. xanthus* (Fig. 3-2, 3-3, 3-4, 3-5, 3-6 and 3-7). Although HAMP domains are associated mostly with transmembrane proteins (Aravind and Ponting, 1999; Williams and Stewart, 1999), our results suggest that the HAMP-like region of FrzCD may function to transmit whatever signals or changes from the N-terminus to the

signaling module at the C-terminus. These findings further underscore the versatility and plasticity of the HAMP domain in signal transduction in diverse organisms, pathways and signaling architectures (Hulko *et al.*, 2006; Taylor, 2007).

Experimental procedures

Bacterial strains, plasmids and culture conditions

The strains and plasmids used in this study are listed in Table 3-1. *M. xanthus* was grown using either CTT or CYE media at 32ºC. CF plates were used as the development-inducing medium for *M. xanthus*. *E. coli* strain XL1-Blue (Stratagene), used for routine cloning and plasmid construction, was grown using Luria-Bertani (LB) media at 37ºC. Liquid cultures were grown using a rotary shaker at 300 rotations per minute (rpm). Plates contained 1.5% agar unless noted otherwise. When necessary, kanamycin was added to media at 100 μg/ml.

Construction of plasmids and mutants

pXQ724, a vector containing the *dif* promoter, was constructed for heterologous gene expression in *M. xanthus*. The *dif* promoter was amplified from *M. xanthus* (DK1622) genomic DNA using oligonucleotides (5'-CCCAAGCTTATCTGCCGCGTTGGATGT-3' and 5'-GGGGTACCCGCATGGGTTGCTTTCGGGGGAT-3'), digested with *Hind*III and *Kpn*I and cloned into the same sites in pWB200 (Xu *et al.*, 2005). To construct the *narX-frzCD* chimeras, the DNA encoding NarX N-terminus was first amplified from the genomic DNA of *E. coli* strain XL1-Blue using oligonucleotides (5'-CGGGATCCTGAAGGAAGAGGTTTACATGCT-3' and 5'-GCCATGGCCAGCAGTTGCCGC-3'). The PCR product was digested with B*am*HI and cloned into *Bam*HI and *Sma*I of pUC18 (Norrander *et al.*, 1983) to create pXQ701. The DNA encoding the FrzCD C-terminus was amplified from *M. xanthus* (DK1622) genomic DNA using oligonucleotides (5'-TTGGTGGCCATCATCGGCCTGGTGCGCGAG-3' and 5'-CGAATTCAGCGCTAGTCGGCCTTGAAC-3'), digested with *Eco*RI and *Msc*I and cloned

into the same sites in pXQ701 to generate pXQ703 with a *narX-frzCD* chimeric gene. pXQ703 was digested with *Bam*HI and *Eco*RI to release the 1.7 kb *narX-frzCD* fragment which was cloned into the same sites of pXQ724 to produce pXQ726. pXQ726, the P*dif-narX-frzCD* plasmid, enables the expression of *narX-frzCD* chimeric gene $nazD_F$ from the *dif* promoter (Table 3-1). To construct $nazD_R$, a 0.3 kb *Bam*HI-*Xho*I fragment from pAD56(G51R) (Ward *et al.*, 2006) was cloned into the same sites of pXQ726 to create pXQ728 [P*dif-narX-frzCD*(G51R)] (Table 3-1). All plasmid constructs were confirmed by DNA sequencing and/or restriction enzyme digestions. The $NazD_F$- and $NazD_R$-expressing strains, YZ1701 and YZ1702, were generated by electroporating (Kashefi and Hartzell, 1995) pXQ726 and pXQ728 into the Δ*frzCD* mutant DZ4480.

Fractionation and cellular localization of NarX-FrzCD chimeras

The fractionation procedure was performed according to McBride *et al*. (1992). Cells were grown in CYE liquid to mid-log phase at approximately 4.0 x 10^8 cells/ml. Ten milliliter aliquots were centrifuged at 10,000 *g* for 2 min and resuspended to 2 x 10^9 cells/ml in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.2) with salt supplementation (250 mM NaCl). Cells were disrupted by sonication and cell debris was removed by centrifugation at 10,000 *g* for 15 minutes. The resulting cell lysate was subject to ultracentrifugation at 100,000 *g* for 1 h. The supernatant was separated from the pellet. Ten microliters of the supernatant and resuspended pellet were separated by SDS-PAGE and probed with polyclonal anti-FrzCD antibodies (McCleary *et al.*, 1990).

Examination of reversal frequency

Reversal frequency was examined similarly as described previously (Shi and Zusman, 1994; Kearns and Shimkets, 1998). Cells were grown in CTT liquid culture to 1.0 ~ 1.5 x 10^8

cells/ml and resuspended to 5.0 x 10^7 cells/ml in TPM buffer [10 mM Tris (hydroxymethyl) aminomethane HCl (pH 7.6), 8 mM $MgSO_4$ and 1 mM K_2HPO_4-KH_2PO_4 (pH 7.6)]. Ten microliters of the cell suspension was spotted onto a TPM plate (0.7% agar) supplemented with indicated chemicals. After 30 s, excess liquid was removed as described (Shi and Zusman, 1994). Cell movements were recorded using an Olympus IX81 inverted microscope for 30 to 360 minutes at 200 x magnification. Recording was set at 1/60 of real time and played back at normal speed. To determine reversal frequency of a strain, cells were selected prior to the start of the movie and the reversals of 20 isolated and fully motile cells were analyzed.

Examination of colony expansion and development

M. xanthus was first grown in CTT liquid to approximately 1.0 ~ 2.0 x 10^8 cells/ml. Cells were harvested, washed, and resuspended in TPM buffer at approximately 5.0 x 10^9 cells/ml. For analysis of colony expansion on hard agar, 5 μl aliquots were spotted onto CTT plates (1.5% agar) with or without IAA and nitrate (KNO_3). Colonies were photographed after 3 days of incubation at 32°C. To obtain quantitative results, colony diameters were measured immediately after being spotted and after 3 days of incubation. Colony expansion was measured by the net increase in colony diameter. For the examination of fruiting body formation, 5 μl of cell suspension at 5.0 x 10^9 cells/ml was spotted onto CF plates with or without IAA and nitrate (KNO_3). Fruiting body formation was examined after 3 days of incubation at 32°C using a Motic dissecting microscope. All plates were sealed with parafilm during the incubation.

Colony expansion in IAA gradients

The experimental setup for examining *M. xanthus* movement in IAA gradients was similar to that by Shi *et al* (1993). Briefly, 30 ml of CTT with 1.5% agar was poured into one side of a Petri plate with two compartments. The other side contained the same medium except that IAA was supplemented at indicated concentrations. After solidification of the medium in both compartments, about 8 ml of CTT with 0.4% agar was poured on the top as a thin layer covering the entire plate. Plates were allowed to air dry for 20 hours with their lids slightly open. *M. xanthus* cells, grown overnight in CTT liquid to approximately 2.0 ~ 3.0 x 10^8 cells/ml, were harvested, washed, and resuspended at 2.5 x 10^{10} cells/ml in TPM buffer containing 10% India ink. Ten microliters of the cell suspension was applied on the top of the divider between the two sides. Plates were incubated at 32°C for about 24 hours before being photographed.

