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Phenotypic analyses of *frz* and *dif* double mutants of *Myxococcus xanthus*

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Abstract

Myxococcus xanthus is a Gram-negative gliding bacterium that aggregates and develops into multicellular fruiting bodies in response to starvation. Two chemosensory systems (*frz* and *dif*), both of which are homologous to known chemotaxis proteins, were previously identified through characterization of various developmental mutants. This study aims to examine the interaction between these two systems since both of them are required for fruiting body formation of *M. xanthus*. Through detailed phenotypic analyses of *frz* and *dif* double mutants, we found that both *frz* and *dif* are involved in cellular reversal and social motility; however, the *frz* genes are epistatic in controlling cellular reversal, whereas the *dif* genes are epistatic in controlling social motility. The study suggests that the integration of these two chemotaxis systems may play a central role in controlling the complicated social behaviors of *M. xanthus*. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Social motility; Chemotaxis; Fruiting body formation; *Myxococcus*

1. Introduction

Myxococcus xanthus is an unusual Gram-negative bacterium that undergoes multicellular development and primitive differentiation [1,2]. When deprived of nutrients, cells aggregate to form fruiting bodies that contain approximately 100 000 cells. With continued starvation, the aggregated cells develop into metabolically dormant myxospores [1,2]. The bacterium also exhibits social motility in which hundreds of thousands of cells move together in a large group [1–3]. It is not surprising that multiple sensory systems are involved in controlling these complicated social behaviors. Indeed, through genetic analyses, two chemosensory systems (*frz* and *dif*), both of which are homologous to known chemotaxis proteins, have been identified and are required for these social behaviors [4,5].

The *frz* chemosensory system was identified through characterizing a group of mutants that formed tangled frizzy filaments under fruiting conditions instead of the normal fruiting bodies [6]. Molecular cloning and sequence analysis revealed that most of the *frz* gene products are homologous to chemotaxis gene products from the enteric bacteria [7–9]. For example, FrzA is homologous to CheW, FrzE is homologous to both CheA and CheY, FrzF is homologous to CheR, FrzG is homologous to CheB, and FrzCD is homologous to the methyl-accepting chemotaxis proteins (MCPs). The *frz* mutants have altered cellular reversal frequency which leads to defective chemotaxis [10,11]. Whereas wild-type *M. xanthus* cells reverse moving direction every 4–6 min, most *frz* mutants rarely reverse. One particular *frzCD* mutant (called *frzD*), which has a Tn5 insertion at the C-terminal portion of the *frzCD* gene, reverses much more frequently than the wild-type [10]. Many *frz* gene products show biochemical functions similar to the chemotaxis proteins of *Escherichia coli*. For example, FrzCD is homologous to the C-terminal part of the MCPs of enteric bacteria, especially Tar, the receptor for aspartate in *E. coli* [7]. Like MCPs in enteric bacteria, FrzCD can be methylated by *S*-adenosylmethionine. Further studies indicated that methylation and demethylation of FrzCD corresponds to stimulation by attractants and repellents, respectively [11–13]. Thus, modification of FrzCD has often been used as an indicator for cellular response to chemotactic signals [11,14].

The *dif* locus was initially identified through the characterization of a mutant defective in fruiting body formation [5]. Molecular cloning, DNA sequencing and se-

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quence analyses indicate that the *dif* locus encodes a new set of chemosensory genes homologous with MCPs, CheW, CheY and CheA. Genetic analyses and phenotypic characterization indicate that the *M. xanthus dif* locus is required for social (S) motility and production of extracellular matrix called fibrils [5,15].

Since both the *frz* and *dif* genes are required for fruiting body formation, they may interact with each other. This study aims to investigate these possible interactions through double mutation analyses.

