FrzCD, a Methyl-Accepting Taxis Protein from *Myxococcus xanthus*, Shows Modulated Methylation during Fruiting Body Formation

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The frizzy (*frz*) genes of *Myxococcus xanthus* are required to control directed motility during vegetative growth and fruiting body formation. FrzCD, a protein homologous to the methyl-accepting chemotaxis proteins from enteric bacteria, is modified by methylation in response to environmental conditions. Transfer of cells from rich medium to fruiting medium initially caused rapid demethylation of FrzCD. Subsequently, the amount of FrzCD increased, but most remained unmethylated. At about the time of mound formation (9 h), most of the FrzCD was converted to methylated forms. Dispersal of developing cells (10 h) in buffer led to the demethylation of FrzCD, whereas concentration of these cells caused methylation of FrzCD. Some mutants which were unable to form fruiting bodies still modified their FrzCD during incubation under conditions of starvation on a surface.

Myxococcus xanthus is a gram-negative bacterium that exhibits a complex life cycle (18, 19). Cells of *M. xanthus* cannot swim in liquid media, but they are able to glide over moist solid surfaces. The mechanism of gliding motility is not known, but it does not involve flagella. Myxobacterial cells travel as large swarms which cooperatively digest other organisms and complex substrates. When the available food source is depleted, the cells aggregate to form fruiting bodies. Within the fruiting bodies, the cells develop into metabolically dormant myxospores. The ability of myxobacteria to form elaborate fruiting bodies suggests that intercellular communication occurs to coordinate individual cell movements. Evidence for cell-cell interactions also comes from the study of mutants (4). Mutants of the *asg*, *bsg*, *csg*, and *dsg* classes do not sporulate when cultured alone. However, these mutants do sporulate in the presence of wild-type cells or of cells of another mutant class; rescue of development occurs extracellularly. These mutants appear to be defective in signal production but are still able to respond to extracellular signals. Several of the signal molecules have been identified. The CsgA protein rescues development of *csg* mutants (7), and any of a variety of amino acids or short peptides rescue development of *asg* mutants (9).

The *frz* mutants are also deficient in cell-cell interactions. Unlike *asg*, *bsg*, *csg*, and *dsg* mutants, *frz* mutants sporulate, but they do not form fruiting bodies (22). Instead, the cells aggregate into tangled filaments and swirls. The *frz* genes control the frequency with which cells reverse their direction of gliding (1, 12, 14, 23). Most *frz* mutants rarely reverse. As the cells migrate over the agar surface, they preferentially follow in their own slime trails and have a tendency to form swirls. The *frz* genes are homologous to chemotaxis (*che*) genes of flagellated swimming bacteria, and the individual gene products share many biochemical similarities (13-15). We postulate that these genes are im-

portant for controlling cell movements during the cooperative feeding of vegetative swarms and during the construction of fruiting bodies.

FrzCD is homologous to the cytoplasmic portion of the enteric methyl-accepting chemotaxis proteins (MCPs) (13). The enteric MCPs are transmembrane proteins that detect specific chemicals in the periplasm and transduce this information into a signal that ultimately affects the swimming behavior of the cell (11). The MCPs are reversibly modified by methylation at specific glutamate residues. These modifications allow adaptation to various levels of stimulation. The extent of methylation increases dramatically following the addition of an attractant and decreases following the addition of a repellent.

FrzCD is not an integral membrane protein (12), but it shares many features with the cytoplasmic portion of the enteric MCPs. Among these, the amino acid sequence of FrzCD contains four or five sites that strongly resemble the consensus sequences for methylation sites of the enteric MCPs (13). FrzCD is reversibly methylated *in vivo*, and FrzF, the homolog to the enteric methyl transferase, CheR, is required for methylation (14). The chemical stabilities of the methylated residues are consistent with their being glutamate methyl esters (14). The extent of methylation of enteric MCPs, or of FrzCD, affects their mobility during sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). We have used this phenomenon to identify factors that interact with the *frz* signal transduction system and alter the level of methylation of FrzCD (12). Vegetative cells respond to a variety of low-molecular-weight alcohols by demethylating FrzCD and respond to Casitone, yeast extract, and several lipids by methylating FrzCD. Not surprisingly, several of these chemicals, in addition to influencing the methylation state of FrzCD, also influence the motile behavior of vegetative cells; short-chain alcohols, for example, cause repeated reversal of cells. The *frz* genes are expressed during vegetative growth and during development, and *frz* mutations affect the behavior of both vegetative and developmental cells. Cells may use the *frz* system to respond to different signals at different stages of their life

