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A new set of chemotaxis homologues is essential for *Myxococcus xanthus* social motility

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Summary

Myxococcus xanthus **cells aggregate and develop into multicellular fruiting bodies in response to starvation. A new *M. xanthus* locus, designated *dif* for defective in fruiting, was identified by the characterization of a mutant defective in fruiting body formation. Molecular cloning, DNA sequencing and sequence analysis indicate that the *dif* locus encodes a new set of chemotaxis homologues of the bacterial chemotaxis proteins MCPs (methyl-accepting chemotaxis proteins), CheW, CheY and CheA. The *dif* genes are distinct genetically and functionally from the previously identified *M. xanthus frz* chemotaxis genes, suggesting that multiple chemotaxis-like systems are required for the developmental process of *M. xanthus* fruiting body formation. Genetic analysis and phenotypical characterization indicate that the *M. xanthus dif* locus is required for social (S) motility. This is the first report of a *M. xanthus* chemotaxis-like signal transduction pathway that could regulate or co-ordinate the movement of *M. xanthus* cells to bring about S motility.**

Introduction

Co-ordinated cell migration and cell–cell interactions are fundamental to developmental biology. As a Gram-negative gliding bacterium, *Myxococcus xanthus* provides one of the most genetically accessible systems for studies of these intercellular interactions. Under nutrient-limiting conditions, *M. xanthus* cells undergo a multicellular developmental process involving both co-ordinated cell movement, termed social motility, and complex intercellular interactions (Dworkin and Kaiser, 1993). During this well-orchestrated developmental process, hundreds of thousands of *M. xanthus* cells aggregate to form a multicellular fruiting body. Rod-shaped vegetative cells eventually differentiate into spherical, stress-resistant myxospores within mature fruiting bodies. Once conditions become favourable for growth, myxospores can germinate and initiate vegetative growth.

M. xanthus cells move by gliding, a form of translocation on solid surfaces in which rod-shaped bacteria move smoothly in the direction of their long axes without the aid of flagella (Burchard, 1981; Pate, 1985; Reichenbach *et al*., 1986). Two distinct systems, the A (adventurous) and the S (social) motility systems, govern *M. xanthus* gliding motility (Hodgkin and Kaiser, 1979a, b). Mutations in A- or S-motility genes inactivate the corresponding systems, but cells are still motile by virtue of the remaining system. A-motile (A^+S^-) cells can move as well-isolated cells or small groups, whereas S-motile (A^-S^+) cells require cell proximity to move and can move only as cell groups. A^-S^- *M. xanthus* cells show no or very little net movement and, consequently, fail to aggregate and to form fruiting bodies under developmental conditions (Hodgkin and Kaiser, 1979b). Mounting evidence indicates that S motility is important, if not essential, for *M. xanthus* development, because the majority of S-motility mutants and all of the well-characterized ones are defective in fruiting body formation (Hodgkin and Kaiser, 1979b; Dana and Shimkets, 1993; Hartzell and Youderian, 1995; Wu and Kaiser, 1995; Wu *et al*., 1997; Yang *et al*., 1998).

Many genetic loci that are required for S motility have been identified and characterized in detail. Among these, the *pil* genes and the *tgl* gene are associated with the production and/or function of *M. xanthus* type IV pili, which are known to be required for S motility (Kaiser, 1979; Wu and Kaiser, 1995; Rodriguez-Soto and Kaiser, 1997; Wu *et al*., 1997; Wall and Kaiser, 1998). A wild-type *dsp* locus is necessary for S motility, cell cohesion and development. The *dsp* mutants are defective in the production of extracellular fibrillar material (Shimkets, 1986; Arnold and Shimkets, 1988; Behmlander and Dworkin, 1991). The defective cohesion and development of *dsp* mutants can be rescued by the addition of fibrillar material isolated from wild-type cells (Chang and Dworkin, 1994). The *sglK* gene encodes an *M. xanthus* DnaK homologue required for S motility (MacNeil *et al*., 1994a, b; Yang *et al*., 1998). Unlike the *dnaK* genes from other bacteria, the *M. xanthus* homologue is not temperature and heat shock regulated.

