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Purification and Characterization of the *Myxococcus xanthus* FrzE Protein Shows that It Has Autophosphorylation Activity

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***Myxococcus xanthus* exhibits multicellular interactions during vegetative growth and fruiting body formation. Gliding motility is needed for these interactions. The frizzy (*frz*) genes are required to control directed motility. FrzE is homologous to both CheA and CheY from *Salmonella typhimurium*. We used polyclonal antiserum raised against a fusion protein to detect FrzE in *M. xanthus* extracts by Western immunoblot analysis. FrzE was clearly present during vegetative growth and at much lower levels during development. A recombinant FrzE protein was overproduced in *Escherichia coli*, purified from inclusion bodies, and renatured. FrzE was autophosphorylated when it was incubated in the presence of [γ-^{32}P]ATP and $MnCl_2$. Chemical analyses of the phosphorylated FrzE protein indicated that it contained an acylphosphate; probably phosphoaspartate. FrzE was phosphorylated in an intramolecular reaction. Based on these observations, we propose a model of the mechanism of FrzE phosphorylation in which autophosphorylation initially occurs at a conserved histidine residue within the "CheA" domain and then, via an intramolecular transphosphorylation, is transferred to a conserved aspartate residue within the "CheY" domain.**

Myxococcus xanthus is a gram-negative soil bacterium that moves by gliding motility (30). The mechanism of gliding motility is not known. Cells move very slowly, about 2 to 4 μm/min, and prefer to glide in preexisting slime trails but can also move in their absence. *M. xanthus* cells are extremely flexible (individual cells can bend to make a right or even a U turn) and are frequently reoriented as they encounter irregularities in the substratum. To control their direction of movement, in spite of such reorientations, individual cells adjust the frequency with which they reverse direction.

M. xanthus has a primitive multicellular life cycle (30, 43). When nutrients are abundant, large numbers of rod-shaped vegetative cells cooperatively feed by secreting antibiotics and hydrolytic enzymes that attack nearby microorganisms or macromolecules. Nutrient depletion triggers the developmental phase of the life cycle, which begins with cellular aggregation and culminates in the formation of spore-filled fruiting bodies. During fruiting body morphogenesis, cell motility is temporally coordinated and spatially directed as about 100,000 cells glide toward aggregation centers, where the cells accumulate in mounds. Within mounds, rod-shaped developmental cells differentiate into spherical, environmentally resistant myxospores. About 10 to 20% of the starved cells follow a different developmental fate. These cells, called peripheral rods, remain as rod-shaped cells around and between fruiting bodies (K. A. O'Connor and D. R. Zusman, unpublished data).

The frizzy (*frz*) mutants show aberrant patterns of cellular aggregation during fruiting body morphogenesis (42; D. R. Zusman, M. J. McBride, W. R. McCleary, and K. A. O'Connor, Symp. Soc. Gen. Microbiol., in press). Upon starvation, *frz* cells aggregate into filaments that at high cell density appear tangled or frizzy and at low cell density appear as donut-shaped swirls. The Frz aggregation phenotype results from an inability to control the frequency with which cells reverse their gliding direction (2). Among the six *frz* genes that have been identified, mutations in *frzA*, *-B*, *-C*, *-E*, and *-F* result in a dramatically reduced frequency at which cells reverse direction. Mutations in the C terminus of the *frzCD* gene (formally called *frzD* [2]) and, to a lesser extent, mutations in *frzG* (22) cause cells to reverse direction more frequently than wild-type cells.

We recently determined the nucleotide sequence of the entire *frz* region (21–23). DNA sequence analysis revealed that most of the *frz* gene products are homologous to proteins involved in enteric chemotaxis (for several excellent reviews of enteric chemotaxis, see references 16, 20, and 34). FrzA is homologous to CheW, FrzCD is homologous to the methyl-accepting chemotaxis proteins, FrzE is homologous to both CheA and CheY, FrzG is homologous to CheB, and FrzF is homologous to CheR. The enteric chemotaxis proteins constitute a signal transduction pathway that transmits information concerning the environment of the bacteria to their flagella, thereby controlling swimming behavior (36). In the absence of a gradient to a chemoeffector, enteric bacteria swim randomly by alternating between periods of smooth swimming and episodes of tumbling. Smooth swimming occurs when flagella rotate counterclockwise; tumbles result from clockwise flagellar rotation. Chemotaxis is achieved by adjusting the time interval between counterclockwise and clockwise flagellar rotation.

FrzE is particularly interesting because it is homologous to two chemotaxis proteins. CheA and CheY are members of the conserved two-component regulatory systems (29, 35, 36). CheA is a histidine kinase that autophosphorylates and then transfers its phosphate group to an aspartate residue of CheY (11–13, 25, 36, 41). It is currently thought that phospho-CheY interacts with the flagellar switch, causing the flagella to reverse their rotation from counterclockwise to clockwise (25, 26, 28). Phosphorylation reactions similar to those observed for CheA and CheY have also been demonstrated for other two-component regulatory pairs: NtrC is phosphorylated by NtrB (15), and EnvZ is phosphorylated by OmpR (9). Recently, it was shown that VirA from

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Agrobacterium tumefaciens is capable of autophosphorylation on a histidine residue (14).