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TABLE 1. *M. xanthus* strains used

Strain	Genotype	Phenotype	Source or reference
DZF1	<i>sglA</i> ^{leaky}	Fru ⁺	16
DK1622	Wild type	Fru ⁺	6
DK5057	<i>asgA</i> (DK1622::Tn5 Ω 4560)	Fru ⁻	10
DK5209	<i>bsgA</i> (DK1622::Tn5-132 Ω M380)	Fru ⁻	3
DK2630	<i>csg</i> (DK1622::Tn5 Ω 519)	Fru ⁻	20
DK3261	<i>dsg-439</i> (DK1622::Tn5 Ω 1867)	Fru ⁻	2

cycle. Previously, we focused our attention on the functioning of the *frz* system in vegetative cells of *M. xanthus* (12). In this report, we address the functioning of the *frz* system during fruiting body formation.

Strains, media, and assays. The *M. xanthus* strains used in this study are listed in Table 1. DZF1, which carries a leaky mutation in the *sglA* gene, was our wild-type, fruiting-competent strain. The *sglA* gene is required for social motility. Strain DK1622 is fully motile and fruiting competent. The fruiting-deficient signalling mutants DK5057, DK5209, DK2630, and DK3261 were all derived from DK1622. *M. xanthus* cells were grown in a medium consisting of 10 g of Casitone per liter, 5 g of yeast extract per liter, and 8 mM MgSO₄ in 10 mM Tris buffer (pH 7.6) (CYE) at 33°C on a rotary shaker at 225 rpm. Development was initiated by concentrating vegetatively grown cells to 1.3 × 10¹⁰ cells per ml in 10 mM N-2-hydroxyethyl-N'-2-ethanesulfonic acid (HEPES)-8 mM MgSO₄ (HM), after which 0.5 ml was evenly spread onto 150-mm-diameter clone fruiting (CF) agar plates (4). Cells were harvested from CF agar by scraping with a razor blade in 0.2 ml of HM and frozen at -70°C until needed. Cell extracts were prepared by sonication in buffer containing 5 mM EDTA, 0.7 µg of pepstatin per ml, 1.0 mM o-phenanthroline, and 0.6 mM phenylmethylsulfonyl fluoride and then immediately boiled in SDS loading buffer as previously described (14) to minimize the risk of proteolysis. Samples containing 10 µg of protein (determined with the bicinchoninic acid reagent; Pierce Chemical Co., Rockford, Ill.) were separated by SDS-PAGE, fractionated proteins were transferred to nitrocellulose, and FrzCD was detected with antisera as previously described (14).

Developmental expression of FrzCD and modification of FrzCD during development. The *frz* mutants of *M. xanthus* were originally isolated as developmental mutants (they are unable to form fruiting bodies) (22). We were interested in determining the role that the *frz* signal transduction system plays during development. Cells were plated on a fruiting medium and harvested after various lengths of incubation, and FrzCD was analyzed by SDS-PAGE and Western immunoblot analysis as described above. Increased levels of methylation cause the protein to migrate more rapidly through SDS-PAGE, although there is little change in the actual molecular weight of the protein (14). Western blot analysis therefore provides information on both the level of FrzCD protein and the degree of methylation of FrzCD. Figure 1A demonstrates that FrzCD was present in vegetative cells and throughout development. (It should be noted that these experiments measured only the amount of FrzCD in cells which had not yet developed into spores. SDS extraction of intact spores releases only a few surface proteins [5].) The amount of FrzCD increased during development, reaching a peak at about the time of mound formation (9 to 12 h), and remained at relatively high levels

FIG. 1. Developmental expression and modification of FrzCD. (A) Vegetative cells were plated at high cell density under starvation conditions on a solid surface (CF agar) to initiate development. At various times (0 h, 1 h, etc.), cells were harvested and extracted, and cell proteins were separated by PAGE, blotted to nitrocellulose, and probed with antibody against FrzCD. Under these conditions of development, cells formed mounds by 9 to 12 h and fruiting bodies by 20 to 24 h. Lanes: 1, vegetative cells harvested from CYE medium; 2, cells that were spread on CF agar and immediately harvested; 3 to 12, cells harvested from CF agar after 1 to 96 h of incubation. (B) Cells were harvested after 96 h of incubation on CF agar and examined for the presence of FrzCD. Lanes: 1, sonication-sensitive cells (rods); 2, sonication-resistant spores.

