Myxococcus xanthus fibril appendages are essential for excitation by a phospholipid attractant

Daniel B. Kearns, Blakemore D. Campbell, and Lawrence J. Shimkets*

Department of Microbiology, University of Georgia, Athens, GA 30602

Edited by A. Dale Kaiser, Stanford University School of Medicine, Stanford, CA, and approved August 11, 2000 (received for review October 18, 1999)

Isolated (A-motile) *Myxococcus xanthus* cells glide over solid surfaces and display excitation, a suppression of direction reversals, when presented with phosphatidylethanolamine (PE) purified from its own membranes or synthetic dilauroyl PE and dioleoyl PE. Although the mechanism of PE signal transduction is unknown, we hypothesized that *M. xanthus* might use surface-associated factors to detect exogenous PE to prevent endogenous lipids from self-stimulating the sensory system. Peritrichous protein and polysaccharide appendages called fibrils were correlated with dilauroyl PE excitation. Wild-type cells treated with Congo red, an inhibitor of fibril assembly, and mutants defective in fibril biosynthesis showed an elevated reversal period, which suggested that fibrils regulate the gliding motor. Furthermore, the loss of fibrils resulted in loss of excitation to dilauroyl PE but not dioleoyl PE. Restoration of fibril production to these mutants restored the dilauroyl PE response. In addition, the *dif* cytoplasmic signal transduction system and starvation conditions were required for dilauroyl PE excitation. The chemically specific nature of the response and the dependence on the *dif* system suggests that fibrils define a novel sensory organelle whose evolution may have been necessary to prevent autostimulation by endogenous membrane lipids. Because the hydrophobic nature of dilauroyl PE would be inaccessible to periplasmic chemosensors, we suggest that fibrils act as extracellular signal transducers to probe surfaces for insoluble chemical signals.

Myxococcus xanthus uses two distinct motility systems to glide over solid surfaces (1). The adventurous or A-motility system enables cells to move as individuals. The social or S-motility system requires cell-cell contact because S motile cells (A^-S^+) cannot move as single cells (2). At high cell densities both motility systems operate, and mutation of both systems is required to render the cells nonmotile. The mechanism of A motility is not understood. S motility bears extensive similarity to twitching motility in its dependence on type IV pili (3–5). *M. xanthus* displays chemotaxis toward phosphatidylethanolamine (PE) purified from its own cell membrane and chemically synthesized dilauroyl (di C12:0) and dioleoyl (di C18:1 ω9c) PE (6). PE is the first discrete chemoattractant for the gliding bacteria and is the first lipid attractant for any bacterium. How the PE chemotaxis sensory systems are coupled to the operation of the A and S motors is a new frontier.

Escherichia coli cells swim through liquid media by using flagella and are chemotactic toward several amino acids and sugars. These attractants are detected by integral membrane methyl-accepting chemotaxis proteins (MCPs) to regulate flagellar rotation (7). Chemotaxis is divided into two processes, excitation and adaptation. *E. coli* regularly punctuates smooth swimming with periods of erratic tumbling but during excitation by an attractant the tumbling behavior is suppressed. Excitation results in directed movement because cells run smoothly for a longer period while traveling up an attractant gradient (8). Chemotaxis ceases when a uniform concentration of attractant is attained because an adaptive response restores the basal frequency of tumbles (9).

Despite its motility differences with *E. coli*, the *M. xanthus* response toward PE demonstrates similar excitation and adaptation behaviors. Isolated A-motile *M. xanthus* cells move along their long axis (equivalent to a run) and reverse their direction of movement (equivalent to a tumble) once every 6.8 min (10). In the excitation assay the behavior of isolated A-motile cells is examined in response to a uniform concentration of an attractant. *M. xanthus* cells increase their reversal period, the time between reversal events, when presented with an attractant, and the basal reversal frequency is restored by adaptation after 45 min of PE exposure (6). This finding suggests that although *E. coli* and *M. xanthus* move differently, they use similar strategies to mediate chemotaxis.