We have previously proposed that the Frz proteins constitute a signal transduction pathway that controls the frequency with which *M. xanthus* cells reverse their gliding direction (22). Based on protein homology, we predicted that FrzE is autophosphorylated on a histidine residue and, through an intramolecular phosphotransfer reaction, shuttles a phosphate to an aspartate residue on its CheY domain (23). Phosphorylated FrzE would then be proposed to interact with the gliding apparatus to generate a reversal. In this study, we found additional support for this hypothesis by purifying the FrzE protein and demonstrating that it is autophosphorylated in the presence of ATP. FrzE is present vegetatively and at times during development when control of directional motility is required.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from New England BioLabs, Inc. (Beverly, Mass.). The Klenow fragment of DNA polymerase I and T4 ligase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). [γ-^{32}P]ATP (>5,000 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, Ill.). Sartorius nitrocellulose was purchased from E & K Scientific Products, Inc. (Saratoga, Calif.), and Immobilon-P membranes were purchased from Millipore Corp. (Bedford, Mass.). Medium components were obtained from Difco (Detroit, Mich.).

Bacterial strains and growth media. *M. xanthus* DZF1(FB) (8) was used in this study. *Escherichia coli* N4830-1 (Pharmacia) was used for overproduction of the protein A-FrzE fusion construct. This strain encodes the temperature-sensitive *c*I857 lambda repressor for use in thermoinduction from the lambda p_R promoter. *E. coli* BL21(DE3) containing the plasmid pLysS (kindly provided by F. W. Studier) was used for overproduction of the recombinant FrzE product from the T7 promoter. BL21(DE3) contains a single chromosomal copy of the T7 RNA polymerase gene under control of the *lacUV5* promoter. The plasmid pLysS is a pACYC184 derivative that encodes the T7 RNA polymerase inhibitor T7 lysozyme.

M. xanthus was grown in CYE broth (7) or on clone fruiting plates (CF) containing 1.5% agar as previously described (22). *E. coli* strains were grown in LB (24) supplemented with ampicillin (100 μg/ml) when needed.

Plasmid constructions. pBM16 (Fig. 1B) is a derivative of the protein A fusion vector pRIT2T (Pharmacia LKB Biotechnology, Piscataway, N.J.). It encodes a protein A-FrzE fusion protein under control of the lambda p_R promoter. pBM16 was constructed by ligating a 5.4-kbp *Pvu*II fragment from pBB20 (1) that contained *frzE* into the *Sma*I site of the multiple cloning region of pUC118 (38). A 5.0-kbp *Eco*RI-*Pst*I fragment was excised from this plasmid and ligated into pRIT2T that had previously been digested with *Eco*RI and *Pst*I. The resulting construct, pBM16, contained most of the *frzE* gene inserted in frame into the protein A coding region.

pBM33 (Fig. 1C) contains the entire *frzE* gene fused to 9 codons from the multiple cloning region of pJES307, which is positioned downstream of the phage T7 ϕ-10 promoter and ribosome-binding site. pJES307 (A kind gift from the S. Kustu laboratory) contains the early T7 terminator inserted at the *Bst*BI site of pT7-7 (10, 37). pBM33 was constructed by filling in the ends of a 3.7-kbp *Nco*I fragment containing the *frzE* gene from pBB12 (3) with the Klenow fragment. This fragment was inserted into pJES307 that had been digested with *Bam*HI and filled in with the Klenow fragment.

Antisera. Antiserum against a protein A-FrzE fusion protein was raised in rabbits. Expression of the fusion protein was induced by bringing the N4830-1(pBM16) culture to 42°C for 1.5 h. Cells were harvested by centrifugation and disrupted by sonic oscillation. The overexpressed insoluble protein was enriched by centrifugation at 10,000 × *g*. Fusion protein (2 mg) was electroeluted with an Elutrap (Schleicher & Schuell, Inc., Keene, N.H.) from a preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel. A rabbit was injected subcutaneously at multiple sites with 100 μg of fusion protein in Freund complete adjuvant, followed by two 100-μg boosts in incomplete adjuvant.

Western immunoblot analysis. *M. xanthus* DZF1 cells were grown in CYE to 3 × 10^8 to 4 × 10^8 cells per ml and then harvested by centrifugation at 12,000 × *g* for 10 min at 4°C. The cell pellets were suspended in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2)–8 mM $MgSO_4$ to a density of 1.3 × 10^{10} cells per ml. A 0.5-ml vegetative sample of these cells was spun for 1 min in a microfuge, the supernatant was removed, and the cells were rapidly frozen at −70°C. Development was initiated by plating 0.5 ml of the concentrated cells onto 150-mm-diameter CF agar plates. Cells from two plates were scraped with a razor blade, transferred to an Eppendorf tube, spun for 3 s in a microfuge, and frozen at −70°C until needed. Sonication buffer (600 μl; 10 mM HEPES [pH 7.2], 10 mM EDTA, 0.7 μg of pepstatin per ml, 0.5 μg of leupeptin per ml, 1 mM *O*-phenanthroline, 1 mM phenylmethylsulfonyl fluoride) was added to the frozen cell pellets, and the cells were immediately disrupted by sonication for 3 min on ice. Then 400 μl of extract was added to 100 μl of 5× protein loading buffer (0.4 M Tris hydrochloride [pH 6.8], 1% [wt/vol] SDS, 50% [vol/vol] glycerol), and each sample was boiled for 3 to 5 min. Protein assays were promptly performed by the BCA (bicinchonic acid) method (Pierce Chemical Co., Rockford, Ill.) on samples from the remaining 200 μl of extract. Samples (30 to 40 μg of protein) were loaded onto 15% SDS–polyacrylamide gels and separated at 30 mA (constant current). Proteins were transferred onto nitrocellulose membranes with a semidry electroblotter (Integrated Separation Systems, Hyde Park, Mass.) for 2 h at ca. 1.2 to 1.5 mA/cm^2 as indicated by the manufacturer. FrzE was detected as described previously (22) with ^{125}I-labeled protein A (Amersham).

