

The Dif chemosensory pathway is directly involved in phosphatidylethanolamine sensory transduction in *Myxococcus xanthus*

Pamela J. Bonner,¹ Qian Xu,² Wesley P. Black,² Zhuo Li,² Zhaomin Yang² and Lawrence J. Shimkets^{1*}

¹Department of Microbiology, University of Georgia, Athens, GA 30602, USA.

²Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA.

Summary

Myxococcus xanthus cells glide on solid surfaces and are chemotactically stimulated by certain phosphatidylethanolamine species. The *dif* gene cluster consists of six genes, *difABCDEG*, five of which encode proteins homologous to known chemotaxis proteins. DifA and DifE are required for the biosynthesis of fibrils, an extracellular matrix comprised of polysaccharide and protein. Chemotactic stimulation by 1,2-*O*-Bis[11-(*Z*)-hexadecenoyl]-*sn*-glycero-3-phosphatidylethanolamine (16:1 PE) and dilauroyl PE (12:0 PE) requires fibrils. Although previous work has shown that *difA* and *difE* mutants are not stimulated by 12:0 PE, these results do not distinguish between a dependence on fibrils or a direct role in chemosensory transduction. Here we provide evidence that the Dif chemosensory pathway directly mediates PE sensory transduction. First, stimulation by and adaptation to 16:1 PE requires all of the *dif* genes, including *difBDG*, which are not essential for fibril biogenesis. Second, a specific residue within the first putative methylation domain of DifA is required for stimulation by 16:1 PE but not fibril biogenesis. Transmembrane signalling through a chimeric NarX-DifA chemoreceptor is required for fibril formation but not for stimulation by or adaptation to 16:1 PE. Third, *difD* and *difE* are required for stimulation by dioleoyl PE (18:1 PE) although the response does not require fibrils. Taken together these results argue that the Dif pathway mediates both matrix formation and lipid chemotaxis.

Introduction

Myxococcus xanthus is a member of the δ -proteobacteria

that requires co-ordinated cell movement for two distinct aspects of its lifestyle, predation and fruiting body development. During vegetative growth, *M. xanthus* feeds upon other bacteria, utilizing the protein and lipid fractions as carbon and energy sources. Under starvation conditions, tens of thousands of cells form a fruiting body in which cells differentiate into desiccation and heat-resistant myxospores (Dworkin, 1996). When nutrients become available, the spores germinate to produce vegetative cells.

Myxococcus xanthus cells glide along linear paths at rates of several micrometres per minute and periodically reverse direction. Cell movement involves two genetically distinct translocation systems, adventurous (A) and social (S). A-motility genes govern the movement of isolated cells, while S-motility genes are necessary for group translocation (Hodgkin and Kaiser, 1979). The mechanism of A-motility may involve propulsion via slime extrusion (Wolgemuth *et al.*, 2002). S-motility utilizes type IV pili (Kaiser, 1979; Wu and Kaiser, 1995) and is similar to twitching motility, in which pilus extension and retraction mediate cell movement, except that cell–cell contact is necessary for group translocation (Merz *et al.*, 2000; Sun *et al.*, 2000; Skerker and Berg, 2001).

Chemotaxis could play important roles in locating prey during growth and migrating to an aggregation centre during fruiting body development. Chemotaxis towards 1,2-*O*-Bis[11-(*Z*)-hexadecenoyl]-*sn*-glycero-3-phosphatidylethanolamine (16:1 PE), a major component of the *M. xanthus* cell membrane (P. Curtis, unpublished) but rare in other bacterial species, may be involved in self-recognition (Kearns *et al.*, 2001). Chemotaxis towards dioleoyl PE (18:1 PE), which is absent from the *M. xanthus* cell membrane but a common component of the cell membranes of other soil-dwelling bacteria, may be involved in prey recognition (Kearns *et al.*, 2000). Cells can direct their movement up gradients of PE and modulate the reversal period which is defined as the amount of time between reversals (Kearns and Shimkets, 1998). Chemotaxis towards dilauroyl PE (12:0 PE) and 16:1 PE requires starvation conditions (Kearns *et al.*, 2000; 2001), perhaps to induce the production of fibrils, an extracellular matrix comprised of carbohydrate and protein (Behmlander and Dworkin, 1994a,b). At least one fibril-bound protein, FibA, a homologue of the M4 family of zinc metalloproteases, must be expressed for stimulation by these attractants

Accepted 20 June, 2005. *For correspondence. E-mail shimkets@uga.edu; Tel. (+1) 706 542 2681; Fax (+1) 706 542 2674.

(Kearns *et al.*, 2002). Conversely, 18:1 PE acts as an attractant in the presence of nutrients (Kearns *et al.*, 2000) and does not require the presence of fibrils or FibA (Kearns *et al.*, 2002). Thus at least two lipid-sensing systems exist and operate during different phases of the life cycle.