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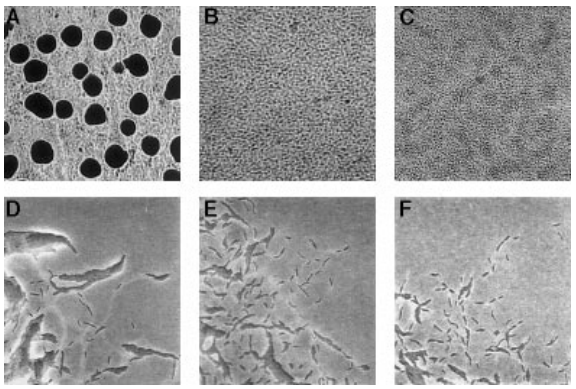

Fig. 1. Developmental and colony edge morphologies of the *dif* mutants. Fruiting body development (top) was examined and photographed under light microscopy with a 4× object lens as described in *Experimental procedures*. The edges of *M. xanthus* colonies (bottom) were photographed under phase-contrast microscopy with a 40× object lens.
A and D. DK1622 (wt).
B and E. SW505 (*difA*).
C and F. SW501 (*difE*).

All of the *M. xanthus* lipopolysaccharide O-antigen mutants tested are defective in S motility (Bowden and Kaplan, 1998). It is not yet clear what specific functions each of these gene products serve and how they interact to bring about the intercellular co-ordination required for S motility.

The cellular reversal of *M. xanthus* gliding is regulated by a set of *M. xanthus* chemotaxis homologues encoded by the *frz* genes (Shi and Zusman, 1995; Ward and Zusman, 1997). *frz* mutant cells either seldom reverse their gliding direction or show hyper-reversal (Blackhart and Zusman, 1985). They fail to respond to certain chemotactic stimuli (Shi and Zusman, 1994) and form characteristic frizzy filaments under starvation conditions. Based on the structural homology and functional analysis, Frz proteins are believed to function as the chemotaxis proteins in *M. xanthus* (Shi and Zusman, 1995; Ward and Zusman, 1997).

In this paper, we report the identification and characterization of an additional locus important for *M. xanthus* S motility. This locus, *dif* for defective in fruiting, was identified in a genetic screen for mutants defective in fruiting body formation. Molecular cloning, DNA sequencing and sequence analysis indicate that the *dif* locus encodes proteins homologous to the bacterial chemotaxis proteins, the MCPs (methyl-accepting chemotaxis proteins), CheW, CheY and CheA. The *dif* genes are genetically and functionally distinct from the previously known *frz* chemotaxis genes. Mutational analysis and behavioural studies indicate that the *dif* genes are required for *M. xanthus* S motility. We propose that the Dif proteins constitute a signal transduction pathway, which functions to co-ordinate the movement of *M. xanthus* cells necessary for S motility.

Results

Isolation of the dif *mutant by transposon mutagenesis*

To identify additional genes necessary for *M. xanthus* development, Tn*5* transposon mutants were screened for fruiting body formation deficiencies (Yang *et al*., 1998). One mutant, SW101, containing a Tn*5*kan903Ω101 insertion, was found to be defective in developmental aggregation. A 100% linkage of the SW101 aggregation defect and the Tn*5* insertion was demonstrated by Mx4-mediated transduction, in which the donor was SW101 and the recipient was the parent strain DZF1. In addition, using the wild-type strain DK1622 as the recipient, the mutant strain SW505 was generated, and its ability to form fruiting bodies was examined. After 2 days of incubation, DK1622 formed visible fruiting bodies on MOPS plates (Fig. 1). In contrast, SW505, similar to SW101, showed little or no aggregation under the same conditions even after prolonged incubation (Fig. 1).

