JOURNAL OF BACTERIOLOGY, July 1992, p. 4246–4257
0021-9193/92/134246-12$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 174, No. 13

Methylation of FrzCD, a Methyl-Accepting Taxis Protein of *Myxococcus xanthus*, Is Correlated with Factors Affecting Cell Behavior

MARK J. McBRIDE,† THILO KÖHLER, AND DAVID R. ZUSMAN*

Department of Molecular and Cell Biology, University of California, 401 Barker Hall, Berkeley, California 94720

Received 2 March 1992/Accepted 27 April 1992

***Myxococcus xanthus*, a nonflagellated gliding bacterium, exhibits multicellular behavior during vegetative growth and fruiting body formation. The frizzy (*frz*) genes are required to control directed motility for these interactions. The *frz* genes encode proteins that are homologous to all of the major enteric chemotaxis proteins, with the exception of CheZ. In this study, we characterized FrzCD, a protein which is homologous to the methyl-accepting chemotaxis proteins from the enteric bacteria. FrzCD, unlike the other methyl-accepting chemotaxis proteins, was found to be localized primarily in the cytoplasmic fraction of cells. FrzCD migrates as a ladder of bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, reflecting heterogeneity due to methylation or demethylation and to deamidation. FrzCD was shown to be methylated in vivo when cells were exposed to yeast extract or Casitone and demethylated when starved in buffer. We used the methylation state of FrzCD as revealed by Western blot (immunoblot) analyses to search for stimuli that are recognized by the *frz* signal transduction system. Common amino acids, nucleotides, vitamins, and sugars were not recognized, but certain lipids and alcohols were recognized. For example, the saturated fatty acids capric acid and lauric acid stimulated FrzCD methylation, whereas a variety of other saturated fatty acids did not. Lauryl alcohol and lipoic acid also stimulated methylation, as did phospholipids containing lauric acid. In contrast, several short-chain alcohols, such as isoamyl alcohol, and some other solvents caused demethylation. The relatively high concentrations of the chemicals required for a response may indicate that these chemicals are not the relevant signals recognized by *M. xanthus* in nature. Isoamyl alcohol and isopropanol also had profound effects on the behavior of wild-type cells, causing them to reverse continuously. Cells of *frzB*, *frzF*, and *frzG* mutants also reversed continuously in the presence of isoamyl alcohol, whereas cells of *frzA*, *frzCD*, or *frzE* mutants did not. On the basis of the data presented, we propose a model for the *frz* signal transduction pathway in *M. xanthus*.**

Myxococcus xanthus is a gram-negative bacterium that exhibits a complex life cycle (23, 24). Vegetative cells move by gliding on a solid surface. The mechanism of gliding motility is not understood but does not involve flagella (5, 21). Cells generally 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. Some cells have a different developmental fate (18, 28). They remain as rod-shaped cells around and between fruiting bodies. They are believed to be important in the natural environment by providing a flexible subpopulation which can feed on low or intermittent amounts of nutrients (19). The complex behavior of individual cells and of swarms of cells suggests that intercellular communication must occur to coordinate cell movements (9).

Frizzy (*frz*) mutants of *M. xanthus* produce wild-type levels of spores but do not form fruiting bodies (26). Instead, cells aggregate into tangled filaments. The *frz* genes were cloned and found to be clustered on the chromosome (3). Mutations in any of the six *frz* genes alter the gliding behavior of individual cells. Wild-type cells glide at a rate of approximately 2.0 μm/min (a rate which varies with the environmental conditions and genetic background) and reverse their direction (the leading end of the cell becomes the lagging end) about every 7 min. Net movement occurs because cells spend more time gliding in one direction than in the other. Mutations in *frzA*, *frzB*, *frzCD*, *frzE*, or *frzF* result in cells that rarely reverse their direction of gliding. These cells reverse, on average, once in 2 h (4). This behavior results in the formation of tangled filaments and swirls composed of thousands of cells. A small subset of mutations near the C terminus of *frzCD* (*frzD* mutations) result in cells that reverse their direction of movement more frequently than wild-type cells (4, 12). *frzD* mutants reverse their direction of gliding so frequently and demonstrate so little bias that they make no net progress and form nonspreading colonies that appear similar to those of nonmotile mutants (4). These *frzD* mutants produce truncated forms of FrzCD (12). *frzG* mutants are only slightly aberrant in gliding behavior and are able to produce fruiting bodies although they are irregular in appearance. The behavior patterns described above are analogous to those of chemotaxis mutants of enteric bacteria, which swim smoothly and rarely tumble or tumble and rarely swim smoothly (10).

Cells of *Escherichia coli* and *Salmonella typhimurium* respond to a variety of environmental stimuli by altering their swimming behavior to allow them to migrate towards a more favorable environment. The behavior of the cells is determined by the direction of rotation of the flagella. Counterclockwise rotation leads to the formation of a flagel-

* Corresponding author.
† Present address: Department of Biological Sciences, University of Wisconsin, Milwaukee, WI 53211.

lar bundle that propels the cell. When the flagella switch to clockwise rotation, the bundle is dispersed and the cell tumbles (10). Individual cells display periods of swimming of various lengths of time punctuated by periods of tumbling. During periods of tumbling, cells are randomly reoriented for the next period of swimming. Cells tumble less frequently when moving in a favorable direction (toward an attractant or away from a repellant), and thus their direction of overall movement is biased.

The components of the enteric chemotaxis signal transduction system include CheA, CheB, CheR, CheY, and CheZ and the methyl-accepting chemotaxis proteins (MCPs). The MCPs are transmembrane receptors that bind chemoeffectors in the periplasm and transmit a signal through the other Che proteins that influences the direction of rotation of the flagella and thus determines the swimming behavior of the cell. Following stimulation, cells adapt over a period of seconds to minutes to their prestimulus behavior and are thus primed to respond to a new change in stimulus level. During adaptation, the cytoplasmic domains of the MCPs are reversibly modified by methylation at several glutamate residues. This apparently resets the MCPs to their prestimulus signalling state.

The nucleotide sequence of the *M. xanthus frz* region has been determined previously (11–13). Sequence analysis revealed that five of the *frz* gene products are homologous to enteric chemotaxis proteins. FrzA is homologous to CheW, FrzCD is homologous to the enteric MCPs, FrzE is homologous to both CheA and CheY, FrzF is homologous to the methyltransferase CheR, and FrzG is homologous to the methylesterase CheB. FrzB is not similar to any characterized protein.

In this paper, we focus our attention on FrzCD, the *Myxococcus* analog of the enteric MCPs. Enteric MCPs are transmembrane proteins that are anchored in the membrane by two stretches of hydrophobic amino acids. FrzCD is similar only to the cytoplasmic portion of the MCPs. Its deduced amino acid sequence does not contain the long stretches of hydrophobic amino acids that are characteristic of integral membrane proteins. FrzCD does contain four or five sites that resemble the potential methylation sites of the MCPs. We have demonstrated that FrzCD is methylated in vivo and that this methylation is dependent on FrzF, the homolog of the enteric methyltransferase CheR (12). Methylation of FrzCD causes the protein to migrate more rapidly during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as has been observed for enteric MCPs (15). Methylated FrzCD migrates as a ladder of bands with apparent molecular sizes of 40 to 45 kDa.

The myxobacteria are nonmotile in liquid media. Chemotaxis in the myxobacteria has never been clearly demonstrated despite several carefully performed investigations (7). The relatively slow movement of this organism on solid surfaces suggests that chemotaxis by temporal sensing of readily diffusible compounds is unlikely to be effective (7). Myxobacteria may respond to slowly diffusible or insoluble chemical stimuli in their paths. It is also formally possible that the myxobacteria sense a spatial rather than a temporal gradient of chemicals. Previous results of experiments in our laboratory suggest that the *frz* system controls cell movements in response to external stimuli. The nature of these stimuli remains to be elucidated. Preliminary experiments designed to identify chemicals that might influence the movements of *M. xanthus* showed that the assays used to analyze chemotaxis of rapidly swimming bacteria are unsuitable for the myxobacteria (unpublished data). We have therefore explored new assays to identify potential chemoeffectors. In this study, we utilized FrzCD for this purpose. In enteric bacteria, the extent of methylation of the MCPs increases dramatically following the addition of an attractant and decreases following the addition of a repellant. The effects of attractants and repellants are different in some other swimming bacteria (1, 2); in particular, in *Bacillus subtilis* there is no significant change in the level of methylation of MCPs following binding of an attractant, but there is an increased turnover of methyl groups. In any event, we postulated that there was a reasonable possibility that chemoeffectors for *M. xanthus* would influence the methylation state of FrzCD. Our strategy was to examine the electrophoretic mobility of FrzCD before and after exposure of cells to various conditions. Using this approach, we have identified several classes of chemicals that strongly affect the methylation state of FrzCD. At least two of these chemicals also influence the frequency with which cells reverse their direction of movement. We have used this information to gain insight into the nature of likely signals for cell-cell interactions in *M. xanthus* and to understand the roles of the various Frz proteins in the *frz* signal transduction system.