In the presence of PE, cells are first stimulated leading to an increase in the reversal period and then adapt by returning to pre-stimulus basal reversal periods (Kearns and Shimkets, 2001). The *dif* chemosensory system plays a role in PE sensory transduction (Kearns and Shimkets, 1998; Kearns *et al.*, 2000). The *dif* chemosensory pathway consists of six genes, *difABCDEG*, five of which encode proteins homologous to chemotaxis proteins (Yang *et al.*, 2000; Black and Yang, 2004). DifA, a methyl-accepting chemotaxis protein (MCP), DifC, a coupling protein (CheW homologue), and DifE, a histidine kinase (CheA homologue) form a ternary signalling complex required for fibril biosynthesis, S-motility, and fruiting body development (Yang *et al.*, 1998; 2000; Bellenger *et al.*, 2002; Yang and Li, 2005). DifA and DifE are also required for a stimulation by 12:0 PE (Kearns *et al.*, 2000). DifD, a response regulator (CheY homologue), and DifG, which is similar to CheC, negatively regulate fibril polysaccharide production (Black and Yang, 2004). DifB has no known function and is not known to interact with other Dif proteins (Black and Yang, 2004; Yang and Li, 2005). Mutations in *difB*, *difD* or *difG* did not severely impact development or S-motility (Black and Yang, 2004).

We investigated the role of the *dif* genes in PE chemotaxis to determine whether the *dif* chemosensory pathway plays a role in PE chemotaxis other than regulation of fibril biogenesis. Our results revealed a surprisingly complex sensory pathway that mediates both matrix production and lipid chemotaxis.

Results

difACE impact the basal reversal period

Myxococcus xanthus cells glide along straight paths and periodically reverse direction. Wild-type cells reverse direction approximately every 7 min on average, which is the basal reversal period (Blackhart and Zusman, 1985). The behaviour of strains containing an in-frame deletion of each *dif* gene was quantified by time lapse video microscopy. *difA*, *difC* and *difE* mutants have a basal reversal period of 30–40 min suggesting that their protein products regulate the basal reversal period (Fig. 1A). The *difB*, *difD* and *difG* mutants have basal reversal periods of 12, 10 and 7 min respectively (Fig. 1A).

difD is required for stimulation by 16:1 PE

At physiological concentrations 16:1 PE is a potent

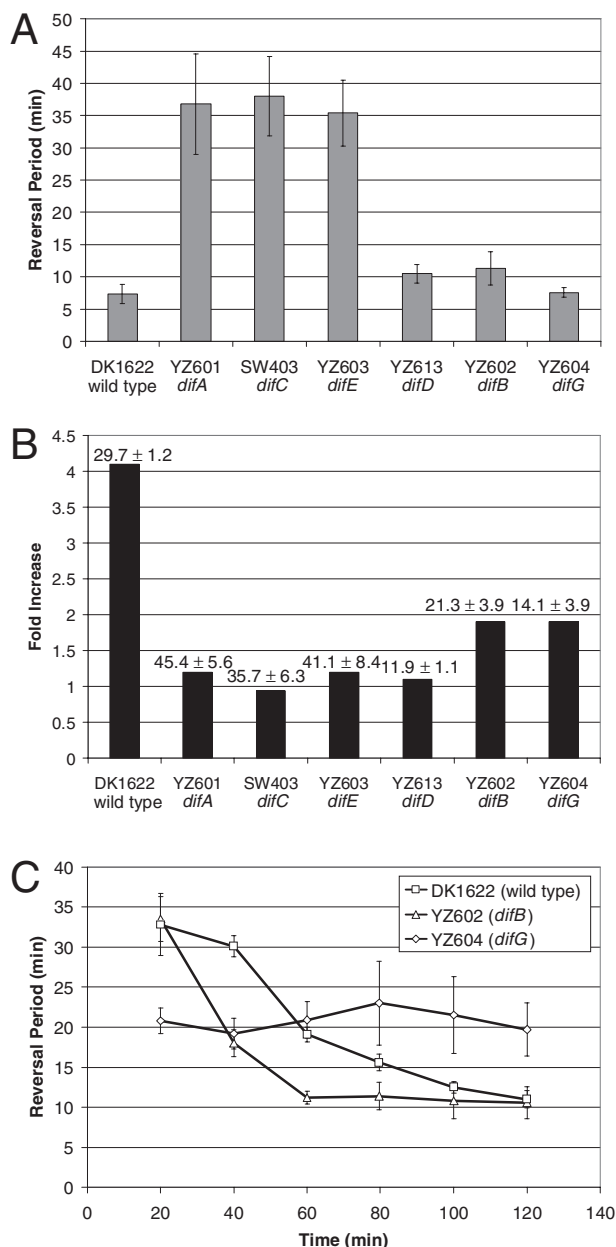


Fig. 1. Basal reversal periods, and stimulation and adaptation responses of *dif* mutants to 16:1 PE.

A. Reversal periods in the absence of an attractant (basal reversal period) for DK1622 (wild type), YZ601 (*difA*), SW403 (*difC*), YZ603 (*difE*), YZ613 (*difD*), YZ602 (*difB*) and YZ604 (*difG*). Error bars are the standard deviations of three replicates.

B. Chemotactic stimulation by 16:1 PE for each strain was determined as the fold increase in reversal period during a 45 min assay. Fold increase is measured as the reversal period in the presence of 16:1 PE/basal reversal period. The reversal period and standard deviation for each strain in the presence of 4 ng of 16:1 PE are shown above each bar.