Homology between the dif *ORFs and bacterial chemotaxis proteins*

To identify the locus disrupted by the Tn*5* insertion, the Kan^r marker within Tn*5*kan903 was used to clone an 11.3 kb *Eco*RI fragment containing the inserted Tn*5* and the flanking regions as described in *Experimental procedures*. Analysis of the DNA sequence of the cloned fragment indicated that the locus, designated *dif* for defective in fruiting, encodes five potential open reading frames (ORFs) designated DifA, DifB, DifC, DifD and DifE (Fig. 2). The Tn*5*kan903 insertion in SW101 occurred at the 3′ end of the *difA* gene as indicated (Fig. 2). These sequence data reported in this paper have been submitted to the GenBank database under accession number AF076485.

Four of the ORFs, DifA, DifC, DifD and DifE, show significant identity to the bacterial chemoreceptors (MCPs), CheW, CheY and CheA proteins respectively (Stock and Surette, 1996). The putative DifA protein possesses all the typical structural features of MCPs: two transmembrane

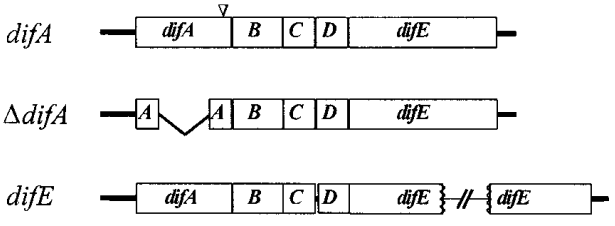

Fig. 2. Structure of the *dif* locus and different *dif* mutant alleles. The open reading frames are labelled. DifA shows homology to MCPs, DifC to CheW, DifD to CheY and DifE to CheA. The *difA* mutants SW101 and SW505 contain a Tn*5*Kan903 insertion at the C-terminus of the *difA* gene as indicated by the inverted triangle (▽); Δ*difA*, an in frame deletion of the *difA* gene; *difE*, an insertion in the *difE* genes. See text for details.

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TlpB    SSGDLTEVI--DI HSK-NEFGQLGESFN EMSASLRSVIGVIQT SVENVASSSEELTAS AAQTSKAIEHITLAI EQFSDGNEAQSEKLE   404
DifA    SQGDLSKPVAAEG GSKRDEIDELTGAIT RMQENLRELVGKIQE TAKSVADTAIDLQRS AENVNGSTEEVGSSM EKIAGGAESQSQLVS   177
FrzCD   REGDLSRW---NT TTEDPQLGPLLEGFG KVIETLRTFVREINE AALRLSSSANQVLAA STQHETSSTEQAAAI HETTATME----ELK   184

TlpB    TSSNHLSQMNEGISK VAQASSTITKSSIQS SEAAGSGEKLVEHTV GQMKTIDQSVQKAEA VVKGLETKSQDITSI LNVINGIADQTNLLA  496
DifA    KASKVITEMAGSIQR TTASAEDAARTTAET SSAAEDGSKAARLAG DKVKKVFNRIESASQ QVFAFGEKTQEISKI VDAITQVAQQTNLLA  267
FrzCD   HASAQIAENAGSVAR -------VAEETLGA ARAGRGAIGEFIQAM QQIRSDGVAVADS-- -IAKLSKRVERIGTV VEVIDEIADRSDLLA  264

TlpB    LNAAIEAARAGEYGR GFSVVAEEVRKLAVQ SADSAKEIEGLIQEI VREIST----SLSMF QSVNHEVKEGLQITD QTAESFKQIYEMTTQ  580
DifA    LNATIEAARAGEYGR GFAVVADEVRKLAES AGRSAEQ----ISKL ARDISGQ---STSVV SAMKEGIAELAEGRE DLTNIVRSMGAITDT  350
FrzCD   LNAALEGSRAGEAGK GFSIVAAEMRRLAEN VLDSTKE----IKNL ITEIREATAAAAGAA EASKSATESGEKLGA VAAQAVEGILAGVQE  350

