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Nitrate-Dependent Activation of the Dif Signaling Pathway of *Myxococcus xanthus* Mediated by a NarX-DifA Interspecies Chimera

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***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.**

Myxococcus xanthus is a gram-negative bacterium with a multicellular developmental process and distinct motility systems (12, 34). 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 (17, 28).

Fibril exopolysaccharides (EPS) (4, 7, 33, 40, 45) have been demonstrated to be crucial for S-motility. It was proposed that fibril EPS may mediate the retraction of type IV pili (26), the likely motor for S-motility (38). The regulation of fibril EPS clearly requires multiple genetic loci, including *tgl* (10), *stk* (10, 22), *sglK* (40, 42), *eps* and *eas* (27), *nla24* (24), and *dif* (4, 7, 45). 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 (7, 43). Deletion of most *dif* genes results in perturbation of EPS production as well as defects in S-motility and fruiting body formation (4, 7, 43, 45). 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 (2, 35). It is proposed that in the regulation of EPS production, DifA perceives signals and activates the downstream DifE kinase through the coupling protein DifC (4, 7, 44). A recent study showed that DifC can indeed mediate interactions between DifA and DifE to form a ternary signaling complex (44). 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 capable of direct ligand binding, as with classical bacterial chemoreceptors (43, 44).

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 (1, 3, 6, 13, 23, 37, 39). The structural basis behind the functionality of these chimeras is that transmembrane signaling mechanisms are well conserved among bacterial MCPs and sensor kinases (39). 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 (25). 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 the wild type, NafA restored fruiting body formation, EPS production, and S-motility to *difA* mutants in the presence

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of nitrate; without nitrate, NafA failed to complement *difA* deletions. Examination of *nafA* in *difA difC*, *difA difD*, *difA difE*, and *difA difG* double deletion backgrounds indicates that the NafA chimera signals through the Dif pathway in response to nitrate. The results suggest that the 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 (8). The functionality of NafA in *M. xanthus* also implies that DifA likely shares similar transmembrane signaling mechanisms with other bacterial chemoreceptors and sensor kinases (11). In addition, observations from this and previous studies (7, 10) suggest that overproduction of EPS may lead to defects in *M. xanthus* S-motility.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The plasmids and *M. xanthus* strains used in this study are listed in Table 1. *M. xanthus* was grown on Casitone-Tris (CTT) plates or in CTT liquid medium (20) at 32°C on a rotary shaker at 300 rpm. The exception was that Casitone-yeast extract (9) plates were used for strain maintenance. CF plates were used as the development-inducing medium for *M. xanthus* (15). The *Escherichia coli* strain XL1-Blue (Stratagene), used for routine cloning and plasmid construction, was grown on Luria-Bertani (LB) plates or LB liquid (29) at 37°C. 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 (14) and cloned into pZErO-2 (Invitrogen), which had been digested with BglI and DraIII and filled in with T4 DNA polymerase (New England BioLabs). pWB200 contains *ccdB* for lethal selection in *E. coli* and the Mx8 ATT for integration in *M. xanthus*. To construct pXQ723, the HindIII and XbaI sites of pACYC184 (30) were first removed by digestion with these enzymes, filled in with T4 DNA polymerase, and then religated to generate pXQ703. A 0.9-kb AflIII-DraIII fragment from pBluescript II SK(+) (Stratagene) was cloned into AvaI- and SacII-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 *lacZ*α 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 PCR (31) and cloned into SmaI of pBJ113 (19). This deletion construct removed the complete *difA* open reading frame (43) 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′-GGAATT CTGATGGCCTCGCTGGTGATG-3′ and 5′-GGAATTCACCTTCATGGGC GGCGCGTC-3′), digested with EcoRI, and cloned into the same site of pXQ723.

To construct pXQ713, a 1.1-kb *difA* C-terminal fragment was PCR amplified, and codon 96 (CGC) of *difA* was changed to CAT in this fragment to create an NdeI site (CATATG). This *difA* C-terminal fragment was cloned into the EcoRV site of pWB200 in the same orientation as the *E. coli lac* promoter to first generate pXQ706. A 2.0-kb EcoRI-NdeI fragment encoding the NarX N terminus and the upstream *tar* promoter from pAD56 (39) was cloned into the same sites of pXQ706 to create pXQ713. pXQ713 was digested with BamHI, filled in with T4 DNA polymerase, and then digested with HindIII; a 0.5-kb PCR fragment containing the *dif* promoter was digested with HindIII 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 (36). To construct *difA* deletion mutants, pWB116 was electroporated (21) into DK1622 (wild type), SW403 (*difC*) (4), YZ603 (*difE*), YZ613 (*difD*), and YZ604 (*difG*) (7) and selected by kanamycin resistance. Mutants of *difA* (YZ601), *difA difC* (YZ720), *difA difE* (YZ719), *difA difD* (YZ653), and *difA 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 (21) to produce *nafA*-carrying strains (Table 1). To construct *M. xanthus aglU* insertion mutants, pXQ730 was used to transform DK1622 (wild type), YZ601 (*difA*), and YZ724 (*difA*/*Pdif*) to generate YZ735 (*aglU*), YZ736 (*difA aglU*), and YZ738 (*difA aglU*/*Pdif*).