C. Adaptation to 16:1 PE was determined for DK1622 (squares), YZ602 (triangles) and YZ604 (diamonds). *M. xanthus* cells were observed for 2 h in the presence of 5 ng of 16:1 PE. Reversal periods were determined over 20 min intervals and plotted at the end of the interval. Error bars are the standard deviation of three replicates.

chemoattractant (Kearns *et al.*, 2001). The concentration of 16:1 PE that generates the greatest increase in reversal period was determined empirically to be 4–5 ng (data not shown). In the presence of 4 ng of 16:1 PE, the reversal period of wild-type cells increases about fourfold to approximately 30 min (Fig. 1B). *difA*, *difC* and *difE* mutants are not stimulated by 16:1 PE (Fig. 1B) but as fibrils are required for stimulation by 16:1 PE (Kearns *et al.*, 2001), these results do not distinguish between a dependence on fibril production or a direct role in sensory transduction. However, the *difD* mutant is not stimulated by 16:1 PE in spite of enhanced fibril production (Black and Yang, 2004). In addition, *difB* and *difG* display reduced stimulation by 16:1 PE in spite of normal and enhanced fibril production respectively (Fig. 1B; Black and Yang, 2004). The requirement for DifD and partial dependence on DifB and DifG provide the first line of evidence that Dif is a chemosensory pathway for 16:1 PE as matrix production is not inhibited in these mutants.

difB and *difG* are involved in adaptation to 16:1 PE

Myxococcus xanthus cells adapt to the stimulus over a period of 1–2 h (Kearns and Shimkets, 1998), such that the reversal period returns to basal levels. The adaptation of wild-type, *difB* and *difG* cells in the presence of 16:1 PE was monitored by determining the cellular reversal period at 20 min intervals for 2 h. In the absence of an attractant, the basal reversal periods of wild-type, *difB* and *difG* cells remain constant (data not shown). Wild-type cells stimulated by 5 ng of 16:1 PE begin adapting after 20 min and achieve complete adaptation in about 120 min (Fig. 1C). DifG is homologous to CheC (Black and Yang, 2004), which affects adaptation by controlling MCP methylation (Rosario *et al.*, 1995; Rosario and Ordal, 1996) and acting as a CheY phosphatase in *Bacillus subtilis* (Szurmant *et al.* 2004). The *difG* mutant is defective in chemotactic adaptation to 16:1 PE (Fig. 1C). *difG* is partially stimulated by 16:1 PE with a reversal period of about 20 min that remains elevated for the entire 2 h. When stimulated by 16:1 PE *difB* mutants adapt unusually fast (Fig. 1C). Although the *difB* mutant appears to be only partially stimulated by 16:1 PE in the standard 45 min chemotactic stimulation assay (Fig. 1B), *difB* cells are stimulated by 16:1 PE to wild-type levels when the response is examined at 20 min intervals.

N-terminus of *DifA* is not required to perceive 16:1 PE

A periplasm-exposed region is typically found between the transmembrane domains in MCP proteins and is involved in ligand binding (Mowbray and Sandgren, 1998). DifA has approximately 10 amino acids between the transmembrane domains (Yang *et al.*, 1998) implying a

mechanism of stimulus sensing that is independent of ligand binding to the periplasmic domain. In order to determine whether the DifA transmembrane and periplasmic regions are required for PE chemotaxis, we examined the behaviour of *M. xanthus* cells containing a chimeric chemoreceptor, NafA, which contains the transmembrane and periplasmic domains of NarX and the highly conserved and methylation domains of DifA (Xu *et al.*, 2005). In the presence of nitrate, fruiting body development, S-motility, and fibril polysaccharide production are restored to NafA cells ($\Delta difA$) (Xu *et al.*, 2005). The production of fibril polysaccharide within the time frame required for chemotactic adaptation was examined by the binding of the fluorescent dye calcofluor white (Dana and Shimkets, 1993). In either the presence or absence of nitrate, wild-type cells fluoresced when illuminated by UV light following 2 h of incubation (data not shown). In the absence of nitrate, NafA cells did not fluoresce suggesting an inability to produce fibrils, which in turn likely accounts for the high basal reversal period of about 20 min and the lack of a chemotactic response to 16:1 PE (Fig. 2A). In the presence of at least 5 μ M nitrate, dye binding was evident within 2 h (data not shown) suggesting that fibril production has been restored. When the reversal period assay was conducted with plates containing 35 μ M nitrate, NafA-expressing cells had a low basal reversal period of about 7 min (Fig. 2B). The basal reversal period increased over the next 40 min until reaching a 14 min reversal period and remained constant throughout the remainder of the assay. This finding suggests that in NafA-expressing cells, nitrate mediates a repellent response and then the cells adapt. The basal reversal period of wild-type cells was not affected by the presence of nitrate (data not shown) suggesting that the nitrate response is due specifically to the presence of NafA. The restoration of fibril biosynthesis when nitrate is present may lower the basal reversal period from about 20 min to 14 min (compare Fig. 2A and B).

In the presence of 35 μ M nitrate and 5 ng of 16:1 PE, the reversal period of NafA-expressing cells is elevated to about 21 min, a 3.1-fold increase (Fig. 2B). The reversal period decreases to 14 min within the next 40 min and then remains relatively constant. This is consistent with the 14 min reversal period of NafA-expressing cells following adaptation to nitrate. NafA-expressing cells are stimulated by and adapt to 16:1 PE suggesting that the DifA transmembrane and periplasmic domains are not required for chemotaxis. Transmembrane signalling is, however, essential for fibril biogenesis (Xu *et al.*, 2005).