CHAPTER 4

Independence and interdependence of Dif and Frz, two chemosensory pathways in *Myxococcus xanthus*

Summary

The two *Myxococcus xanthus* chemosensory pathways, Dif and Frz, are required for the excitation and adaptation of chemotaxis to phosphatidylethanolamine (PE) respectively. Hence, the two pathways likely communicate with each other in the chemotactic responses to PE, such as 12:0 PE and 16:1 PE, which require the intact Dif and Frz pathways. In this study we investigated the methylation of MCPs (DifA and FrzCD), the major mechanism for adaptation, during signaling of the chemotactic response in this bacterium. Here we provided evidence to indicate that DifA does not undergo methylation upon stimulations by chemoeffectors including 12:0 PE. However, using mutants expressing a NarX-DifA (NafA) chimera, which is specifically activated by nitrate, it was found that a signal transducing through DifA, DifC (CheW) and DifE (CheA) modulates FrzCD methylation. Surprisingly, the *bone fide* attractant 12:0 PE can modulate FrzCD methylation by two mechanisms, which can be differentiated by dependency on the putative complex of DifA, DifC and DifE (DifACE). The DifACE-independent mechanism, possibly resulting from specific sensing by FrzCD, increases FrzCD methylation as expected. Strikingly, the DifACE-dependent mechanism decreases FrzCD methylation, as opposed to the chemotaxis paradigm that an attractant (repellent) increases (decreases) MCP methylation. This "opposite" FrzCD methylation by DifACE-dependent signaling in 12:0 PE response was nevertheless corroborated by results of NafA-expressing mutants, because nitrate functions as a repellent and increases FrzCD methylation for these mutants. Based on these findings, we proposed a model for the chemotaxis toward 12:0 PE (and 16:1 PE), in which DifA and FrzCD both sense the same signal and activate the pathways of excitation (Dif) and adaptation (Frz) respectively. The two pathways communicate with each other via methylation crosstalk from DifACE to FrzCD so that excitation and adaptation can be coordinated.

Introduction

Myxococcus xanthus is a bacterium that utilizes its motility in both the vegetative and developmental stages of its life cycle (Zusman *et al.*, 2007). During vegetative growth, this Gram-negative bacterium moves on solid surfaces to seek nutrients in the form of organic matter or other bacteria as preys (Reichenbach, 1999; Berleman *et al.*, 2006). Under nutrient limitation, *M. xanthus* initiates a developmental process in which up to 10^5 cells move to aggregate on surfaces and form a multicellular structure called a fruiting body. Approximately 20% of the cells eventually differentiate into myxospores within fruiting bodies (Wireman and Dworkin, 1975). The myxospores, which are more resistant to environmental stress such as desiccation, UV irradiation and heat than vegetative cells, can germinate and re-enter the vegetative cell cycle when conditions become conducive for growth. The surface motility of *M. xanthus,* known as gliding, involves no flagella (Shimkets, 1990). It is controlled by two distinct systems: the adventurous (A-) and the social (S-) motility systems. The A-motility engine powers the movement of isolated cells and the S-motility engine functions only when cells are in close proximity or in cell groups (Hodgkin and Kaiser, 1977; Hodgkin and Kaiser, 1979a, b). The two motility systems appear to function synergistically to enable surface translocation of *M. xanthus* (Kaiser and Crosby, 1983; Spormann and Kaiser, 1999).

Chemotaxis, movement of cells toward attractants or away from repellents, is best studied in the enteric bacteria such as *Eschrichia coli* and *Salmonella typhimurium* (Falke *et al.*, 1997; Szurmant and Ordal, 2004). These bacterial cells alternate between two types of motility behaviors: straight run and tumble. A run enables a cell to move a certain distance in one direction whereas a tumble results in no net movement and a change in direction for the ensuring run. Chemotaxis in these swimming bacteria is achieved by a temporal sensing

mechanism through the regulation of tumbling frequency by the chemotaxis pathway. Tumble is suppressed by attractants and promoted by repellents upon initial exposure. These chemotaxis stimuli are detected by transmembrane sensors known as methyl-accepting chemotaxis proteins (MCPs), which transduce signals to downstream components of the chemotaxis pathway to regulate the tumbling frequency. The immediate change of cell behavior, or excitation, is followed by a temporal adaptation that eventually resets the tumbling frequency back to the prestimulus level. This adaptation, achieved mainly through MCP methylation, provides a "memory" such that cells respond to concentration differences rather than an absolute concentration of a stimulus (Falke *et al.*, 1997; Szurmant and Ordal, 2004). MCP methylation is stimulated by attractants and suppressed by repellents in enteric bacteria (Falke *et al.*, 1997).

Based on sequence homology, *M. xanthus* has at least eight chemosensory pathways (Zusman *et al.*, 2007), of which the Frz and Dif systems are two of the best studied. The Frz pathway controls cellular reversal frequency (Blackhart and Zusman, 1985; McBride *et al.*, 1992) and mediates tactic response to various chemostimuli including small alcohols and certain phosphatidylethanolamine (PE) species (Shi *et al.*, 1993; Kearns and Shimkets, 1998). Although the Dif pathway plays a central role in regulating the production of exopolysaccharide (EPS) (Yang *et al.*, 1998b; Bellenger *et al.*, 2002; Black and Yang, 2004), it is also involved in the regulation of cell reversal in response to PE. Previous results suggested the Dif system is essential for excitation while the Frz system is essential for adaptation in the response to PE (Kearns and Shimkets, 1998; Bonner *et al.*, 2005). Therefore, the functions of the Dif and Frz pathways must converge at the regulation of cell reversal behavior as was proposed (Kearns and Shimkets, 2001). We are interested in using the Dif and the Frz pathways as examples to explore how the activities of different chemosensory pathways may be coordinated in *M. xanthus*.

In particular, we investigated the impact of the Dif pathway on the methylation of FrzCD, the MCP homolog of the Frz system. Our initial experiments indicated that DifA, the MCP homolog of the Dif system, does not undergo methylation in either the regulation of EPS production or in the response to PE. On the other hand, FrzCD methylation, easily detectable, is clearly influenced by signaling through the Dif pathway. The flow of information from DifA to FrzCD requires DifC (CheW-like) and DifE (CheA-like), suggesting an essential role for the DifA-DifC-DifE (DifACE) ternary complex in the process. Surprisingly, the modulation of FrzCD methylation by signals from the DifACE complex is the opposite of expectation: attractants sensed by the Dif system decreased FrzCD methylation whereas repellents signaling through Dif increase FrzCD methylation. Interestingly, 12:0 PE, which is an attractant that signals through the Dif pathway, still increased FrzCD methylation in all the *dif* mutants examined. Hence, there are apparently two mechanisms for sensing PE in *M. xanthus*: one is DifACE-dependent and the other DifACE-independent and that the latter results in adaptation by increasing FrzCD methylation. The signal from the former is divided into two branches, one directing a decrease in cell reversal and the other reducing FrzCD methylation to slow the rate of adaptation for the slow moving *M. xanthus*.

Table 4-1. *M. xanthus* strains and plasmids used in this study.

