COMMENTARY

Developments in Defining *dif*[▽]

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The Gram-negative soil bacterium *Myxococcus xanthus* has a complex life cycle that involves vegetative growth and, when nutrients are limiting, formation of spore-containing fruiting bodies. The progression of the developmental cycle depends in part upon the ability of *M. xanthus* cells to sense and respond to their environment and to coordinate two different motility systems to achieve directed movements. The *dif* genes were originally isolated by Yang et al. (13) as part of a search for mutants defective in fruiting body formation. It was later determined that they encode a chemosensory pathway that regulates the production of extracellular polysaccharides (EPS), which are essential for *M. xanthus* social (S) motility and fruiting body formation (9, 14). Social motility is powered by the extension and retraction of type IV pili (TFP) (8, 11); when pili bind to EPS found on the surface of cells or on slime trails, they trigger retraction, pulling cells forward (10). Single and combinatorial *dif* mutants show that, despite the strong homology of *dif*-encoded proteins to core components of bacterial chemosensory systems, the Dif pathway does not regulate EPS production directly through a phosphorylated response regulator protein but rather through unidentified intermediates (1, 5, 14).

To clarify Dif signaling, Black et al. (2) have now studied *in vitro* phosphorylation and dephosphorylation of purified Dif proteins to establish the relationships between these proteins and their respective biochemical properties. These results provide a solid foundation upon which to base future work to untangle the various and varied aspects of Dif regulation. As shown in Fig. 1, five of the six *dif* genes show good homology to characterized chemosensory proteins: DifA is a membrane-bound receptor similar to other methyl-accepting chemotaxis proteins (MCPs), DifC is a CheW-like coupling protein, DifE contains a CheA-like autokinase domain, DifD is a CheY-like response regulator protein, and DifG is a homolog of the *Bacillus subtilis* CheY phosphatase, CheC.

Yeast two- and three-hybrid experiments indicate that DifA, DifC, and DifE form a ternary complex similar to those found in other chemosensory systems (15). Yeast two-hybrid studies also revealed binding affinities between DifE and DifD and between DifD and DifG (15). Mutational inactivation of *difA*, *difC*, or *difE* caused the loss of EPS production by *M. xanthus* (1, 14). However, inactivation of DifD or DifG caused EPS overproduction (5). To explain their data, Black and col-

leagues proposed that the receiver domain protein DifD serves as a phosphate sink, with DifG acting as a phosphatase to regenerate the population of phosphate-accepting DifD (Fig. 1). Thus, the DifA-DifC-DifE ternary complex positively regulates EPS production, while DifD and DifG act in concert as negative regulators.

The biochemical studies now published by Black et al. (2) provide clear confirmation that DifE is indeed an autokinase that can efficiently autophosphorylate *in vitro*. Furthermore, the incubation of phosphorylated DifE (DifE~P) with DifD results in the rapid transfer of phosphate to DifD, as expected. However, DifD~P proved unusually stable with a half-life of 30 min; dephosphorylation of DifD was greatly accelerated by relatively low concentrations of DifG, showing that DifG is an efficient phosphatase.

DifD and DifG are likely to serve as a phosphate sink for DifE. However, the biochemical activities established here do not account for the phenotype of a *difD difG* double mutant, which produces more EPS than do *difD* and *difG* single mutants (4). DifG may influence the Dif pathway downstream of DifE phosphorylation since DifG does not affect DifE autokinase activity *in vitro*.

Interestingly, DifE autokinase activity was inhibited by the addition of DifA and DifC. In other systems, including the *M. xanthus* Frz chemosensory system, the MCP and coupling protein strongly enhance the level of autophosphorylation of the CheA histidine kinase homolog (7). The authors suggest that this modification may reflect the diversity of inputs for the Dif system. For example, the Dif pathway regulates the ability of *M. xanthus* to respond to phosphatidylethanolamine (PE) (6), in addition to regulating EPS production. EPS production is subject to positive stimulation of the Dif system by type IV pili (TFP), which are responsible for powering S motility (4). Conversely, the Dif pathway inhibits cell reversals when cells respond to PE (6). The authors propose that one way that Dif could accommodate these distinct inputs is by maintaining populations of DifA in different conformations. Further work is needed to determine if and how DifE autokinase activity might be altered by its association with DifA and DifC based on the nature of the Dif stimulus.

Understanding Dif function will require the assimilation of observations concerning both EPS production and PE responses. The various *dif* mutants produce differing effects on PE-based behaviors. For example, wild-type cells show a reduction in cell reversal frequency when exposed to PE; mutants lacking DifA, DifC, or DifE do not. This phenotype might at first be attributed to the failure of these mutant strains to produce EPS; however, the DifD mutant, which produces an

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FIG. 1. Chemosensory gene clusters in *M. xanthus*. The genome of *M. xanthus* encodes eight sets of chemosensory genes, including Frz, Dif, and Che7. The Dif system includes a membrane-bound MCP and homologs to a CheA histidine kinase, a CheW coupling protein, a CheY response regulator, and a CheC phosphatase. Phosphorelay between DifE and an unidentified factor (DifX) results in upregulation of EPS production, while DifD and DifG downregulate EPS production by serving as a phosphate sink. EPS production and cell reversal behavior in response to PE are regulated by complex signaling events that include cross talk between Dif, Frz, and Che7.

excess of EPS, is also unable to respond to PE. To complicate things further, the enigmatic DifG is not essential for changes in cell reversal frequency but is defective in adaptation. Finally, *difB* mutant cells show no obvious defects in the regulation of EPS production and respond to PE like wild-type cells do by reducing reversal frequency. However, the *difB* mutant adapts more quickly than does the wild type (6). The DifB protein bears homology to a conserved family of proteins of unknown function.

The complexity of *dif* mutant phenotypes may be due in part to cross talk between Dif and members of the seven other chemosensory systems encoded in the *M. xanthus* genome. For example, responses to PE require both Dif and Frz signaling: Dif signaling is required for excitation while the Frz pathway controls adaptation (12). Interestingly, it has been shown that signaling through DifA, DifC, and DifE affects the methylation state of FrzCD, the MCP of the Frz pathway (12). Additionally, interactions between Dif and members of the *M. xanthus* Che7 pathway (Fig. 1) have been implicated by EPS⁺ suppressors of *difA* mutants (3).

As our knowledge of the Dif pathway expands, so too does the degree to which Dif signaling is found to differ from the established paradigm of other chemosensory systems. For example, DifA does not appear to be methylated in response to TFP or PE stimuli, raising the question of how this MCP homolog relays its signal to downstream partners. In addition, the ternary complex of DifA, DifC, and DifE forms as predicted but appears to inhibit DifE autokinase activity rather than enhance it. The subsequent phosphorelay from DifE to DifD acts as a negative regulator of EPS production by drawing phosphate away from DifE. With DifD functioning to silence rather than propagate DifE~P signal, there must be an

as-yet-unidentified protein, “DifX,” that serves to link Dif signaling to various downstream targets. The biochemical studies by Black et al. establish key aspects of the Dif pathway and will assist in further defining the mechanisms of Dif signaling, including the identity of “DifX.”

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