TlpB    I--SGELQNL--NAT VEQLSAGSQEVSSAV EDISAVAKESSAGIQ DIAASAEEQLASMEE ISSSAETLANMAEEL QDITKKFKIES    662
DifA    IRKGSEKVHLISESA REQLK-GSEEMVTAI EEIKLVARNNASSTE AIQAVIQEQTAAVSR MTSLASELTNLSVEL QSVVRSFRLGP    435
FrzCD   TSDAARVINL---AT QQQRT-ATEQVVASM AEIEDVTR------- ------QTTQAS-KQ ATGAAAELTQLAGRL AELIKRFKAD-    417
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Fig. 3. Alignment of the predicted DifA cytoplasmic domain with *B. subtilis* TlpB and *M. xanthus* FrzCD. Identical residues are indicated by shading. The sequences between the two arrows (↓) constitute the signalling domain. The flanking sequences constitute the two methylation helices. The DifA cytoplasmic domain is 33% identical to TlpB and 23% identical to FrzCD.

domains, the linker region, the methylation helices and the signalling domain (Figs 3 and 4). The predicted DifA is most strongly identical to *Bacillus subtilis* TlpB (33% over the entire predicted cytoplasmic domains; Fig. 3) (Hanlon and Ordal, 1994) and shows homology to FrzCD, the only *M. xanthus* MCP in the databases (23% identity over the methylation helices and the signalling domain; Fig. 3) (McBride *et al*., 1989). Although the exact function of *B. subtilis* TlpB is not known, *tlpB* mutant cells show an unusual tendency to adhere together and resist disaggregation (Hanlon and Ordal, 1994). The putative DifA appears to have a very short periplasmic span of 10 amino acids between the two transmembrane regions in the N-terminal receptor domain based on hydrophobicity analysis (Fig. 4). The *difE* gene is predicted to encode a polypeptide of 770 amino acids with homology to the CheA histidine protein kinases from various bacteria. It is most identical in the conserved regions in the N-terminus and the C-terminus to *Thermotoga maritima* CheA (40%; Swanson *et al*., 1996) and to *M. xanthus* FrzE (37%; McCleary and Zusman, 1990) (Fig. 5). The homology of DifC to CheW and DifD to CheY are over the entire ORFs (data not shown). The predicted DifC is 27% and 24% identical to CheWs from *Rhodospirillum centenum* (Jiang and Bauer, 1997) and *B. subtilis* (Hanlon *et al*., 1992) respectively. The homology of DifD to CheY proteins is the highest of all, over 60% identical to CheY proteins from *T. maritima* (Swanson *et al*., 1996) and *B. subtilis* (Bischoff and Ordal, 1991). The deduced amino acid sequence of DifB shows no homology to proteins in the databases.

Further mutational analysis of the dif *locus*

The chromosomal clustering of these *M. xanthus* chemotaxis homologues suggested that these genes might encode a new signal transduction pathway necessary for *M. xanthus* development. However, it was unclear whether *difA* itself was important for *M. xanthus* development or whether the *difA* Tn*5* insertion had polar effects on the downstream genes. It was also not clear whether the downstream genes were involved in the same developmental process. To clarify these points, we constructed and characterized two additional *dif* locus mutants. One *dif* mutant, SW504, contains a *difA* in frame deletion, and the other, SW501, contains a *difE* insertion (Table 1, Fig. 2). Both SW501 (Fig. 1C) and SW504 (data not shown) showed the same developmental defects as SW505, indicating that both the MCP and the CheA homologues are essential for fruiting body development in *M. xanthus*.

Differences between dif *and* frz *mutants*

The *dif* genes appear to be genetically and functionally distinct from the previously identified *frz* chemotaxis homologues. First, the *dif* mutants show no or very little aggregation under developmental conditions (Fig. 1). This is in striking contrast to the *frz* mutants, which form very distinctive frizzy filaments under development (Zusman, 1982). Secondly, individual cells of the *dif* mutants

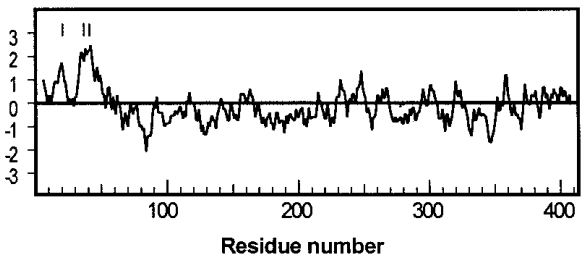

Fig. 4. Hydrophobicity analysis of the predicted DifA protein. The amino acid sequence of DifA was provided by the PEPPLOT program (Devereux *et al*., 1984), and the Kyte–Doolittle (Kyte and Doolittle, 1982) hydropathy profile was calculated. Roman numerals indicate the two predicted transmembrane domains.