Designation	Genotype or description	Source or reference
Strains		
DK1622	Wild type	(Kaiser, 1979)
YZ601	Δ*difA*	(Xu *et al.*, 2005)
YZ690	Δ*pilA*	This study
DZ4480	Δ*frzCD*	(Bustamante *et al.*, 2004)
YZ724	Δ*difA*/*nafA*	(Xu *et al.*, 2005)
YZ1012	Δ*difA* Δ*pgi*/*nafA*	This study
YZ730	Δ*difA* Δ*difC*/*nafA*	(Xu *et al.*, 2005)
YZ732	Δ*difA* Δ*difE*/*nafA*	(Xu *et al.*, 2005)
YZ754	Δ*difA* Δ*difB*/*nafA*	This study
YZ731	Δ*difA* Δ*difD*/*nafA*	(Xu *et al.*, 2005)
YZ733	Δ*difA* Δ*difG*/*nafA*	(Xu *et al.*, 2005)
YZ602	Δ*difB*	(Black and Yang, 2004)
SW403	Δ*difC*	(Bellenger *et al.*, 2002)
SW406	Δ*difD*	(Black and Yang, 2004)
YZ603	Δ*difE*	(Black and Yang, 2004)
YZ604	Δ*difG*	(Black and Yang, 2004)
YZ811	Δ*sglK*	This study
LS2200	*fibA*	(Kearns *et al.*, 2002)
YZ1001	Δ*pgi*	This study
YZ1701	Δ*frzCD*/*nazD*$_F$	(Xu *et al.*, 2007)
YZ1702	Δ*frzCD*/*nazD*$_R$	(Xu *et al.*, 2007)
Plasmids		
pXQ719	*narX-difA* (*nafA*) chimeric gene in pWB200; Kan^r	(Xu *et al.*, 2005)
PWB505	*pilA* in-frame deletion in pBJ113	This study
pLZ406	*sglK* in-frame deletion in pBJ113	This study
P7425	*pgi* in-frame deletion in pBJ113	This study

Results

DifA does not undergo methylation upon stimulations

First, the methylation of DifA, the MCP homolog in the Dif pathway, was examined for its involvement in EPS production. It has been shown that type IV pili (TFP) function upstream of the Dif pathway in EPS regulation and mutations in *pilA*, which encodes the pilus subunit pilin, lead to a TFP^- and EPS^- phenotype (Black *et al.*, 2006). DifA methylation in a *pilA* mutant (YZ690) was analyzed in comparison with the wild type (DK1622) by alkaline treatment and immunobloting (see **Experimental procedures**). As shown in Figure 4-1 A, DifA appeared as a single band in both the mutant and the wild type whether samples

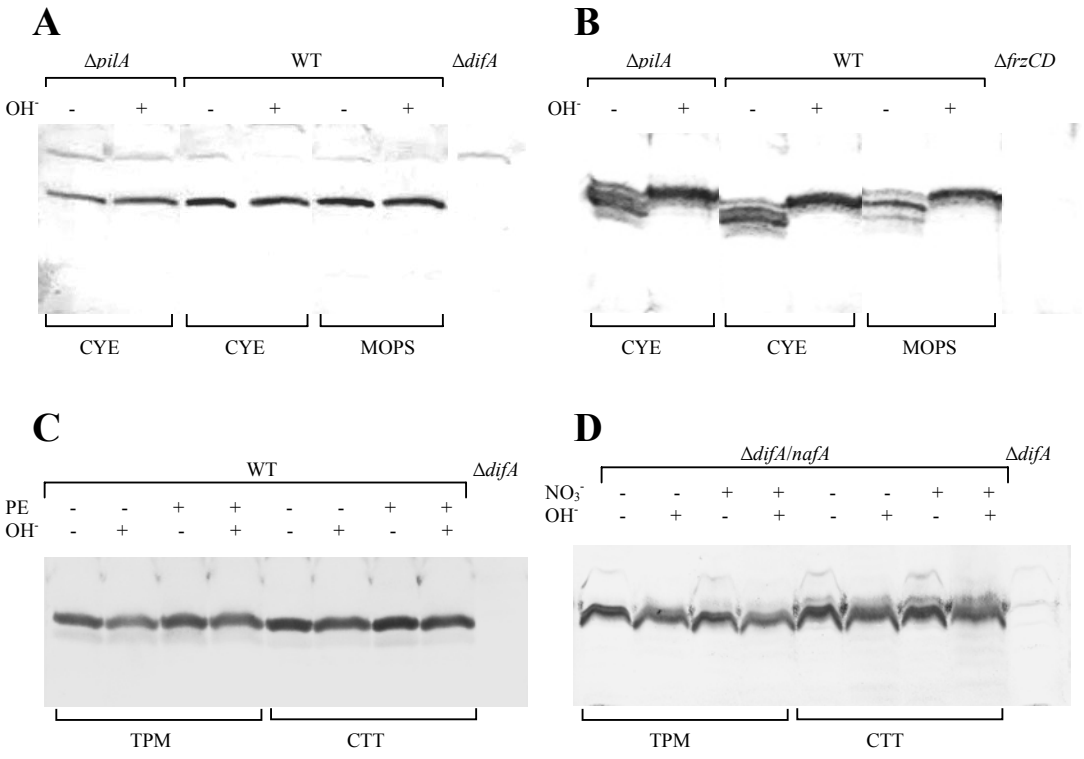

Figure 4-1. Examination of DifA methylation. Whole cell lysates from 1.5 x 10^8 cells were separated by SDS-PAGE and probed with polyclonal antibodies against DifA (A, C, D) or FrzCD (B). To identify base-labile modifications, each cell lysate was additionally treated with 0.1 M NaOH (OH^-) for 30 min, followed by neutralization. (A, B) Cells were incubated in 4 ml CYE or MOPS liquids for 3 h. (C) 2.5 ml cell suspensions in TPM or CTT liquids were supplemented with 25 µl solvent only (-) or 25 µl solvent containing 1.25% (w/v) 12:0 PE (+), and incubated for 100 minutes. (D) 4 ml cell suspension in TPM or CTT liquid was incubated with or without 350 µM nitrate (NO_3^-) for 3 h. Different methylation species of a MCP are typically displayed as a ladder of bands, which is clearly manifested by FrzCD with the lower bands correspond to increased methylation (B). See **Experimental procedures** for details.

were treated with NaOH or not. This initial experiment was performed in a nutrient medium (CYE, see **Experimental procedures**) in which the *pilA* deletion eliminated EPS production (Black *et al.*, 2006). It is known that nutrient deprivation up-regulates EPS production (Shimkets, 1986; Behmlander and Dworkin, 1991; Bonner and Shimkets, 2006). DifA methylation in the wild type was additionally examined in MOPS (see **Experimental procedures**), a buffer without any nutrients (Fig. 4-1 A). Again, the banding pattern of DifA looked the same in MOPS as in CYE with no signs of methylation. As controls, FrzCD from these same samples showed clear indications of methylation (Fig. 4-1 B) as reported previously (McCleary *et al.*, 1990; McBride *et al.*, 1992). These results (Fig. 4-1 A and 4-1 B) suggested that DifA may not undergo methylation during regulation of EPS production.

Next, the involvement of DifA in chemotaxis was investigated. Methylation of MCPs is usually associated with adaptation in chemotaxis (Falke *et al.*, 1997; Szurmant and Ordal, 2004). Since EPS production is not chemotaxis, lack of DifA methylation in EPS regulation was not unexpected. On the other hand, DifA and the Dif pathway are involved in the taxis of *M. xanthus* towards certain PE molecules, which does display adaptation (Kearns *et al.*, 2000; Bonner *et al.*, 2005). The possible methylation of DifA in response to 12:0 PE, one of the known attractants, was examined using the same assays as in Figure 4-1 A. Again, DifA appeared as a single band, regardless of the presence of PE, alkaline treatment or nutrient conditions (Fig. 4-1 C). These results indicated that DifA may not be methylated even during regulation of chemotaxis in *M. xanthus*.