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

Strain	Genotype	Phenotype	Source or reference
DZF1	*frz*$^+$ *sglA*(leaky)	Fru$^+$	14
DZF1084	*frzA*	Frz	26
DZF1227	*frzE*	Frz	26
DZF1262	*frzE*	Frz	26
DZF1313	*frzF*	Frz	26
DZF1359	*frzF*	Frz	26
DZF1421	*frzF*	Frz	26
DZF1434	*frzF*	Frz	26
DZF1444	*frzE*	Frz	26
DZF3373	*frzA*:Tn*5*Ω214	Frz, Kan^r	3
DZF3377	*frzE*:Tn*5*Ω226	Frz, Kan^r	3
DZF3460	*frzD*:Tn*5*Ω224	Nonspreading, Kan^r	4
DZF3593	*frzF*Tn*5lac*Ω540	Frz, Kan^r	25
DZF4017	*frzCD*:Tn*5tac*1Ω4017	Frz, Kan^r	This study
DZF4020	*frzG*:Tn*5tac*Ω4020	Fru$^+$, Kan^r	This study
DZF4021	*frzG*:Tn*5*Ω239	Fru$^+$, Kan^r	12
DZF4022	*frzG*:Tn*5*Ω243	Fru$^+$, Kan^r	This study
DZF4023	*frzF*:Tn*5*Ω245	Frz, Kan^r	12
DZF4059	(*frzCD-F* deletion)	Frz, Kan^r	This study
DZF4123	*frzB*:Kan insert	Frz, Kan^r	This study
DZF4142	*frzF*Ω248	Frz, Kan^r	3

MATERIALS AND METHODS

Strains and culture conditions. The *M. xanthus* strains used in this study are listed in Table 1. Strains containing Tn*5*, Tn*5tac*1, or kanamycin resistance inserts were constructed essentially as previously described (3, 12, 16). Strain DZF4123 was constructed by inserting the kanamycin resistance gene from pUC4K at the *Eco*T221 restriction site within the *frzB* gene. Strain DZF4059 contains the same kanamycin resistance cartridge inserted in place of 6.4 kb of DNA between the *Pst*I restriction sites located in *frzCD* and downstream of *frzF*. Strains DZF4017 and DZF4020 contain Tn*5tac*1 (6) inserted into *frzCD* and *frzG*, respectively. Tn*5tac*1 contains an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible outward-reading promoter, which allows expression of downstream genes and thus eliminates the polarity effects of transposon insertion. All constructs were

generated and maintained as plasmids in *E. coli*. Generalized transduction with bacteriophage P1 was used to introduce plasmids into *M. xanthus* (16). The plasmids do not replicate in *M. xanthus*, but recombination results in the replacement of the wild-type gene with the mutant gene or the formation of a merodiploid containing two copies of the gene. When only merodiploids were obtained, we used generalized transduction with the myxophage Mx4 to generate the gene replacements as previously described (17). *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 $MgSO_4$ in 10 mM Tris buffer (pH 7.6) (CYE) at 33°C on a rotary shaker at 225 rpm. CF agar, used to test fruiting competence and motility, was prepared as previously described (8).

For routine analysis of potential chemoeffectors, starvation in broth was initiated by harvesting vegetative cells (grown to a cell density of 3×10^8 to 5×10^8 cells per ml) by centrifugation and resuspending them at 3×10^8 cells per ml in a medium consisting of 8 mM $MgSO_4$, 1.5 mM $(NH_4)_2SO_4$, 1 mM KH_2PO_4, and 10 mM Tris (pH 7) (CFS) supplemented with 9.1 mM pyruvate and 0.4 mM methionine (CFSPM). Cultures were incubated with shaking at 33°C for 60 min. Test compounds were added to starved cells, and incubation was continued for an additional 60 min. Cells were concentrated by centrifugation for 3 min at $10,000 \times g$, frozen in a dry ice-ethanol bath, and stored at −70°C until needed. When the test compounds were insoluble lipids, we formed emulsions of the lipids in water by sonication and rapidly added an aliquot of the emulsion to the cells.

Western blotting (immunoblotting). The cell pellets described above were resuspended in SDS loading buffer, and samples (containing 10 μg of protein) were separated by SDS-PAGE as previously described (12). Fractionated proteins were transferred to nitrocellulose and stained with Ponceau S (Sigma Chemical Co.). The blots were blocked with 10% dried milk in a buffer consisting of 50 mM Tris-HCl [pH 7.5] and 150 mM NaCl overnight at 4°C. Antiserum against FrzCD (12) was added (1:1,000 dilution) and allowed to adsorb for 60 min. Bound antibody was detected with the Amersham detection kit for rabbit antibodies (biotinylated donkey anti-rabbit antibody and streptavidin-linked alkaline phosphatase) or with Protein A-alkaline phosphatase (Pierce Chemical Co).

Localization of FrzCD. Cells were grown vegetatively in CYE to mid-log phase (approximately 5×10^8 cells per ml). Aliquots were centrifuged at $10,000 \times g$ for 2 min and resuspended to 2×10^9 cells in 1 ml of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.2) with or without salt supplementation (250 mM NaCl). The cells were disrupted by sonication (3 × 30 1-s bursts; power level, 2) with a Branson Sonifier 450. Cell debris was pelleted by centrifugation for 15 min at $10,000 \times g$. After ultracentrifugation at $100,000 \times g$ for 1 h, the supernatant was removed and the pellet was redissolved in an equivalent volume of 10 mM HEPES buffer (pH 7.2). Twenty-microliter samples of supernatant and of resuspended pellet were separated by SDS-PAGE, and the amount of FrzCD protein was estimated by Western blotting as described above.

Pulse-chase analysis. Vegetative cells were grown overnight at 33°C with shaking in 50 ml of a medium consisting of 5 g of Casitone per liter, 1.0 mM KH_2PO_4, 8.0 mM $MgSO_4$, and 10 mM Tris buffer (pH 7.6) with 0.5 mCi of [^{35}S]methionine (Amersham; 1,000 Ci/mmol) to a final cell density of 5×10^8 cells per ml. The cells were harvested by centrifugation, washed free of exogenous label, and incubated at the same

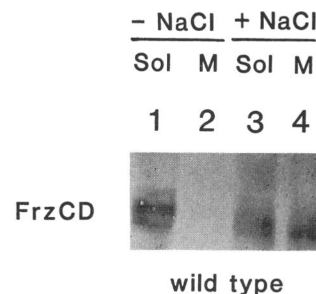

FIG. 1. Localization of FrzCD. Vegetatively grown cells were disrupted by sonication in HEPES buffer (lanes 1 and 2) or HEPES buffer plus 250 mM NaCl (lanes 3 and 4). Cellular debris was removed by centrifugation at $10,000 \times g$ for 15 min. Insoluble material was pelleted from the supernatant fraction by centrifugation at $100,000 \times g$ for 1 h. The pellet was dissolved in HEPES buffer, the proteins in both the $100,000 \times g$ pellet (lanes 2 and 4) and supernatant (lanes 1 and 3) fractions were separated by SDS-PAGE and transferred to nitrocellulose, and FrzCD was detected with anti-FrzCD antiserum.

cell density for 15 min in CYE growth medium at 33°C with shaking. At this time (T_0), a sample of the cells was harvested by centrifugation and stored as a cell pellet at −70°C. The remaining cells were harvested by centrifugation and resuspended in the starvation buffer (CFSPM). After 45 min of incubation at 33°C, yeast extract was added to a final concentration of 5.0 g/liter, and the incubation was continued for an additional 45 min. Aliquots of these cells were harvested and frozen for later analysis as described above. The cells were lysed, and FrzCD was immunoprecipitated as described previously (12). The immunoprecipitate was suspended in loading buffer, the proteins were separated by SDS-PAGE, and the labelled bands were visualized by fluorography.

In vivo labelling of FrzCD with [^{3}H]SAM. Cells were grown in CYE broth to about 5×10^8 cells per ml, harvested by centrifugation, and resuspended in CFSPM at the same cell density. After 45 min of incubation at 33°C, 25 μCi of *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]SAM) was added, and incubation was continued for another 15 min. Lauryl alcohol (final concentration, 1 mg/ml) was added to one sample, and incubation was continued for another hour. Cells were harvested by centrifugation and lysed, and FrzCD was immunoprecipitated, separated by SDS-PAGE, and detected as previously described (12).

Video microscopy. The motility behavior of cells on CFS medium supplemented with 9.1 mM pyruvate (CFSP) was examined by the method previously described (12), except that agarose was used instead of agar and the temperature of incubation was 27°C.

RESULTS

Localization of FrzCD in cell extracts. The FrzCD protein (417 amino acids) contains a region of about 250 amino acids which is similar to the C-terminal portion of the enteric MCPs but lacks regions similar to the N-terminal transmembrane and periplasmic domains (11). As a first step toward understanding the function of FrzCD, we determined its localization in cell extracts. Cells were suspended in 10 mM HEPES buffer and disrupted by sonication. Insoluble material was sedimented by centrifugation at $10,000 \times g$ for 15 min and at $100,000 \times g$ for 60 min. As demonstrated in Fig.