TABLE 1. *M. xanthus* strains and plasmids used in this study

Designation	Genotype or description	Source or reference
Strains		
DK1622	Wild type	20
SW403	*difC*	4
YZ601	*difA*	This study
YZ603	*difE*	7
YZ604	*difG*	7
YZ613	*difD*	7
YZ720	*difA difC*	This study
YZ653	*difA difD*	This study
YZ719	*difA difE*	This study
YZ654	*difA difG*	This study
YZ716	*difA*/P*tar-nafA*; Kan^r	This study
YZ724	*difA*/P*dif-nafA*; Kan^r	This study
YZ722	*difA difC*/P*tar-nafA*; Kan^r	This study
YZ659	*difA dif*D/P*tar-nafA*; Kan^r	This study
YZ721	*difA difE*/P*tar-nafA*; Kan^r	This study
YZ660	*difA difG*/P*tar-nafA*; Kan^r	This study
YZ730	*difA difC*/P*dif-nafA*; Kan^r	This study
YZ731	*difA dif*D/P*dif-nafA*; Kan^r	This study
YZ732	*difA difE*/P*dif-nafA*; Kan^r	This study
YZ733	*difA difG*/P*dif-nafA*; Kan^r	This study
YZ735	*aglU*; Tet^r	This study
YZ736	*difA aglU*; Tet^r	This study
YZ738	*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; *lacZ*α for screening; Kan^r	14
pWB200	Mx8 phage ATT site; *ccdB* for lethal selection; Kan^r	This study
pACYC184	Tet^r Cm^r	30
pXQ703	pACYC184 with HindIII and XbaI sites removed	This study
pBluescript II SK(+)	Cloning vector with *lacZ*α for screening; Amp^r	Stratagene
pXQ723	*lacZ*α for screening; Tet^r	This study
pBJ113	Gene replacement vector with KG cassette; Kan^r	19
pWB116	*difA* in-frame deletion in pBJ113	This study
pAD56	P*tar-nart*; Amp^r	39
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

Examination of NafA expression. *M. xanthus* was cultured in CTT liquid overnight with or without KNO_3 to approximately 1.0×10^8 to 1.5×10^8 cells/ml. About 2.5×10^8 cells were harvested, washed with cold 50 mM Tris-HCl (pH 7.4), and resuspended in 50 μl of loading buffer (2% sodium dodecyl sulfate, 5% mercaptoethanol, 8.5% glycerol) (31). Ten microliters of these samples, after being boiled for 5 min, was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% acrylamide gel. Immunoblotting was performed as described elsewhere (31) using antibody against cytoplasmic domains of DifA (24; Z. Yang, unpublished data).

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

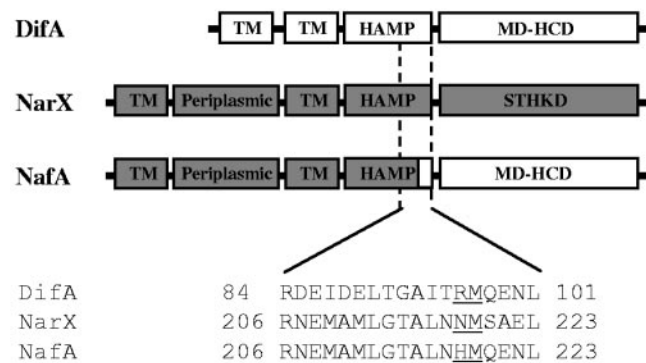

FIG. 1. Construction of NarX-DifA (NafA) chimera. TM, transmembrane domain; HAMP, HAMP linker region; STHKD, signal transduction histidine kinase domain; MD-HCD, methylation and highly conserved (signaling) domains. The diagram is not drawn to scale. The amino acid sequences at the bottom are from the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

mM MOPS, 2 mM $MgSO_4$, pH 7.6) at approximately 5×10^9 cells/ml. Five-microliter aliquots 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 32°C.

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) (10) and resuspended to approximately 2.5×10^8 cells/ml in agglutination buffer with 0, 5, 35, or 100 μM nitrate. Optical density at 600 nm (OD_{600}) was recorded every 30 min for 2 h and was normalized against the initial OD reading. Calcofluor white binding (7, 10) was used to evaluate EPS production. Cells from liquid cultures were washed and resuspended in MOPS buffer at appropriate cell densities. Five-microliter aliquots 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 32°C before they were examined and documented using a Nikon COOLPIX 4500 digital camera under the illumination of UV light (365 nm).

For analysis of S-motility on soft agar, cells from liquid cultures were washed and resuspended in MOPS buffer at approximately 1×10^{10} cells/ml. Five-microliter aliquots 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 32°C. 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 KNO_3. After 2 days of incubation at 32°C, colony edges were photographed using phase-contrast microscopy.