Finally, we took advantage of a NarX-DifA (NafA) chimera (Xu *et al.*, 2005) to examine the effect of direct signaling through DifA on its own methylation. The primary reason for performing the experiments with NafA is because the exact function of DifA in either EPS regulation or PE taxis is yet to be elucidated. It may be argued that the inability to detect DifA methylation in the experiments in Figures 4-1 A and 4-1 C was because DifA is

not a direct signal transducer under those experimental conditions. The NafA chimera contains the sensory module of NarX, the nitrate sensor kinase from *E. coli*, and the signaling module of DifA; it was shown to activate EPS production and change cellular reversal frequency in a nitrate-dependent manner (Bonner *et al.*, 2005; Xu *et al.*, 2005). This NafA chimera, which directly signals through the Dif pathway (Xu *et al.*, 2005), contains the predicted methylation domains of DifA and is recognized by anti-DifA polyclonal antibodies (Yang *et al.*, 1998b; Bonner *et al.*, 2005). As shown in Figure 4-1 D, NafA appeared as a single band in the methylation assays whether or not nitrate was supplemented to the medium. Since NafA in this case is known to sense nitrate directly and to transduce the signal downstream through the Dif pathway (Xu *et al.*, 2005), these results indicated that DifA is not modified by methylation even when it is directly involved in signal transduction. We conclude from the collective results in Figure 4-1 that DifA is unlikely to be methylated despite its homology to bacterial MCP transducers.

Signaling through the Dif pathway modulates FrzCD methylation

The NarX-DifA (NafA) chimera and nitrate (Bonner *et al.*, 2005; Xu *et al.*, 2005) were also utilized to examine the modulation of FrzCD methylation by signaling through the Dif pathway. Previous studies indicated that the tactic response of *M. xanthus* to PE involves both the Frz and the Dif chemosensory pathways: the Dif pathway is crucial for excitation to or perception of PE, and the Frz pathway is essential for temporal adaptation (Kearns and Shimkets, 1998). It is plausible that signal input through the Dif pathway may modulate the methylation state of FrzCD, a proven methyl-accepting chemoreceptor (McCleary *et al.*, 1990), to bring about adaptation. As shown in Figure 4-2 A, nitrate had no effect on mobility of FrzCD in the wild type, but clearly increased the methylation of FrzCD in YZ724, a *difA* deletion mutant containing the NafA chimera (Δ*difA*/*nafA*). This indicated that there is

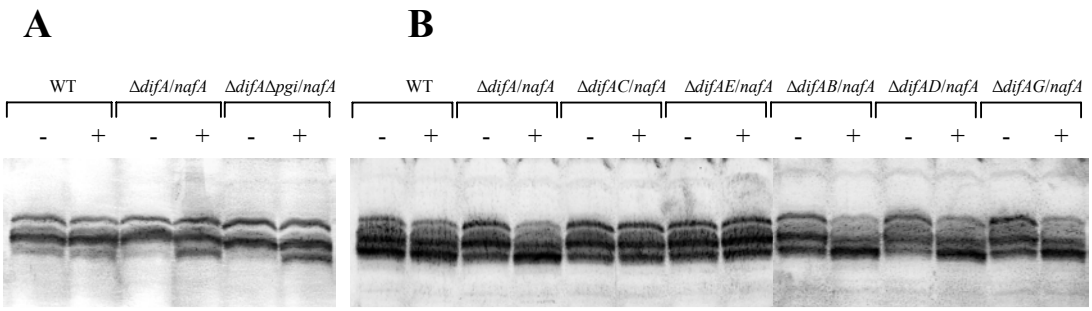

Figure 4-2. FrzCD methylation in NafA-expressing mutants with or without nitrate exposure. 4 ml cell suspension in CTT liquid was incubated with (+) or without (-) 350 μM nitrate for 3 h. Whole cell lysates from 1.5 x 10^8 cells were separated by SDS-PAGE and probed with polyclonal antibodies against FrzCD. The lower bands of FrzCD represent increased methylation.

crosstalk from the Dif pathway to the Frz pathway at the level of FrzCD methylation. It is known that nitrate also results in EPS production in YZ724 (Δ*difA*/*nafA)* (Xu *et al.*, 2005). To examine whether the Dif-Frz methylation crosstalk is the result of direct signaling through DifA or through EPS production, *pgi,* a gene essential for EPS biosynthesis in *M. xanthus* (Cadieux and Yang, unpublished data), was deleted from the Δ*difA*/*nafA* mutant. The resulting Δ*difA* Δ*pgi*/*nafA* mutant (YZ1012) displayed similar changes in FrzCD methylation in response to nitrate as its parental strain YZ724 (Δ*difA*/*nafA*) (Fig. 4-2 A). These results demonstrate that it is the signaling through NafA and the Dif pathway, not EPS production *per se*, can lead to the modulation of FrzCD methylation. Since both pathways regulate cell behavior in the PE response, regulation of FrzCD methylation by the Dif pathway provides a possible mechanism for the coordination of the Dif and the Frz signal transduction pathways.

DifA requires DifC and DifE, but not DifB, DifD and DifG, to affect FrzCD methylation

Each of the *dif* genes (*difB, difC, difD, difD and difG*) was deleted from the Δ*difA*/*nafA* mutant to examine their effect on the crosstalk from DifA to FrzCD. The resulting mutants (Table 4-1) were examined for the nitrate-induced FrzCD methylation. As shown in Figure 4-2 B, the deletions of *difB*, *difD* and *difG* had no appreciable effect on this crosstalk: the double mutants with Δ*difA* containing the NafA chimera still increased FrzCD methylation in

the presence of nitrate. In contrast, mutants harboring either a *difC* or a *difE* deletion showed no increase in FrzCD methylation in response to nitrate: both mutants displayed the same FrzCD banding patterns with or without nitrate. The results (Fig. 4-2 B) here indicated that DifC and DifE, but not DifB, DifD and DifG, are required by DifA for its modulation of FrzCD methylation. Since DifA, DifC and DifE likely form a ternary complex in *M. xanthus* (Yang and Li, 2005), these results suggest that it is this DifACE signaling complex that is essential for this crosstalk from Dif to Frz.

The Frz pathway can sense PE independently of the Dif pathway

As eluded to earlier, a simple scenario assumes that the perception of PE by the Dif pathway will lead to changes in cell reversal first and adaptation will ensure thereafter due to increased FrzCD methylation (McBride *et al.*, 1992; Kearns and Shimkets, 1998). To examine these assumptions, FrzCD methylation was analyzed in various *dif* mutants in response to 12:0 PE. To our surprise, PE treatment led to FrzCD methylation in all the *dif* mutants including Δ*difA,* Δ*difB,* Δ*difC,* Δ*difD,* Δ*difE* and Δ*difG* mutants (Fig. 4-3). First, these results indicated that 12:0 PE can modulate FrzCD methylation independently of the DifACE complex. This was surprising because DifACE has been implicated in PE perception or excitation and an attempted adaptation to a signal that the cells could not detect was not