2. Materials and methods

The bacterial strains used in this study are listed in Table 1. *M. xanthus* was grown and maintained at 32°C in CYE medium [16]. Other media used in this study include MOPS medium (10 mM MOPS, pH 7.6; 8 mM $MgSO_4$), and CF medium [17]. Myxophage Mx4 was used for generalized transduction [18] to construct the strains listed in Table 1. For fruiting body formation, cells at about 5×10^8 cells ml^{-1} were placed on MOPS or CF plates (1.5% agar) and incubated at 32°C for 2–3 days. Colony edge morphology and cell motility were assayed using video microscopy as described by Shi and Zusman [19]. The assays for FrzCD methylation were performed according to the methods described previously [4]. For the auto-agglutination assay, *M. xanthus* was grown and analyzed in CYE. As described by Shimkets [20] and Wu and Kaiser [21], agglutination was determined continuously at room temperature by a change with the optical density (OD) measured at 600 nm with a Shimadzu BioSpec-1601 spectrophotometer.

Table 1
Bacterial strains used in this study

Strains	Genotype	Reference
DK1622	wild-type	[23]
DK1253	*tglI* (A^+S^-)	[23]
DK1300	*sglG1* (A^+S^-)	[23]
DK1217	*aglB1* (A^-S^+)	[23]
DK1218	*cglB2* (A^-S^+)	[23]
SW501	*difE::kan*^{r}	[5]
SW504	Δ*difA*	[5]
SW506	*aglB1 difE::kan*^{r}	[5]
SW600	*frzE*::Tn5*tet*Ω234 in DK1622 background	this study
SW601	*frzD*::Tn5*tet*Ω224 in DK1622 background	this study
SW602	Δ*difA frzE*::Tn5*tet*Ω234	this study
SW603	Δ*difA frzD*::Tn5*tet*Ω224	this study
SW604	*difE::kan*^{r} *frzE*::Tn5*tet*Ω234	this study
SW605	*difE::kan*^{r} *frzD*::Tn5*tet*Ω224	this study
SW607	*sglG1 frzE*::Tn5*tet*Ω234	this study
SW608	*aglB1 frzE*::Tn5*tet*Ω234	this study
SW609	*cglB2 frzE*::Tn5*tet*Ω234	this study
SW610	*cglB2 difE::kan*^{r}	this study
SW611	*tglI frzE*::Tn5*tet*Ω234	this study

3. Results

In contrast to wild-type *M. xanthus*, which forms fruiting bodies in response to starvation conditions (Fig. 1A), the *frzE* mutant formed 'frizzy' filaments when starved (Fig. 1B). The *difE* mutant, known to be defective in social