In this work we examine the cellular components required for the response of A-motile cells to PE by using the excitation assay. We were puzzled by the fact that lipids extracted from the membranes of cells repress reversals and direct cell movements when presented exogenously, but do not autostimulate the producer cell. The use of an integral membrane MCP as the sole chemoreceptor does not seem a likely solution to the problem of autostimulation because it is surrounded by membrane lipid. We searched for cell surface components that might be required for PE chemotaxis and discovered that fibrils, flexible appendages that extend away from the cell surface, are required for excitation by dilauroyl PE. We suggest that the fibrils are extracellular signal receptors/transducers for detecting insoluble chemical signals.

Methods

Strains and Conditions. *M. xanthus* DK1622 (wild type), DK3482 (*tglA*), LS1119 (*tglA*/*stk*), DK3470 (*dsp*), SW501 (*difE*), and SW504 (*difA*) were grown in CYE broth [10 g/liter Difco casitone, 5 g/liter yeast extract, 10 mM 3-(*N*-morpholino)-propanesulfonic acid (Mops, pH 7.6), and 4 mM $MgSO_4$] with vigorous shaking at 32°C.

Excitation Assay. The excitation assay measures changes in the reversal period in response to exposure to a uniform concentration of attractant (6). A TPM agar plate [10 mM Tris (hydroxymethyl) aminomethane HCl (pH 7.6), 8 mM $MgSO_4$, 1 mM $KHPO_4$-KH_2PO_4, and 1.5% Difco agar] was dried for 10 min at 37°C. Dioleoyl PE and dilauroyl PE were dissolved in chloroform and chloroform/methanol (1:1), respectively, to 0.005 mg/ml, 0.05 mg/ml, and 0.5 mg/ml concentrations. Four microliters of PE solution was applied to TPM agar in an area of about 0.4 cm^2 and dried for 20 min at 37°C. Five microliters of *M. xanthus* cells diluted to 5×10^6 cells/ml in Mops buffer

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PE, phosphatidylethanolamine; MCP, methyl-accepting chemotaxis protein.

*To whom reprint requests should be addressed at: 527 Biological Sciences Building, Department of Microbiology, University of Georgia, Athens, GA 30602. E-mail: shimkets@arches.uga.edu.

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.210448597. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.210448597