FrzE overproduction and purification. A recombinant FrzE protein was expressed by inducing exponentially growing BL21(DE3) cells harboring both pLysS and pBM33 with 0.6 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested after 3 h by centrifugation and then disrupted by sonication. The cell extracts were centrifuged at 8,000 × *g* for 10 min. Pellets were washed three times in 10 mM HEPES (pH 7.2)–5 mM EDTA–0.1% (vol/vol) Triton X-100. The protein pellet was dissolved in 50 mM $NaPO_4$ (pH 6.8)–6 M guanidine hydrochloride. The denatured protein (20 ml at 100 μg/ml) was slowly renatured by dialyzing against 500 ml of 50 mM $NaPO_4$ (pH 6.8)–10 mM $MgCl_2$–1 mM dithiothreitol–50% (vol/vol) glycerol. The dialysis buffer was changed four times. Protein was stored in working samples at −20°C.

Phosphorylation assays. Protein samples (0.2 μg) were added to a reaction buffer (50 mM HEPES [pH 7.2], 10 mM $MnCl_2$, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 10 μM [γ-^{32}P]ATP [2.2 × 10^8 to 4.4 × 10^8 cpm/nmol]) for 5 to 10 min at 22°C or as otherwise specified below. Reactions were stopped by the addition of 0.33 volume of 4× stop buffer

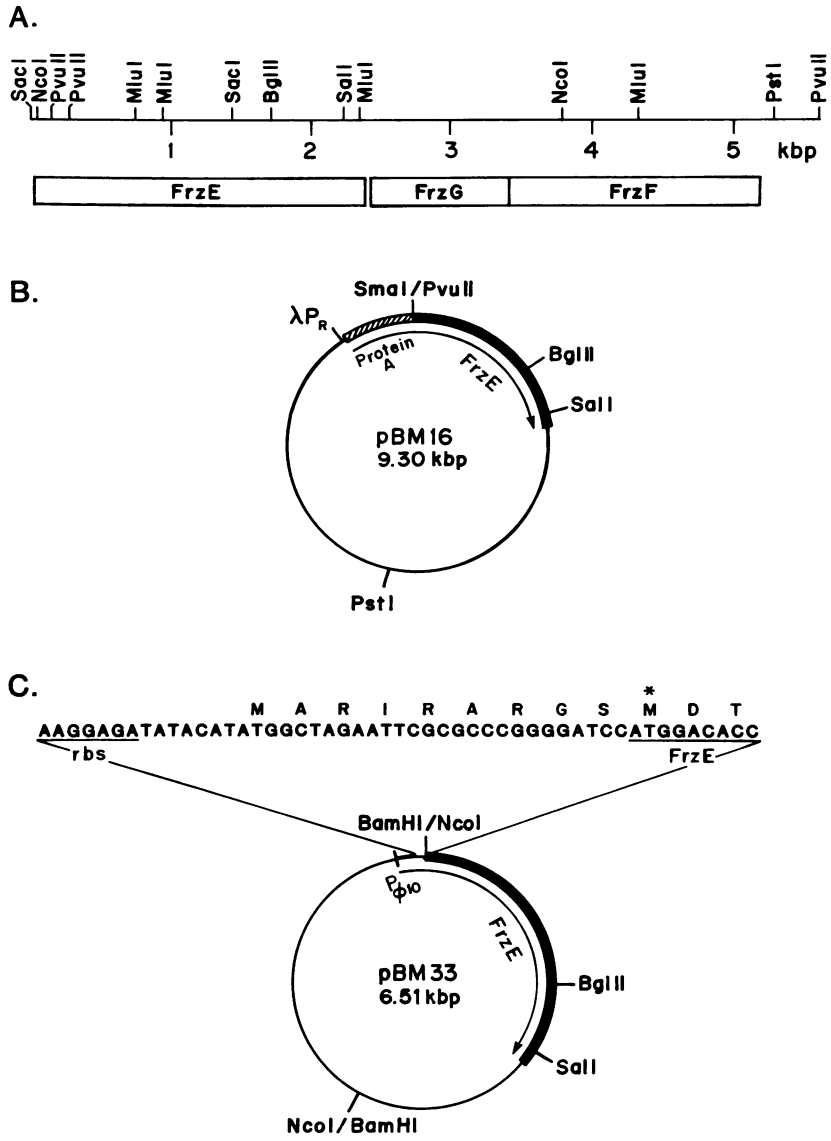

FIG. 1. (A) Physical map of the *frzEGF* region of the *M. xanthus* chromosome. (B) Structure of the plasmid pBM16. This plasmid encodes a protein A-FrzE fusion protein whose expression is under control of the lambda p_R promoter. (C) Structure of the FrzE expression vector pBM33. This plasmid encodes a recombinant FrzE protein containing nine amino acid residues encoded by the multiple cloning site of pJES307 and the entire predicted *frzE* gene product. This protein-coding region is downstream of the bacteriophage T7 gene 10 ribosome-binding site and is under control of the strong ϕ-10 T7 RNA polymerase promoter.