RESULTS

Construction and expression of NarX-DifA (NafA) chimera in *M. xanthus*. 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 (43, 44) (Fig. 1). 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. 1). An NdeI site was introduced into *narX* by replacing alanine 218 with a histidine (39), 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 1). These two constructs were transformed into YZ601, which contains a new and more complete deletion of *difA*. The resulting strains YZ716 (*difA*/P*tar*) and YZ724 (*difA*/P*dif*) (Table 1) were used throughout this study. The expression of NafA was examined by immunoblotting using polyclonal antibodies against the C terminus of DifA (24; Yang, unpublished) 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 of nitrate on *M. xanthus* strains containing *nafA* should not be attributed to any regulation of NafA expression by nitrate.

The DifA N terminus is likely essential for its function. *M. xanthus difA* mutants are known to be defective in formation of fruiting bodies (43, 45). The newly constructed *difA* mutant (YZ601) showed no development under starvation conditions that prompted normal development of the wild type (DK1622) (Fig. 2A, first row). YZ716 and YZ724 also exhibited no development under these conditions (Fig. 2A, 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. 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 (8). 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 (39). Although nitrite and nitrate both are sensed by NarX and Nart (25, 39), 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 (25).

Nitrate clearly influenced the development of the two strains harboring the *nafA* constructs (Fig. 2A). Both YZ716 (*difA*/P*tar*) and YZ724 (*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 compared to the wild type. They appeared to be less compact and irregular in shape, with more cells remaining outside of the aggregates.

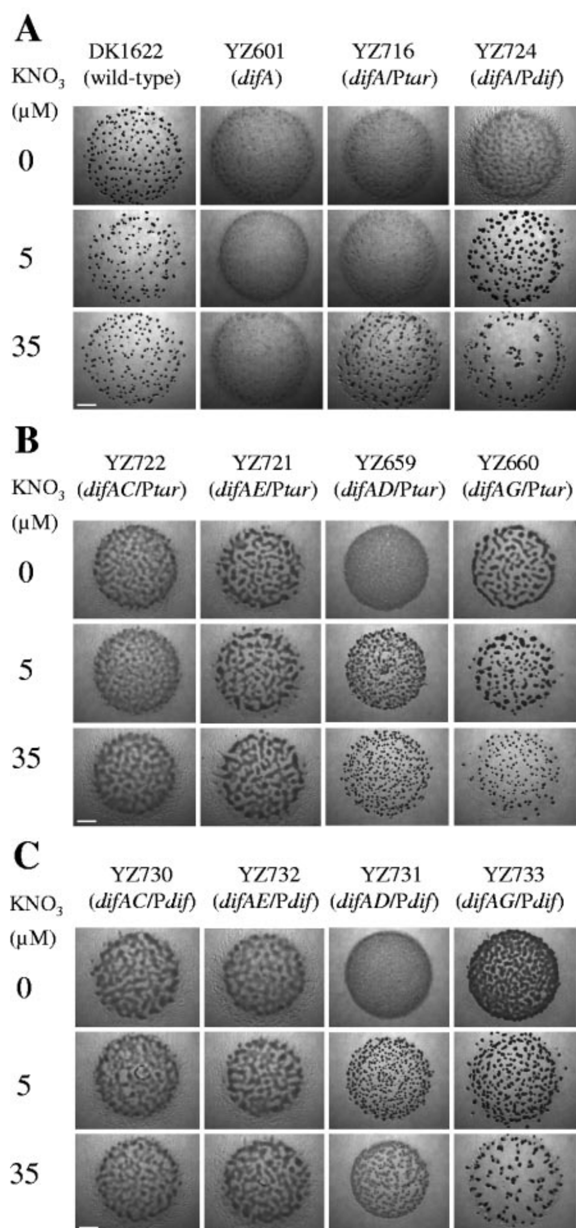

FIG. 2. Fruiting body formation on CF plates supplemented with KNO_3. A. Wild type, *difA* mutant, and two *nafA*-carrying strains in *difA* background. B. Mutants with the P*tar* construct (pXQ713) in double deletion backgrounds of *difA difC*, *difA difE*, *difA difD*, and *difA difG*. C. Mutants with the P*dif* construct (pXQ719) in double deletion backgrounds of *difA difC*, *difA difE*, *difA difD*, and *difA difG*. Five-microliter aliquots of cell suspension (approximately 5×10^9 cells/ml) in MOPS buffer were spotted onto CF plates with KNO_3 at the indicated concentrations. Pictures were taken after incubation at 32°C 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 control of the *E. coli tar* promoter and *M. xanthus dif* promoter, respectively.

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 Fig. 2A indicate that nitrate concentration affects fruiting body morphology formed by strain YZ724. At 5 μM nitrate the fruiting bodies 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 (also see later sections) 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 (7, 10).

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 (4, 7, 43, 44, 45). DifD and DifG, which are negative regulators of the Dif pathway (7), 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 *difA difC*, *difA difD*, *difA difE*, and *difA difG* double deletion strains.