throughout the remainder of the experiment. These results are in general agreement with those reported previously (21) except that in these experiments, we did not observe a drop in the amounts of FrzCD late in development. Proteolysis of FrzCD in extracts of developmental cells may have caused problems in the previous analysis of FrzCD. It is clear from the number and relative mobility of the bands in Fig. 1A that FrzCD was present in different methylation states at different times during development. In this and in all following figures, the arrows numbered 1 to 6 correspond to bands of 45, 44, 42.5, 41.5, 41, and 40 kDa, respectively, as described previously (12). Band 2 is most likely the unmodified form of the protein (12, 14). Band 1 probably corresponds to a form in which a glutamine has been deamidated to a glutamate, thus generating another site for methylation. Bands 3 to 6 correspond to methylated forms of FrzCD. Mutants which lack FrzF (the putative methyltransferase) did not produce these bands during incubation on CF agar (data not shown). The proteins in the faster-migrating bands are presumed to contain more methylated glutamate residues. FrzCD from vegetative cells was present as a ladder of bands (Fig. 1A, lane 1), but the slowest-migrating band (band 1) was absent. Transfer of these cells to CF agar (a low-nutrition medium) resulted in dramatic changes in the banding pattern. During the first hours of incubation, there was a shift to the slower-migrating (less methylated) bands (lanes 2 to 5). This shift began within minutes of transfer to nutrient-poor conditions (lane 2), long before any multicellular aggregates or fruiting bodies were observed. We have reported elsewhere (12) that this same shift occurred following starvation of cells in liquid. (Under those conditions, cells did not form fruiting bodies or spores.) These results suggest that during the first hour of starvation, FrzCD was demethylated and/or deamidated. Most likely this is a means for the bacterium to adapt to the new (lower) level of nutrients. After approximately 6 to 9 h of incubation, mounds of cells began to appear on the agar surface. At this time, band 1 decreased in intensity while bands 5 and 6 increased in intensity (lane 6). This shift toward faster-migrating forms of FrzCD became more prominent over the next few hours and then remained relatively stable throughout the rest of the experiment. We interpret

these results to indicate that FrzCD was methylated (by FrzF) at this time. The *frz* transduction system controls the migration of cells by altering the frequency of cell reversals. We hypothesize that during mound formation, FrzCD senses some chemical produced by other cells or by itself and sends a signal via the other *frz* proteins that promotes continuation of movement in the same direction or cell reversal. This allows the cells to control their movements and aggregate into mounds. As the cells pack together into mounds, they are exposed to increased concentrations of these chemicals, which probably accounts for the increased methylation of FrzCD.

Analysis of spores for FrzCD. *M. xanthus* produces two different cell types during development, peripheral rods and spores (17). The procedures described above were useful only for detecting FrzCD from the rods. Analysis of spore proteins requires physical disruption of spores. Intact spores retain their integrity, although not their viability, and release very few proteins during SDS extraction (5). To determine whether FrzCD was present in spores, cells were allowed to develop for 96 h on CF agar. The peripheral rods and some immature spores were disrupted by sonication, leaving the mature spores intact. The spores were collected by centrifugation, washed in buffer, and then disrupted by agitation with zirconium beads as previously described (17). Equal amounts (10 μ g) of rod and spore cell extracts were separated by SDS-PAGE, and FrzCD was detected by Western blot analysis as described above. FrzCD was present in the extract of the rod-shaped cells, but little or no FrzCD was detected in extracts of the sonication-resistant spherical myxospores (Fig. 1B).

Effect of high cell densities or cell-cell contacts on the methylation of FrzCD. Previously, we reported that vegetative cells harvested from a rich medium (CYE) responded to incubation in a low-nutrient medium such as CF broth by demethylating their FrzCD (12). Even after incubation for 24 h in this medium, FrzCD remained in its slower-migrating, unmethylated forms. Addition of some chemicals or nutrient mixtures to these cells resulted in increased methylation of FrzCD. As demonstrated by Fig. 1, the behavior of cells incubating on an agar surface of the same medium was quite different. These cells initially showed decreased methylation of FrzCD but showed increased methylation upon further incubation, as the cells began to aggregate to form mounds and fruiting bodies. Because of this difference in behavior, we were interested in determining how developmental cells harvested from the low-nutrient CF agar would respond to suspension in CF broth (CF medium lacking agar). In this and in all succeeding experiments, cells harvested from CF agar or from liquid cultures were concentrated by centrifugation for 3 min at $10,000 \times g$, frozen in a dry ice-ethanol bath, and stored at -70°C . They were resuspended directly in loading buffer and boiled for 5 min, and the proteins were separated by SDS-PAGE, transferred to nitrocellulose, and analyzed as described above. Cells harvested directly from CF agar after 10 h of development had FrzCD primarily in its methylated forms (Fig. 2, lane 1; note the prevalence of bands 2 to 5). When these cells were suspended in CF broth at a density of 3×10^8 cells per ml and incubated with shaking at 33°C , there was a shift to the slower-migrating forms of FrzCD (lane 2; note the disappearance of band 5 and the increased intensity of band 1). This shift began within minutes of suspension of the cells and was essentially complete by 1 h (data not shown). There are several possible reasons why transfer from one low-nutrient medium (CF agar) to another (CF broth) resulted in decreased methylation of FrzCD.

