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Methylation of FrzCD Defines a Discrete Step in the Developmental Program of *Myxococcus xanthus*

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Myxococcus xanthus* is a gram-negative soil bacterium which undergoes fruiting body formation during starvation. The *frz* signal transduction system has been found to play an important role in this process. FrzCD, a methyl-accepting taxis protein homologue, shows modulated methylation during cellular aggregation, which is thought to be part of an adaptation response to an aggregation signal. In this study, we assayed FrzCD methylation in many known and newly isolated mutants defective in fruiting body formation to determine a possible relationship between the methylation response and fruiting morphology. The results of our analysis indicated that the developmental mutants could be divided into two groups based on their ability to show normal FrzCD methylation during development. Many mutants blocked early in development, i.e., nonaggregating or abnormally aggregating mutants, showed poor FrzCD methylation. The well-characterized *asg*, *bsg*, *csg*, and *esg* mutants were found to be of this type. The defects in FrzCD methylation of these signaling mutants could be partially rescued by extracellular complementation with wild-type cells or addition of chemicals which restore their fruiting body formation. Mutants blocked in late development, i.e., translucent mounds, showed normal FrzCD methylation. Surprisingly, some mutants blocked in early development also exhibited a normal level of FrzCD methylation. The characterized mutants in this group were found to be defective in social motility. This indicates that FrzCD methylation defines a discrete step in the development of *M. xanthus* and that social motility mutants are not blocked in these early developmental steps.

Myxococcus xanthus is a gram-negative bacterium which commonly grows in damp soil, on animal dung, or in other natural habitats rich in organic matter (4). The bacteria lyse, digest, and live on other microorganisms (e.g., *Escherichia coli*) but can also be grown on a mixture of amino acids or complex peptides. When nutrients are abundant, the bacteria swarm as a thin spreading colony on a solid surface. When deprived of nutrients, the cells aggregate to form mounds of approximately 100,000 cells. With continued starvation, the aggregated cells develop into metabolically dormant spherical myxospores.

The developmental process of *M. xanthus* involves directed cell movements which are controlled by the *frz* signal transduction system (4, 22). The *frz* system was discovered through characterization of a group of mutants which formed tangled frizzy filaments under fruiting conditions instead of the normal fruiting bodies (28). Sequence analysis revealed that the *frz* genes are homologous to chemotaxis genes (12, 14, 15). For example, FrzA was homologous to CheW, FrzE was homologous to both CheA and CheY, FrzF was homologous to CheR, and FrzG was homologous to CheB. FrzCD is homologous to the C-terminal part of methyl-accepting chemotaxis proteins of enteric bacteria, especially Tar, the receptor for aspartate in *E. coli* (12). The methylation of these receptor proteins in enteric bacteria is catalyzed by a specific methyltransferase, CheR, which modifies the methyl-accepting chemotaxis proteins at specific glutamate residues with *S*-adenosylmethionine as a methyl donor (7). FrzCD was found to be methylated at the homologous glutamate residues and *S*-adenosylmethionine was found to be the methyl donor. The methylation was catalyzed by the CheR homologue, FrzF (14).

A correlation between directed cell movement and chemical modification of FrzCD was established (11, 17, 18). Attractants were found to cause methylation of FrzCD, while repellents cause demethylation of FrzCD. Furthermore, it was found that over the course of development, cells aggregated; at this time, FrzCD became more methylated, indicating that a signal(s) might be produced and sensed by starved *M. xanthus* cells (13, 17). Even though the chemical nature of the developmental attractant(s) is still unknown, studies suggested that the putative developmental signal(s) was produced by developmental cells during fruiting body formation in a cell density-dependent manner and that the signals were sensed by the *frz* system to suppress cellular reversal frequencies and make cells aggregate together (20). Recently, Sogaard-Andersen and Kaiser (24) reported that the *csg* mutant did not exhibit FrzCD methylation during development, suggesting an interesting relationship between the *frz* mutants and other developmental mutants. In this study, we further investigated the role of FrzCD methylation in development by screening many known and newly isolated mutants defective in fruiting body formation. The results indicated that the methylation of FrzCD defines a discrete step in the developmental program of *M. xanthus*; some mutants were blocked before that step and some were blocked after.