DifA E110 is required for stimulation by 16:1 PE

Although the variable N-termini of MCPs are typically involved in sensing various stimuli and the conserved C-

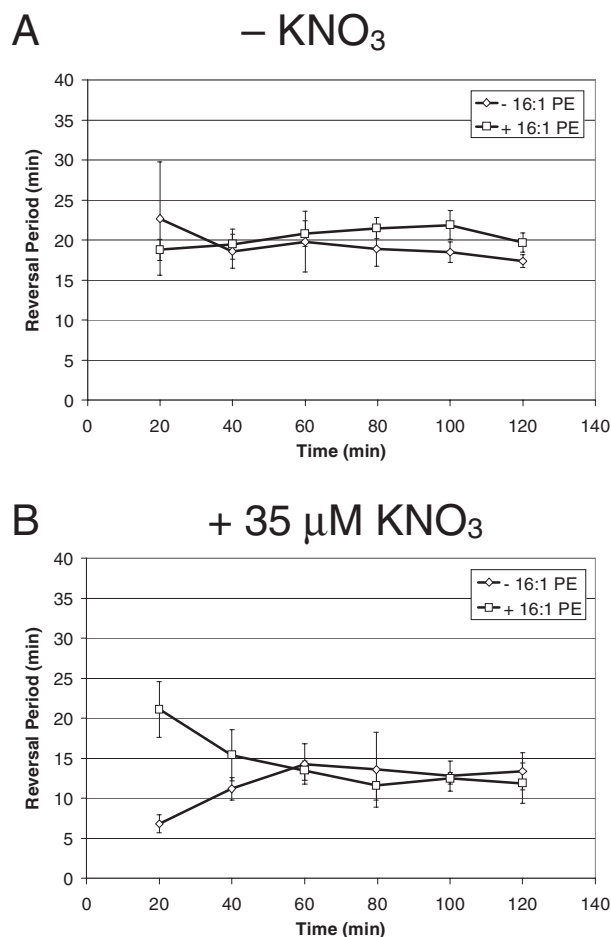


Fig. 2. N-terminus of DifA is not required for PE perception. YZ724 (*difA P_{dif}-nafA*) *M. xanthus* cells were incubated on TPM agar (A) or TPM agar supplemented with 35 μM KNO₃ (B) for 2 h in the absence (diamonds) and presence (squares) of 5 ng of 16:1 PE. Reversal periods were determined over 20 min intervals and plotted at the end of the interval. Error bars are the standard deviation of three replicates.

terminal multidomain module is typically involved in protein signalling and adaptation (Zhulin, 2001), several MCPs lack transmembrane and periplasmic domains, including FrzCD of *M. xanthus*. The methylation domains of FrzCD have been proposed to be sensors for chemotactic stimuli (Bustamante *et al.*, 2004). To determine whether the putative DifA methylation domains are important for stimulation by or adaptation to 16:1 PE, strains harbouring mutations in potential methylation sites were constructed in the *difA* deletion strain, YZ601, and the modified *difA* genes were integrated at the *M. xanthus* Mx8 *attB* site. Expression of some genes is downregulated by insertion at the *attB* site (Li and Shimkets, 1988; Fisseha *et al.*, 1996) and this appears to be the case for expression of *difA*. Western blot analysis using a polyclonal anti-DifA antibody revealed a decrease in expression of DifA from the *attB* site (data not shown). However,

the expression of wild-type *difA* from the *attB* site in YZ601 is sufficient to restore production of FibA (Fig. 3H, lane A). As FibA is required for stimulation by 16:1 PE (Kearns *et al.*, 2002) each potential methylation site mutant was tested for the production of FibA using Western blot analysis with monoclonal antibody (mAb) 2105 (Fig. 3H). Expression of *difA* containing the Q346D mutation does not produce detectable levels of FibA (Fig. 3H, lane E). Expression of *difA* containing other point mutations in the potential methylation sites did not significantly alter the production of FibA (Fig. 3H, lanes B–D, F and G).

Cells expressing wild-type *difA* from the *attB* site are stimulated by 16:1 PE 2.5-fold to a 25 min reversal period (Fig. 3A), significantly less than the fourfold increase observed with wild-type cells (Fig. 1B) and adaptation is nearly complete after only 1 h (Fig. 3A). Strains expressing DifA carrying point mutations in potential methylation sites expressed from the *attB* site were examined for stimulation by and adaptation to 16:1 PE. YZ713 (Q346D; Fig. 3E) is defective in stimulation by 16:1 PE and has an elevated basal reversal period. Both results can be explained by the absence of FibA (Fig. 3H, lane E), which is also indicative of an absence of fibril polysaccharide. YZ712 (E110D) produces normal levels of FibA (Fig. 3H, lane D), has a low basal reversal period, and yet is not stimulated by 16:1 PE (Fig. 3D). This suggests that the N-terminal region of the first putative methylation domain (MD1) is required for 16:1 PE chemotaxis.