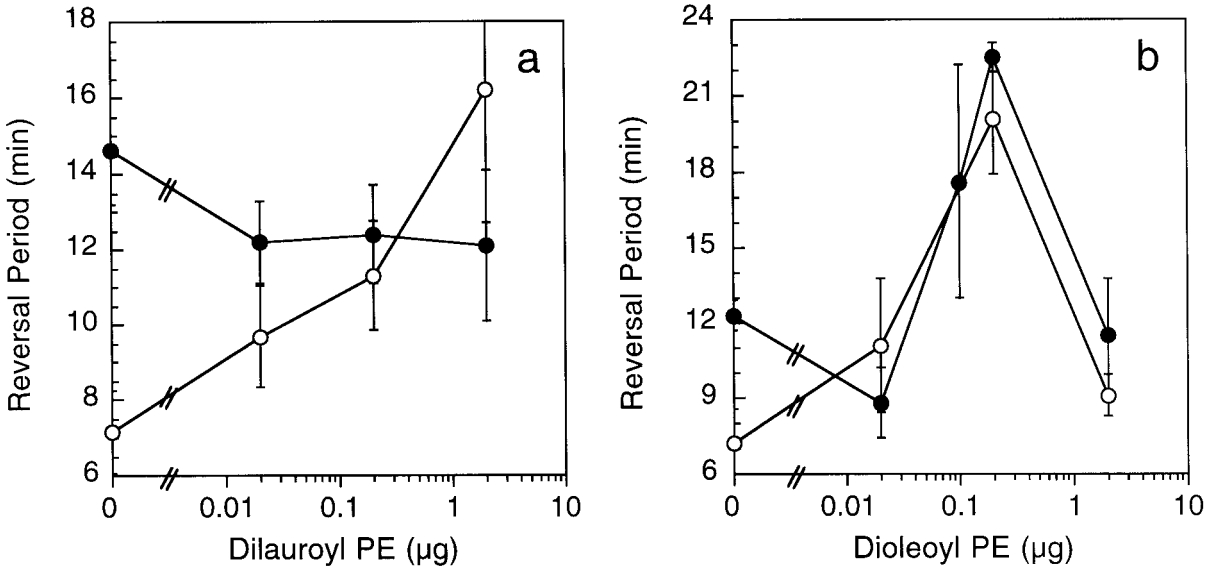

Fig. 1. Elimination of fibrils inhibits excitation by dilauroyl PE. Wild-type DK1622 *M. xanthus* reversal periods with increasing amounts of dilauroyl PE (*a*) and dioleoyl PE (*b*). Reversal periods were determined with (●) and without (○) 57 μM Congo red dye added to the agar. Error bars are the standard deviation of three replicates.

(10 mM Mops, pH 7.6/8 mM $MgSO_4$), was dried on top of the test compound for 15 min at 32°C to allow the liquid grown cells to adjust to the surface. Plate cultures were observed at room temperature with a Leitz Laborlux D microscope for 45 min at ×640 magnification. Stop-motion digital movies were produced by using a microscope-mounted Sony Power HAD 3CCD color video camera and a Macintosh 9500 with Adobe PREMIER software (frame capture rate: 12 frames/min). To determine cellular reversal period, the paths of 20 isolated cells were followed, and reversals were manually enumerated.

In some experiments, purified fibrils were added to 5×10^7 cells in 1 ml of cohesion buffer (10 mM Mops, pH 6.8/1 mM $CaCl_2$/1 mM $MgCl_2$) at a final concentration of 3.2×10^{-7} μg carbohydrate/cell and rotated at 32°C for 20 min before spotting cells on top of test compound.

Fibril Isolation and Quantitation. Fibrils were purified from developmental *M. xanthus* cells by using a protocol modified from Clemans, Chance, and Dworkin (11). *M. xanthus* DK1622 was grown to mid-log phase in 200 ml of CYE broth, harvested, and resuspended in Mops buffer to 5×10^9 cells/ml. A total of 4.2 ml of cell suspension was spread on each of two 500-ml TPM agar trays (17″ × 11″), covered, and incubated for 24 h at 32°C. The cells then were scraped in a beaker and stirred in 5 ml of ice-cold TNE buffer [10 mM Tris (hydroxymethyl) aminomethane HCl, pH 7.5/100 mM NaCl/5 mM EDTA] for 10 min. Five milliliters 1% SDS in TNE buffer was added and stirred for 30 min at room temperature. The solution was centrifuged at 12,000 × *g* for 10 min at 4°C, and the pellet was resuspended in 2.5 ml of ice-cold TNE and 2.5 ml of 1% SDS TNE and stirred for 1 h at room temperature. The pellet was recovered and then washed once in 5 ml of TNE buffer, twice in 5 ml of 10 mM Mops buffer (pH 6.8), and twice in 5 ml of cohesion buffer. Finally, the pellet was resuspended in 1 ml of cohesion buffer and stored at −20°C. Fibrils were quantified as a function of carbohydrate content (12).

Results

Lipids extracted from the membranes of *M. xanthus* cells repress direction reversals when presented exogenously, but do not autostimulate the producer cell (6). We reasoned that to prevent autostimulation the detection system must not be in direct contact with the membrane lipids. *M. xanthus* has two types of appendages that extend away from the cell surface: type IV pili and fibrils. Pili are rigid filaments of repeating pilin monomers that extend from one cell pole and are thought to provide the power for S motility (3, 4). Fibrils are long, flexible appendages composed of a polysaccharide matrix decorated with proteins (13–15). Although the genes involved in fibril biogenesis have not been identified and sequenced, there are several ways to remove fibrils from the cell surface. One involves treating cells with the dye Congo red, which appears to prevent crystallization of the fibril polysaccharide matrix. Congo red-treated cells lack fibrils when examined by electron microscopy (16). Treatment of wild-type cells with 57 μM Congo red elevated the *M. xanthus* basal reversal period from 6 to 15 min, suggesting that fibrils influence the operation of the A-motility motor. More important, Congo red inhibited excitation to dilauroyl PE over a wide concentration range (Fig. 1*a*) but had no effect on the response to dioleoyl PE (Fig. 1*b*). It appeared that fibrils regulate the excitation of A motility in a chemically specific manner.