(0.32 M Tris hydrochloride [pH 6.8], 0.1 M EDTA, 8% [wt/vol] SDS, 40% [vol/vol] glycerol). Reaction mixtures were separated by electrophoresis on 10% SDS–polyacrylamide gels. Proteins were blotted onto Immobilon-P membranes with a semidry blotting apparatus. After blotting, the filters were washed for 5 min in 50 mM $NaPO_4$ (pH 6.8)–6 M guanidine hydrochloride to remove nonspecifically bound label and stained briefly (1 min) with Ponceau S (31). Filters were briefly rinsed in 50 mM $NaPO_4$ (pH 6.8), air dried, and subjected to autoradiography for 18 h with two intensifying screens on preflashed film. For quantitation of phosphorylation, the bands from the Immobilon-P filters were excised with a razor blade and placed in scintillation vials containing Ecolume (ICN Radiochemicals, Irvine, Calif.) and counted in a Beckman LS 6000IC scintillation counter (Beckman Instruments, Fullerton, Calif.).

RESULTS

Identification of FrzE and expression during development. The product of the *frzE* gene is necessary for proper cellular aggregation during fruiting body morphogenesis. From DNA sequence analysis (23), we predicted that *frzE* encodes a large protein that contains two functional domains that are homologous to the enteric CheA and CheY proteins. At the boundary between the "CheA" and "CheY" domains there is an ATG codon preceded by a possible ribosome binding site (nucleotides 1986 to 2001 [23]). We were intrigued by the possibility of a potential alternate or secondary translational start site for this gene just upstream of the "CheY" domain. Initiation of protein synthesis from this site would produce a 14.5-kDa polypeptide.

To identify FrzE in *M. xanthus* by Western blot analysis

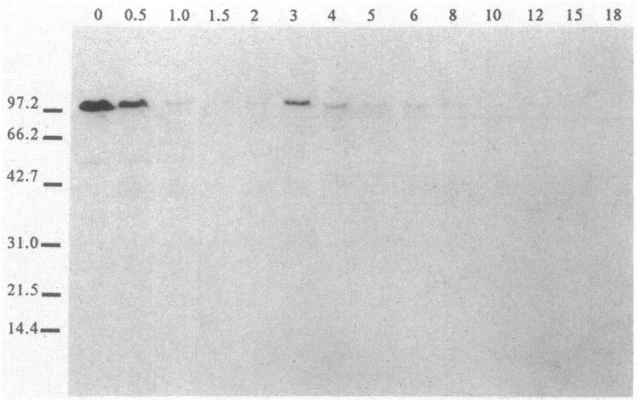

FIG. 2. Western immunoblot demonstrating the presence of FrzE and its expression during development. Cells were harvested at the following times after the initiation of development: 0, 0.5, 1.0, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 15, and 18 h. Cell extracts were prepared in the presence of five protease inhibitors. Proteins were separated on a 15% SDS–polyacrylamide gel and transferred onto a nitrocellulose membrane. FrzE was detected by using polyclonal antiserum to a protein A-FrzE fusion protein and ^{125}I-labeled protein A. Molecular mass standards (kilodaltons) are labeled.

and to determine whether it was present in multiple forms, we raised polyclonal antisera to a *Staphylococcus aureus* protein A-FrzE fusion protein (see Fig. 1B for a diagram of the plasmid used to prepare the protein A-FrzE fusion protein). Early attempts to examine FrzE by Western blot analysis sometimes showed several hybridizing bands. However, our results became very reproducible as we took extraordinary precautions to reduce proteolysis. The antisera recognized a major band with an apparent molecular mass of 93 kDa (Fig. 2, lane 1). This band was missing in strains containing a Tn*5* insertion within *frzE* (data not shown). The apparent molecular mass of this protein was larger than the 83.1-kDa product predicted from DNA sequence analysis but is in close agreement to the size previously determined from SDS-polyacrylamide gels of *E. coli* maxicells expressing *frzE*. We were only able to detect the full-length protein product; no smaller proteins corresponding to secondary translational products were observed.

A developmental time course of FrzE expression is shown in Fig. 2. In this experiment, cells were plated onto CF agar and harvested at different times during development. Equal amounts of protein were separated by polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and probed with FrzE antiserum. FrzE is most abundant in vegetative cells; its level decreases at early times in development and subsequently increases. A peak level of expression is observed at 3 to 5 h after the initiation of development. After this period of increased production, FrzE appears to be present in very low amounts. The pattern of FrzE accumulation in cells was very different from that predicted by previous experiments with a reporter gene (see Discussion).