As shown in Fig. 2B and C, nitrate did not restore development in the *difA difC* or *difA difE* strains in the presence of *nafA*. In contrast, both P*tar* and P*dif* constructs supported developmental aggregation of *difA difD* and *difA difG* mutants in the presence of nitrate. Although 5 μM nitrate and the P*tar* construct together did not restore developmental aggregation to the *difA* mutant (YZ716) (Fig. 2A), they did result in some aggregation of the *difA difD* (YZ659) and the *difA difG* (YZ660) double mutants (Fig. 2B). The *difA difD* double mutant containing the P*dif* construct (YZ731) (Fig. 2C) showed severe defects in aggregation at 35 μM nitrate, and the *difA difG* double mutant with the construct (YZ733) (Fig. 2C) formed fewer and more irregularly shaped aggregates at 35 μM than at 5 μM nitrate (Fig. 2C). The results agree with those of Black and Yang (7) in showing that deletion of *difD* or *difG* does not eliminate development but rather alters the appearance of fruiting bodies and that mutations in *difD* result in more severe defects than those in *difG*. 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 (7, 44). In addition, the results here suggest that neither DifD nor DifG functions downstream of DifA, because the *difA difD* and the *difA difG* double mutants showed similar developmental phenotypes as the *difA* single mutant under all conditions (Fig. 2B and 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 (4, 7, 43, 45). An apparent explanation for the nitrate-induced fruiting body formation by *nafA*-containing strains was the restoration of EPS production. Since *M. xan-*

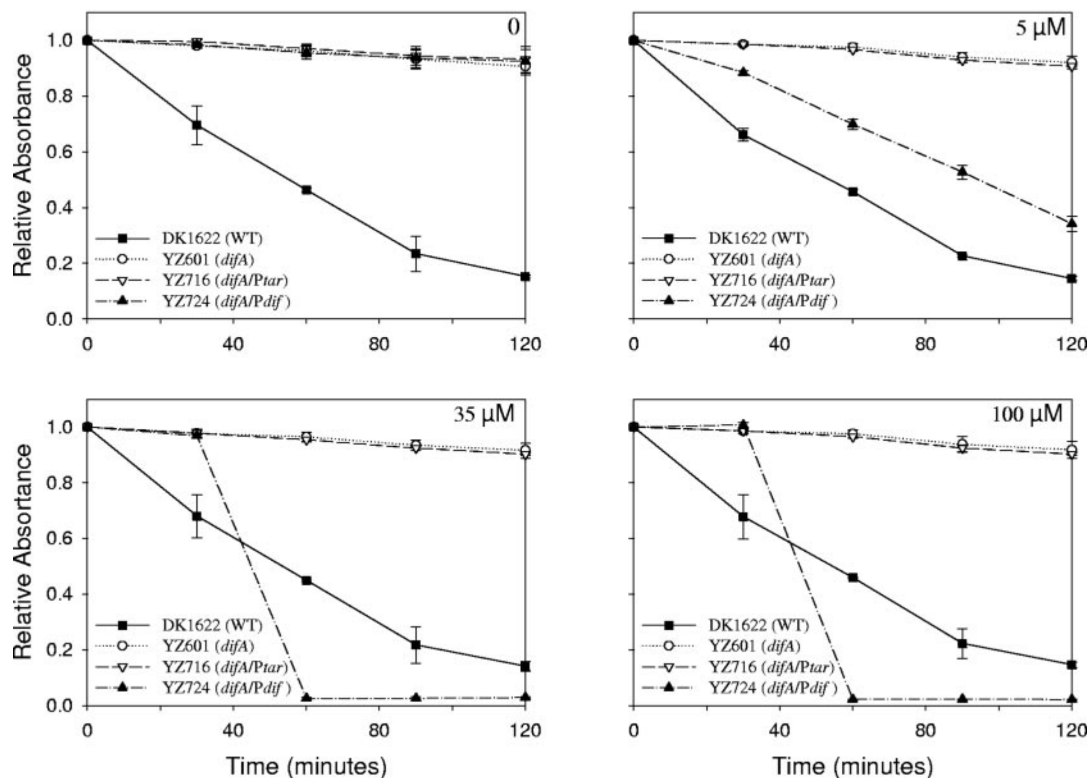

FIG. 3. Agglutination assay. Cells grown overnight in CTT were washed and resuspended to approximately 2.5×10^8 cells/ml in agglutination buffer with KNO_3 at concentrations indicated at the upper right of each panel. The OD was measured every 30 min for 2 h. Relative absorbance was obtained by dividing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

thus cellular cohesion requires EPS (7, 33, 45), agglutination assays were performed to examine whether nitrate could induce cellular cohesion (Fig. 3). Cells of strain DK1622 agglutinated similarly under all conditions tested as indicated by decreasing 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 YZ724 (*difA*/P*dif*) showed agglutination patterns similar to those of YZ601 in the absence of nitrate, it agglutinated at all three nitrate concentrations examined (5, 35, and 100 μM) (Fig. 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 (*difA*/P*tar*) did not agglutinate at all nitrate concentrations, 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) and agglutination (Fig. 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 Materials and Methods after 7 days of incubation (Fig. 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 clear fluorescent signals at 35 and 100 μM nitrate although not at 5 μM. 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