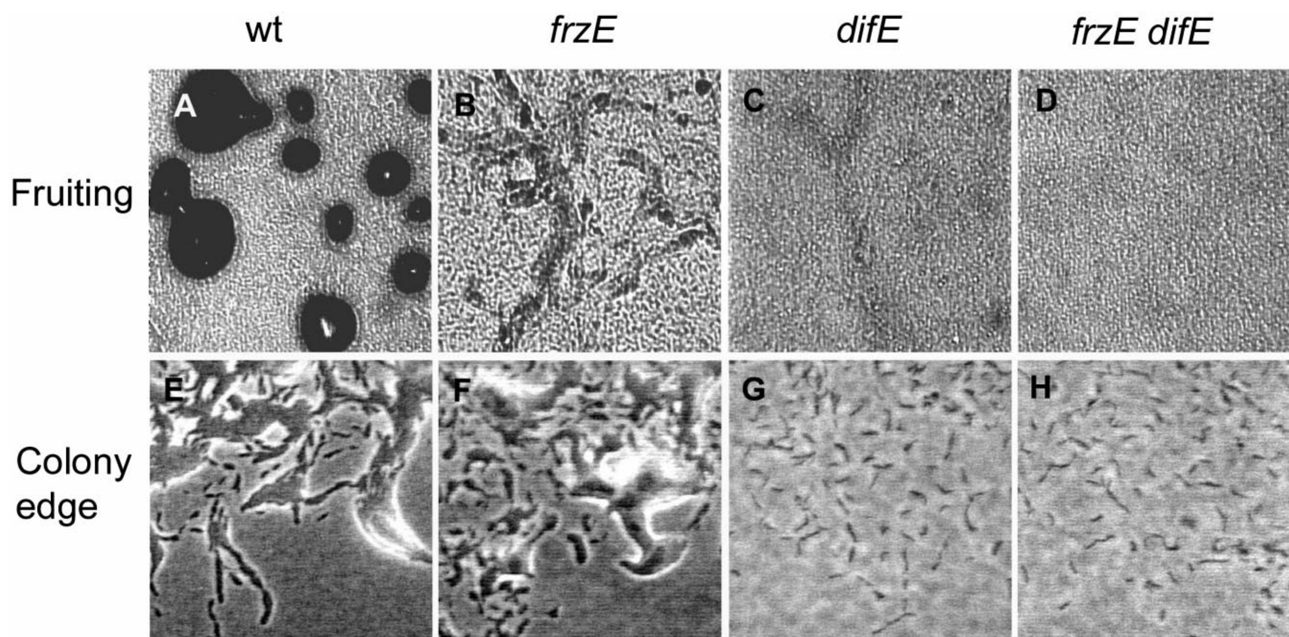

Fig. 1. Fruiting body formation and morphology of colony edges. Fruiting body formation (top panel) was examined and photographed under light microscopy with a 4× objective lens. The edges of colonies (bottom panel) were photographed under phase contrast microscopy with a 40× objective lens. A and E, DK1622 (wild-type); B and F, SW600 (*frzE*); C and G, SW501 (*difE*); D and H, SW604 (*frzE difE*). SW602, SW603 and SW605 exhibited similar phenotypes as SW604 (data not shown).

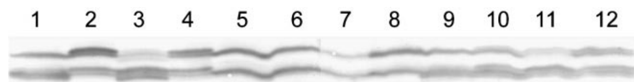

Fig. 2. FrzCD modification in response to various stimuli. FrzCD methylation was analyzed with Western blotting using anti-FrzCD antibodies as previously described [4,20]. The lower bands are methylated FrzCD. Lanes 1, 5, and 9: DK1622, SW600, and SW501 cells, respectively, treated with fresh CYE medium for 1 h; lanes 2, 6, and 10: DK1622, SW600, and SW501 cells, respectively, treated with MOPS medium for 1 h; lanes 3, 7, and 11: DK1622, SW600, and SW501 cells, respectively, treated with MOPS medium for 24 h; lanes 4, 8, and 12: DK1622, SW600, and SW501 cells, respectively, treated with 0.1% isoamyl alcohol. SW601 had similar phenotypes as SW600 and SW504 had similar phenotypes as SW501 (data not shown).

motility, also failed to form fruiting bodies, but did not form 'frizzy' filaments (Fig. 1C). Because the formation of the 'frizzy' filaments is thought to be related to abnormal FrzCD modifications in response to various environmental stimuli and altered cellular reversal frequency [12,22], we first tested the FrzCD methylation response of the wild-type and the *frzE* and *difE* mutants to various stimuli using Western blotting analyses. The wild-type and *difE* mutant methylated FrzCD in the presence of CYE (Fig. 2, lanes 1 and 9) and in the late phase of fruiting body formation (Fig. 2, lanes 3 and 11). In response to isoamyl alcohol, these strains demethylated FrzCD (Fig. 2, lanes 4 and 12). In contrast, the *frzE* mutant showed no response to these stimuli (Fig. 2, lanes 5–8). These results suggest that, although the *frzE* mutation abrogates the FrzCD modification response, the *difE* mutation has a minimal effect of the *frz* sensory transduction system.

Secondly, we examined the cellular reversal frequency of the wild-type and various *frz* and *dif* mutant strains of *M. xanthus* (Table 2). The *frzE* mutant showed an inability to reverse direction, whereas the *frzD* mutant reversed more frequently than the wild-type. The cellular reversal frequency of the *dif* mutants was also affected, but not to the same degree as the *frz* mutants. Both the *difA* and *difE* mutants reversed their direction approximately half as frequently than did the wild-type, suggesting that the *dif* chemosensory system does have some effect on 'cellular motors'.