YZ707 (E135D; Fig. 3B), YZ708 (E380D; Fig. 3C) and YZ714 (E352D; Fig. 3F) are stimulated by 16:1 PE to about 25 min, like YZ619 (wild-type DifA; Fig. 3A). While the magnitude of the response is similar for each strain, the actual increase in reversal period varies from 2.6-fold (YZ707) to 1.4-fold (YZ714). The amount of time it takes for each strain to adapt to their basal reversal period varies from 40 to 120 min. Whether this variation indicates a role in adaptation is not clear as it could result from subtle differences in basal reversal period. The phenotype of strain YZ715 (E359D; Fig. 3G) may suggest a role in adaptation. This strain has an elevated basal reversal period and is partially stimulated by 16:1 PE (1.6-fold) but does not adapt throughout the 2 h of observation. Unlike all the other strains, there was large variability in the reversal period of this mutant when presented with 16:1 PE.

By looking at the specific locations of the point mutations, along with their corresponding phenotypes, some trends become apparent (Fig. 4). The E110D mutation that prevents stimulation by 16:1 PE is located within MD1. Mutation of the other potential methylation site within MD1 produced no discernible phenotype with regard to lipid chemotaxis (Figs 3 and 4). Three of the four mutations in potential methylation sites in the second putative methylation domain (MD2) have elevated basal

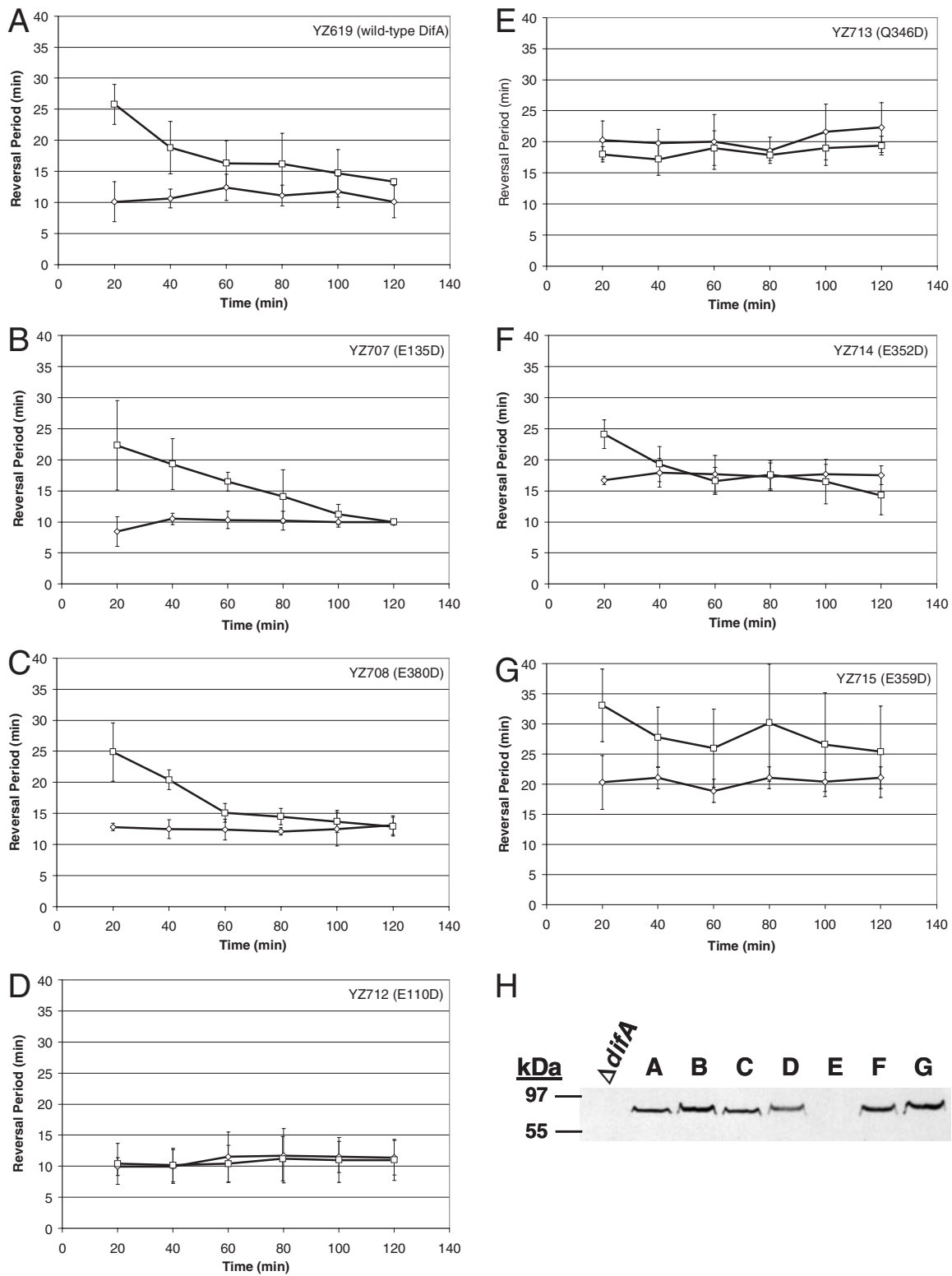


Fig. 3. Specific DifA residues impact basal reversal period, stimulation by and adaptation to 16:1 PE, and FibA production. A–G. YZ619 (wild-type DifA) (A), YZ707 (E135D) (B), YZ708 (E380D) (C), YZ712 (E110D) (D), YZ713 (Q346D) (E), YZ714 (E352D) (F) and YZ715 (E359D) (G) *M. xanthus* cells were observed for 2 h in the absence (diamonds) and presence (squares) of 5 ng of 16:1 PE. Reversal periods were determined over 20 min intervals and plotted at the end of the interval. Error bars are the standard deviation of three replicates. H. Western blot analysis for FibA production. Whole-cell lysates were prepared from 5×10^7 cells, separated by SDS-PAGE and probed with anti-FibA mAb 2105. Lanes: $\Delta difA$ (YZ601); A, YZ619; B, YZ707; C, YZ708; D, YZ712; E, YZ713; F, YZ714; G, YZ715.