tglA cells are defective in the assembly of pili and consequently the ability to move by S motility (17, 18). The *tglA* mutation is pleiotropic, as *tglA* mutants are also defective in fibril production (19). *tglA* cells retain A motility but lack excitation to both dilauroyl and dioleoyl PE (Fig. 2). *tglA* can be partially complemented by a second mutation in the *stk* locus that restores fibril production, but not piliation or S motility (19). Suppression of the fibril defect restored excitation to dilauroyl PE (Fig. 2*a*), which argues that fibrils, and not pili, are necessary for dilauroyl PE excitation. The *stk* mutation did not restore excitation to dioleoyl PE (Fig. 2*b*), suggesting that *tglA* must lack additional elements necessary for dioleoyl PE excitation. The loss of fibrils by Congo red treatment increased the basal reversal period 2- to 3-fold (Fig. 1*a*) but *tglA* did not exhibit a similar behavior. We suspect this loss is caused by the pleiotropic nature of *tglA*; the loss of pili, components involved in dioleoyl PE perception, or other unknown defects may suppress an elevated reversal period

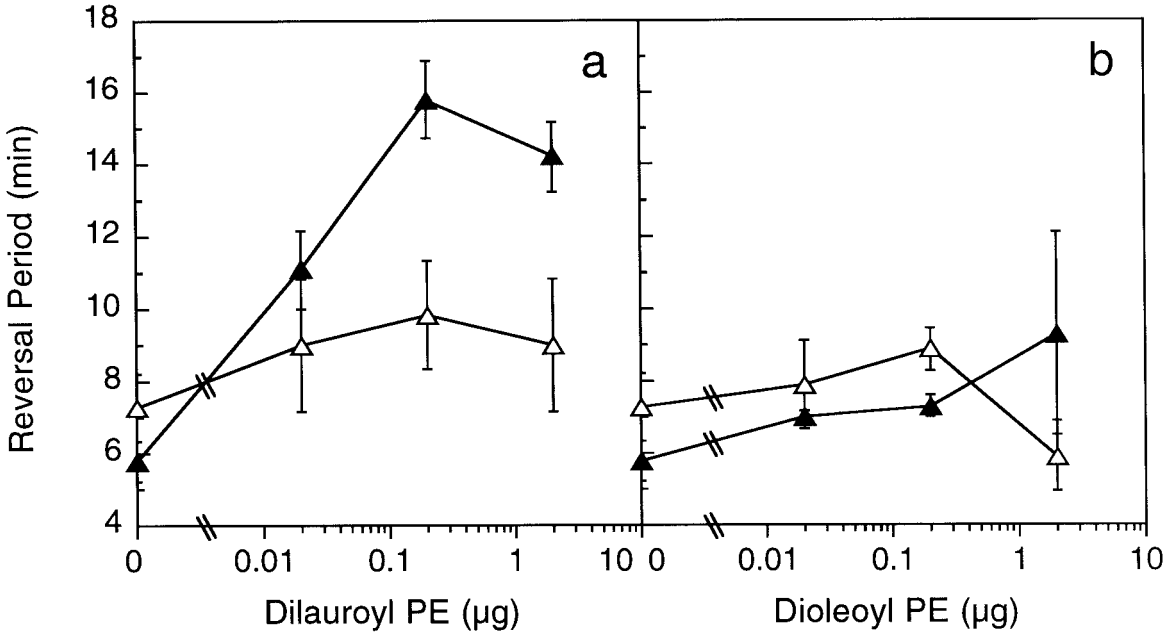

Fig. 2. Genetic complementation of the fibril defect restores excitation to dilauroyl but not dioleoyl PE in a *tglA* mutant. DK3482 *tglA* (△) and LS1119 *tglA*/*stk* (▲) *M. xanthus* reversal periods with increasing amounts of dilauroyl PE (*a*) and dioleoyl PE (*b*). Error bars are the standard deviation of three replicates.

caused by the lack of fibrils. Interestingly, complementation of fibrils by the *stk* second site suppressor decreased the *tglA* reversal period by 30%, suggesting that fibrils are involved in maintaining a lower reversal period (Fig. 2).