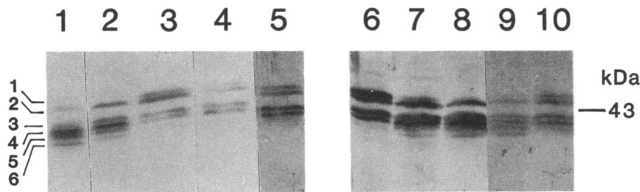

FIG. 2. Factors affecting the modification state of FrzCD. Cells were harvested from CYE broth (lane 1), transferred to CYE agar (lane 2), CF agar (lane 3), CF liquid (lane 4), or starvation buffer (HM) (lane 5), and incubated for 1 h at 33°C. Cells were collected by centrifugation and suspended in SDS loading buffer, and proteins were separated by SDS-PAGE, transferred to nitrocellulose, and detected with anti-FrzCD. For samples in lanes 6 to 8, cells were incubated in CFSPM for 1 h to cause the shift to the more slowly migrating forms of FrzCD and then incubated for an additional hour with no addition (lane 6), 10 g of Casitone per liter (lane 7), or 5 g of yeast extract per liter (lane 8). For samples in lanes 9 and 10, cells were incubated in CFSPM for 1 h, harvested by centrifugation, and resuspended in vegetative growth medium (CYE) (lane 9) or spent CYE (which had previously supported growth of *M. xanthus*) to a density of 2×10^9 cells per ml (lane 10). Multiple forms of FrzCD corresponding to bands 1 to 6 are labelled on the left (see Results).

1, most of the FrzCD remained in the soluble (S100) fraction. The addition of 250 mM NaCl to extracts caused a substantial amount of FrzCD to sediment at $100{,}000 \times g$. The addition of other salts (LiCl, KCl, or NaH_2PO_4) gave the same result (data not shown). The pelleting of FrzCD was not caused by elevated salt concentrations alone but required the presence of the membrane fractions. When we added salt to S100 fractions containing FrzCD and then repeated the centrifugation at $100{,}000 \times g$, FrzCD remained in the soluble fraction (data not shown).

Modification of FrzCD in response to incubation of cells in different media. FrzCD migrates as a ladder of bands on high-resolution SDS-PAGE, as do the MCPs from enteric bacteria. Modification of FrzCD by methylation/demethylation and deamidation is responsible for much or all of this heterogeneity (12). Increased levels of methylation apparently cause the protein to migrate more rapidly through SDS-polyacrylamide gels, although there is little change in the actual molecular weight of the protein. We were interested in using the migration patterns of FrzCD as an assay of the extent of methylation of the protein. The FrzCD protein was visualized by the Western blot procedure with antiserum prepared against FrzCD. Figure 2 shows that extracts prepared from cells growing exponentially in CYE, a very rich medium, contained multiple forms of FrzCD corresponding to bands 2 to 6 (Fig. 2, lane 1). In this blot and in all following blots, the numbered bands correspond to the following molecular sizes (in kilodaltons): 1, 45; 2, 44; 3, 42.5; 4, 41.5; 5, 41; and 6, 40. Previously reported data indicate that the most likely interpretation of the bands is as follows. Band 2 is most likely the unmodified form of the protein. Band 1 probably corresponds to a form in which one or several glutamine residues have been deaminated to glutamate, generating potential sites for methylation. Bands 3, 4, 5, and 6 correspond to methylated forms of FrzCD. These bands migrate to the same apparent molecular weights as the methylated bands previously observed (12). Presumably, the faster-migrating bands contain more methyl groups.

Since FrzCD is involved in controlling cell movements and cells move only when they are present on a solid surface, we tested the effect of transfer of cells to such surfaces on the modification state of FrzCD. Transfer of cells from CYE broth to CYE agar and incubation for 1 h had little or no effect on the pattern of bands of FrzCD when analyzed by SDS-PAGE (Fig. 2, lane 2). However, when we transferred cells from CYE broth to the nutritionally poor CF agar (a medium that we routinely use for studying motility and to induce the development of fruiting bodies) and incubated these cells for 1 h, there was a change in the modification state of FrzCD (lane 3). Bands 5 and 6 were no longer present, and band 1 was now clearly evident. This constitutes a shift from faster-migrating (presumably more-methylated) forms of FrzCD to more slowly migrating (presumably less-methylated) forms. Transfer of cells from CYE broth to CF broth (lane 4) resulted in a shift in the banding pattern identical to that observed during incubation on CF agar. CF broth could be replaced by CF salts (Casitone, pyruvate, and citrate omitted from CF broth; data not shown), 10 mM HEPES buffer (pH 7.2) containing 10 mM $MgCl_2$ (lane 5), or CFSPM (lane 6), with the same result. The shift in banding pattern induced by transfer from rich media to buffer was reversible. When we transferred cells from CFSPM to growth medium (CYE) (lane 9), band 1 disappeared and bands 4 and 5 became more intense. When we resuspended the cells in "spent" CYE, which had supported growth of *M. xanthus* to a density of 2×10^9 cells per ml, FrzCD was not modified (lane 10). We tested several of the components of CYE and found that the addition of Casitone to a final concentration of 10 g/liter (lane 7) or yeast extract to 5 g/liter (lane 8) resulted in increased methylation of FrzCD (compare with lane 6).

Figure 3 illustrates the length of time over which the modifications of FrzCD took place. Transfer of cells from CYE to CFSPM resulted in an almost immediate shift in the banding pattern (compare lanes 1 and 2 of panel A), and modification was apparently complete or in steady state after 60 min of incubation at 33°C. Similarly, addition of yeast extract to cells in CFSPM resulted in a shift in the banding pattern, which occurred over a period of approximately 60 min.

The mobility changes in FrzCD described above could be the result of modification of preexisting FrzCD or could be due to degradation of preexisting FrzCD and synthesis of new forms of the protein. To address this point, we performed a pulse-chase experiment. Cells were labelled with [^{35}S]methionine as described in Materials and Methods. The labelled cells were starved in CFSPM for 45 min. Yeast extract was added to a concentration of 5 g/liter, and the incubation was continued for an additional 45 min. Aliquots were removed at various times during this experiment, and the cells were concentrated by centrifugation and frozen at −70°C for later analyses. At the conclusion of the experiment, the cells were lysed and FrzCD was immunoprecipitated, suspended in SDS loading buffer, and separated by SDS-PAGE. In vegetative cells, bands 1 and 2 were very weak and bands 3, 4, 5, and 6 were prominent, with the lowest band (band 6) being the most intense (Fig. 4, lane 1). During incubation in CFSPM for 45 min, there was a shift to the more slowly migrating forms of FrzCD. Bands 5 and 6 disappeared, and bands 1 and 2 increased in intensity (lanes 2 and 3). After the addition of yeast extract, there was a shift back to the faster-migrating forms. Bands 1 and 2 disappeared, and bands 5 and 6 reappeared (lanes 4 and 5). These results indicate that the nutritional shifts resulted in modification of preexisting FrzCD.

The effect of various chemicals on methylation/demethyl-

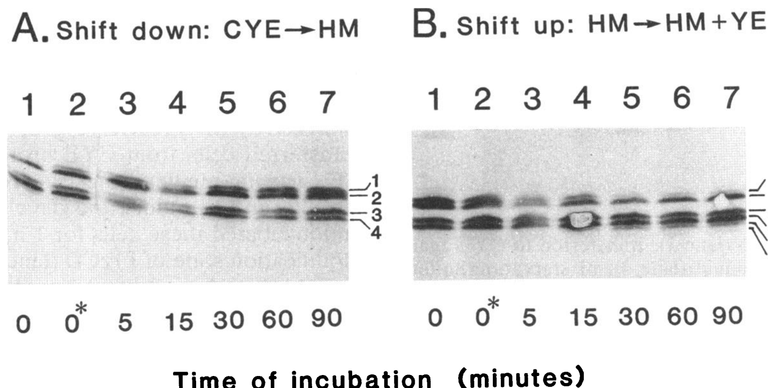

FIG. 3. Time course of response to starvation and of addition of yeast extract. (A) Vegetative cells were harvested from CYE and analyzed by Western blotting (lane 1) or resuspended in starvation buffer (HM), which consists of 10 mM HEPES buffer (pH 7.2) containing 8 mM $MgSO_4$. Samples in HM were removed for Western blot analysis immediately after resuspension (0*, lane 2) or after 5, 15, 30, 60, and 90 min of incubation at 33°C (lanes 3 to 7). (B) Cells were incubated for 1 h in HM (lane 1). Yeast extract (YE) was added, and samples were removed for analysis immediately (lane 2) or after 5, 15, 30, 60, and 90 min (lanes 3 to 7). The bands labelled 1 to 4 (A) and 1 to 6 (B) correspond to the multiple forms of FrzCD as described in Results.

ation of FrzCD. The effects of yeast extract and Casitone on the modification of FrzCD prompted us to test the effect of addition of a wide variety of substances to cells in CFSPM. As mentioned above, addition of yeast extract at 5 to 10 mg/ml resulted in increased mobility of FrzCD. However, at lower concentrations (1.0 mg/ml) or higher concentrations (20 mg/ml), it had little or no effect. Casitone caused increased mobility of FrzCD when added at concentrations in excess of 5 mg/ml (5.0 to 40 mg/ml tested) but had no effect when added at a concentration of 1.0 mg/ml. Vitamin-free Casitone (Difco) had the same effect as Casitone, whereas Casamino Acids (10 or 20 g/liter) gave no response. The addition of each of the common 20 amino acids at 0.6 mM individually and of a pool of all of the amino acids at 0.1 mM each had no effect on FrzCD mobility. Similarly, the addition of 2.5 mM adenine, uracil, thymine, guanine, cytosine, adenosine, guanosine, cytidine, uridine, cyclic guanosine monophosphate, or cyclic adenosine monophosphate; 0.2 or 2 mM SAM; 2.5 mM D-alanine, *N*-acetylglucosamine, or diaminopimelic acid; 10 or 50 mM glucosamine or mannosamine; or a vitamin mixture containing (milligrams per liter) ascorbic acid (0.5), folic acid (0.5), thiamine (0.5), pyridoxine HCl (0.5), riboflavin (0.2), niacinamide (0.2), pantothenic acid (0.2), cyanocobalamin (0.05), and biotin (0.05) had no effect on FrzCD modification. Several intermediary metabolites such as fumarate, acetate, ketoglutarate, glycerophosphate (all tested at 0.1 and 1.0 g/liter) had no effect on FrzCD modification. We also tested several macromolecules since myxobacteria are specialized for growth on such complex substrates. The addition of 1 g of salmon sperm DNA per liter, 10 g of bovine serum albumin per liter, or 1 g of *S. typhimurium* lipopolysaccharide per liter had no effect on the banding pattern of FrzCD.