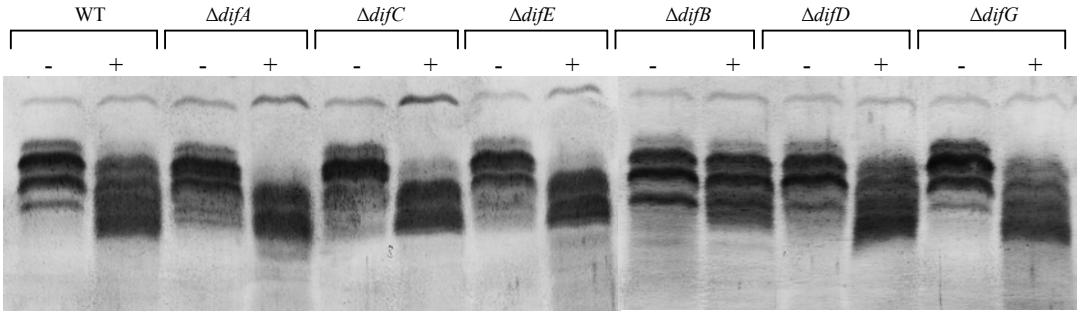

Figure 4-3. **FrzCD methylation in the wild type and *dif* mutants with or without 12:0 PE exposure.** 2.5 ml cell suspensions in TPM liquid were supplemented with 25 µl solvent only (-) or 25 µl solvent containing 1.25% (w/v) 12:0 PE (+), and incubated for 100 minutes. Whole cell lysates from 2.5 x 10^8 cells were separated by SDS-PAGE and probed with polyclonal antibodies against FrzCD. The lower bands of FrzCD represent increased methylation.

the expectation. Second, the similar FrzCD methylation levels in the Δ*difB* and Δ*difG* mutants, as in the wild type, were somewhat unexpected because DifD and DifG along with FrzCD have been implicated in adaptation to PE (Bonner *et al.*, 2005; Bonner and Shimkets, 2006). Since the Dif pathway is involved in sensing PE and FrzCD methylation is at least partially responsible for adaptation to PE (McCleary *et al.*, 1990; McBride *et al.*, 1992; Shi *et al.*, 1993; Shi and Zusman, 1994), the results here suggest that there is likely a Dif-independent sensing and adaptation response to PE by the Frz pathway in *M. xanthus*.

Signaling through the Dif system affects FrzCD methylation in a manner opposite of expectation

The results in Figures 4-2 and 4-3 also suggested that signaling through the DifACE ternary complex affected FrzCD methylation in a manner opposite of what was expected. Figure 4-3 shows that FrzCD becomes more methylated in the presence of 12:0 PE than in its absence in all strains; this is expected for the exposure to an attractant. However, the most unmethylated forms of FrzCD are missing from Δ*difA,* Δ*difC* and Δ*difE* mutants but not from the wild type after PE treatment. These results indicated that the signaling from 12:0 PE, an attractant, through the Dif pathway would normally reduce FrzCD methylation in the wild type.

The results in Table 4-2 and Figure 4-2 further corroborate this observation. It was shown previously the Δ*difA*/*nafA* strain perceives nitrate as a repellent since nitrate increased the reversal frequency of this strain (Bonner *et al.*, 2005). Yet, nitrate clearly increased FrzCD methylation in this mutant (Fig. 4-2 A) and in the Δ*difAB*/*nafA,* Δ*difAD*/*nafA* and Δ*difAG*/*nafA* mutant strains (Fig. 4-2 B). In other words, nitrate, apparently a repellent from behavior analysis (Bonner *et al.*, 2005), increases FrzCD methylation. One complicating factor in the previous behavior assays (Bonner *et al.*, 2005) was that nitrate also stimulates

the Δ*difA*/*nafA* strain to produce EPS (Xu *et al.*, 2005) which is known to influence reversal frequency of *M. xanthus* cells (Kearns *et al.*, 2000). To verify that nitrate is a *bona fide* repellent for NafA-expressing cells, the Δ*difA* Δ*pgi*/*nafA* (YZ1012) strain, which produces no EPS with or without nitrate, was examined for its behavioral response to nitrate. The results showed that the reversal frequency of YZ1012 cells was increased by 350 μM nitrate to about 1.7 fold of the basal level without nitrate (Table 4-2), indicating that nitrate does elicit a repellent-like response from NafA-expressing cells independently of EPS production. Furthermore, 350 μM nitrate also reduced the colony expansion of YZ1012 by 32% while showing no effect on that of the Δ*difA* Δ*pgi* mutant and the wild type (data not shown). This is consistent with a recent study indicating that repellents can reduce colony expansion of *M. xanthus* due to its lack of adaptation to negative stimuli (Xu *et al.*, 2007). Thus, these results (Fig. 4-2 and 4-3, Table 4-2, Bonner *et al.*, 2005) collectively support a model wherein chemoeffectors signaling through DifACE impact FrzCD methylation in an unorthodox manner: a repellent increases whereas an attractant decrease FrzCD methylation.

Table 4-2. Reversal frequency (rev/min) with stimulation (nitrate or 12:0 PE) on CTT agar.

Chemical	Δ*difA*Δ*pgi*/*nafA*	Δ*difA*/*nafA*
0	0.071 ± 0.046	0.145 ± 0.057
Nitrate	0.123 ± 0.046	N.D.[a]
12:0 PE	N.D.[a]	0.052 ± 0.035

[a] N.D.: not determined.

EPS$^-$ and *fibA* mutants overmethylate FrzCD in response to PE

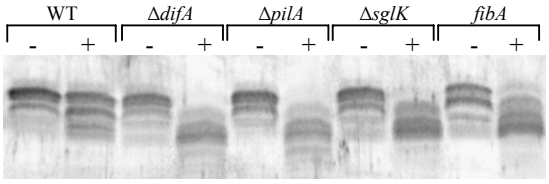

Figure 4-4. **FrzCD methylation in fibril mutants with or without 12:0 PE exposure.** The experiment was performed similarly as in Fig. 4-3.

It has been reported that the EPS matrix and its associated protein FibA mediate the signal input from 12:0 PE to the Dif pathway (Kearns *et al.*, 2000; Kearns *et al.*, 2002). The above model would predict that EPS$^-$ and *fibA* mutants would behave similarly as the *dif* mutants in response to PE at the level of FrzCD methylation. Δ*pilA* (YZ690) and Δ*sglK* (YZ811) mutants, both EPS$^-$, as well as the *fibA* mutant LS2200 were examined for FrzCD methylation upon PE treatment. As shown in Figure 4-4, FrzCD in these three mutants was more methylated than in the wild type after PE treatment similarly as in the Δ*difA* mutant. These results confirmed the above conclusion that signaling from attractants through the Dif pathway in general functions to decrease or reduce FrzCD methylation in the wild type.

Effect of signaling directly through the Frz pathway on FrzCD methylation

To examine the effect of direct signaling through the Frz pathway on FrzCD modification, we took advantage of two NarX-FrzCD chimeras constructed recently (Xu *et al.*, 2007). These two chimeras, $NazD_F$ and $NazD_R$, contain the sensory module of NarX fused to the signaling and methylation domains of FrzCD. $NazD_F$ contains the wild-type NarX N-terminus and $NazD_R$ harbors a G51R mutation in the

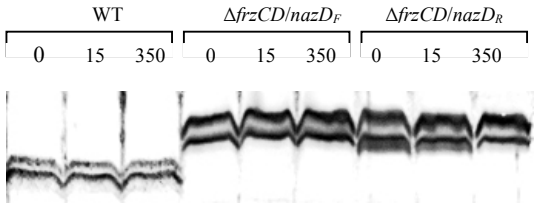

Figure 4-5. Methylation of NarX-FrzCD chimeras in the presence of different concentrations of nitrate. 4 ml cell suspension in TPM liquid was incubated with 0, 15 or 350 μM nitrate for 3 h. Whole cell lysates from 1.5 x 10^8 cells were separated by SDS-PAGE and probed with polyclonal antibodies against FrzCD.