Another S-motility mutant, *dsp*, lacks fibrils but retains type IV pili (16). As with the Congo red treatment, the lack of fibrils in the *dsp* mutant resulted in an increase in the basal reversal period from 7 min to at least 16 min (Fig. 3). More significantly, *dsp* cells did not suppress reversals in the presence of dilauroyl PE but displayed normal excitation to dioleoyl PE (Fig. 3*a*). Unlike *tglA* cells, *dsp* cells are unable to form fibrils with the *stk* mutation (19) and lack the fibril proteins (20). However, *dsp* can be complemented by the addition of purified wild-type fibrils, which presumably self-assemble on the *dsp* cell surface (21). Using a similar approach, we incubated *dsp* cells with purified wild-type fibrils for 20 min before initiating the excitation assay. The addition of purified fibrils lowered the unstimulated reversal period of *dsp* closer to that of the wild type and restored excitation to dilauroyl PE (Fig. 3*b*).

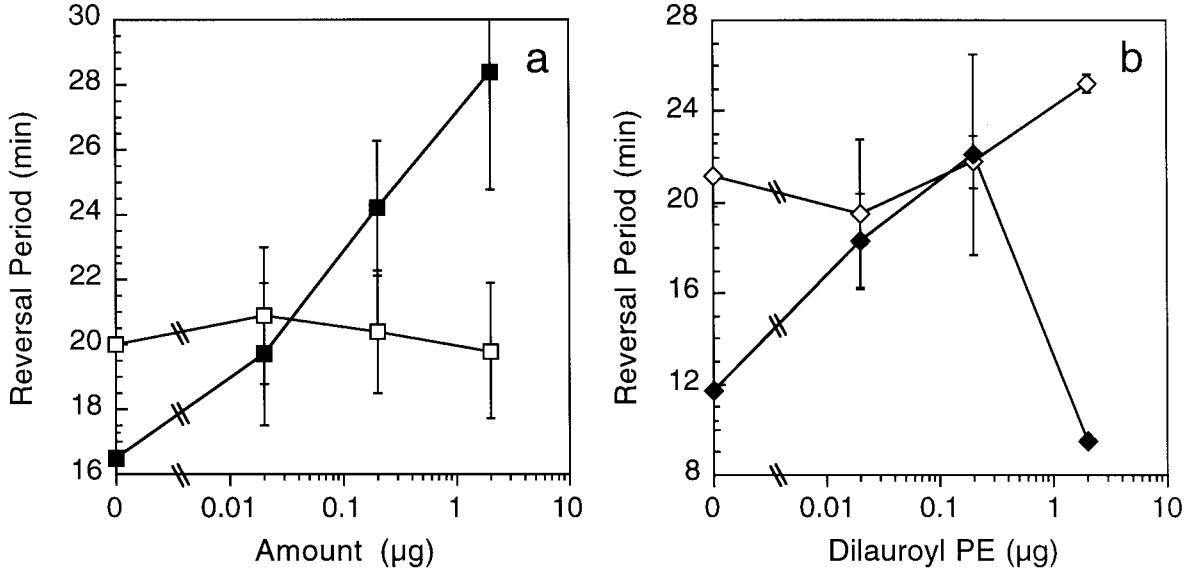

Fig. 3. Purified fibrils restore dilauroyl PE excitation in an *M. xanthus dsp* mutant. (*a*) DK3470 *dsp M. xanthus* reversal periods with increasing amounts of dilauroyl (□) and dioleoyl PE (■). (*b*) Reversal periods of *dsp* cells with increasing amounts of dilauroyl PE (◇). Purified fibrils were added exogenously to *dsp* before observation (◆). Error bars are the standard deviation of three replicates.