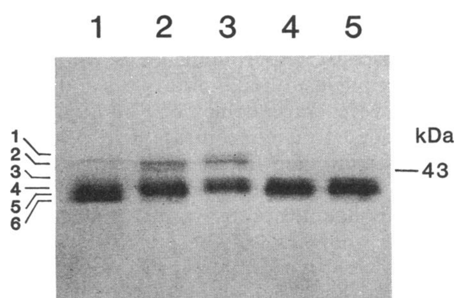

FIG. 4. Pulse-chase analysis of FrzCD modification. Cells were labelled with [^{35}S]methionine, washed free of exogenous label, and incubated in CYE growth medium for an additional 15 min. At this time (T_0), cells were collected by centrifugation, resuspended in CFSPM, and incubated for 45 min. Yeast extract was added to a final concentration of 5.0 g/liter, and incubation was continued for an additional 45 min. Aliquots were removed at the times indicated below. The cells were concentrated by centrifugation and lysed, and FrzCD was immunoprecipitated and analyzed by SDS-PAGE. Lanes: 1, T_0; 2, 15 min after transfer to CFSPM; 3, 45 min after transfer to CFSPM; 4, 15 min after addition of yeast extract; 5, 45 min after addition of yeast extract. Multiple forms of FrzCD corresponding to bands 1 to 6 are labelled on the left (see Results).

An analysis of the factor(s) in yeast extract that results in modification of FrzCD revealed that this material was somewhat hydrophobic, so we tested the effect of various lipids on FrzCD modification. In our initial screening, we found two phospholipids (bovine cardiolipin and phosphatidic acid) which caused increased mobility of FrzCD when added to cells at a concentration of 1 g/liter. In contrast, many other lipids, including several other phospholipids, had no effect. We tested the component parts of the phospholipids and found that several fatty acids (lauric acid and capric acid at concentrations of 0.2 to 1.0 g/liter; Fig. 5, lanes 3 and 5) also caused a shift toward the more rapidly migrating forms of FrzCD. Lower concentrations of these fatty acids (0.1 g/liter) had little or no effect. A variety of other fatty acids had no effect on FrzCD mobility (Table 2). Addition of lauryl alcohol had the same effect as addition of lauric acid (Fig. 5, lane 4). Chemically synthesized phosphatidylethanolamine (PE) containing lauric acid caused increased mobility of FrzCD, whereas PE containing myristic acid or palmitic acid did not (Fig. 5, lanes 6 to 8). A detergent containing lauric acid (polyoxyethylenesorbitan monolaurate, Tween 20) also resulted in increased mobility of FrzCD, whereas detergents containing palmitic acid (polyoxyethylenesorbitan monopalmitate, Tween 40), stearic acid (polyoxyethylenesorbitan monostearate, Tween 60), or oleic acid (polyoxyethylenesorbitan monooleate, Tween 80) did not (Fig. 5, lanes 9 to 12). Of a variety of other lipids tested, including sphingomyelin,

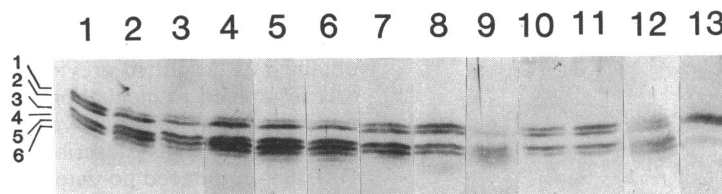

FIG. 5. Effect of lipids, solvents, and detergents on FrzCD modification. Cells were incubated in CFSPM for 1 h and then transferred to test tubes containing the chemicals to be tested. After incubation for an additional hour, the cells were collected and the mobility of FrzCD was analyzed as described in Materials and Methods. Lanes: 1, no addition; 2, yeast extract (5 g/liter); 3, lauric acid (1 g/liter); 4, lauryl alcohol (1 g/liter); 5, capric acid (1 g/liter); 6, PE-dilauroyl (1 g/liter); 7, PE-dimyristoyl (1 g/liter); 8, PE-dipalmitoyl (1 g/liter); 9, Tween 20 (1 g/liter); 10, Tween 40 (1 g/liter); 11, Tween 60 (1 g/liter); 12, Tween 80 (1 g/liter); 13, isoamyl alcohol (1 g/liter). Multiple forms of FrzCD corresponding to bands 1 to 6 are labelled on the left (see Results).

squalene, phosphatidylserine (bovine brain), phosphatidylinositol (soybean), lysolecithin, and lipoic acid (all tested at 1 g/liter), only lipoic acid resulted in increased mobility of FrzCD (Table 2). None of the lipids tested was effective at concentrations below 0.1 g/liter.

Additions of propanol, isopropanol, butanol, isobutanol, or isoamyl alcohol at concentrations of 1 g/liter caused decreased mobility of FrzCD (Fig. 5, lane 13, and data not shown). Ethanol gave a similar effect but only at higher concentrations (3.0 g/liter). Isoamyl alcohol gave the strongest response, so we determined the minimum concentration of this alcohol required for an observed effect. A concentration of 0.1 g/liter (1.1 mM) resulted in a noticeable change in FrzCD mobility, whereas a concentration of 0.05 g/liter did not. Some other solvents also resulted in decreased mobility of FrzCD (Table 2).

Table 2 summarizes the effects of these various chemicals on the modification state of FrzCD. The alcohols that resulted in decreased mobility of FrzCD (demethylation) did not affect cell morphology at the concentrations tested. Isopropanol and isoamyl alcohol, which were chosen for further studies, allowed cell movement and growth at the concentrations employed in these experiments. In contrast, except for yeast extract and Casitone, all of the compounds that resulted in increased mobility of FrzCD (increased methylation) were toxic to the cells. These compounds inhibited motility and caused cell death upon extended exposure. For example, the addition of lauric acid or lauryl alcohol (1.0 g/liter) caused many of the cells to become spherical in shape, apparently forming spheroplasts. These cells eventually lysed after several hours of incubation. This effect was observed to a lesser extent with the detergent Tween 20. The other detergents, fatty acids, and lipids which did not alter the mobility of FrzCD were also toxic to the cells. They inhibited motility and caused cell protoplasting and eventually cell disruption.

The bands observed in the experiments mentioned above correspond in apparent molecular weight to those previously observed and demonstrated to be methylated forms of FrzCD. We conducted several experiments to determine that these bands did correspond to different methylated states, rather than proteolytic fragments of FrzCD. The methyl groups on FrzCD are base labile (12). Figure 6 demonstrates the effect of base treatment of protein samples on their migration pattern in SDS-PAGE. In each case, bands 3 to 6 were chased to bands 1 and 2 by base treatment, as expected if bands 3 to 6 corresponded to methylated forms of FrzCD. As further proof of methylation, the addition of lauryl alcohol to cells incubated with [^{3}H]SAM resulted in increased levels of methylation of FrzCD (Fig. 7). These methylated bands migrated at the same apparent molecular weights as bands 3 to 6. Increased labelling of

TABLE 2. Summary of effects of various chemicals on modification of FrzCD[a]

Increased mobility (methylation)	Decreased mobility (demethylation)	No effect[b]
Na laurate (0.2–1.0)	Ethanol (3.0)	Methanol (1.0–3.0)
Na caprate (0.5–1.0)	Propanol (1.0)	Na propionate (1.0)
Lauryl alcohol (1.0)	Isopropanol (1.0)	Na butyrate (1.0)
Lauroyl coenzyme A (1.0)	Butanol (1.0)	Na caproate (1.0)
PE-dilauroyl (1.0)	Isobutanol (1.0)	Na caprylate (0.16)
Tween 20 (0.5–1.0)	Isoamyl alcohol (0.1–3.0)	Na myristate (1.0)
Lipoic acid (1.0)	Chloroform (1.0)	PE-dimyristoyl (1.0)
Casitone (10–40)	Phenethyl alcohol (1.0)	PE-dipalmitoyl (1.0)
Yeast extract (5–10)	Dimethyl sulfoxide (30.0)	Tween 40 (0.1–2.0)
		Tween 60 (0.1–2.0)
		Tween 80 (1.0–2.0)
		Glycerol (37.5)
		Ethanolamine (0.55–5.5)
		Procaine (0.27–2.7)

[a] Chemicals were added to cells, and the mobility of FrzCD on SDS-PAGE was determined as described in the text. The concentrations (in grams per liter) in parentheses indicate the concentrations tested which gave the indicated responses.