NarX sensory module. $NazD_F$ mediates an attractant response and $NazD_R$ initiates a repellent-like response to nitrate. The methylation of $NazD_F$ and $NazD_R$ after nitrate exposure was examined. Unexpectedly, $NazD_F$ (Fig. 4-5) showed no obvious change in methylation upon nitrate treatment although the $NazD_F$–expressing strain does adapt to nitrate as an attractant (Xu *et al.*, 2007). On the other hand, $NazD_R$ displayed decreased methylation with increasing concentrations of nitrate. The latter might be taken as evidence that direct signaling through the Frz pathway can affect FrzCD methylation in a more conventional manner: a repellent leads to decreased methylation of a MCP receptor. This is also supported by the observation that isoamyl alcohol (IAA), a negative stimulus sensed by the Frz pathway, also decreases FrzCD methylation (McBride *et al.*, 1992). However, it must be noted that *M. xanthus* does not show adaptation to negative chemostimulants in general despite their ability to cause increased FrzCD methylation (Xu *et al.*, 2007).

Discussion

In this study, we examined the methylation of DifA and FrzCD, two MCP homologs from the *M. xanthus* Dif and Frz chemosensory pathways, respectively. No DifA methylation could be detected in various genetic backgrounds and under different environmental conditions that are known to impact signaling through the Dif pathway (Fig. 4-1). In contrast, FrzCD methylation was easily detectable as expected (Fig. 4-1 B; McBride *et al.*, 1992). Interestingly, 12:0 PE, an attractant known to require the Dif pathway to elicit excitation responses from *M. xanthus*, can induce FrzCD methylation in the absence of the Dif pathway (Fig. 4-3). It was additionally discovered that signaling through the Dif pathway can modulate FrzCD methylation, but in a manner opposite of expectation (Fig. 4-2, 4-3 and 4-4). That is, attractant signaling through the Dif pathway reduces FrzCD methylation whereas repellent signaling through Dif increases methylation of FrzCD. In summary (Fig. 4-6), this study revealed three important observations. First, there exist two independent sensing mechanisms for PE in *M. xanthus*; second, there are unexpected patterns of DifACE-directed modulation of FrzCD methylation; third, there is not strict correlation between between FrzCD methylation and chemotactic adaptation; last but not least, DifA is likely an unmethylated MCP homolog.

Involvement of FrzCD methylation during chemotactic adaptation in *M. xanthus*

Modulation of FrzCD methylation has been considered critical for chemotactic adaptation in *M. xanthus* (Zusman *et al.*, 2007). DMSO and IAA as well as spent media, all potential repellents for *M. xanthus*, decrease FrzCD methylation (McBride *et al.*, 1992). Both PE and nutrients, attractants for *M. xanthus*, increase FrzCD methylation (McBride *et al.*, 1992). It was proposed that in PE taxis, the Frz proteins constitute a pathway essential for adaptation but not excitation (Kearns and Shimkets, 1998). The observation that PE led to

more FrzCD methylation in various strains (Fig. 4-3 and 4-4) is consistent with the previous model and supports the notion that increases in FrzCD methylation are directly involved in adaptation to the PE attractants. Surprisingly, *dif, pil, sglK* and *fibA* mutants, presumably deficient in sensing PE or transducing the excitation signal (Fig. 4-4; Kearns and Shimkets, 1998; Kearns *et al.*, 2000), showed increased FrzCD methylation in the presence of PE (Fig. 4-3 and 4-4). In other bacteria, chemotaxis adaptation is always the consequence of excitation and shows absolute dependency on the sensing mechanism for excitation. It is therefore quite remarkable that in *M. xanthus*, there are two independent sensing mechanisms for PE, one for the excitation pathway involving Dif and the other for the Frz-mediated adaptation response (Fig. 4-6).

Roles of Dif-dependent FrzCD methylation

Despite the two independent sensing mechanisms for PE, the Dif and the Frz pathways do communicate with each other: signaling through the Dif pathway clearly influences FrzCD methylation. As shown in Figure 4-2, nitrate, a repellent-like signal mediated by the NarX-DifA chimera (Table 4-2; Bonner *et al.*, 2005), increased FrzCD methylation. In contrast, the attractant 12:0 PE suppressed FrzCD methylation when the signal is relayed through the DifACE complex: FrzCD is more methylated in *difA*, *difC* and *difE* mutants than in the wild type in the presence of PE (Fig. 4-3). Mutations in components proposed to be upstream of Dif in PE taxis (Kearns *et al.*, 2000) also showed similar effect on FrzCD methylation (Fig. 4-4). These results indicate interdependence between the Dif and the Frz pathways despite their ability to sense the PE signal independently. The signal appears only to be transduced in the direction of Dif to Frz since there are no quantitative differences between the wild type and various *frz* mutants, null or otherwise, in EPS production that is regulated by the Dif pathway (data not shown). The signal originated from the Dif pathway must branch three

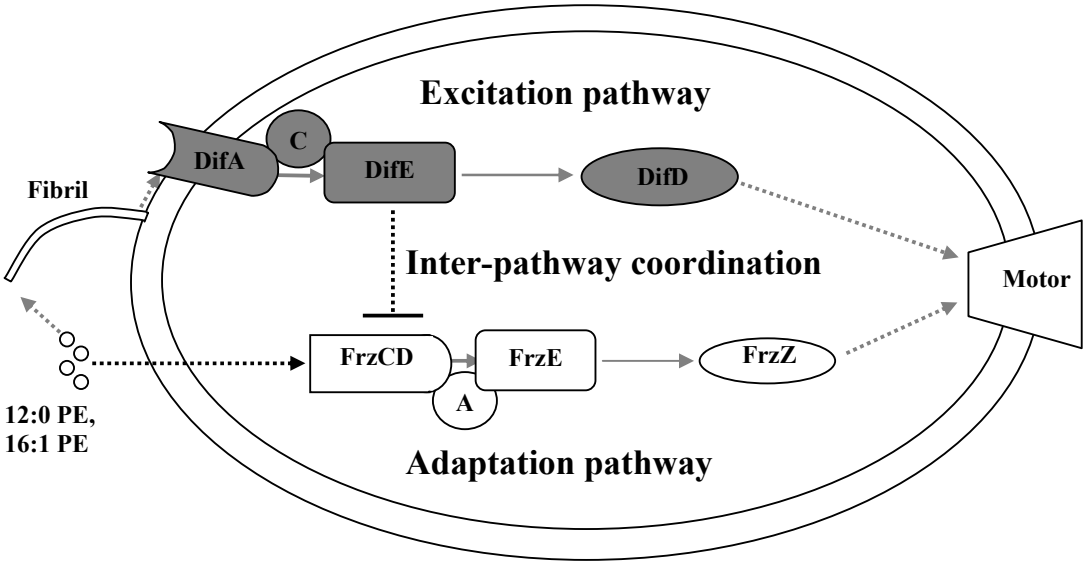

Figure 4-6. A working model of co-regulating chemotaxis towards 12:0 PE and 16:1 PE by the Dif and Frz pathways. The Dif and Frz pathways can both specifically sense PE, which results in excitation and adaptation, respectively. As the two signaling events function separately, the methylation crosstalk from DifACE to FrzCD coordinates the excitation and adaptation pathways. Arrows and bars represent stimulatory and inhibitory effects, respectively. Solid lines illustrate direct interactions while dashed lines indicate indirect or hypothetical interactions.

ways after DifACE (Fig. 4-6). One branch regulates EPS through unknown mechanisms (Black and Yang, 2004); another modulates the gliding motors through DifD (Bonner *et al.*, 2005); a third communicates with the Frz pathway by modulating FrzCD methylation. One prediction from this model is the suppression or reduction of EPS production by PE which has yet to be examined.