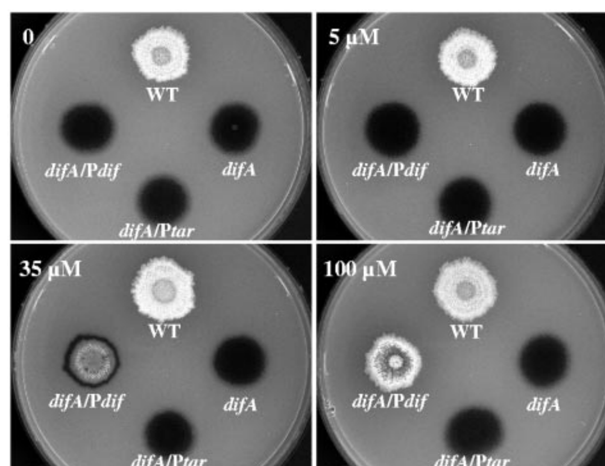

FIG. 4. EPS production under different nitrate concentrations. Five-microliter aliquots of cells at $\sim 5 \times 10^7$ cells/ml in MOPS buffer were spotted onto CTT plates containing 50 μg/ml of calcofluor white and KNO_3 at different concentrations as indicated in the upper left of each picture. After incubation at 32°C for 7 days, the plates were photographed right side up without the lid under the illumination of UV light (365 nm). The diameter of the plates shown is 9 cm. WT, DK1622; *difA*, YZ601; *difA*/P*tar*, YZ716; *difA*/P*dif*, YZ724.

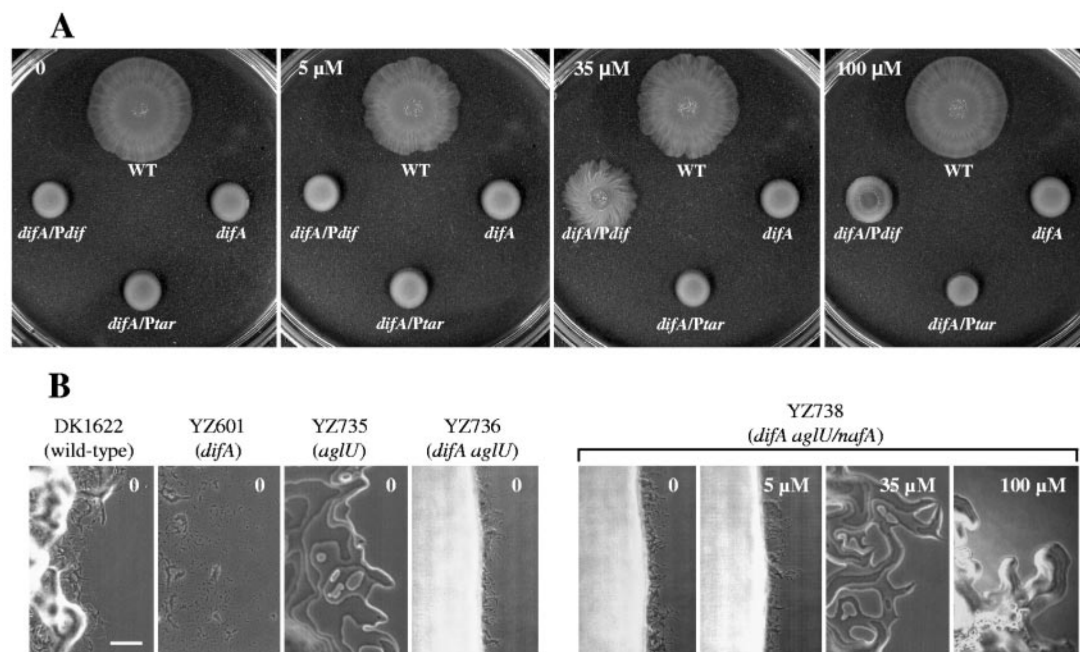

FIG. 5. Examination of S-motility using soft (0.4% agar) (A) and hard (1.5% agar) (B) CTT plates. A. Five-microliter aliquots of cells at ~1 × 10^{10} cells/ml were spotted onto soft CTT plates containing KNO_3 at concentrations as indicated at the upper left of each picture. Plates were photographed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

restoration of S-motility to *difA* mutants expressing *nafA*. S-motility was examined semiquantitatively using swarming assays on soft CTT plates (0.4% agar). As shown in Fig. 5A, DK1622 (wild type) spread considerably and produced rough flares at the colony edge in the presence and absence of nitrate. YZ601 (*difA*) and YZ716 (*difA*/P*tar*) colonies expanded insignificantly and had a glossy appearance and smooth edges under all conditions examined. At 0 and 5 μM nitrate, YZ724 (*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. 5A), 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 (16, 17), an A-motility mutation *aglU* (46) was introduced into strain YZ724 (see Materials and Methods) and the motility of the resultant strain was compared with that of parental strains using hard CTT plates (1.5% agar). Figure 5B shows that DK1622 (wild type) moved both as large cell groups (S-motility) and well-isolated cells (A-motility). Strain YZ601 (*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 (*difA aglU*), which lacks genes essential for both motility systems, displayed only residual motility as reported previously (4, 41). 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. 5B). 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. 5A) 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. 5A) 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 Fig. 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 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 Fig. 6 shows the fluorescence at the sixth day (132 h, bottom) when the plate was viewed upside down with UV illumination.