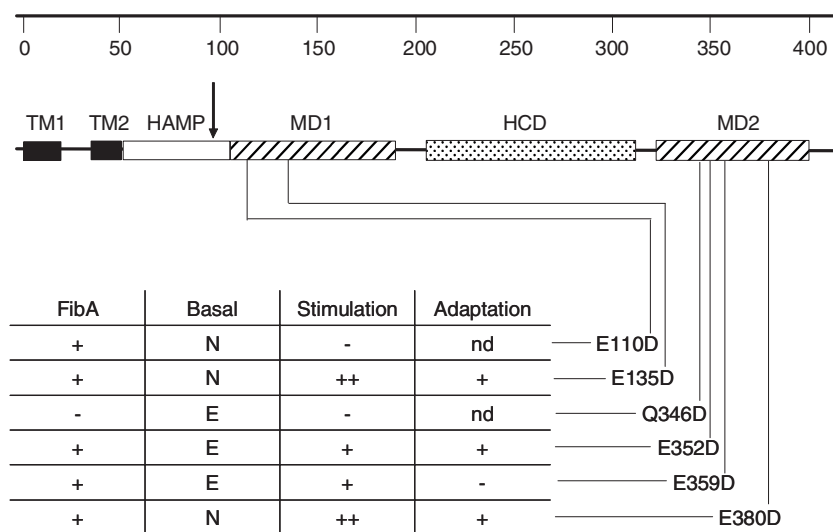


Fig. 4. Phenotypes of DifA point mutations. DifA domains include transmembrane helices (TM1 and TM2), the linker (HAMP), methylation domains (MD1 and MD2) and the highly conserved domain (HCD). The numbers on the bar above the schematic indicate amino acid residue. The arrow indicates the first DifA residue following NarX (M97) in NafA in YZ724. The production of FibA (+) was determined by reactivity with mAb 2105. Basal reversal periods of strains are considered normal (N) if they are less than 15 min; basal reversal periods greater than 15 min are elevated (E). Stimulation by 16:1 PE was scored relative to strain YZ619. A score of ++ indicates that the reversal period increased more than twofold in the presence of 16:1 PE; + indicates an increase less than twofold; - indicates no increase over the basal reversal period. Adaptation (+) occurs if there is a marked decrease in reversal period with time, approaching the basal reversal period within 2 h. If there is no stimulation, adaptation cannot be determined (nd).

reversal periods in excess of 15 min (Figs 3 and 4). Furthermore, specific mutations in MD2 affect chemotactic adaptation and fibril production. The fact that mutagenesis of three sites within a span of 14 amino acids produces three distinct phenotypes suggests that this region plays critical roles in regulating the DifA output.

difD and *difE* are required for stimulation by 18:1 PE

18:1 PE is a less potent chemoattractant than 16:1 PE; peak stimulation requires about 100-fold greater lipid concentrations and elicits a weaker 2.5-fold increase in reversal period (Kearns *et al.*, 2000; 2001). The reversal period of wild-type cells in the presence of 200 ng of 18:1 PE increases 2.6 times to nearly 20 min (Fig. 5A). The *difA* mutant is stimulated by 18:1 PE (twofold increase) resulting in a reversal period in excess of 70 min (Fig. 5A). Similar results were obtained for the *difC* mutant (Fig. 5A). Although the magnitudes of the *difA* and *difC* responses far exceed that observed with wild-type cells, the fold increase relative to the basal reversal period is similar to wild type. 18:1 PE sensory transduction occurs despite the fact that the *difA* and *difC* mutants are compromised in fibril production and S-motility. *difD* and *difE* mutants are not stimulated by 18:1 PE (Fig. 5A) suggesting that the histidine kinase-response regulator pair is required for the response. Activation of these proteins may occur through another MCP, possibly encoded by one of the seven other chemotaxis-like gene clusters or 13 additional genes predicted to encode MCPs found within the *M. xanthus* genome (J. Kirby, unpublished). The *difB* and *difG* mutants are stimulated by 18:1 PE, increasing their reversal periods 2.3- and 2.1-fold respectively.

While wild-type cells are stimulated by 200 ng of 18:1 PE, cells begin to stop moving after approximately 1 h

making it difficult to assess adaptation. It was determined that 50 ng of 18:1 PE elicits a stimulation response similar to that obtained using 200 ng of PE and does not impact cell movement (data not shown). Wild-type cells exposed to 50 ng of 18:1 PE begin adapting within 20 min and adaptation is nearly complete within 120 min (Fig. 5B). Adaptation of *difB* and *difG* cells to 18:1 PE is comparable to wild type (Fig. 5B), suggesting that DifB and DifG are not required for adaptation to 18:1 PE, unlike adaptation to 16:1 PE (Fig. 1C).