What is the function of the DifACE- or the excitation-dependent modulation of FrzCD methylation? As being indicated early, the Dif-directed FrzCD methylation change opposes adaptations since an attractant (12:0 PE) decreases (Fig. 4-3 and 4-4) and a repellent (nitrate and NafA) increases (Fig. 4-2) FrzCD methylation. One possibility is that this DifACE-dependent modulation is to augment the response to the signal from the Dif pathway. It is well known that the Frz system plays a primary role in regulating cell motility behavior in *M. xanthus*. Although the Dif signal transduction pathway is able to effect changes in cell reversal independently of the Frz pathway, Dif is the primary system that regulates the

production of surface- or exo-polysacchacchride (EPS) (Yang *et al.*, 2000; Bellenger *et al.*, 2002; Black and Yang, 2004). As such, the Dif pathway may conceivably require augmentation from the Frz system to mount an effective excitation response. Indeed, a *frzCD*^{c} (constitutively active) mutant is unresponsive to PE (Kearns and Shimkets, 1998), suggesting that the signal from the Frz pathway can overwhelm those from Dif in the regulation of cell reversal. However, the magnitude of suppression of reversal by PE is similar in the wild type as in a *frzCD* null mutant (Kearns and Shimkets, 1998). This indicates that the Dif system can effectively regulate cell reversal behaviors independently of its effect on FrzCD methylation. We therefore favor a model where the DifACE-dependent modulation of FrzCD methylation is to tamper with the adaptation response instead of augmentation of the excitation response.

We argue that this "tampering" of adaptation is crucial for proper chemotaxis responses in the slowly moving *M. xanthus*. Temporal sensing is at the heart of bacterial chemotaxis and the timing of adaptation is of ultimate importance for effective chemotaxis (Macnab and Koshland, 1972; Bray *et al.*, 2007; Lovdok *et al.*, 2007). The rate or speed of adaptation has to match the speed of bacterial movement. Too quick of an adaptation is as good as no sensing at all and too slow of an adaptation leads cells to stray from desirable territories or to be trapped in unfavorable environments. The gliding *M. xanthus* moves quite slowly, about 1/1000 of the swimming speed of flagellated bacteria (Dworkin and Eide, 1983). Adapting too quickly relative to its slow speed would lead to ineffective taxis at best. Depending on the strength of stimulations, adaptation in flagellated bacteria takes from seconds to minutes (Macnab and Koshland, 1972; Brown and Berg, 1974; Goldman and Ordal, 1981). In comparison, it takes *M. xanthus* about one and half hours to adapt to PE or other attractants (Kearns and Shimkets, 1998; Xu *et al.*, 2007). There is indeed evidence that the timing of adaptation in *M. xanthus* is regulated in the PE response. It took a *frzE* mutant more time and a *difB* mutant less time to adapt to PE (Kearns and Shimkets, 1998; Bonner *et al.*, 2005). We

propose that the DifACE-dependent modulation of FrzCD (Fig. 4-6) is to slow down adaptation to match the slow movement of *M. xanthus* for effective taxis so that adaptation to chemostimuli sensed by the Dif pathway does not occur too quickly. This is also somewhat reminiscent of the roles proposed for CheV phosphorylation and dephosphorylation in *B. subtilis* chemotaxis (Karatan *et al.*, 2001). It was suggested that the slow phosphorylation of CheV and the increased stability of CheV-P is to allow an excitatory signal enough time for a significant period of response before adaptation.

Mechanisms of Dif-directed FrzCD methyation

How may the signaling from DifACE regulate FrzCD methylation? The usual suspects include the putative methyltransferase FrzG and methylesterase FrzF (McCleary *et al.*, 1990). In flagellated bacteria, the methylesterase CheB, with which FrzF shares similarity, is regulated by the phosphorylation of its response regulator domain by the CheA kinase: phosphorylation of CheB activates its methylesterase. However, FrzF has no homology to response regulators. It is unlikely, however reasonable, that the activity of FrzF is regulated through phosphorylation by the putative DifE kinase. If the regulation of FrzCD methylation by the Dif pathway is through FrzF, the mechanism may have to be phosphorylation-independent. On the other hand, although the CheR methyltransferase in flagellated bacteria is not regulated, the modulation of FrzG activity by the Dif pathway is a viable alternative in *M. xanthus*. Unlike the classical single-domained CheR, FrzG has an N-terminal TPR domain in addition to the C-terminal methyltransferase domain. It has been proposed that in the Frz system, FrzG, not FrzF, is regulated one and responsible for modulating the level of FrzCD methylation. It remains to be investigated whether FrzF, FrzG or other proteins serve as liaisons for the DifACE-directed regulation of FrzCD methylation.

FrzCD methylation-independent adaptation mechanism in *M. xanthus* chemotaxis

The results in this study indicated that there is no strict correlation between FrzCD methylation and adaptation. First, there is no behavioral adaptation by the $NazD_R$-expressing mutant to nitrate (Xu *et al.*, 2007); yet $NazD_R$ exhibits decreased methylation in response to nitrate (Fig. 4-5). Similarly, although IAA and other small alcohols clearly reduce FrzCD methylation, evidence indicates that *M. xanthus* adapts to none of them (McBride *et al.*, 1992; Xu *et al.*, 2007). What is even more intriguing is that nitrate does not appreciably change $NazD_F$ methylation (Fig. 4-5) although the $NazD_F$-expressing strain adapts to nitrate as an attractant (Xu *et al.*, 2007). Taken together, these results revealed that in the regulation of *M. xanthus* chemotaxis, FrzCD methylation is not the only game in town: changes in its methylation do not necessarily mean adaptation and lack of changes in its methylation does not mean lack of adaptation.

What are the possible FrzCD-independent mechanisms for adaptation in *M. xanthus*? One possibility is through methylation of other *M. xanthus* MCP homologs. In *E. coli*, there are high-abundance MCPs (Tsr and Tar) and low-abundance MCPs (Trg and Tap) (Hazelbauer and Engstrom, 1981; Wang *et al.*, 1982). The methylation of the low-abundance MCPs depends on the presence of the high-abundance ones (Barnakov *et al.*, 1998). Nevertheless, methylation of any of these receptors can influence the activity of the chemotaixs pathway by their effects on the CheA kinase. In *M. xanthus*, there are over 20 MCP homologs and eight known chemosensory systems (Zusman *et al.*, 2007). Although there is no DifA methylation (Fig. 4-1; Xu and Yang, unpublished data), it is possible that the methylation of some other receptors can influence the signaling through the Frz or other chemosensory pathways to facilitate chemotaxis adaptation in *M. xanthus*.

Alternatively, there may be methylation-independent chemotaxis adaptation in *M. xanthus* as in other bacteria. In *B. subtilis*, there are two proposed adaptation mechanisms that

are methylation-independent albeit receptor-dependent. The receptor-decoupling mechanism involves CheV, as was eluded to earlier. CheV has two domains, a CheW-like domain at the N-terminus and a response regulator domain at the C-terminus. Phosphorylated CheV appears to uncouple receptor signaling from CheA kinase activity in attractant response (Karatan *et al.*, 2001). The receptor-deactivating mechanism, functional in adaptation to attractants, involves CheC, CheD and CheY (Muff and Ordal, 2007). It is known that CheD interacts with both CheC and MCP receptors. It was shown that only phosphorylated CheY (CheY-P) can form a complex with CheC. In the model proposed by Muff and Ordal (2007), this CheC-CheY binary protein complex can bind and sequester CheD to reduce its binding to chemoreceptors. The decrease in the association of receptors with CheD leads to a reduction in the activation of CheA and adaptation to attractants. In addition, there could be receptor-independent adaptation mechanisms. The Frz system has proteins with domains of unknown function. Besides a kinase domain, FrzE contains a response regulator domain at its C-terminus. FrzZ, the component proposed to function downstream of FrzE, contains two response regulator-like domains instead of one. It is certainly possible that these "extra" domains may function in the methylation-independent adaptation in the Frz pathway.