Overexpression and purification of FrzE. The Western blot data presented above indicated that FrzE was present at very low levels and was also extremely labile. This suggested that its purification from *M. xanthus* would not be easy. Previous work in our laboratory (3) demonstrated that the *frz* proteins were also produced at very low levels in *E. coli* when expressed from the strong *trp* promoter. We therefore constructed an expression vector that encoded a recombinant FrzE protein. Its protein-coding region was positioned downstream of the translational start site for the highly expressed T7 gene 10 protein and was under control of the strong ϕ-10 T7 RNA polymerase promoter. Figure 3 shows a Coomassie blue-stained SDS-polyacrylamide gel of extracts prepared from this strain. After induction, FrzE was the most abundant cellular protein (lane 2). This protein was highly expressed and was found in an insoluble fraction (inclusion bodies). The protein could be greatly enriched by simply collecting and then washing the low-speed pellet of sonicated cell extracts in a buffer containing 0.1% Triton X-100. The protein pellet (which was almost pure FrzE) was solubilized in 6 M guanidine hydrochloride and slowly renatured by dialysis at a low protein concentration.

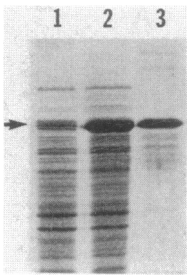

FIG. 3. Coomassie blue-stained SDS polyacrylamide gel showing expression and purification of FrzE. Lanes: 1, uninduced cell extract from strain harboring pBM33; 2, isopropyl-β-D-thiogalactopyranoside-induced cell extracts from strain harboring pBM33; 3, low-speed pellet from isopropyl-β-D-thiogalactopyranoside-induced cells.

Autophosphorylation activity of purified FrzE. To determine whether this renatured, recombinant form of FrzE was capable of autophosphorylation, we incubated it in the presence of [γ-^{32}P]ATP. Reaction mixtures were separated on 10% SDS–polyacrylamide gels and transferred onto Immobilon-P membranes. This procedure separated FrzE from other radioactive components. Figure 4 shows a typical phosphorylation blot. FrzE was not phosphorylated when only Mg^{2+} and [γ-^{32}P]ATP were present. However, autokinase activity was detected when Mn^{2+} was included in the reaction buffer (lanes 2, 3, and 4). No phosphorylation was observed when Ca^{2+}, Zn^{2+}, Cu^{2+}, or Fe^{2+} was added in the absence of Mn^{2+} (data not shown). There was a slight increase in activity when both manganese and magnesium were present in the reaction buffers (compare lanes 2 and 3).

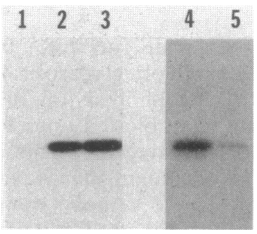

FIG. 4. Autophosphorylation of FrzE. Purified FrzE was incubated in 50 mM HEPES (pH 7.2) with [γ-^{32}P]ATP (2.2×10^8 cpm/nmol) at 22°C for 10 min with the following additions (lanes): 1, 10 mM $MgCl_2$; 2, 10 mM $MnCl_2$; 3 and 4, 10 mM $MgCl_2$ plus 10 mM $MnCl_2$; 5, same conditions as lanes 3 and 4 except that FrzE was boiled in 1% SDS for 3 min before incubation with [γ-^{32}P]ATP. Reaction products were separated on 10% SDS–polyacrylamide gels, electroblotted onto Immobilon-P membranes, and exposed to preflashed X-ray film with two intensifying screens.

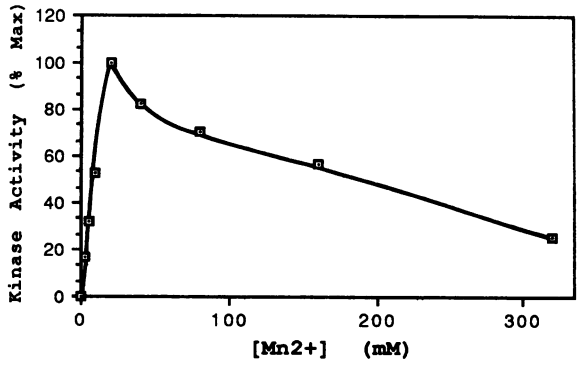

FIG. 5. Effect of Mn^{2+} concentration on FrzE autophosphorylation. Standard reactions were performed with the indicated amounts of $MnCl_2$. Reactants were separated by SDS-polyacrylamide gel electrophoresis, blotted, and visualized by autoradiography. Bands from the filter were excised and counted. A value of 100% was given to the activity measured with 20 mM $MnCl_2$.

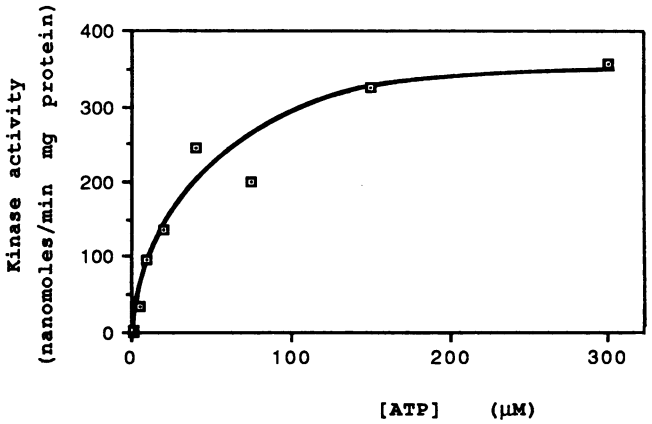

FIG. 6. Initial rate of FrzE autophosphorylation as a function of ATP concentration. FrzE (0.2 μg) was incubated with the indicated amounts of [γ-^{32}P]ATP at 22°C as described in Materials and Methods. Kinase activities were calculated from the amount of ^{32}P incorporated into FrzE in a 5-min reaction. These results indicate that FrzE autokinase shows half-maximal velocity with ATP concentrations between 25 and 35 μM.