Discussion

In this work, we have determined that the role of the Dif pathway in PE chemotaxis is distinct from the role of the Dif pathway in the production of fibril polysaccharide. A model for the roles of the Dif proteins in 16:1 and 18:1 PE chemotaxis is shown in Fig. 6. Under vegetative conditions, 18:1 PE is sensed independently of DifA and DifC (Fig. 5A), suggesting that another lipid-sensing system exists. However, DifD and DifE are required for stimulation by 18:1 PE (Fig. 5A). This finding indicates that DifE may integrate signals among various sensory systems. The integration of multiple signals is especially intriguing from a biological and mechanistic standpoint as this organism may use the PE sensory transduction systems to distinguish between self (16:1 PE) and prey (18:1 PE).

When nutrients become limiting the production of fibril polysaccharide is increased by the Dif chemosensory pathway (Yang *et al.*, 1998; Bellenger *et al.*, 2002; Black and Yang, 2004). Stimulation by 16:1 PE occurs only when the fibril polysaccharide and associated proteins are present and requires the core DifACDE proteins. Furthermore, two lines of evidence suggest that DifA functions in lipid sensory transduction in a manner that is distinct from

domain helps co-ordinate A- and S-motility (Li *et al.*, 2005). Attractants presumably lead to an increase in unphosphorylated DifD leading to an increase in reversal period.

The predicted adaptation pathway for 16:1 PE appears to be novel and methylation independent. This notion is supported by the fact that mutagenesis of all but one of the candidate methylation sites in DifA did not dramatically effect adaptation (Figs 3 and 4). Dif-mediated adaptation involves DifB and DifG. Homologues of DifB and DifG are not found elsewhere in the *M. xanthus* genome (P. Bonner, unpublished) indicating that the DifBG mechanism of adaptation may be unique to the Dif system. The *difG* mutant fails to adapt in the presence of 16:1 PE (Fig. 1C). DifG is homologous to CheC, suggesting that *M. xanthus* may utilize signal removal to mediate adaptation to 16:1 PE. This notion is supported by the observation that DifG interacts with DifD (Yang and Li, 2005). The *difB* mutant has a unique phenotype and appears to be involved in slowing adaptation to 16:1 PE (Fig. 1C). This is the first time a hyperadaptation phenotype has been described. The time frame for adaptation in *M. xanthus* is slow relative to adaptation in the enteric bacteria, which may reflect the longer reversal period of gliding cells compared with the run period of swimming cells. Curiously, *difB* and *difG* mutations had no effect on adaptation to 18:1 PE. If 18:1 PE is perceived by an MCP, the MCP may be subject to

an alternate mechanism of adaptation, allowing adaptation to occur independently of DifBG, or in addition to the DifBG regulation.

In conclusion, the Dif chemosensory system has several novel features worthy of future investigation. Foremost are the two mechanisms of DifA sensory input; transmembrane signalling is required for fibril formation but not for 16:1 PE lipid chemotaxis. Second is an unusually slow adaptation process utilizing a novel protein, DifB, which controls the rate of adaptation. Finally, DifE appears to receive independent inputs for fibril biogenesis, stimulation by 16:1 PE and by 18:1 PE. DifE also has multiple outputs, interacting with DifD to control motility and presumably with an unidentified protein to control fibril biogenesis. Together the results suggest an extraordinary level of complexity for this sensory pathway which simultaneously regulates biofilm formation, self-recognition (16:1 PE) and prey recognition (18:1 PE).

Experimental procedures

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. All *M. xanthus* strains were grown in CYE broth [10 g l⁻¹ Difco Casitone, 5 g l⁻¹ yeast extract, 10 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS; pH 7.6) and 4 mM MgSO₄] with vigorous shaking at 32°C.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or description	Source or reference
<i>M. xanthus</i> strains		
DK1622	Wild type	Kaiser (1979)
SW403	DK1622 $\Delta difC$	Bellenger <i>et al.</i> (2002)
YZ601	DK1622 $\Delta difA$	Xu <i>et al.</i> (2005)
YZ602	DK1622 $\Delta difB$	Black and Yang (2004)
YZ603	DK1622 $\Delta difE$	Black and Yang (2004)
YZ604	DK1622 $\Delta difG$	Black and Yang (2004)
YZ613	DK1622 $\Delta difD$	Black and Yang (2004)
YZ619	YZ601 with <i>difA</i> (wild type); Kan ^r	This study
YZ707	YZ601 with <i>difA</i> (E135D); Kan ^r	This study
YZ708	YZ601 with <i>difA</i> (E380D); Kan ^r	This study
YZ712	YZ601 with <i>difA</i> (E110D); Kan ^r	This study
YZ713	YZ601 with <i>difA</i> (Q346D); Kan ^r	This study
YZ714	YZ601 with <i>difA</i> (E352D); Kan ^r	This study
YZ715	YZ601 with <i>difA</i> (E359D); Kan ^r	This study
YZ724	YZ601 P <i>dif-nafA</i> ; Kan ^r	Xu <i>et al.</i> (2005)
Plasmids		
pWB200	<i>Myxococcus</i> gene integration vector with Mx8 attachment site and backbone from pZero; Kan ^r	Xu <i>et al.</i> (2005)
pWB210	pWB200 with <i>difA</i> (wild type)	This study
pWB230	pWB210 with two point mutations (a silent mutation, CTG to CTC, at <i>difA</i> codon 103 to introduce a SacI site; a C to G at -43 to introduce a SmaI site)	This study
pXQ707	pWB230 with <i>difA</i> (E135D)	This study
pXQ708	pWB230 with <i>difA</i> (E380D)	This study
pHN001	pWB230 with <i>difA</i> (E110D)	This study
pHN002	pWB230 with <i>difA</i> (Q346D)	This study
pHN003	pWB230 with <i>difA</i> (E352D)	This study
pHN004	pWB230 with <i>difA</i> (E359D)	This study
pBJ113	Gene replacement vector; <i>galk</i> , Kan ^r	Julien <i>et al.</i> (2000)
pLZ405	pBJ113 with sequences upstream and downstream of <i>difD</i>	This study
pXQ724	<i>Myxococcus</i> gene integration vector with Mx8 attachment site and <i>difA</i> promoter; Kan ^r	This study