DifA, an unmethylated "MCP" signal transducer?

DifA does not appear to be methylated despite its homology to methyl-accepting chemoreceptors. As shown in Figure 4-1, DifA does not display a ladder of bands that reflects MCP methylation. In addition, mutations on potential methylation sites of DifA do not change the band pattern or cause a mobility shift as determined by SDS-PAGE (Xu and Yang, unpublished). This is not unexpected since among the chemosensory systems in *M. xanthus*, Dif is the only one that lacks CheB- and CheR-like proteins. In contrast, the only CheC homolog in *M. xanthus*, DifG, is encoded by the *dif* locus. The possible methylation-

independent signaling of DifA might be pertinent to the regulation of EPS production, which needs to be sustained for adequate length of time. Using a *difA* mutant expressing NafA, it was discovered that agglutination, an indication of EPS biogenesis, occurs 1 h after the stimulation of the Dif pathway (Xu *et al.*, 2005). Because adaptation normally takes effect about 1.5 h after the activation of chemosensory systems in *M. xanthus* (Kearns and Shimkets, 1998; Bonner *et al.*, 2005; Xu *et al.*, 2007), the involvement of a chemotaxis-like adaptation in EPS regulation may turn off EPS production before a sufficient level is produced. It is thus plausible that by using a separate pathway for adapting to PE stimulation, the Dif pathway can regulate multiple functions including EPS biogenesis. It is also likely that EPS production, a biologically costly event, is eventually shut down once sufficient level is reached. Hence, a chemosensory pathway that presumably generates temporal output in response to a persistent signal (Zusman *et al.*, 2007), rather than a simple two component system, is responsible for EPS regulation. Despite the identification of several negative EPS regulators encoded by *stk* (Dana and Shimkets, 1993; Kim *et al.*, 1999), *difD* and *difG* (Black and Yang, 2004), how a putative negative feedback loop in EPS regulation without involving receptor methylation is achieved remains to be explored.

Experimental procedures

Strains, plasmids and growth conditions

The *M. xanthus* strains and plasmids used in this study are listed in Table 4-1. *M. xanthus* was grown on agar plates of CYE (1% Casitone, 0.5% yeast extract, 0.1% $MgSO_4 \cdot 7H_2O$, 10 mM MOPS pH 7.6) (Campos and Zusman, 1975) and in liquid media of CYE or CTT (1% casitone, 8 mM $MgSO_4$, 10 mM Tris·HCl, 1 mM KPO_4, pH 7.6) (Kaiser, 1979) at 32ºC on a rotary shaker at 300 rotations per min (rpm). The *E. coli* strain XL1-Blue (Stratagene), used for routine cloning and plasmid construction, was grown on Luria-Bertani (LB) (Miller, 1972) plates or LB liquid at 37ºC. Plates contained 1.5% agar unless noted otherwise. When necessary, kanamycin was added to media at 100 μg/ml.

Construction of mutants

Mutants with in-frame deletions in *pilA, sglK* and *pgi* genes were constructed by using the positive-negative kanamycin/galactose (KG) method (Ueki *et al.*, 1996). The *nafA* chimeric gene was introduced into the *M. xanthus* genome as described previously (Xu *et al.*, 2005).

Methylation examination

To examine DifA methylation in different genetic backgrounds or under different nutrient conditions, cells were grown in CYE liquid to approximately 2.0 ~ 3.0 x 10^8 cells/ml before being harvested, washed and resuspended in CYE liquid or MOPS (morpholinepropanesulfonic acid) buffer (10 mM MOPS, 2 mM $MgSO_4$, pH 7.6) to 2.5 x 10^8 cells/ml. Cell suspensions were incubated in the shaker for 3 h. About 1.0 x 10^9 cells were washed and resuspended with cold 50 mM Tris-HCl (pH 7.4) and resuspended in 200 μl of

loading buffer (2% SDS, 5% mercaptoethanol, 8.5% glycerol). The samples were boiled for 5 min. Thirty microliters of these samples were separated by a special sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) designed to detect methylation (McCleary *et al.*, 1990). The gel was run in the 16 cm Protean II xi apparatus (Bio-Rad Laboratories, Hercules, Calif.) at a constant current of 45 mA for 8 h. Immunoblotting was performed as described (Sambrook and Russell, 2001) using polyclonal antibodies against DifA (Xu *et al.*, 2005).

To examine receptor methylation after nitrate exposure, cells were grown in CTT liquid to approximately 2.0 ~ 3.0 x 10^8 cells/ml before being harvested, washed and resuspended in CTT liquid or TPM buffer [10 mM Tris (hydroxymethyl) aminomethane HCl (pH 7.6), 8 mM $MgSO_4$ and 1 mM K_2HPO_4-KH_2PO_4 (pH 7.6)] to 2.5 x 10^8 cells/ml. Nitrate (KNO_3) was supplemented at 0, 15 or 350 μM into the cell suspensions, which were incubated in the shaker for 3 h before cells were collected. Immunoblotting was performed as described above using using polyclonal antibodies against FrzCD (McCleary *et al.*, 1990).

To examine receptor methylation after exposure of 12:0 PE, cells were grown in CTT liquid to approximately 1.0 ~ 2.0 x 10^8 cells/ml before being harvested, washed and resuspended in CTT liquid or TPM buffer to 6.0 x 10^8 cells/ml. One percent (v/v) of solvent (50% chloroform + 50% methanol) that contains 0 or 1.25% (w/v) 12:0 PE was added into cell suspensions, which were incubated in the shaker for 100 min before cells being collected. Immunoblotting was performed similarly as described above using polyclonal antibodies against DifA (Xu *et al.*, 2005) or FrzCD (McCleary *et al.*, 1990).

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Reversal frequency examination

The reversal frequency of the Δ*difA*Δ*pgi*/*nafA* mutant in response to nitrate was examined as previously described (Xu *et al.*, 2007). Cells were grown to approximately 1.0 ~

1.5 x 10^8 cells/ml in CTT liquid containing 50 mM glucose, washed and resuspended to 5.0 x 10^7 cells/ml in CTT liquid. Ten microliters of the cell suspension was spotted onto a TPM plate (0.7% agar) containing 0 or 350 μM KNO_3. After 30 s, excess liquid was removed as described (Shi and Zusman, 1994). To determine reversal frequency, cells were selected prior to the start of the movie and the reversals of 50 isolated and fully motile cells were analyzed.

The reversal frequency of the Δ*difA*/*nafA* mutant in response to 12:0 PE was performed similarly as described by Kearns and Shimkets (1998). 12:0 PE was dissolved in (50% chloroform + 50% methanol) at 0.03% (w/v). Four microliters of test compound (solvent only or solvent containing 12:0 PE) was applied to an area of about 0.4 cm^2 and dried for 10 min on a thin layer of 0.7% CTT agar in a Petri dish. Cells were grown in CTT liquid containing 25 μM KNO_3 to approximately 1.0 ~ 1.5 x 10^8 cells/ml, before being washed and resuspended to 5.0 x 10^7 cells/ml in CTT liquid. Five microliters of such cell suspension was dried on top of the test compound and incubated at room temperature for 10 min. Recording and determination of reversal frequency was performed as above.

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