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Tm CheA -----MMEEYLGVFV DETKEYLQNLNDTLL ELEKNPEDMELINEA FRALHTLKGMAGTMG FSSMAKLCHTLENIL DKARNSEIKITSDLL  85
Mx DifE --MTMDMSRYLGLFI SEATDHLEALGRDLV ELEREGS-SSAVDSM FRHAHSVKGMASSMG FEPIAIVAHRVEDLV DAVRQDRGRLDRDLV  87
Mx FrzE MDTEALKKSLLKKFQ EVTADRLQKIQLGVL DLEKETA-DQAAEDV ARELHTMKGEARMLG LAAIGQLAHAAEDVL RAEREGK-TATEVAT  88

Tm CheA DKIFAGVD  93 ~~ ~~ SQTVRVDIEKLG NLMDLMGELVIARSR ILET--LKKYN---- IKELDESLSHLSRIT LDLQNVVMKIRMVPI 358
Mx DifE DLLLTAAD  95 ~~ ~~ SRTVRVRTELLD YFLDTVGELMLATAR LREVGKVLPENT--- RPALEEGVYRLHTLV KDLHDKVMTARMTPL 542
Mx FrzE DVLLRACD  96 ~~ ~~ SR-LGDRFLRLA EEIDISNEVREQLDR VESDLHMLRDDAFRF VRRNDDGINTLHGNL AKMADHVAEARLVPL 326

Tm CheA SFVFNRFPRMVRDLA KKMNKEVNFIMRGED TELDRTFVEEIGEPL LHLLRNAIDHGIEPK EERIAKGKPPIGTLI LSARHEGNNVVIEVE 448
Mx DifE SLITDRLPRAARDIA RRKEREVDLVITGAE IELDRAILEELSDPL LHLLRNCIDHGLEAP EDRAAAKKGPRGRVL VAVKRARDRVIIELE 632
Mx FrzE STVFDAFPRAVREMS RTQGKEVDLVIENAD IGVDRSMLGDVRDAL VHLLRNSVDHGVESP DTRQQLGKPLNGRIR IRVRVDGDMLHIEVE 416

Tm CheA DDGRGIDKEKIIRKA IEKGLIDESKAATLS DQEILNFLFVPGFST KEKVSEVSGRGVGMD VVKNVVESLNGSISI ESEKDKGTKVTIRLP 538
Mx DifE DDGRGMDPAKLKNAA VSRGLLSAEAAVRLT DREAFMLSCLPGVST AKDITDISGRGVGMD AVKRVVENVGGTLEI DSERGRGTRFTLRLR 722
Mx FrzE DDGRGIDPERLRQAA ISKRLINAVQAAALS EREAIELIFRPGFST RDQVSELSGRGVGMD VVKRKVETLGGSVGV SSRIGRGSTITLRLP 506
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Fig. 5. Alignment of deduced *M. xanthus* (Mx) DifE to *T. maritima* (Tm) CheA and to *M. xanthus* FrzE. Identical residues among the sequences are shaded. DifE is over 40% identical to *T. maritima* CheA and 37% to *M. xanthus* FrzE over the aligned sequences.

(SW501, SW504 and SW505), similar to wild-type cells, reverse gliding direction about every 6–8 min compared with the *frz* mutant cells, which either seldom reverse or hyper-reverse (Blackhart and Zusman, 1985). Thirdly, physical mapping located the *dif* genes to almost the opposite side of the *frz* genes on the *M. xanthus* circular chromosome (D. Xu and H. B. Kaplan, unpublished).