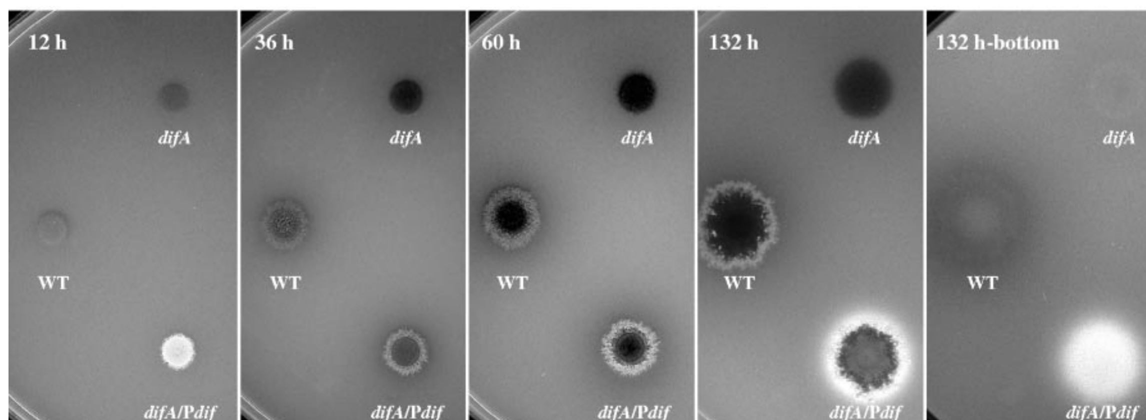

FIG. 6. EPS production at different time points upon exposure to 100 μM nitrate. Cells grown without nitrate were washed and resuspended at $\sim 1 \times 10^{10}$ cells/ml in MOPS buffer. Five-microliter aliquots of the cell suspension were spotted onto CTT plates containing 50 μg/ml of calcofluor white and 100 μM KNO_3. After incubation at 32°C for the indicated times (hours), the plates were photographed as in Fig. 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; *difA*, YZ601; *difA*/P*dif*, YZ724.

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. 5A), possibly suggesting a cause-and-effect relationship. It should be noted that there are differences between the experiments in Fig. 4 and 6: cell suspension at $\sim 5 \times 10^7$ cells/ml was used as inoculum for Fig. 4, and $\sim 1 \times 10^{10}$ cells/ml was used for Fig. 6, because sufficient numbers of cells had to be present for the detection of fluorescence for the experiments in Fig. 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 (35). 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 *difA difD* double mutant was complemented by the *nafA* plasmids similarly as the *difA* mutant (Fig. 2B 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 (44) as MCPs, CheW and CheA, in bacterial chemotaxis systems, DifD is therefore unlikely downstream of DifE in the regulation of EPS in *M. xanthus* (7).

Most prokaryotic chemoreceptors have the same general transmembrane topology as the classical *E. coli* MCPs (Tar, Tsr, Trg, and Tap) (47). 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 (6, 18). The *E. coli* aerotaxis receptor Aer detects the redox state of the cell through a flavin adenine dinucleotide that binds to the N terminus of the transducer (5, 6). *H. salinarium* HtrI senses light through interactions of its N-terminal transmembrane domain with its cognate sensory rhodopsin, SRI (18). 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. 2A) and higher nitrate concentrations (data not shown) display obvious defects. Similarly, S-motility during vegetative swarming by this strain on soft agar is very sensitive to nitrate concentration (Fig. 5A). 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. 6). We argue that growth defects are unlikely because the expansion of YZ724 colonies on hard agar, which depends more heavily on A-motility (32), is not appreciably diminished by 100 μM nitrate (Fig. 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 (7, 10). 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-expressing strain requires higher concentrations of nitrate than the restoration of development. Development can be partially restored to strain YZ716 (*difA*/P*tar*) at 35 μM nitrate (Fig. 2A), but S-motility and detectable EPS production could not be restored even at 100 μM for this strain (Fig. 4 and 5A). Similarly, YZ724 (*difA*/P*dif*) requires 35 μM nitrate to restore detectable EPS production and S-motility (Fig. 4 and 5A) but only 5 μM to restore development (Fig. 2A). One possible explanation is that S-motility requires a higher level of EPS production than development. Alternatively, because EPS production was measured only under vegetative conditions in this study (Fig. 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*.