Phosphorylation was dependent on the renatured FrzE structure. When FrzE was boiled in 1% SDS before incubation with [γ-^{32}P]ATP, no phosphorylation above the background was observed (lane 5). We also detected phosphorylation of a truncated form of FrzE missing its 50 C-terminal amino acid residues (data not shown). We were unable to detect phosphorylation of FrzE in crude extracts of *M. xanthus* because of the extremely high background, presumably from other more abundant or more active kinases. The background level of phosphorylation observed in reactions with heat-inactivated FrzE (lane 5) or in reactions without manganese (lane 1) was very similar to that observed when bovine serum albumin was incubated in the presence of manganese and [γ-^{32}P]ATP (data not shown) and must therefore be considered nonspecific.

To investigate the stimulatory effects of manganese on FrzE phosphorylation, we incubated FrzE in the presence of [γ-^{32}P]ATP with various concentrations of manganese. Maximum activity was observed when 20 mM $MnCl_2$ was present in the reaction buffer (Fig. 5). The phosphorylation activity increased dramatically between 0 and 20 mM $MnCl_2$ and declined between 20 and 320 mM $MnCl_2$. The nonphysiological level of manganese dependence suggests that it is substituting for other factors present in vivo which normally regulate its activity (see Discussion).

Chemical stability of phosphorylated FrzE. The chemical stability of the phosphate bond in phosphorylated FrzE was assayed to determine the class of amino acid residue that was phosphorylated (6). For example, phosphoserine, phosphothreonine, and phosphotyrosine are stable in acid. Phosphohistidine and phospholysine are not stable in acid but are stable in base. Phosphoaspartate and phosphoglutamate are not stable in acid, base, or hydroxylamine.

Equal amounts of FrzE were phosphorylated with [γ-^{32}P]ATP and blotted onto Immobilon-P membranes as described in Materials and Methods. Individual bands were excised from the membranes, placed in test tubes, containing 10 ml of the solutions indicated below, and incubated at 42°C for 2 h. Each band was rinsed in 50 mM $NaPO_4$ (pH 6.9), briefly air dried, and counted. The percentages of radioactivity remaining in the FrzE bands after 2 h of incubation as compared with a control that was not subjected to any treatment were as follows: with 50 mM $NaPO_4$ (pH 6.9), 39.1%; with 0.8 M hydroxylamine (pH 6.9), 11.9%; with 2N NaOH, 6.7%; with 1 M HCl, 9.2%. These data show that the phosphate group was labile under neutral conditions with an approximate half-life of 1.5 h. However, it was even more labile under both acidic and alkaline conditions as well as in 0.8 M hydroxylamine at pH 6.8. These characteristics are consistent with the phosphorylated amino acid residue being an acyl phosphate, such as phosphoaspartate or phosphoglutamate.

Kinetics of FrzE phosphorylation. We investigated the initial FrzE phosphorylation rates at various ATP concentrations. Reactions were performed at 22°C, stopped after 5 min by the addition of 4× stop buffer, and placed at −70°C until being loaded on SDS-polyacrylamide gels. Preliminary experiments (data not shown) indicated that reaction rates were linear for at least 8 min. A plot of initial reaction rates versus ATP concentration is presented in Fig. 6. Under these reaction conditions, we estimate that FrzE has a half-maximum velocity with ATP at concentrations between 25 and 35 μM.

The initial phosphorylation rates of an intramolecular reaction should remain constant when the enzyme is diluted under constant substrate concentrations as long as the substrate is in vast molar excess over enzyme concentration; in contrast, intermolecular reactions are very sensitive to dilution. To test whether FrzE phosphorlyation was an intramolecular reaction, we incubated 2 pmol of FrzE for 5 min in 20-, 50-, and 120-μl reactions containing 10 μM ATP. Reactions were again stopped and placed at −70°C until SDS-polyacrylamide gel electrophoresis. FrzE was phosphorylated to approximately equivalent levels in each lane of Fig. 7, indicating that each of the initial reaction rates was similar. Two trials of this experiment showed exactly the same pattern of phosphorylation. The initial rates for the 50- and 120-μl reactions were equivalent and were about 30% higher than the rates for the 20-μl reactions. In no case was the initial reaction rate reduced because of enzyme dilution. The lower initial phosphorylation rates observed in the 20-μl reactions might be due to higher levels of glycerol.