[b] See the text for the specific compounds of sugars, amino acids, nucleotides, and vitamins and the concentrations tested.

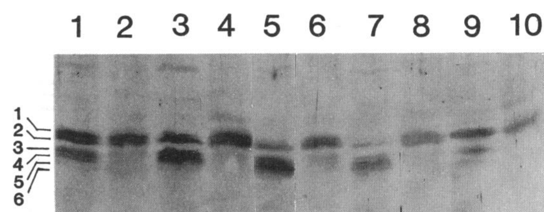

FIG. 6. Base lability of FrzCD modification. Cells were incubated in CFSPM for 1 h and then incubated in CFSPM containing the test compounds for an additional hour. At that time, cells were harvested and disrupted by sonication. Half of each extract was transferred immediately to SDS loading buffer (lanes 1, 3, 5, 7, and 9), and the other half was incubated with 0.5 N NaOH for 30 min at 37°C prior to neutralization and transfer to loading buffer (lanes 2, 4, 6, 8, and 10). The mobility of FrzCD was analyzed as described in Materials and Methods. Lanes: 1 and 2, cells incubated in CFSPM; 3 and 4, yeast extract added; 5 and 6, lauryl alcohol added; 7 and 8, Tween 20 added; 9 and 10, isoamyl alcohol added. Multiple forms of FrzCD corresponding to bands 1 to 6 are labelled on the left (see Results).

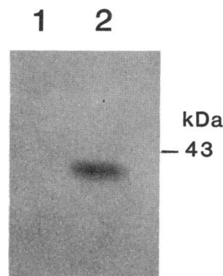

FIG. 7. Increased methylation of FrzCD in response to lauryl alcohol. Cells were incubated in CFSPM for 45 min, at which time [^{3}H]SAM was added. After 15 min of incubation, the cells were transferred to tubes containing no additions (lane 1) or 1 mg of lauryl alcohol per ml (lane 2), and incubation was continued for an additional hour. Cells were harvested and lysed, and FrzCD was immunoprecipitated, separated by SDS-PAGE, and detected by fluorography.

FrzCD was not due to increased uptake of [^{3}H]SAM, since in a control experiment, equivalent amounts of label were taken up by cells in the presence or absence of lauryl alcohol (data not shown).

The effect of mutations in the *frz* genes on the methylation/demethylation of FrzCD. In order to gain further insights into the mechanism of *frz* signal transduction, we examined the ability of the *frz* mutants to modify FrzCD. Since the *frz* genes form one or two operons (25), some of the mutants generated by Tn*5* transposition were likely to be polar and thus affect the expression of other *frz* genes. We therefore prepared extracts of various *frz* mutants and performed Western blot analysis using antisera specific to FrzCD and FrzE (12) and antisera against FrzA and FrzB, which were obtained as described previously (12). Wild-type cells (Fig. 8A) responded to incubation in buffer (lane 2) and to additions of isoamyl alcohol, yeast extract, and lauryl alcohol (lanes 3 to 5) as described above. The *frzA* mutant, DZF1084, produced no detectable FrzA and reduced levels of FrzB and wild-type levels of FrzCD (data not shown). These cells produced several forms of FrzCD but did not alter the level of methylation of FrzCD in response to the stimuli tested (Fig. 8B, compare lanes 4 and 5 with lane 2). The cells did modify FrzCD in response to isoamyl alcohol (demethylation/deamidation), but the response was less dramatic than that observed with wild-type cells (Fig. 8A and B, compare lanes 3 with lanes 2). The *frzB* mutant (DZF4123) made no detectable FrzB but made normal levels of FrzA and FrzCD (data not shown). The *frzB* mutant demethylated FrzCD in response to addition of isoamyl alcohol (Fig. 8C, compare lane 3 with lane 2) but did not methylate FrzCD in response to yeast extract or lauryl alcohol (compare lanes 4 and 5 with lane 2). We tested a variety of mutations in *frzE*. DZF1227, DZF1262, and DZF1444 produced approximately wild-type levels of FrzE protein, whereas the Tn*5* insertion mutant DZF3377 produced a truncated form of FrzE. All produced several methylated forms of FrzCD but did not alter this pattern following stimulation with isoamyl alcohol, lauryl alcohol, or yeast extract (Fig. 8D). The *frzG* mutant responded to stimuli in a manner indistinguishable from the wild type. Additions of yeast extract or lauryl alcohol (Fig. 8E, lanes 4 and 5) resulted in increased methylation of FrzCD, whereas addition of isoamyl alcohol (lane 3) resulted in demethylation. The *frzF* mutant (DZF4023), as expected,

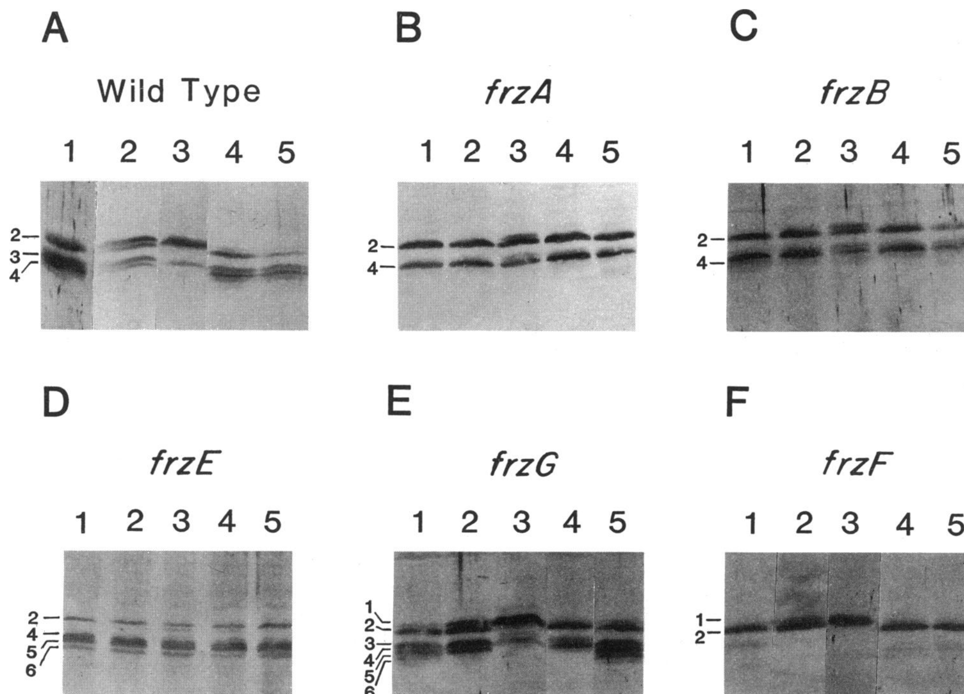

FIG. 8. Effect of mutations in various *frz* genes on modification of FrzCD. Cells were harvested from CYE (lanes 1) and incubated for 2 h in CFSPM (lanes 2), incubated for 1 h in CFSPM and then for 1 h in CFSPM plus isoamyl alcohol (lanes 3), incubated for 1 h in CFSPM and then for 1 h in CFSPM plus yeast extract (lanes 4), or incubated for 1 h in CFSPM and then for 1 h in CFSPM plus lauryl alcohol (lanes 5). (A) Wild-type cells; (B) DZF1084, *frzA*; (C) DZF4123, *frzB*; (D) DZF3377, *frzE*; (E) DZF4021, *frzG*; (F) DZF4023, *frzF*. Multiple forms of FrzCD are labelled on the left (see Results).

TABLE 3. Summary of ability of *frz* mutants to modify FrzCD in response to yeast extract or isoamyl alcohol[a]

Gene (strain)	Response to:	
	Yeast extract (methylation)	Isoamyl alcohol (demethylation/deamidation)
Wild type (DZF1)	+++	+++
frzA (DZF1084)	–	+
frzB (DZF4123)	–	++
frzE (DZF3377)	–	–
frzG (DZF4021)	+++	+++
frzF (DZF4023)	+	+++

[a] Yeast extract and isoamyl alcohol were added to the cells, and the mobility of FrzCD on SDS-PAGE was determined as described in the text.

was deficient in its ability to methylate FrzCD in response to additions of yeast extract or lauryl alcohol. It did not produce bands 3 to 6 under any conditions tested (Fig. 8F). There was a slight shift from band 1 to band 2 following the addition of yeast extract (compare lanes 2 and 4 or 5), which may indicate some low level of residual methylation activity or some other modification. Essentially the same results were obtained with six other *frzF* mutants (DZF3593, DZF4142, DZF1313, DZF1359, DZF1421, and DZF1434) (data not shown). The addition of isoamyl alcohol to the *frzF* mutants resulted in the shift from band 2 to band 1 (compare lanes 2 and 3 in Fig. 8F) as observed with the wild type. The data described above are summarized in Table 3.