FIG. 2. Factors affecting modification state of FrzCD from developmental cells. Lanes 1 to 6, developmental cells harvested from CF plates after 10 h of incubation (untreated cells [lane 1]; cells incubated for 1 h in CF broth and then for an additional hour in CF broth [lane 2] or CF broth plus 5 g of yeast extract per liter [lane 3]; cells replated on the same CF agar from which they were harvested [lane 4]; cells plated on fresh CF agar [lane 5]; cells pelleted by centrifugation and incubated as a cell pellet under CF broth [lane 6]). Lanes 7 to 10, vegetative cells grown in CYE (untreated cells [lane 7]; cells incubated for 1 h as a cell pellet in CYE broth [lane 8]; cells incubated for 2 h in CF broth [lane 9]; cells incubated for 1 h in CF broth and for 1 h as a cell pellet under CF broth [lane 10]).

The CF agar might have accumulated some substance which caused the modification of FrzCD to the faster-migrating forms. Transfer into fresh media would remove this factor and result in a shift to the slower-migrating forms of FrzCD. Alternatively, cell-cell contacts may have been responsible for the presence of the rapidly migrating forms of FrzCD from cells harvested from CF agar. Dilution of the cells into buffer would disrupt these contacts and could have resulted in the shift to the slower-migrating forms of FrzCD. We performed several experiments to distinguish between these possibilities (Fig. 2). Developmental cells which had been incubated in CF broth for 1 h (lane 2) were transferred to the CF agar from which the cells were originally harvested (lane 4) or to fresh CF agar (lane 5) and incubated at 33°C for 1 h. In both cases, there was a shift to the faster-migrating (more methylated) forms of FrzCD. The pattern of FrzCD migration was nearly identical to that obtained from developmental cells freshly harvested from CF agar (compare lane 1 with lanes 4 and 5). This is in contrast to the behavior of vegetative cells placed on CF agar (as shown in Fig. 1, FrzCD decreased in mobility during the first hour and did not increase in mobility until 9 h of incubation). The rapid shift in FrzCD modification upon transfer of developmental cells to CF agar caused us to suspect that cell contacts or cell density might be important in determining this response. To test this possibility, we incubated developmental cells in CF broth for 1 h and then concentrated them by centrifugation and incubated them for an additional hour as a cell pellet under CF broth (Fig. 2, lane 6). Incubation as a cell pellet resulted in modification of FrzCD to its more rapidly migrating forms (compare lanes 2 and 6). Apparently the cells sense and respond to some signals associated with other cells or to soluble signals which are present in a concentrated form in the cell pellets. Direct cell-cell contact or at least high cell density appears to be necessary for the cells to perceive these signals.

We performed the same experiment with vegetative cells to determine whether they would respond similarly to close packing. The pattern of bands of FrzCD obtained from extracts of vegetative cells grown in CYE was nearly identical to that obtained from cells that were incubated for 1 h as a pellet under CYE (Fig. 2, lanes 7 and 8). Incubation of vegetative cells in CF broth for 1 h resulted in the accumulation of the slower-migrating (less methylated) forms of FrzCD (lane 9). Incubation of these cells as a pellet under CF broth did not alter the pattern of FrzCD bands (compare lanes 9 and 10). We interpret these results to indicate that 10-h developmental cells send signals to and respond to

signals from their immediate neighbors and that cell contact or high signal strength is required for this transmission, whereas vegetative cells, or cells which have been starved for 1 h and therefore may have just entered development, either produce lower levels of these signals or are less able to perceive them.

We have previously reported that vegetative cells respond to starvation by demethylating FrzCD and to addition of yeast extract by methylating FrzCD. It is not surprising that vegetative cells respond to some factor present in yeast extract, since these bacteria survive by lysing and digesting other cells. The cells probably sense some cues produced by living and/or lysed cells to find their prey or to remain with their prey once they have made contact. We wanted to know whether developmental cells would recognize some of the same signals as vegetative cells. Developmental cells were harvested from CF agar and incubated for 1 h in CF broth. At this time, yeast extract was added to some of the cells. Figure 2, lane 3, demonstrates that the cells did respond to the addition of yeast extract in the same manner as vegetative cells. Following yeast extract addition, FrzCD became modified to the more rapidly migrating (more heavily methylated) forms (bands 4 to 6).