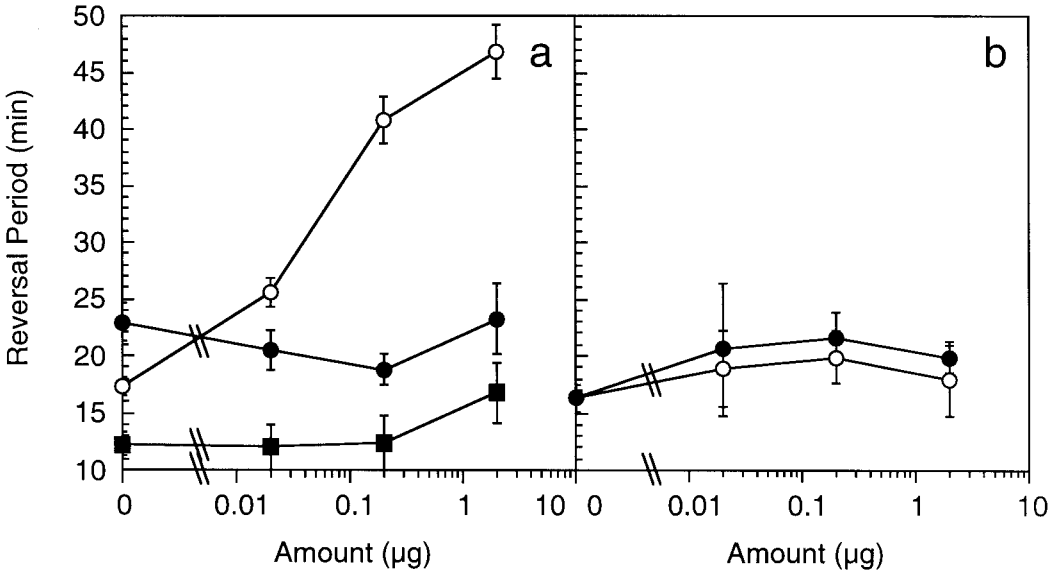

Fig. 4. The Dif cytoplasmic sensory transducers are essential for dilauroyl PE excitation. (*a*) The reversal periods of SW504 *difA* in response to increasing amounts of dioleoyl PE (○) and dilauroyl PE with (■) and without (●) the addition of purified wild-type fibrils. (*b*) The reversal periods of SW501 *difE* in the presence of increasing amounts dioleoyl PE (○) and dilauroyl PE (●). Error bars are the standard deviation of three replicates.

The *dif* locus maps close to *dsp* on the *M. xanthus* chromosome, and *dif* mutants have similar defects in S motility (22). The *dif* locus consists of at least five genes homologous to the enteric chemotaxis proteins (23). Recent results suggest that *dif* mutants also lack fibrils, suggesting a cognate relationship between fibrils and a cytoplasmic signal transduction system (22). We tested mutants in *difA*, an MCP homolog, and *difE*, a *cheA* homolog, for their excitation response to the two attractants. Both mutants displayed elevated basal reversal periods, which is consistent with the observation that they lack fibrils (Fig. 4). The *difA* mutant lacked excitation to dilauroyl PE but the *difE* mutant failed to increase its reversal period in the presence of either attractant. The addition of purified wild-type fibrils to *difA* cells resulted in a decrease in the basal reversal period as with *dsp*. Unlike *dsp*, exogenous fibrils did not restore excitation to dilauroyl PE (Fig. 4*a*). These results suggest that both DifA and fibrils are essential for the response.