Bacterial strains, culture conditions, and experimental procedures. The bacterial strains used in this study are listed in Table 1. *M. xanthus* was grown and maintained at 32°C in CYE medium (1). Other media used in this study include MOPS (morpholinepropanesulfonic acid) medium (10 mM MOPS, pH 7.6, and 8 mM $MgSO_4$) and CF medium (6). Either P1::Tn*5 lac* or P4::Tn5kan903 (courtesy of Bryan Julien at Stanford University) was used for transposon mutagenesis as

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TABLE 1. Bacterial strains and FrzCD methylation during development

Strain	Phenotype or genotype	FrzCD methylation during development[a]	Reference
DZ2	Wild type	+	
DK1622	Wild type	+	
DK5057	*asg*; nonaggregating	−	10
DK5209	*bsg*; nonaggregating	−	5
DK2630	*csg*; abnormally aggregating	−	23
JD300	*esg*; nonaggregating	−	2
SW201	Nonaggregating	−	This study
SW280	Nonaggregating	−	This study
SW282	Nonaggregating	−	This study
SW129	Abnormally aggregating	−	This study
SW174	Abnormally aggregating	−	This study
SW178	Cell density-dependent aggregation	−	This study
SW160	Translucent mounds	+	This study
SW194	Translucent mounds	+	This study
SW107	Defective in aggregation and social motility	+	26
SW164	Defective in aggregation and social motility	+	26
SW101	Defective in aggregation and social motility	+	27
SW131	Nonaggregating	+	This study
SW103	Abnormally aggregating	+	This study
SW127	Abnormally aggregating	+	This study
SW115	Cell density-dependent aggregation	+	This study

[a] See the legend to Fig. 2 for a description of the FrzCD methylation assay. +, methylation of FrzCD after 24 h of incubation in MOPS medium; −, no methylation of FrzCD after 24 h of incubation in MOPS medium.

described previously (9). Myxophage Mx4 was used for generalized transduction (16). For fruiting body formation, cells (~5×10^8/ml were placed on MOPS or CF plates (1.5% agar) and incubated at 32°C for 2 to 3 days. For the examination of developmental spores, *M. xanthus* cells were spotted on to CF plates and incubated at 32°C for 7 days. Spore formation was then examined by light microscopy. The spores are refractile spherical cells which are resistant to 1% sodium dodecyl sulfate. Cell motility was assayed by time-lapse video microscopy as described by Shi and Zusman (21). The assays for FrzCD methylation were performed by the methods described previously (14, 19).

Isolation and phenotypic characterization of mutants defective in fruiting body formation. Mutants defective in fruiting body formation were isolated following transposon mutagenesis. Strain DZF1 is wild type with regard to fruiting body formation but contains a leaky *sglA* locus, a gene involved in social gliding motility (1). The strain was used initially for transposon mutagenesis because it is a better host for phages. Using P4::Tn*5*kan903 and P1::Tn*5 lac*, more than 10,000 Tn*5* insertional mutants were isolated. These mutants were streaked on CF plates and examined for cellular aggregation and fruiting body development. The linkage between the fruiting defects and the Tn*5* insertions was established by introducing the Tn*5* mutations back to DZF1 and to DZ2 by Mx4-mediated generalized transduction. About 200 mutants with various degrees of defects in fruiting body formation were identified. Table 1 and the legend to Fig. 1 list some of the representative mutant strains: some mutants (e.g., SW131) did not undergo any cellular morphogenesis even though they are fully motile; some mutants (e.g., SW127 and SW174) exhibited abnormal aggregation and rested at intermediate steps of fruiting body formation; some mutants (e.g., SW115) exhibited cell density-dependent behavior (no fruiting body formation at low cell density but normal at high density); some mutants (e.g., SW160) exhibited translucent mounds (forming aggregates but not spores). A number of mutants were found to be nonmotile or to exhibit frizzy filaments (data not shown). We are in the process of characterizing the genetic nature of these mutations (26, 27). In this study, these mutants, together with several other known developmental mutants, were used for analysis of FrzCD methylation.

Two different patterns of FrzCD methylation among developmental mutants. As reported previously (13), we found that FrzCD of wild-type cells became demethylated after 2 hours of incubation in MOPS medium (Fig. 2). After several hours of starvation, *M. xanthus* cells underwent developmental aggregation to form fruiting bodies. FrzCD extracted from wild-type cells after 24 h was fully methylated (Fig. 2), indicating that cells were being stimulated. We screened a number of known and newly isolated developmental mutants for FrzCD methylation in the hope of obtaining information on a possible correlation between mutant phenotypes and the FrzCD methylation step in the developmental program. As shown in Fig. 2 and Table 1, developmental mutants could be divided into two groups: the first group showed defective FrzCD methylation