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REFERENCES

1. **Appleman, J. A., L.-L. Chen, and V. Stewart.** 2003. Probing conservation of HAMP linker structure and signal transduction mechanism through analysis of hybrid sensor kinases. J. Bacteriol. **185:**4872–4882.
2. **Armitage, J. P., I. B. Holland, U. Jenal, and B. Kenny.** 2005. "Neural networks" in bacteria: making connections. J. Bacteriol. **187:**26–36.
3. **Baumgartner, J. W., C. Kim, R. E. Brissette, M. Inouye, C. Park, and G. L. Hazelbauer.** 1994. Transmembrane signalling by a hybrid protein: communication from the domain of chemoreceptor Trg that recognizes sugar-binding proteins to the kinase/phosphatase domain of osmosensor EnvZ. J. Bacteriol. **176:**1157–1163.
4. **Bellenger, K., X. Ma, W. Shi, and Z. Yang.** 2002. A CheW homologue is required for *Myxococcus xanthus* fruiting body development, social gliding motility, and fibril biogenesis. J. Bacteriol. **184:**5654–5660.
5. **Bibikov, S. I., L. A. Barnes, Y. Gitin, and J. S. Parkinson.** 2000. Domain organization and flavin adenine dinucleotide-binding determinants in the aerotaxis signal transducer Aer of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **97:**5830–5835.
6. **Bibikov, S. I., A. C. Miller, K. K. Gosink, and J. S. Parkinson.** 2004. Methylation-independent aerotaxis mediated by the *Escherichia coli* Aer protein. J. Bacteriol. **186:**3730–3737.
7. **Black, W. P., and Z. Yang.** 2004. *Myxococcus xanthus* chemotaxis homologs DifD and DifG negatively regulate fibril polysaccharide production. J. Bacteriol. **186:**1001–1008.
8. **Bustamante, V. H., I. Martinez-Flores, H. C. Vlamakis, and D. R. Zusman.** 2004. Analysis of the Frz signal transduction system of *Myxococcus xanthus* shows the importance of the conserved C-terminal region of the cytoplasmic chemoreceptor FrzCD in sensing signals. Mol. Microbiol. **53:**1501–1513.
9. **Campos, J. M., and D. R. Zusman.** 1975. Regulation of development in *Myxococcus xanthus*: effect of 3′:5′-cyclic AMP, ADP, and nutrition. Proc. Natl. Acad. Sci. USA **72:**518–522.
10. **Dana, J. R., and L. J. Shimkets.** 1993. Regulation of cohesion-dependent cell interactions in *Myxococcus xanthus*. J. Bacteriol. **175:**3636–3647.
11. **Draheim, R. R., A. F. Bormans, R. Z. Lai, and M. D. Manson.** 2005. Tryptophan residues flanking the second transmembrane helix (TM2) set the signaling state of the Tar chemoreceptor. Biochemistry **44:**1268–1277.
12. **Dworkin, M.** 1996. Recent advances in the social and developmental biology of the myxobacteria. Microbiol. Rev. **60:**70–102.
13. **Feng, X., J. Baumgartner, and G. Hazelbauer.** 1997. High- and low-abundance chemoreceptors in *Escherichia coli*: differential activities associated with closely related cytoplasmic domains. J. Bacteriol. **179:**6714–6720.
14. **Guo, D., M. G. Bowden, R. Pershad, and H. B. Kaplan.** 1996. The *Myxococcus xanthus rfbABC* operon encodes an ATP-binding cassette transporter homolog required for O-antigen biosynthesis and multicellular development. J. Bacteriol. **178:**1631–1639.
15. **Hagen, D. C., A. P. Bretscher, and D. Kaiser.** 1978. Synergism between morphogenetic mutants of *Myxococcus xanthus*. Dev. Biol. **64:**284–296.
16. **Hodgkin, J., and D. Kaiser.** 1979. Genetics of gliding motility in *Myxococcus xanthus* (Myxobacterales): genes controlling movement of single cells. Mol. Gen. Genet. **171:**167–176.
17. **Hodgkin, J., and D. Kaiser.** 1979. Genetics of gliding motility in *Myxococcus xanthus*: two gene systems control movement. Mol. Gen. Genet. **171:**177–191.
18. **Hoff, W. D., K.-H. Jung, and J. L. Spudich.** 1997. Molecular mechanism of photosignaling by archaeal sensory rhodopsins. Annu. Rev. Biophys. Biomol. Struct. **26:**223–258.
19. **Julien, B., A. D. Kaiser, and A. Garza.** 2000. Spatial control of cell differentiation in *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **97:**9098–9103.
20. **Kaiser, D.** 1979. Social gliding is correlated with the presence of pili in *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **76:**5952–5956.
21. **Kashefi, K., and P. L. Hartzell.** 1995. Genetic suppression and phenotypic masking of a *Myxococcus xanthus frzF-* defect. Mol. Microbiol. **15:**483–494.
22. **Kim, S. H., S. Ramaswamy, and J. Downard.** 1999. Regulated exopolysaccharide production in *Myxococcus xanthus*. J. Bacteriol. **181:**1496–1507.