DISCUSSION

Model of FrzE autophosphorylation. The most interesting result reported in this paper is that the *frzE* gene encodes a

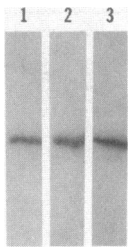

FIG. 7. Effects of FrzE dilution on initial autophosphorylation rates. Equal amounts of FrzE (0.2 μg) were incubated under standard conditions (see Materials and Methods) for 5 min at 22°C in the following reaction volumes (lanes): 1, 20 μl; 2, 50 μl; 3, 120 μl. Reactants were separated on a 3-mm-thick 10% SDS–polyacrylamide gel, blotted, and exposed to preflashed X-ray film for 24 h at −70°C with one intensifying screen.

protein that is autophosphorylated. In this respect FrzE is similar to the enteric CheA protein (13). However, unlike CheA, FrzE appears to have a phosphate on an aspartate residue, rather than on a histidine. In this respect it is like CheY (32). The simplest explanation is that FrzE is a hybrid molecule that autophosphorylates at a histidine and then transfers the phosphate, in an intramolecular reaction, to an aspartate residue. Figure 8 presents our current working model of FrzE phosphorylation. We hypothesize that FrzE autophosphorylates on the conserved histidine residue of its "CheA" domain and, through an intramolecular reaction facilitated by an alanine- and proline-rich hinge (23), transfers this phosphate group to the conserved aspartate residue of its "CheY" effector domain.

The following lines of evidence support this model. (i) The predicted FrzE amino acid sequence shows significant homology to both CheA and CheY (23). There are no stop codons between the two functional domains of FrzE. The conserved protein sequence motifs between FrzE and CheA and CheY suggest conserved protein functions. It has been shown that CheA is a histidine kinase that autophosphorylates on a histidine residue (His-48) (11, 13, 36) that is

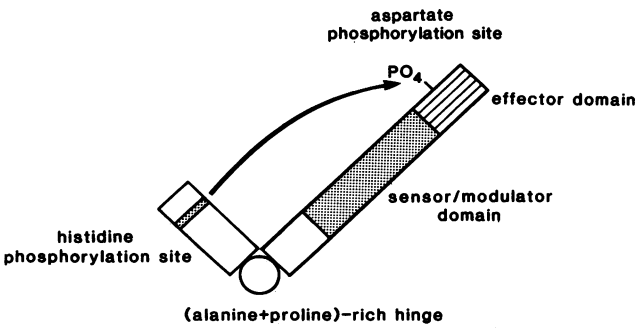

FIG. 8. Model of FrzE autophosphorylation. FrzE has two domains that are homologous to CheA (shaded regions): a histidine phosphorylation site and a sensor-modulator domain. It also contains one domain that is homologous to CheY (striped region). The center circle consists of an alanine- and proline-rich segment that is hypothesized to act as a flexible hinge. In this model FrzE is autophosphorylated on the conserved histidine residue of its 'CheA' domain. Through a rapid, intramolecular, reaction (facilitated by the alanine- and proline-rich hinge) the phosphate group is transferred from the 'CheA' domain to the conserved aspartate residue of the 'CheY' domain. Presumably, as a result of phosphorylation, FrzE becomes activated and generates a cell reversal.

conserved between CheA and FrzE. It has also been demonstrated that CheA transfers a phosphate group to the Asp-57 residue of CheY (12, 32, 41). This aspartate residue not only is conserved between CheY and FrzE but also is invariant throughout the entire effector class of the two-component regulatory proteins (36). In addition, the predicted FrzE amino acid sequence contains an unusual 68-amino-acid segment that is 72% alanine plus proline. This region is similar in amino acid composition to several flexible segments found in *E. coli* pyruvate dehydrogenase (27) and would be expected to confer a high degree of flexibility to the FrzE protein. (ii) The Western immunoblot data presented in this paper showed that *frzE* encodes a single protein that corresponds in size to the predicted *frzE* gene product. In fact, the size estimated from SDS-polyacrylamide gels (93 kDa) is somewhat larger than that predicted from the DNA sequence (83.1 kDa). This discrepancy in size might, in part, be accounted for by the predicted alanine- and proline-rich region, which may cause FrzE to migrate slower than other globular proteins of that size in SDS-polyacrylamide gels. (iii) The chemical stability of phosphorylated FrzE suggests that it, like CheY, contains an acyl phosphate. CheA contains a phosphohistidine that is very stable under alkaline conditions (6). Phosphorylated FrzE was unstable in alkali. We cannot distinguish between phosphoaspartate and phosphoglutamate. However, in light of the chemical characteristics of phospho-FrzE and the sequence homology between FrzE and CheA and CheY, we feel that it is most likely that the phosphorylated residue is an aspartate. (iv) The kinetics of FrzE phosphorylation at the aspartate residue indicates that the rate limiting aspect of this reaction is intramolecular. The autophosphorylation of CheA has been shown to be followed by a second transphosphorylation reaction between CheA and CheY or between CheA and CheB (12, 36, 41). Since FrzE contains the conserved histidine phosphorylation site of CheA and the conserved aspartate phosphorylation site of CheY and CheB, we wanted to investigate whether the presumed transphosphorylation between the histidine and aspartate residues within FrzE represents an intramolecular reaction. If FrzE is autophosphorylated by an intermolecular reaction, then one would expect to see a dramatic decrease in initial reaction rates as FrzE was diluted. This was not observed and indicates an intramolecular transfer. The reaction conditions for this experiment were chosen so that substrate levels were in vast molar excess over enzyme concentrations. Under these conditions we feel that it is likely that an intermolecular reaction would be rate limiting. A rapid intramolecular transfer might preclude phosphorylation of other proteins by FrzE. CheA activates the methylesterase activity of CheB by phosphorylation (19). The regulatory domain of CheB shares the same sequence motif that is found in CheY and is presumably phosphorylated by a similar mechanism. It is interesting that among the sequence differences between FrzG (the predicted methylesterase) and CheB there is a substitution of an aspartate residue at position 10 of CheB with a lysine in FrzG. When the analogous position in CheY is changed from an aspartate to a lysine, a dominant, constitutively active form of CheY is produced (5). We predict that FrzE does not phosphorylate FrzG but that FrzG is normally present in an active form. Further experiments are necessary to address this question.