Motility and frequency of reversal of *M. xanthus* can be affected by chemicals which cause methylation/demethylation of FrzCD. Since FrzCD is a methyl-accepting protein needed for directional control of cell movement, it seemed likely that stimuli which alter the modification state of FrzCD should have an effect on the frequency of cell reversal. The alcohols isopropanol and isoamyl alcohol were previously found to cause demethylation of FrzCD (Fig. 5). In the presence of isoamyl alcohol, wild-type cells showed a dramatic increase in cell reversals (Table 4). The addition of isopropanol had essentially the same effect. In both cases, cells moved about 0.5 to 1 cell length between reversals and thus did not migrate from their initial location during several hours of incubation. Cell motility was inhibited by many of the substances which promoted methylation of FrzCD (lauric acid, lauryl alcohol, Tween 20, and yeast extract). However, this is unlikely to be due to a specific effect on the *frz* system since these substances, with the exception of yeast extract, were toxic to cells and eventually caused cell lysis. Casitone at 10 g/liter, also an effective stimulator of FrzCD methylation, did not significantly inhibit motility but had no effect on the reversal frequency of cells. Cells transferred from CYE growth medium to CFSP-agarose reversed 15.7 (standard deviation, 5.2) times per h, whereas cells transferred to CFSP-agarose with 10 g of Casitone per liter reversed 17.7 (standard deviation, 3.5) times per h. It should be noted, however, that the cells were incubated on the agarose medium for 30 min before cell reversals were counted. This was necessary because most cells did not move immediately after transfer to agarose. Therefore, a transient effect of the presence or absence of Casitone on cell reversal frequency might have been missed.

TABLE 4. Effect of isoamyl alcohol on frequency of reversals of wild-type cells and *frz* mutants[a]

Gene (strain)	*che* homolog	No. of reversals (SD)/h	
		Agarose slide	Agarose slide with isoamyl alcohol
Wild type (DZF1)		15.1 (3.3)	55.8 (11)
frzA (DZF3373)	*cheW*	1.1 (1.3)	2.3 (2.7)
frzA (DZF1084)	*cheW*	6.1 (4.8)	4.1 (2.3)
frzB (DZF4123)		1.0 (1.5)	39.5 (18)
frzCD (DZF4017)	MCPs	1.0 (1.2)	3.8 (3.4)
frzD (DZF3460)	MCPs	34.5 (4.9)	47.0 (7.9)
frzE (DZF3377)	*cheA cheY*	0.3 (0.6)	1.0 (1.4)
frzE (DZF1444)	*cheA cheY*	2.5 (1.2)	3.7 (3.4)
frzF (DZF4023)	*cheR* (methyltransferase)	1.1 (1.1)	53.6 (13)
frzG (DZF4021)	*cheB* (methylesterase)	8.1 (3.2)	46.5 (6.4)
frzCD, -E, -G, -F deletion (DZF4059)		0.9 (1.3)	4.4 (3.2)

[a] Five microliters of a cell sample (2×10^7 cells per ml) spotted onto CFSP agarose or CFSP agarose containing 1 g of isoamyl alcohol per liter. The spot was allowed to dry, after which cells were covered with an oxygen-permeable membrane (12) and incubated at 27°C. After 30 min of incubation, cells were examined by video microscopy for 1 h. The results shown indicate the average number of reversals per h from observation of at least 15 cells.

If FrzCD behaves like the MCPs from enteric bacteria, then methylation/demethylation of FrzCD should occur during adaptation to a stimulus. Cells deficient in components of the adaptation machinery but containing an intact signal transmission pathway might still respond to stimuli by reversing but never adapt to their prestimulus behavior. We examined the effect of isoamyl alcohol on the behavior of the various *frz* mutants in an attempt to determine which proteins are central to signal transmission and which are more likely involved in adaptation. The results presented in Table 4 indicate that cells of the wild-type strain, *frzB* mutants, *frzG* mutants, and *frzF* mutants all respond to isoamyl alcohol by reversing very frequently. In contrast, cells with mutations in *frzA*, *frzCD*, and *frzE* or cells from which *frzCD*, *-E*, *-F*, and *-G* had been deleted responded to isoamyl alcohol with no significant increase in reversal frequency. *frzD* cells reverse frequently even in the absence of any additions to CFSP-agarose. Addition of isoamyl alcohol caused these cells to increase their frequency of reversals slightly, although given the large standard deviations, this increase in reversals may not be significant. We were concerned about the possibility of polar mutations influencing the expression of downstream genes in these experiments. Whenever possible, we therefore chose strains whose mutations were less likely to exhibit polarity. For example, the *frzA* mutant DZF3373 contains a transposon in *frzA* and produces no FrzB, as determined by Western blot analysis. We therefore also analyzed the motility of *frzA* mutant DZF1084, which produces no FrzA but does make FrzB (although at reduced levels). Similarly, all of our mutants in *frzCD* contained polar mutations which did not allow the expression of *frzE*. We therefore constructed and analyzed a strain (DZF4017) with the transposon Tn*5tac*1 inserted in the *frzCD* gene, to mutate *frzCD* without eliminating *frzE* expression. In the presence of 1.0 mM IPTG, these cells produced approximately wild-type levels of FrzE as determined by Western blot analysis (data not shown). Finally, the *frzE* mutant DZF3377 contains a transposon in the *frzE* gene and, therefore, may be polar on *frzG* and *frzF*. We therefore analyzed *frzE* mutant DZF1444, which makes wild-type levels of apparently nonfunctional FrzE protein and is less likely to be polar on *frzG* and *frzF*.

The analyses described above were performed at low cell

density (10 to 20 cells per field of view) to facilitate observations of cell movements and to avoid cell interactions. In their natural state, however, *M. xanthus* cells are normally in contact with their neighbors in a swarm composed of thousands of cells. We therefore analyzed the effect of the addition of isoamyl alcohol on the behavior of cells at higher cell density. The wild-type strain (DZF1) formed flares of cells in the absence of isoamyl alcohol but not in its presence. Cells of the *frzG* mutants behaved similarly. Cells with mutations in *frzA*, *frzCD*, or *frzE* formed the tangled filaments of cells which are characteristic of the frizzy mutants in the presence or absence of isoamyl alcohol. Cells with mutations in *frzB* or *frzF* formed frizzy filaments in the absence of isoamyl alcohol but did not exhibit this multicellular behavior in its presence, presumably because the cells were continuously reversing in place.

DISCUSSION

Modification of FrzCD provides a handle for the study of sensory transduction in *M. xanthus*. *M. xanthus* moves by gliding motility, a poorly understood motility system that does not involve flagella (5, 21). Evidence of coordinated cell movements are observed during vegetative growth on a solid surface and even more dramatically during aggregation leading to formation of fruiting bodies (23). These coordinated movements require the *frz* genes which are involved in controlling the rate of cell reversals (4). The *frz* genes encode proteins that are homologous to all of the major enteric chemotaxis proteins, with the exception of CheZ. FrzCD protein, which is homologous to a family of methyl-accepting chemotaxis receptors from enteric bacteria, can be methylated in vivo (12). In enteric bacteria, methylation/demethylation of the MCPs is required for adaptation to changes in the environment. We assumed that methylation of FrzCD might perform a similar function. Since little is known about nutrients or chemicals which can be specifically recognized by *M. xanthus*, we attempted to use the methylation state of FrzCD of vegetative cells as an assay for such substances and to test the effects of these chemicals on cell movements. It should be mentioned that it is possible that a different complement of signals is recognized by developmental cells. Vegetative cells probably respond to chemicals which indicate the presence of a food source and also to signals produced by their neighbors, so that they can maintain an effective feeding swarm. Developmental cells may respond to the same or different signals produced by their neighbor cells, to coordinate aggregation and fruiting body formation.

Modification of FrzCD. Cells of *M. xanthus* produced at least six different forms of FrzCD as indicated by discrete bands on one-dimensional SDS-PAGE. Previously reported data indicate that band 2 is probably the unmodified form of the protein (12). Band 1 probably corresponds to a form in which a specific glutamine residue(s) has been deamidated (a reaction most likely catalyzed by FrzG) to generate an additional site(s) for methylation. Bands 3 to 6 correspond to the methylated forms of the protein (they migrate at the same apparent molecular weights as the methylated bands that were previously identified [12]). Presumably the faster-migrating bands contain more methyl groups per protein molecule. The exact pattern of bands observed was dependent on the culture conditions. Cells harvested from rich medium (CYE) contained at least four forms of FrzCD, with the faster-migrating (methylated) forms of the protein predominating. Transfer of cells to buffer caused a shift to the more slowly migrating (less-methylated) forms.

We tested the effect of a variety of potential stimuli on the modification state of FrzCD. The addition of yeast extract or Casitone caused increased levels of the faster-migrating, presumably more-methylated, forms of FrzCD. The results of a pulse-chase analysis indicated that at least some of the modifications to FrzCD, following the addition or removal of yeast extract, were to preexisting FrzCD. However, the extent of modification of preexisting FrzCD appeared to be less than that for total FrzCD. This may be due to the synthesis of additional FrzCD during the incubation period and could indicate that newly synthesized FrzCD was preferentially modified during the nutritional shifts. The addition of lauric acid, capric acid, lipoic acid, lauryl alcohol, Tween 20, or phosphatidylethanolamine containing lauric acid also caused a shift to the faster-migrating forms of the protein. We believe that this shift is due to methylation of FrzCD for the following reasons. (i) The FrzCD sequence contains several stretches of amino acids that resemble the sites surrounding the methylatable glutamates of enteric MCPs. (ii) Methylation of enteric MCPs results in increased mobility on SDS-PAGE. (iii) The modified bands of FrzCD migrate at the same position as the methylated bands previously identified (12). (iv) Glutamate methyl esters are base labile. When we treated samples with base before electrophoresis, the faster-migrating bands were chased into the more slowly migrating bands. (v) Addition of lauryl alcohol resulted in increased incorporation of label from [^{3}H]SAM into FrzCD. In contrast to the compounds listed above, a variety of other fatty acids, lipids, and detergents had no effect on the modification state of FrzCD. The addition of some solvents, or of alcohols with chain lengths between 2 and 5, resulted in increased levels of the more slowly migrating, less-methylated forms of FrzCD.