S-motility defects of the dif *mutants*

In most cases, S-motility defects lead to defects in fruiting body development (Kroos *et al*., 1990; Hartzell and Youderian, 1995). Taking this into account, we tested the *dif* mutants for motility deficiencies. The smaller swarming colony diameter of the mutants on 1.5% agar indicated defects in motility (Fig. 6). Microscopic examination of the advancing edges of the mutant colonies indicated that the mutant cells largely move as individual cells or as small cell groups, suggesting defects in S motility (Fig. 1). On soft agar (0.3%) surfaces, which are preferential for S motility (Shi and Zusman, 1993), the mutants showed very little expansion (data not shown), consistent with defects in S motility.

To determine definitively whether the mutants are defective in S motility, each *dif* mutation was combined with an A-motility mutation to generate double mutants (Table 1). Colonies of the double mutants were examined for swarming on 1.5% agar plates. In contrast to the parental strains, colonies of the A$^-$ *dif* double mutants (SW506, SW507 and SW508) showed no or very little expansion (Fig. 6). S$^-$ *dif* double mutants showed phenotypes similar to the S$^-$ and *dif* parent strains (data not shown). These results indicate that the *dif* genes are required for S motility but not for A motility.

Discussion

In this paper, we report the isolation and characterization of the *dif* locus, which was identified by a Tn*5* insertion mutant defective in fruiting body development. The locus was found to encode homologues of the bacterial chemotaxis proteins MCPs, CheW, CheY and CheA. The original Tn*5* insertion mutant contained an insertion in the end of the first gene (*difA*), which encodes an MCP homologue (Fig. 3). This mutation was subsequently found to cause defective S motility. Both a *difA* in frame deletion and a *difE* (*cheA* homologue) insertion were constructed and found to cause the same defects in development and in S motility as the original *difA* Tn*5* insertion (Figs 1 and 6). This is the first report of components of a chemotaxis-like signal transduction pathway that are required for *M. xanthus* S motility. This suggests that signalling events

Table 1. Bacterial strains used.

Strains	Relevant genotype/description	Source or reference
DZF1	*dif*$^+$ *sglA* (leaky)	Morrison and Zusman (1979)
DK1622	*dif*$^+$ *sglA*$^+$	Kaiser (1979)
DK1217	*dif*$^+$ *aglB1*	Hodgkin and Kaiser (1979b)
MHX1216	*dif*$^+$ *A*::Tn*5-lac*Ω1215	MacNeil *et al*. (1994a)
SW101	*difA*::Tn*5*kan903Ω101 *sglA* (leaky)	This study
SW501	*difE*::kan^r *sglA*$^+$	This study
SW504	*ΔdifA sglA*$^+$	This study
SW505	*difA*::Tn*5*kan903Ω101 *sglA*$^+$	This study
SW506	*difE*::kan^r *aglB1 sglA*$^+$	This study
SW507	*difA*::Tn*5*kan903Ω101 *aglB1 sglA*$^+$	This study
SW508	*ΔdifA A*::Tn*5-lac*Ω1215 *sglA*$^+$	This study