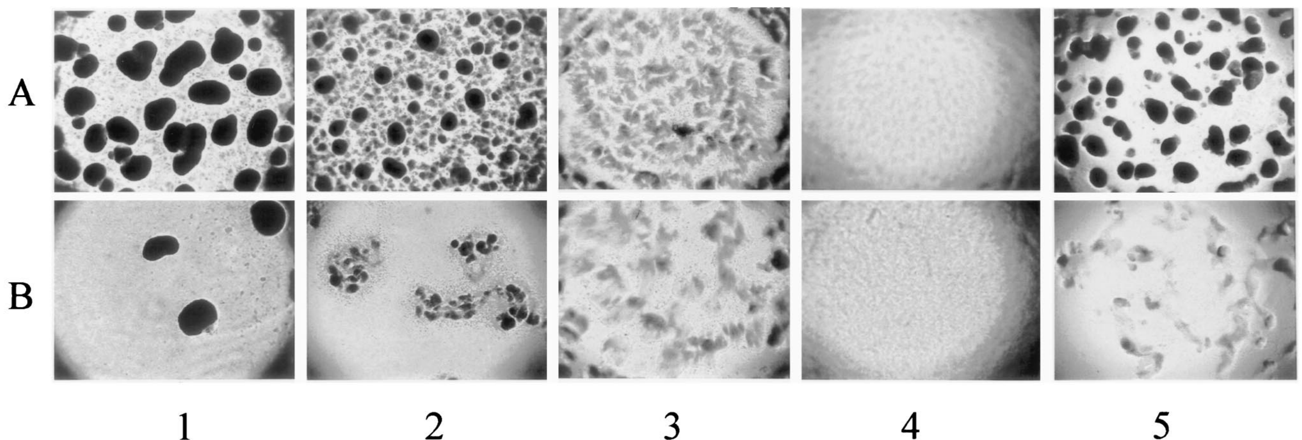

FIG. 1. Phenotypes of representative mutants defective in fruiting body formation. Row A shows fruiting phenotypes at a high cell density (5×10^9 cells/ml), and row B shows fruiting phenotypes at a low cell density (1×10^9 cells/ml). Pictures were taken after cells had been on MOPS medium for 72 h. Panels: A1 and B1, wild-type FB; A2 and B2, SW127; A3 and B3, SW174; A4 and B4, SW131; A5 and B5, SW115.

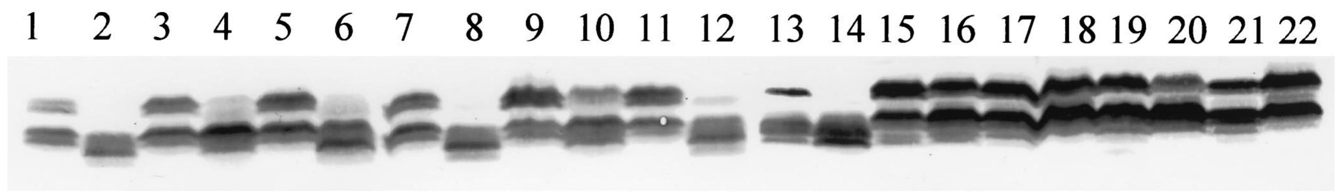

FIG. 2. FrzCD methylation of developmental mutants. Log-phase cells were placed on MOPS buffer for 2 h (odd-numbered lanes) or 24 h (even-numbered lanes). The cells were collected for FrzCD methylation analysis with Western blotting as previously described (19). The lower bands are methylated FrzCD. Lanes: 1 and 2, wild-type FB; 3 and 4, SW131; 5 and 6, SW101; 7 and 8, SW107; 9 and 10, SW201; 11 and 12, SW164; 13 and 14, wild-type DK1622; 15 and 16, *asg*; 17 and 18, *bsg*; 19 and 20, *csg*; 21 and 22, *esg*.