The effects of these chemicals on the methylation state and the mobility of FrzCD in SDS-PAGE are reminiscent of the behavior of *E. coli* MCPs following stimulation by attractants or repellants (10) and are quite different from the results obtained for *B. subtilis*. *B. subtilis* does not alter the absolute level of methylation of its MCPs following stimulation but does alter the rate of turnover of these methyl groups (2).

The factors in yeast extract or Casitone which result in methylation may be signals that are recognized by cells as they digest and feed on their prey organisms. These signals may attract myxobacteria to, or keep them near, their prey. Since spent media did not stimulate methylation of FrzCD, we speculate that the factors in yeast extract and Casitone are consumed or altered during cell growth. We do not know why C_{10} and C_{12} saturated fatty acids, alcohols, and their derivatives result in increased methylation of FrzCD, whereas many other lipids and fatty acids do not. We do not believe that these chemicals are physiologically relevant signals. All of the lipid molecules that resulted in increased methylation were toxic to cells after prolonged incubation. They inhibited motility and caused protoplasting and eventually lysis of the cells. These molecules may be chemically similar to the actual signal molecules that are recognized by *M. xanthus*. They may interact specifically with the receptor protein(s) in the cell membrane which then interacts with FrzCD in the cytoplasm. Alternatively, these lipids may act less specifically by accumulating in the cell membrane and altering the environment around the putative receptor proteins. The chain length of these compounds may be important in determining how they affect these proteins. We do not

believe that the toxic effects of these molecules are responsible for the observed methylation of FrzCD, since a variety of other lipids and detergents that also caused cell protoplasting and/or lysis did not cause methylation of FrzCD. The short-chain alcohols and other solvents which caused decreased mobility of FrzCD were, in general, not toxic to the cells at the concentrations used. It is unlikely that these molecules are physiologically relevant signals, since relatively large amounts of the solvents (millimolar concentrations) were required to observe an effect. These solvent molecules may enter the cell membrane and affect its fluidity or alter the conformation of some receptor proteins in the membrane. Local anesthetics such as procaine and phenethyl alcohol are membrane perturbants that have been demonstrated to influence gene expression in *E. coli*, presumably by interacting with the membrane protein, EnvZ, which is involved in signal transduction (22). Phenethyl alcohol (1.0 g/liter) did cause decreased mobility of FrzCD in *M. xanthus*, but procaine (1.0 or 10 mM) had no effect (Table 2).

Localization of FrzCD. The results of the cell fractionation experiment (Fig. 1) indicate that FrzCD is a soluble protein, unlike the enteric MCPs which are integral membrane proteins. This result confirms the predictions made on the basis of sequence analysis of *frzCD* (11). A considerable amount of FrzCD (30 to 60%) did pellet with the membrane fraction when salt was added to the extracts. This may indicate that FrzCD is loosely associated with the membrane, with membrane proteins, or with large protein complexes via hydrophobic interactions. Increasing the salt content of the extract would be expected to enhance such interactions. In control experiments, we removed the membranes by high-speed centrifugation, leaving FrzCD in solution. When we added salt to these samples and repeated the centrifugation, virtually none of the FrzCD sedimented, indicating that FrzCD is not simply precipitated by the elevated salt concentrations but most probably interacts with some other insoluble component in the presence of salt.

Effects of *frz* mutations on methylation/demethylation of FrzCD. Cells with mutations in *frzA* were apparently able to methylate FrzCD, as judged by the appearance of multiple bands on Western blots, but did not alter the level of modification in response to additions of yeast extract or lauryl alcohol, factors that cause increased methylation of FrzCD in wild-type cells. These cells were able to demethylate/deamidate FrzCD in response to isoamyl alcohol, but the response was much less dramatic than that observed for wild-type cells. It should be noted that the *frzA* mutant DZF1084 contains a mutation that is polar on *frzB*. Cells with mutations in *frzA* produced only traces of FrzB (approximately 10-fold less than wild-type cells, as judged by Western blot analysis). Cells with mutations in *frzB* were apparently able to methylate FrzCD but were unable to alter the level of modification in response to yeast extract or lauryl alcohol. They did respond to the addition of isoamyl alcohol by demethylation and/or deamidation of FrzCD. Cells with mutations in *frzE* were apparently able to methylate FrzCD, as judged by the appearance of multiple bands, but did not alter the level of modification in response to additions of yeast extract, lauryl alcohol, or isoamyl alcohol. Cells with mutations in *frzF* were deficient in their ability to methylate FrzCD, as judged by the absence of bands 3 to 6 and by our previous results (12). Addition of yeast extract or lauryl alcohol did cause a slight shift from band 1 to band 2. We interpret these results to indicate that some of the completely demethylated and deamidated forms of the protein (corresponding to band 1 in Fig. 8) become methylated at one site during this incubation, suggesting some residual *frzF* methyltransferase activity of the mutant. Alternatively, some other enzyme may modify (methylate?) FrzCD at a low level in the absence of FrzF. This second alternative may be more likely, since each of seven *frzF* mutants gave the same results. *frzF* cells did respond to additions of isoamyl alcohol by decreasing the mobility of FrzCD, suggesting that these cells are able to deamidate FrzCD in response to stimuli. Cells with mutations in *frzG* responded to the additions of yeast extract, lauryl alcohol, or isoamyl alcohol by modifying FrzCD in a manner indistinguishable from wild-type cells. This finding was a surprise. We predicted that mutations in *frzG*, the homolog to the chemotactic methylesterase and deamidase CheB, would result in the inability of the cells to demethylate or deamidate FrzCD (12). Our interpretation of the Western blot data, however, suggests that these mutants are still able to perform these enzymatic activities and to regulate them in response to stimuli. This is in conflict with our previous observations which suggested that cells with *frzG* mutations did not demethylate or deamidate their FrzCD (12). The new findings are a direct result of our discovery of conditions which promote demethylation of FrzCD in wild-type cells. *M. xanthus* may have multiple enzymes that can catalyze the demethylation and deamidation reactions so that in the absence of FrzG, some other enzyme is able to partially replace its function. Another possibility, that the *frzG* mutants produce enough active FrzG protein to perform this function, seems unlikely since we obtained the same results with cells carrying Tn*5* insertions in three different regions of the gene. From sequence analyses, we predicted that DZF4021 would produce a peptide that contains only the first eight amino acid residues of FrzG and should, therefore, be unable to perform any catalytic function. The ability of *frzG* mutants to demethylate and deamidate FrzCD may explain why the *frzG* mutants are able to form fruiting bodies while the other *frz* mutants are unable to do so (12).

Effects on cell motility. Isoamyl alcohol and isopropyl alcohol, which caused demethylation of FrzCD, also had dramatic effects on the motile behavior of cells. Wild-type cells reversed continuously in the presence of these alcohols and, as a result, were unable to migrate from their point of origin. Cells with mutations in *frzB*, *frzG*, or *frzF* also continually reversed in the presence of isoamyl alcohol. In contrast, cells with mutations in *frzA*, *frzCD*, or *frzE* responded weakly, if at all, to additions of isoamyl alcohol. Individual cells spent most of their time gliding smoothly in one direction, with only occasional reversals. When the cells were present at high density, this type of movement with few reversals resulted in the familiar frizzy pattern with swirls and long filaments of cells. We interpret these findings to indicate that FrzA, FrzCD, and FrzE are central components of the *frz* system and are necessary for signal transmission. In the absence of any one of these proteins, cells rarely reverse and do not respond well to stimuli which would induce wild-type cells to reverse. In contrast, FrzB, FrzG, and FrzF are probably not directly involved in signal transmission. In the absence of isoamyl alcohol, *frzB* and *frzF* cells rarely reverse, but upon its addition, these cells reverse continuously. Apparently, they are able to respond to this signal. The results presented above indicate that FrzF is necessary for methylation of FrzCD and that FrzB is necessary for increased methylation of FrzCD in response to certain stimuli. It is likely that FrzB and FrzF are involved in adaptation to stimuli rather than in the initial signalling

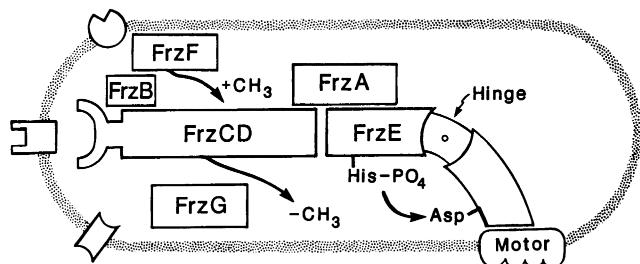

FIG. 9. Model of the *frz* signal transduction system. The model, described in the text, incorporates all of the known *frz* proteins, as well as hypothetical receptor proteins and a hypothetical gliding motor, to account for signal reception and transmission controlling cell movements in *M. xanthus*.

events leading to a change in reversal frequency. Previously, we indicated that in addition to the region of FrzF that is similar to CheR, there is a large C-terminal domain of unknown function (12). We have not identified the function of this region, but it is unlikely to be necessary for signal transmission since mutants lacking FrzF still respond to stimuli by increasing their reversal frequency. FrzG is probably also involved in adaptation, since it is homologous to CheB, the chemotactic methylesterase of enteric bacteria. However, mutations in *frzG*, as noted above, have extremely subtle phenotypic effects. *frzG* mutants are still able to form fruiting bodies, although slightly less efficiently than wild-type cells, and remain able to demethylate FrzCD.