Environmental conditions are also known to regulate fibril biogenesis. At low cell densities, starvation in the presence of divalent cations is necessary for fibril formation (24). Under similar conditions, nutrient-rich media inhibits fibril production (20). The reversal period assay was performed on CTT agar, a nutrient-rich growth medium (1), to suppress fibril biogenesis, and under these conditions the cells did not demonstrate excitation to dilauroyl PE but retained excitation to dioleoyl PE (Fig. 5). Therefore, it appears that although the dilauroyl PE response is starvation-dependent, the dioleoyl PE response is starvation-independent. CTT-grown cells are another case in which fibrils are absent but the basal reversal period is similar to wild type (Fig. 5). The physiology of *M. xanthus* differs greatly between growing and starving cells, and perhaps growing *M. xanthus* has an alternate means of suppressing the fibril-deficient reversal defect. *dif* mutants have a wild-type reversal period of 6–8 min when observed on CTT media, and the *dif* basal reversal defect only becomes apparent under starvation conditions (23) (Fig. 4). Thus, nutrient-rich conditions can suppress the elevated reversal periods we observe.

In summary, Congo red- and CTT-treated wild-type cells and the *tgl*, *dsp*, and *dif* mutants all lack excitation to dilauroyl PE and all lack fibrils. Furthermore, restoration of fibril production to *tglA* with the *stk* mutation restored excitation to dilauroyl PE as did addition of fibrils to the *dsp* mutant. Except for *tglA*, all display a normal response to dioleoyl PE. These results suggest that fibrils are essential for excitation by dilauroyl PE but not dioleoyl PE. To determine whether type IV pili are necessary for the dioleoyl PE response, we examined the excitation of DK10410, which lacks the gene encoding the principle pilin

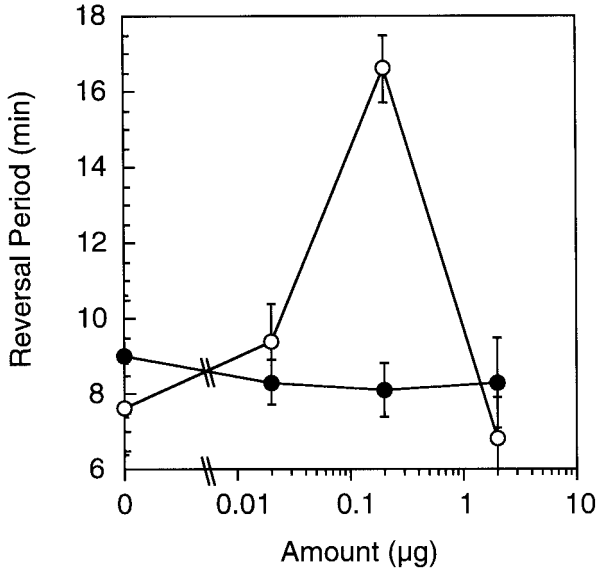

Fig. 5. Starvation is required for excitation to dilauroyl PE. Wild-type DK1622 *M. xanthus* reversal periods on nutrient-rich CTT agar in response to increasing amounts of dilauroyl (●) and dioleoyl PE (○). Error bars are the standard deviation of three replicates.

subunit PilA (4). The *pilA* mutant displayed excitation to dioleoyl PE (data not shown), indicating that pili are not required for this response.

Discussion

In *E. coli*, chemotaxis sensory transduction begins when a hydrophilic chemoattractant or an attractant/protein-complex binds to the periplasmic domain of a transmembrane MCP (7). MCP binding then inhibits a cytoplasmic phosphotransfer relay to control the direction of flagellar rotation (25). However, recognition of a hydrophobic ligand such as PE by the hydrophilic periplasmic domain of an MCP is unlikely and suggests that the *M. xanthus* PE sensory transduction pathway involves novel elements. Here we suggest that appendages called fibrils may be instrumental in carrying information about insoluble chemicals to the A-motility motor.

M. xanthus chemotaxis is the first bacterial system known to use a lipid chemoattractant. Both dilauroyl and dioleoyl PE induce similar responses, but until now it was unclear whether these species were detected separately or whether both attractants stimulated one system with loose specificity. It appears that each species has a unique detection method because fibril appendages are required for excitation by one species but not the other.