In order to analyze the effect of the *frz* and *dif* mutations on cell motility, the colony edge morphology of the wild-type and various mutants on 1.5% agar was examined. As expected, wild-type *M. xanthus* exhibited both individual cell motility (A motility) and cell group motility (S motility) (Fig. 1E). The *difE* mutant, known to lack S motility, showed only A motility (Fig. 1G). Furthermore, A^- *dif* mutants (such as SW506 and SW610) were nonmotile ([23]; data not shown). Similar to the wild-type strain, the *frzE* mutant demonstrated both A and S motility (Fig. 1F), suggesting that the *frz* mutation has little effect on motility. We also found that both the A^- *frzE* and S^- *frzE* mutants retained S and A motility, respectively (Fig. 3D–F), further confirming that the *frz* genes are not directly involved in either type of motility. However, it is interest-

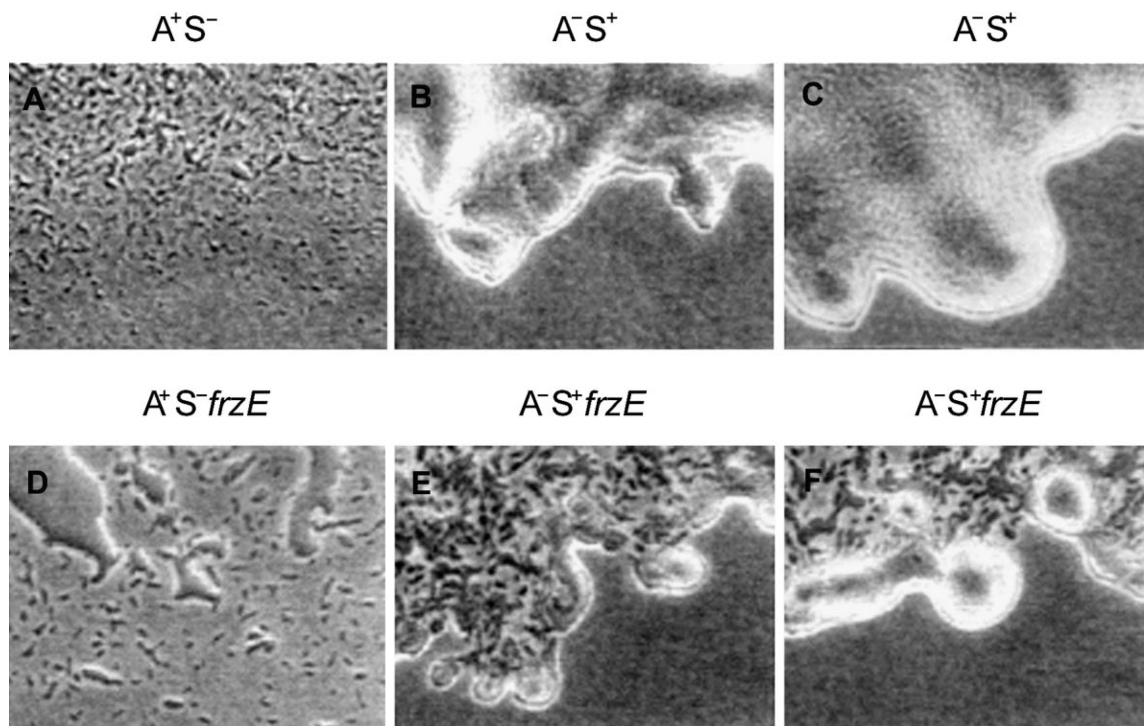

Fig. 3. Analyses of A and S motility of the *frz* mutants with colony edge morphology. The edges of colonies were photographed under phase contrast microscopy with a 40× objective lens. A, DK1300 (*sglG1*); B, DK1217 (*aglB1*); C, DK1218 (*cglB2*); D, SW607 (*frzE sglG1*); E, SW608 (*frzE aglB1*); F, SW609 (*frzE cglB2*). SW611 had similar phenotypes as SW607 (data not shown).

ing to note that the colony edges of the A^- *frzE* strains are unique. Unlike the A^- frz^+ strains, which are packed with cell groups at the colony edges (Fig. 3B and C), the A^- *frzE* mutant strains have only a limited number of small cell groups at the colony edges (Fig. 3E and F). We don't know what the cause of this interesting phenotype is as yet; however, it is possible that the *frz* genes play a role in directing large cell groups to move from the center of the colony (less nutrients) to the edge of the colony (more nutrients). Perhaps the failed directional movement of the A^- *frzE* mutants caused fewer cell groups to move to the colony edge.