during development, while the second group showed normal FrzCD methylation. It is interesting that the well-characterized signaling mutants, i.e., *asg*, *bsg*, *csg*, and *esg* (2, 3, 5, 8, 10, 23), all exhibited the phenotype of the first group, defective in FrzCD methylation (Fig. 2), indicating that these mutants are blocked in the developmental program before the production or the perception of a signal(s) which normally is transduced through the *frz* signal transduction system. Many other nonfruiting mutants (such as SW201, SW280, SW282, SW129, SW174, and SW178) also were blocked before the FrzCD methylation step (Table 1 and Fig. 2). The strains with translucent mounds (SW160 and SW194) exhibited normal FrzCD methylation (Table 1). Interestingly, some developmental mutants (SW107, SW164, SW101, SW131, SW103, SW127, and SW115) exhibited normal FrzCD methylation even though they were not able to form fruiting bodies (Fig. 2). The characterized mutants among this group (SW107, SW164, and SW101) were observed to have defects in social motility (26, 27). We also tested several known social motility mutants and found they have normal FrzCD methylation during development (data not shown). This suggests that the social motility mutants can still produce and detect the putative signal, but because of their defects in coordinated cell movement they are unable to produce the movements needed for cellular aggregation. Some of the nonfruiting mutants listed in Table 1 (SW131, SW103, SW127, and SW115) were not defective in social motility but still showed normal FrzCD methylation. These mutants must be blocked after the signal production and detection step.

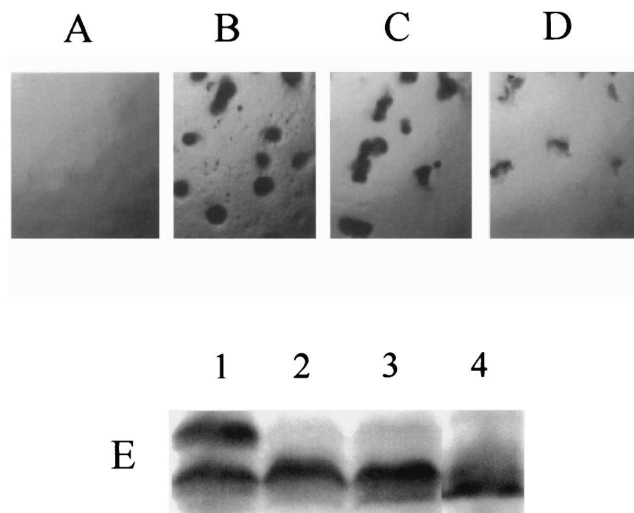

FIG. 3. Correlation between fruiting body formation and FrzCD methylation in an *esg* mutant JD300. Shown are the fruiting phenotypes of JD300 (A), wild-type DK1622 (B), a 1:1 mixture of DK1622 and JD300 (C), and JD300 grown in the presence of 1 mM isovaleric acid (D). (E) FrzCD methylation after 24 h of starvation. Lanes 1 to 4 contain JD300, wild-type DK1622, a 1:1 mixture of DK1622 and JD300, and JD300 grown in the presence of 1 mM isovaleric acid, respectively. The lower bands are methylated FrzCD.

Rescue of FrzCD methylation of an *esg* mutant by extracellular complementation with wild-type cells or addition of isovaleric acid. Sogaard-Andersen and Kaiser (24) reported that the *csg* mutant which is defective in both fruiting body formation and FrzCD methylation could be rescued for both mutant phenotypes by addition of purified C factor. These results suggested that C factor plays a role in signal transduction through the *frz* pathway. We therefore investigated the phenotypic rescue of another signaling mutant, *esg*, which is also blocked in the developmental program (2, 3, 25). The *esg* mutant, like the *csgA* mutant, is defective in fruiting body formation and can be rescued when mixed with wild-type cells (3, 25) (Fig. 3). In addition, the *esg* mutant can be rescued by growth in the presence of isovaleric acid (3, 25) (Fig. 3). As shown in Fig. 3, under the conditions of extracellular complementation with wild-type cells or growth in the presence of isovaleric acid, FrzCD of the *esg* mutant also became methylated. We also found that the defect of FrzCD methylation of *asg* and *bsg* mutants can be partially rescued by extracellular complementation with wild-type cells (data not shown). These results show that many mutants blocked in fruiting body formation are defective in FrzCD methylation during development. Since C signaling depends on prior A signaling, B signaling, and E signaling, it is possible that the defects of FrzCD methylation of *asg*, *bsg*, and *esg* mutants are due to blocked C signaling or a signaling step later than C signaling. In any case, since the methylation of FrzCD proceeds once the block is overcome, it can serve as a very useful developmental marker to evaluate the phenotype of fruiting mutants.

It should be noted that we recently identified a new genetic locus which also encodes genes homologous to chemotaxis genes (27). Mutants defective in these new chemotaxis protein homologues were found to have a normal *frz* signaling pathway (including FrzCD methylation) but to be defective in social motility. We are currently interested in investigating further the FrzCD methylation-dependent and -independent processes and the interactions between them.

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