The loss of fibrils, either genetically or biochemically, alters the behavior of A-motile *M. xanthus* and inhibits excitation to dilauroyl PE. We propose that fibrils initiate a sensory transduction pathway that communicates information about insoluble materials across the cell membrane. In support of this hypothesis, Congo red-treated wild-type cells, the *dsp* mutant and *dif* mutants demonstrated an elevated reversal period in the absence of PE, which indicates a role for fibrils in motility control. Furthermore, the loss of fibrils resulted in the loss of excitation to dilauroyl PE but not dioleoyl PE as one might expect for a chemoreceptor. When considered together, these data suggest that *M. xanthus* fibrils serve as chemosensors for lipid recognition. Fibrils also mediate intercellular cohesion (16) although cohesion and excitation are not obligately interdependent as fibril-mediated excitation is observed in isolated cells. Isolated *M. xanthus* cells are not stimulated by the endogenous attractant in their own membrane, and thus localization of receptors away from the cell surface may have evolved to prevent autoexcitation.

It is not known how fibrils transmit information to the inside of the cell. It is unlikely that fibrils simply degrade PE into soluble components that serve as chemoattractants as the minimum structure with activity is diacylglycerol (6). Fibrils could be physical channels that siphon signal lipids to cytoplasmic membrane receptors. They might be mechanosensors whose freedom of motion is restricted when binding PE. Although these are possible mechanisms, the overriding theme of bacterial chemoreceptors is one of conformational change. In *E. coli*, ligand recognition alters the conformation of MCPs, and this physical change inhibits a cytoplasmic chemical modification relay (25, 26). We therefore favor the notion that fibrils bind PE and recognition is propagated along the length of the fibril by recursive conformational change via either steric or covalent modification. Covalent modification is particularly intriguing as a 29-kDa fibril protein becomes ADP-ribosylated and could play a role in signal transduction (27).

Fibrils may interact with cytoplasmic components to control the A-motility motor. *M. xanthus* motility is regulated by at least two distinct cytoplasmic signal transduction systems, Frz and Dif, homologous to the enteric Che proteins (23, 28). Mutants in the Frz system demonstrate excitation to dilauroyl PE but are defective in chemotaxis because they lack the adaptive response (6). Fibrils therefore are unlikely to interact directly with the Frz system because fibril mutants are defective in excitation and *frz* mutants are not. The Dif system is essential for social motility and *dif* mutants lack fibrils (22). It is not known why signal transduction mutants produce cell surface defects but the phenotype might indicate that the Dif proteins form the cognate cytoplasmic transduction system for fibril-mediated signals. This idea is supported by the fact that the *difA* mutant specifically lost excitation to dilauroyl PE and could not be rescued by exogenous fibrils, thus *difA* is likely the MCP that receives the dilauroyl PE stimulus. The *difE* mutant was defective in excitation to both dilauroyl and dioleoyl PE and suggests that both attractants feed into a common cytoplasmic signal transduction pathway. The MCP for dioleoyl PE is not known. That fibril assembly is coupled to the Dif cytoplasmic signal transduction system further supports the idea that the fibrils may transduce chemical signals.

M. xanthus fibrils are not only responsible for cell cohesion but also, like antennae, appear to probe surfaces for insoluble chemical signals. But how widespread is this type of appendage-mediated perception system? Cell-contact dependent alteration of cell physiology is being observed with increasing frequency in pathogenic bacteria (29). In *Neisseria meningitidis*, adhesion to host cells by type IV pili is required for enhanced gene expression, colony dispersal, effacing lesions and ultimately, virulence (30, 31). Fibril-like appendages are induced after contact of *Salmonella typhimurium* to host cells and are required for subsequent cell invasion (32). P pilus-mediated adherence of *E. coli* to host globoside receptors directly results in induction of virulence-essential iron acquisition genes (33), and *E. coli* induces filamentous appendages before type III-mediated secretion of virulence factors (34). In each case an extracellular appendage, albeit of different types, is required for attachment and, like the fibrils of *M. xanthus*, may transduce a ligand-specific signal to change the physiology of the pathogen. The unification of adhesion and signaling within an intercellular appendage may be the hallmark of a new and diverse class of bacterial extracellular signal transducers.

This work was supported by National Science Foundation Grants MCB9601077 and BIR9413235.

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