Cells with Tn*5* insertions in *frzA*, *frzCD*, or *frzE* or cells deleted for several of these genes were still able to reverse their direction of gliding at a low frequency. We initially assumed that any cell completely lacking FrzE (which we predict to interact with the motor) would never reverse. Such reversals may be due to cross talk from other signal transduction systems in the cell or may indicate that the motor spontaneously reverses at some low frequency.

Model of the *frz* signal transduction pathway. Figure 9 summarizes our current understanding of the *frz* signal transduction system. This model is based on the results presented in this paper and in previous papers (10–13, 27) and on models for chemotactic signal transduction in enteric bacteria (10, 20). In our model, the putative (unidentified) receptor protein(s) in the cell membrane recognizes external stimuli and conveys this information to FrzCD. FrzCD interacts with FrzA and FrzE and presumably affects the phosphorylation state of FrzE. We hypothesize that it is FrzE (or phospho-FrzE) that interacts with some component of the gliding motility machinery and determines whether cells reverse their direction of movement. In our model, FrzF and FrzB are involved in methylation of FrzCD, and FrzG is involved in demethylation of FrzCD. These modifications presumably occur to mediate adaptation to changing levels of stimuli. The transmembrane receptor molecules in our model are purely speculative, and equally plausible models lacking such receptors are possible. For example, FrzCD might detect signals within the cytoplasm without interacting with transmembrane receptor proteins. These signals could reach FrzCD by one of several routes. Relatively lipophilic molecules could pass through the lipid bilayer of the cell membrane and interact with FrzCD in the cytoplasm. Hydrophilic molecules would probably require some type of permease to cross the membrane. Finally, the signals could be generated within the cytoplasm in response to external stimuli. We do not know how many steps lie between FrzCD and the external stimuli so the possibility of such second messengers must be entertained.

The proposed model contains obvious similarities to that proposed for chemotactic signal transduction in the enteric bacteria but also displays many striking differences. For example, (i) FrzCD, unlike the enteric MCPs, is not an integral membrane protein, (ii) FrzE combines the functions of CheA and CheY in a single protein, (iii) we have no CheZ homolog in the *Myxococcus* model, (iv) FrzB does not have a *che* homolog in *E. coli*, and (v) finally, the motor proposed to be involved in gliding motility may have some similarities to the enteric flagellar motor, but most of the components are likely to display many differences, since no flagella are involved in cell propulsion of *M. xanthus*. Continued comparison of the similarities and differences between the *Myxococcus frz* system and the enteric *che* system will allow us to identify those features which are essential and have been conserved during evolution and those which have evolved and changed to suit the particular needs of each organism.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant GM 20509 from the National Institutes of Health and by National Science Foundation grant DMB 8820799. M.J.M. was supported by National Research Service award 1 F32 GM12356-01A1 from the National Institutes of Health. T.K. was supported by a fellowship from the Swiss National Science Foundation.

We thank G. Acuna, E. Berger, K. O'Connor, K. Smith, and K. Trudeau for helpful discussions.

REFERENCES

1. **Armitage, J. P., W. A. Havelka, and R. E. Sockett.** 1990. Methylation-independent taxis in bacteria. Symp. Soc. Gen. Microbiol. **46:**177–197.
2. **Bischoff, D. S., and G. W. Ordal.** 1992. *Bacillus subtilis* chemotaxis: a deviation from the *E. coli* paradigm. Mol. Microbiol. **6:**23–28.
3. **Blackhart, B. D., and D. R. Zusman.** 1985. Cloning and complementation analysis of the "frizzy genes" of *Myxococcus xanthus*. Mol. Gen. Genet. **198:**243–254.
4. **Blackhart, B. D., and D. R. Zusman.** 1985. "Frizzy" genes of *Myxococcus xanthus* are involved in control of frequency of reversal of gliding motility. Proc. Natl. Acad. Sci. USA **82:** 8767–8770.
5. **Burchard, R. P.** 1981. Gliding motility of prokaryotes: ultrastructure, physiology, and genetics. Annu. Rev. Microbiol. **35:**497–529.
6. **Chow, W. Y., and D. E. Berg.** 1988. Tn*5tac1*, a derivative of transposon Tn*5* that generates conditional mutations. Proc. Natl. Acad. Sci. USA **85:**6468–6472.
7. **Dworkin, M., and D. Eide.** 1983. *Myxococcus xanthus* does not respond chemotactically to moderate concentration gradients. J. Bacteriol. **154:**437–442.
8. **Hagen, D. C., A. P. Bretscher, and D. Kaiser.** 1978. Synergism between morphogenetic mutants of *Myxococcus xanthus*. Dev. Biol. **64:**284–296.
9. **Kaiser, D.** 1986. Control of multicellular development: *Dictyostelium* and *Myxococcus*. Annu. Rev. Genet. **20:**539–566.
10. **Macnab, R. M.** 1987. Motility and chemotaxis, p. 732–759. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
11. **McBride, M. J., R. A. Weinberg, and D. R. Zusman.** 1989. "Frizzy" aggregation genes of the gliding bacterium *Myxococcus xanthus* show sequence similarities to the chemotaxis genes of enteric bacteria. Proc. Natl. Acad. Sci. USA **86:**424–428.
12. **McCleary, W. R., M. J. McBride, and D. R. Zusman.** 1990. Developmental sensory transduction in *Myxococcus xanthus*

involves methylation and demethylation of FrzCD. J. Bacteriol. **172**:4877–4887.

13. **McCleary, W. R., and D. R. Zusman.** 1990. FrzE of *Myxococcus xanthus* is homologous to both CheA and CheY of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **87**:5898–5902.
14. **Morrison, C. E., and D. R. Zusman.** 1979. *Myxococcus xanthus* mutants with temperature-sensitive, stage-specific defects: evidence for independent pathways in development. J. Bacteriol. **140**:1036–1042.
15. **Nowlin, D. M., J. Bollinger, and G. L. Hazelbauer.** 1988. Site-directed mutations altering methyl-accepting residues of a sensory transducer protein. Proteins Struct. Funct. Genet. **3**:102–112.
16. **O'Connor, K. A., and D. R. Zusman.** 1983. Coliphage P1-mediated transduction of cloned DNA from *Escherichia coli* to *Myxococcus xanthus*: use for complementation and recombinational analyses. J. Bacteriol. **155**:317–329.
17. **O'Connor, K. A., and D. R. Zusman.** 1986. Genetic analysis of *Myxococcus xanthus* and isolation of gene replacements after transduction under conditions of limited homology. J. Bacteriol. **167**:744–748.
18. **O'Connor, K. A., and D. R. Zusman.** 1991. Development in *Myxococcus xanthus* involves differentiation into two cell types: peripheral rods and spores. J. Bacteriol. **173**:3318–3333.
19. **O'Connor, K. A., and D. R. Zusman.** 1991. Behavior of peripheral rods and their role in the life cycle of *Myxococcus xanthus*. J. Bacteriol. **173**:3342–3355.
20. **Parkinson, J. S.** 1988. Protein phosphorylation in bacterial chemotaxis. Cell **53**:1–2.
21. **Pate, J. L.** 1988. Gliding motility in prokaryotic cells. Can. J. Microbiol. **34**:459–465.
22. **Rampersaud, A., and M. Inouye.** 1991. Procaine, a local anesthetic, signals through the EnvZ receptor to change the DNA binding affinity of the transcriptional activator protein OmpR. J. Bacteriol. **173**:6882–6888.
23. **Rosenberg, E.** 1984. Myxobacteria: development and cell interactions. Springer-Verlag, New York.
24. **Shimkets, L. J.** 1990. Social and developmental biology of the myxobacteria. Microbiol. Rev. **54**:473–501.
25. **Weinberg, R. A., and D. Zusman.** Evidence that the *Myxococcus xanthus frz* genes are developmentally regulated. J. Bacteriol. **171**:6174–6186.
26. **Zusman, D. R.** 1982. Frizzy mutants: a new class of aggregation-defective developmental mutants of *Myxococcus xanthus*. J. Bacteriol. **150**:1430–1437.
27. **Zusman, D. R., and M. J. McBride.** 1991. Sensory transduction in the gliding bacterium *Myxococcus xanthus*. Mol. Microbiol. **5**:2323–2329.
28. **Zusman, D. R., and K. A. O'Connor.** 1991. Development in *Myxococcus xanthus* involves aggregation and sporulation as well as non-aggregation and non-sporulation. Semin. Dev. Biol. **2**:37–45.