23. **Krikos, A., M. P. Conley, A. Boyd, H. C. Berg, and M. I. Simon.** 1985. Chimeric chemosensory transducers of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **82:**1326–1330.
24. **Lancero, H., N. B. Caberoy, S. Castaneda, Y. Li, A. Lu, D. Dutton, X.-Y. Duan, H. B. Kaplan, W. Shi, and A. G. Garza.** 2004. Characterization of a *Myxococcus xanthus* mutant that is defective for adventurous motility and social motility. Microbiology **150:**4085–4093.
25. **Lee, A. I., A. Delgado, and R. P. Gunsalus.** 1999. Signal-dependent phosphorylation of the membrane-bound NarX two-component sensor-transmitter protein of *Escherichia coli*: nitrate elicits a superior anion ligand response compared to nitrite. J. Bacteriol. **181:**5309–5316.
26. **Li, Y., H. Sun, X. Ma, A. Lu, R. Lux, D. Zusman, and W. Shi.** 2003. Extracellular polysaccharides mediate pilus retraction during social motility of *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **100:**5443–5448.
27. **Lu, A., K. Cho, W. P. Black, X. Y. Duan, R. Lux, Z. Yang, H. B. Kaplan, D. R. Zusman, and W. Shi.** 2005. Exopolysaccharide biosynthesis genes required for social motility in *Myxococcus xanthus*. Mol. Microbiol. **55:**206–220.
28. **MacNeil, S. D., A. Mouzeyan, and P. L. Hartzell.** 1994. Genes required for both gliding motility and development in *Myxococcus xanthus*. Mol. Microbiol. **14:**785–795.
29. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Rose, R. E.** 1988. The nucleotide sequence of pACYC184. Nucleic Acids Res. **16:**355.
31. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
32. **Shi, W., and D. R. Zusman.** 1993. The two motility systems of *Myxococcus xanthus* show different selective advantages on various surfaces. Proc. Natl. Acad. Sci. USA **90:**3378–3382.
33. **Shimkets, L. J.** 1986. Correlation of energy-dependent cell cohesion with social motility in *Myxococcus xanthus*. J. Bacteriol. **166:**837–841.
34. **Shimkets, L. J.** 1999. Intercellular signaling during fruiting-body development of *Myxococcus xanthus*. Annu. Rev. Microbiol. **53:**525–549.
35. **Szurmant, H., and G. W. Ordal.** 2004. Diversity in chemotaxis mechanisms among the bacteria and archaea. Microbiol. Mol. Biol. Rev. **68:**301–319.
36. **Ueki, T., S. Inouye, and M. Inouye.** 1996. Positive-negative KG cassettes for construction of multi-gene deletions using a single drug marker. Gene **183:**153–157.
37. **Utsumi, R., R. E. Brissette, A. Rampersaud, S. A. Forst, K. Oosawa, and M. Inouye.** 1989. Activation of bacterial porin gene expression by a chimeric signal transducer in response to aspartate. Science **245:**1246–1249.
38. **Wall, D., and D. Kaiser.** 1999. Type IV pili and cell motility. Mol. Microbiol. **32:**1–10.
39. **Ward, S. M., A. Delgado, R. P. Gunsalus, and M. D. Manson.** 2002. A NarX-Tar chimera mediates repellent chemotaxis to nitrate and nitrite. Mol. Microbiol. **44:**709–719.
40. **Weimer, R. M., C. Creighton, A. Stassinopoulos, P. Youderian, and P. L. Hartzell.** 1998. A chaperone in the HSP70 family controls production of extracellular fibrils in *Myxococcus xanthus*. J. Bacteriol. **180:**5357–5368.
41. **Wu, S. S., J. Wu, and D. Kaiser.** 1997. The *Myxococcus xanthus pilT* locus is required for social gliding motility although pili are still produced. Mol. Microbiol. **23:**109–121.
42. **Yang, Z., Y. Geng, and W. Shi.** 1998. A DnaK homolog in *Myxococcus*

xanthus is involved in social motility and fruiting body formation. J. Bacteriol. **180:**218–224.

43. **Yang, Z., Y. Geng, D. Xu, H. B. Kaplan, and W. Shi.** 1998. A new set of chemotaxis homologues is essential for *Myxococcus xanthus* social motility. Mol. Microbiol. **30:**1123–1130.
44. **Yang, Z., and Z. Li.** 2005. Demonstration of interactions among *Myxococcus xanthus* Dif chemotaxis-like proteins by the yeast two-hybrid system. Arch. Microbiol. **183:**243–252.
45. **Yang, Z., X. Ma, L. Tong, H. B. Kaplan, L. J. Shimkets, and W. Shi.** 2000. *Myxococcus xanthus dif* genes are required for biogenesis of cell surface fibrils essential for social gliding motility. J. Bacteriol. **182:**5793–5798.
46. **Youderian, P., N. Burke, D. J. White, and P. L. Hartzell.** 2003. Identification of genes required for adventurous gliding motility in *Myxococcus xanthus* with the transposable element *mariner*. Mol. Microbiol. **49:**555–570.
47. **Zhulin, I. B.** 2001. The superfamily of chemotaxis transducers: from physiology to genomics and back. Adv. Microb. Physiol. **45:**157–198.