Modification of FrzCD by developmental mutants of *M. xanthus*. Kaiser and colleagues described a series of mutants that are blocked at different stages of development (4, 8). These mutants (called *asg*, *bsg*, *csg*, and *dsg*) can be rescued by the presence of wild-type cells or by cells of another *sg* mutant class, suggesting that the mutations prevent cell-cell signalling from occurring. Several of the gene products associated with these loci have been characterized. Since methylation of FrzCD occurs during development on a solid surface, we speculated that mutations that affect development might alter the expression or modification of FrzCD. We examined the degree of modification of FrzCD in representative *asg*, *bsg*, *csg*, and *dsg* mutants incubated on CF agar at 28°C for various lengths of time. Cells of the wild-type strain DK1622 fruited under these conditions, and fruiting bodies were first observed at about 24 h. We chose time points to capture cells at various stages of development. The *asg*, *bsg*, and *csg* mutants did not form fruiting bodies during 48 h of incubation on CF agar. Cells of strain DK3261 (*dsg*) formed numerous fruiting bodies during incubation on CF agar. Because this strain fruited under our conditions, we will not consider it further except to indicate that FrzCD was present at all times and that the pattern of modified bands was nearly identical to that of the wild type (DK1622) during development (data not shown).

Cells of DK1622 modified their FrzCD during development in a manner similar to DZF1. Cells harvested from CYE had several forms of FrzCD (Fig. 3A, lane 1). After 2 h of incubation on CF agar, there was a slight shift to the slower-migrating forms (note the increased intensity of band 1 in lane 2), suggesting that demethylation had occurred. By 24 or 48 h of incubation, there was a shift back to the faster-migrating forms of FrzCD (lanes 3 and 4; note the decreased intensity of bands 1 and 2 and the increased intensity of bands 5 and 6), suggesting that FrzCD had been methylated. Cells with mutations at the *asg*, *bsg*, and *csg* loci (Fig. 3B to D) also modified their FrzCD during development. However, bands 1 and 2 remained prominent even after extended incubation, probably indicating that FrzCD was less heavily methylated in these strains. Bands 5 and 6 were particularly weak in extracts from the *asg* mutant (Fig. 3B), which may indicate that FrzCD was less methylated in this strain. The *asg* mutation, by blocking development at a

FIG. 3. Modification of FrzCD of wild-type cells and developmental mutants during incubation on CF agar. Cells were grown in CYE broth at 32°C, spread on CF agar, and incubated at 28°C. Cells were harvested at the times indicated and frozen at -70°C. Proteins were extracted and FrzCD was detected as described in the text. (A) Wild-type cells (DK1622); (B) DK5057 (*asgA*); (C) DK5209 (*bsgA*); (D) DK2630 (*csg*). Lanes: 1, vegetative cells harvested from CYE; 2, cells harvested from CF agar after 2 h of incubation; 3, cells harvested from CF agar after 24 h of incubation; 4, cells harvested from CF agar after 48 h of incubation.

very early stage, may have pleiotropic effects, some of which may affect the functioning of the *frz* system and prevent mound formation.

The experiments presented in this report allow us to draw the following conclusions. (i) FrzCD is present in vegetative and in developmental cells but is absent, or present at much lower levels, in spores. (ii) The amount of FrzCD increases during development, reaching a peak at about the time of mound formation (9 to 12 h). (iii) FrzCD is modified during development. FrzCD apparently becomes demethylated early during development (perhaps a starvation response) and becomes heavily methylated at about the time of mound formation. FrzCD appears to be responsive to some signals produced by developing cells of *M. xanthus*. Contact of developmental cells (harvested from mounds) with their neighbors results in methylation of FrzCD, whereas dispersal of the cells in buffer leads to demethylation of FrzCD, suggesting that these signals are cell bound or highly concentrated in cell pellets. (iv) The *asg*, *bsg*, and *csg* mutants, although unable to make fruiting bodies, still modify their FrzCD during incubation on CF agar. Since FrzCD was modified in these mutants, the signal molecules associated with these loci are not necessary to elicit the developmental modification of FrzCD. Further analyses may indicate the nature of the developmental signals recognized by FrzCD and their role(s) in fruiting body development.

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