We also examined the ability of various *frz* and *dif* mutants to self-agglutinate (Fig. 4). The wild-type and the *frzE* mutant show marked agglutination within 3 h. However, like other social motility mutants, the *difE* mutant did not agglutinate in suspension. This suggests that the *frz* mutations have minimal effect on cellular agglutination.

To further study the interactions between *frz* and *dif*, we constructed various double mutants (Table 1). The *dif* mutations had an epistatic effect on the *frz* mutations in terms of social motility and cellular agglutination. The *dif frz* double mutants formed neither fruiting bodies nor the 'frizzy' filaments characteristic of the *frzE* mutant (Fig. 1D). Furthermore, the *frzE difE* double mutant showed only A motility (Fig. 1H). Like the *dif* mutants, the *dif frz* double mutants also failed to self-agglutinate (Fig. 4). These data suggest that social motility is required for expression of the 'frizzy' filaments and lack of agglutination. In contrast, the *frz* mutation was epistatic over the *dif* mutation in terms of cellular reversal and FrzCD modification. All double mutants with the *frzE* mutation were unable to reverse direction, whereas the double mutants with the *frzD* reversed more frequently than the wild-type strain (Table 2). FrzCD methylation of the double mutants was similar to the *frz* mutant in that there was no response to the different stimuli (data not shown).

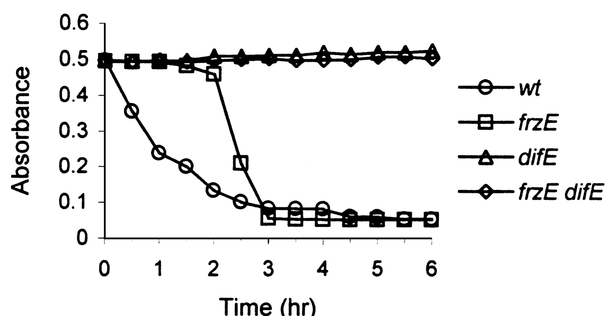

Fig. 4. Cellular agglutination of various *M. xanthus* strains. wt: wild-type DK1622; *frzE*, SW600; *frzE difE*, SW604. The experiments were performed according to Wu et al. [21]. 2.5×10^8 cells ml^{-1} cell suspensions were used for analyses. Absorbance at 600 nm was used as the index for cellular agglutination. SW601 had similar phenotypes as SW600, whereas the SW504, SW602, SW603 and SW605 had similar phenotypes as SW501 and SW604 (data not shown).

Table 2
Cellular reversal interval of various *M. xanthus* strains

Strains	Description	Cellular reversal interval (min per reversal)
DK1622	wild-type	6.24 ± 0.51
SW600	*frzE*::Tn5*tet*Ω234	>60
SW601	*frzD*::Tn5*tet*Ω224	2.06 ± 0.22
SW504	Δ*difA*	12.03 ± 3.39
SW501	*difE*::*kan*^{r}	11.30 ± 2.13
SW602	Δ*difA frzE*::Tn5*tet*Ω234	>60
SW603	Δ*difA frzD*::Tn5*tet*Ω224	2.59 ± 0.25
SW604	*difE*::*kan*^{r} *frzE*::Tn5*tet*Ω234	>60
SW605	*difE*::*kan*^{r} *frzD*::Tn5*tet*Ω224	2.91 ± 0.37

Cellular reversal was assayed with time-lapse video microscopy as described by Shi and Zusman [19]. The data presented are the mean of 50 cells randomly chosen for motility analyses.

4. Discussion

The above phenotypic analyses suggest that the *frz* and *dif* systems function independently of each other to carry out different cellular functions (directional movement and social motility), yet do have some overlapping functions in controlling cellular reversal and coordinated cell movement. The double mutation analyses indicate that the *frz* genes are epistatic in controlling cellular reversal and directional movement, whereas the *dif* genes are epistatic in controlling coordinated social motility. Based on these observations, we would like to propose that fruiting body formation in *M. xanthus* requires two parallel chemosensory systems defined by the *dif* and *frz* genes. The *dif* system is responsible for organizing cells into social groups and for coordinating cell movements within a social group. The function of the *frz* system is to confer chemotactic capability that directs cells into aggregation centers. These dual chemosensory systems, independent of and yet complementary to each other, constitute an integrated signaling network that enables the bacterium to perform various social behaviors.

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