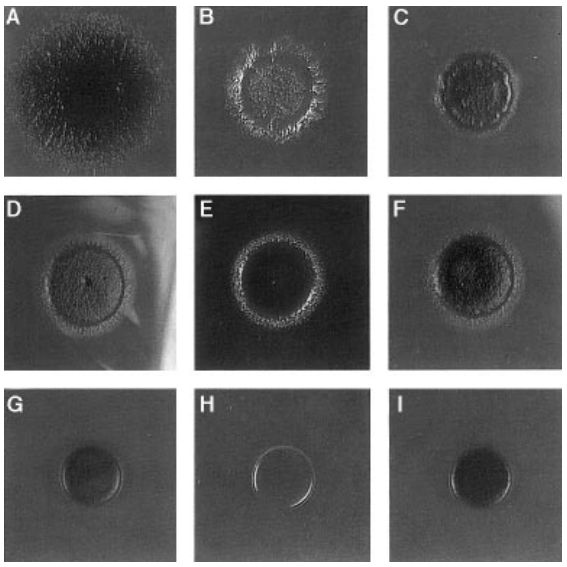

Fig. 6. Swarming defects of the *dif* mutants. The swarming of various strains was analysed on 1.5% agar as described in *Experimental procedures*. The area of the panels shown is approximately $4.3 \times 4.3\,cm^2$. The panels correspond to different strains as follows: A. DK1622; B, MXH1216; C, DK1217; D, SW501; E, SW504; F, SW505; G, SW506; H, SW508; I, SW507.

are essential in the co-ordination of *M. xanthus* group movement and that the *dif* genes comprise part of a novel signal transduction pathway essential for the social motility of *M. xanthus*. The components of this signal transduction pathway may function in a way that is analogous to those of the well-characterized chemotaxis pathways from enteric bacteria (Stock, 1996).

Previous studies have indicated that *M. xanthus* social motility requires the cell surface components pili and extracellular fibrillar material (Kaiser, 1979; Shimkets, 1986; Arnold and Shimkets, 1988; Behmlander and Dworkin, 1991; Wu and Kaiser, 1995). Both pili and fibrils are known mediators of cell–cell interactions, especially of cellular adhesion and agglutination (Kaiser, 1979; Shimkets, 1986; Arnold and Shimkets, 1988; Behmlander and Dworkin, 1991; Wu and Kaiser, 1995). It has been proposed recently that pili may provide the mechanical energy specific for social gliding, or they may transmit a signal for social motility-dependent activation of the gliding motor (Wu *et al*., 1997). In either case, as the *dif* genes are also required for S motility, perhaps functional and/or physical interactions occur between the Dif proteins and these two cell surface components. Interestingly, DifA, the MCP homologue, appears to have a very short periplasmic span of 10 amino acids between the two transmembrane regions in the N-terminal receptor domain. This region is structurally more similar to the *Halobacterium salinarum* HtrI transducer that interacts with the seven-transmembrane phototaxis receptor sensory rhodopsin I (SRI) (Hoff *et al*., 1997) than it is to chemotaxis MCPs, Tsr and Tar, which have about 160-amino-acid periplasmic regions that directly bind signals or their ligand-binding proteins. It is possible that DifA, which is predicted to be a transmembrane protein, may receive signals that originate from pili or the fibrillar material on the cell surface. The signal transmitted by DifA may interact with the putative gliding motor in an S motility-specific manner and regulate co-ordinated cell movement. In such a signalling system, pili and/or fibrils would be upstream of the Dif proteins and the putative gliding motor.

Alternatively, the Dif proteins may transmit a signal that regulates the synthesis, assembly or activity of pili and/or fibrils. In this case, pili and/or fibrils would function downstream of the Dif proteins. It is also possible that pili and fibrils function at different ends of a signalling pathway that contains these Dif chemotaxis homologue proteins in the centre. Interestingly, the twitching motility of *Pseudomonas aeruginosa* also requires a gene cluster encoding proteins with remarkable similarity to the chemotaxis proteins of the enterics (Alm and Mattick, 1997; Darzins and Russell, 1997). These *P. aeruginosa* chemotaxis homologues have been proposed to control twitching motility by regulating and/or interacting with *P. aeruginosa* pili, the same type of pili (type 4) that are required for *M. xanthus* social motility. More studies are needed to elucidate whether *dif* genes function in a similar fashion to regulate *M. xanthus* social motility.

It is important to consider the relationship between the *dif* genes and the *frz* genes, which both function to regulate *M. xanthus* motility. The Frz system is known to control the directed movement of *M. xanthus* social cell groups as well as individual cells (Shi and Zusman, 1993; 1995; Ward and Zusman, 1997), whereas the Dif system is essential for social motility. It is possible that the Frz and the Dif pathways converge before or at the gliding motor. It would be interesting to understand whether these two chemotaxis-like signal transduction pathways interact to integrate multiple environmental and intercellular signals.