Reversal period assays

The assays were modified from Kearns and Shimkets (1998) using stated amounts of 16:1 PE or 18:1 PE dissolved in 4 μ l of chloroform. 16:1 PE contains the fatty acid, 16:1 ω 5c, at both the *sn*-1 and *sn*-2 positions of the glycerol backbone. 18:1 PE contains the fatty acid, 18:1 ω 9c, at both *sn*-1 and *sn*-2 positions of the glycerol backbone. The optimal lipid amount was determined by completion of a dose–response curve in which the stimulation response of DK1622 was determined for a range of lipid amounts. A TPM agar [10 mM Tris (hydroxymethyl) aminomethane HCl (pH 7.6), 8 mM MgSO₄, 1 mM K₂HPO₄–KH₂PO₄ (pH 7.6) and 1.5% agar (Difco)] plate containing 25 ml of media was warmed for 10 min at 37°C. Four microlitres of the test compound were applied to an area of about 0.4 cm² and incubated for 15 min at 32°C. Five microlitres of *M. xanthus* cells diluted to 10⁶ cells ml⁻¹ in MOPS buffer (10 mM MOPS, 8 mM MgSO₄) were dried on top of the test compound and incubated at 32°C for 15 min. For the chemotactic stimulation assay, cultures were observed at approximately 29°C with a Lietz phase contrast microscope (Laborlux D) for 45 min at 400 \times magnification. Digital movies were produced using a Sony Power HAD3CCD colour video camera and Adobe Premiere software with a frame capture rate of 12 frames per minute. To determine the cell reversal period, the paths of 20 isolated cells were followed, and reversals were manually enumerated. The average and standard deviation of the reversal period (time between reversals) were calculated from three assays. For adaptation rates, cells were observed for 2 h. Each movie was divided into 20 min segments and reversal periods were determined for each interval from the behaviour of 20 isolated cells.

Construction of mutants

Construction of *difA* strains was dependent on the integration of plasmids containing either wild-type or mutant *difA* genes under the control of the *difA* promoter at the Mx8 phage attachment site in YZ601. pWB210 contains a 2 kb fragment with the wild-type *difA* gene and upstream promoter cloned into *Spe*I and *Kpn*I sites of pWB200 (Table 1). To construct pWB230, a plasmid with two point mutations but otherwise the same as pWB210, polymerase chain reaction (PCR)-based mutagenesis (Sambrook and Russell, 2001) was used with pWB210 as a template. In pWB230 a cytosine –43 upstream of the *difA* initiation codon (Yang *et al.*, 1998) was mutated to guanine and codon 103 of *difA* was mutated from CTG to CTC. These two mutations create *Sma*I/*Xma*I and *Sac*I sites, respectively, but do not change the DifA polypeptide or abundance as determined by Western immunoblotting analysis with polyclonal anti-DifA (Q. Xu and Z. Yang, unpublished). pWB230 served as a template for subsequent PCR-based mutagenesis to generate plasmids with mutant *difA* genes. The methylation domains of DifA were identified based upon sequence alignment between DifA, McpB from *B. subtilis* and Tsr from *Escherichia coli* (data not shown). Potential methylation sites selected for mutagenesis were the second residue of each characteristic [E/Q]–[E/Q] motif within the methylation domains of DifA. The resultant plasmids, pXQ707 (E135D), pXQ708 (E380D), pHN001 (E110D),

pHN002 (Q346D), pHN003 (E352D) and pHN004 (E359D), as well as those described previously were sequenced and found to contain no errors. The plasmids were transformed by electroporation (Kashefi and Hartzell, 1995) into YZ601 and kanamycin-resistant colonies were selected and purified. Expression of DifA in each resultant strain was detected by Western immunoblot analysis with polyclonal anti-DifA.

Western immunoblot analysis

Cell lysates were prepared from 5 \times 10⁷ cells grown to mid-log phase. Proteins were separated by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto nitrocellulose and probed for the presence of FibA using a 1:500 dilution of primary antibody mAb 2105 and a 1:10 000 dilution of secondary antibody composed of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G. The immunoblot was developed with the ECL luminescence detection kit (Amersham Pharmacia, Piscataway, NJ).

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