Experimental procedures

Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. *M. xanthus* was grown and maintained at 32°C in CYE medium (Campos and Zusman, 1975). Development-inducing media used in this study were MOPS medium and CF medium (Hagen *et al*., 1978). *Escherichia coli* strains were grown and maintained at 37°C in LB medium (Miller, 1972).

Mutant isolation and phenotypical characterization

SW101 was isolated in the genetic screen for developmental mutants described previously (Yang *et al*., 1998). DZF1 was used as the parent strain in the mutagenesis because, compared with wild type, it is more easily infected by transducing

phage, grows more dispersed in liquid culture and forms more compact colonies on agar plates (Campos *et al*., 1978). DZF1 was first mutagenized by a Tn*5* transposon derivative Tn*5*kan903. In the initial screen, Tn*5*kan903 transductants selected on CYE plates containing 100 μg ml^{-1} kanamycin were streaked directly onto CF plates for observation. For subsequent characterization of their developmental phenotypes, cells from overnight cultures were resuspended in MOPS medium at about 5×10^9 cells ml^{-1}, spotted on MOPS or CF plates and incubated at 32°C for 2 days. For the swarming assay, 10 μl of cells at the above density were spotted on CYE plates containing either 1.5% or 0.3% agar and incubated for 2 days at 32°C before documentation (Shi *et al*., 1994). Reversal frequency of *M. xanthus* cells was analysed by time-lapse video microscopy as described previously (Shi *et al*., 1994). Cells were incubated on CYE plates at 32°C for 2 h before recording. At least 10 cells from each strain were followed over a 30 min period.

Molecular cloning and DNA sequencing

An *Eco*RI fragment from SW101 chromosomal DNA containing a Tn*5*kan903Ω101 insertion and flanking chromosomal DNA was cloned into the *Eco*RI site of pUC18 as described previously (Tn*5*kan903 has no internal *Eco*RI site) (Yang *et al*., 1998). DNA sequencing of the *M. xanthus* DNA was performed by the automated DNA sequencing facility at University of California, Davis, CA, USA. Standard techniques were used for cloning and plasmid construction (Sambrook *et al*., 1989). Sequence assembly and analysis were performed with BCM Search Launcher, BLAST and GCG programs (Devereux *et al*., 1984; Altschul *et al*., 1990; Smith *et al*., 1996).

Construction of new M. xanthus *mutants*

The *difA* in frame deletion mutant SW504 was constructed by gene replacement using the positive–negative KG cassettes described by Ueki *et al*. (1996). A 1.29 kb *Pst*I fragment containing the majority of the *difA* ORF was cloned into pBluescript KS+ to give rise to pYG301Z. pYG301Z was digested with *Nar*I to delete 693 bp internal of the *difA* gene and religated to generate pMCPΔ. pBJMCPΔ was generated by cloning the *Pst*I fragment harbouring the *difA* in frame deletion from pMCPΔ into pBJ113 (courtesy of Dr Bryan Julien, Stanford University, USA), which contains both a Kan^r marker for positive selection and a *galK* gene for negative selection. pBJMCPΔ was used to construct the *difA* in frame deletion mutant SW504 by a positive selection followed by a negative selection as described previously (Ueki *et al*., 1996).

For the construction of the *difE* insertion mutant SW501, a *Hin*dIII–*Bam*HI fragment from pBRZK (Trudeau *et al*., 1996) containing the Kan^r marker was first cloned into pBluescript to give rise to pBSkan. A *Sac*I fragment containing codons 256–485 of the *difE* gene and part of the polylinker from pBluescript was cloned into the *Sac*I site of pBSkan to generate pYG402, which was used to disrupt the *difE* gene through single cross-over homologous recombination. Plasmids were transformed into *M. xanthus* by electroporation (Kashefi and Hartzell, 1995). Southern analysis was performed to ensure that the mutants contained the expected structure. Double mutants SW506, SW507 and SW508 were constructed by Mx4-mediated transduction (O′Connor and Zusman, 1986).

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