FrzE autokinase activity requires manganese in vitro. Significant stimulation of FrzE autokinase activity was observed in reactions containing very high manganese levels, between 5 and 20 mM Mn^{2+}. It seems very unlikely that the

high Mn^{2+} levels required to stimulate phosphorylation are physiologically relevant. Recent experiments with the human insulin receptor, which contains a Mn^{2+}-stimulated tyrosine kinase domain, might provide some insights into the mechanism by which FrzE autokinase is activated by Mn^{2+}. Wente et al. (40) showed by electron paramagnetic resonance spectroscopy that the cytoplasmic domain of the human insulin receptor does not bind Mn^{2+} tightly. However, they showed by circular dichroism that the presence of Mn^{2+} causes a conformational change in the protein. They speculated that Mn^{2+} might mimic the action of a physiological activator (40). In a similar manner, Mn^{2+} might stimulate FrzE autokinase activity by inducing a conformational change that mimics a physiological activator. By analogy to the chemotaxis signal transduction pathway, in which CheA is stimulated by both CheW and the methyl-accepting chemotaxis proteins (4, 18, 33), FrzE might also be stimulated by FrzA and/or FrzCD. It should be noted that genetic data indicate that FrzE and FrzCD interact (2).

Developmental expression of FrzE. Myxobacteria show directional movement vegetatively as they "hunt" for food and developmentally when they aggregate to form fruiting bodies. If the *frz* genes control directional movement in *M. xanthus*, then one would predict their presence vegetatively and during development. Studies with the transcriptional reporter Tn*5-lac* showed that the *frz* genes are developmentally regulated (39). Low levels of expression were detected vegetatively, followed by a period of high expression that corresponded to times in which mounds were being formed. We have shown in this study that FrzE is present at high relative levels vegetatively and that its amount rapidly decreases in the early stages of development. We detected peak levels of FrzE after 3 h of development. Significant levels of FrzE were not observed after 15 h of development. How can we rationalize the difference between these two experimental approaches, Western blot analysis versus reporter gene expression? Development is a starvation response in *M. xanthus*. Starvation in bacteria is often coupled with high levels of protein turnover. We predict that although transcription of *frzE* might be low vegetatively, its protein product is relatively stable. To compensate for high protein turnover during the early stages of development, there is an increase in transcription. In support of this proposal, long exposures of the autoradiograms from the FrzE time course Western blot (Fig. 2) showed the presence of many smaller cross-reacting bands (presumably protein degradation intermediates) during development (data not shown). These bands were not present in vegetative samples.

There is a significant difference between the kinetics of transcription as previously determined with the transcriptional reporter Tn*5-lac* (39) and the kinetics of protein accumulation as observed in Western blots. We do not know whether this difference is due to changes in protease levels during development or to other factors. Although the half-life of β-galactosidase decreases during development (39), it is still very stable (ca. 2.5 h). If FrzE has a much shorter half-life during development than does β-galactosidase, results from the reporter might indicate higher levels of FrzE than are actually present. In addition, the timing of expression of *frzE* might be different in the Tn*5-lac* mutants and the wild-type cells.

There are many similarities in the protein components and associated biochemical reactions (phosphorylation and methylation) (17) between the enteric chemotaxis and *frz* sensory transduction systems. However, many specific questions remain about the frizzy system. For example, we do not yet know to what signals the frizzy system is responding during feeding and/or aggregation, nor have we identified the presumed target (gliding motor) of phosphorylated FrzE. We hope that continued study of the *frz* genes and their products will provide insights into these unanswered questions.

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LITERATURE CITED

1. **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.
2. **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.
3. **Blackhart, B. D., and D. R. Zusman.** 1986. Analysis of the products of the *Myxococcus xanthus frz* genes. J. Bacteriol. **166**:673–678.
4. **Borkovich, K. A., N. Kaplan, J. F. Hess, and M. I. Simon.** 1989. Transmembrane signal transduction in bacterial chemotaxis involves ligand-dependent activation of phosphate group transfer. Proc. Natl. Acad. Sci. USA **86**:1208–1212.
5. **Bourret, R. B., J. F. Hess, and M. I. Simon.** 1990. Conserved aspartate residues and phosphorylation in signal transduction by the chemotaxis protein CheY. Proc. Natl. Acad. Sci. USA **87**:41–45.
6. **Buss, J. E., and J. T. Stull.** 1983. Measurement of chemical phosphate in proteins. Methods Enzymol. **99**:7–14.
7. **Campos, J. M., J. Geisselsoder, and D. R. Zusman.** 1978. Isolation of bacteriophage MX4, a generalized transducing phage from *Myxococcus xanthus*. J. Mol. Biol. **119**:167–178.
8. **Dworkin, M.** 1962. Nutritional requirements for vegetative growth of *Myxococcus xanthus*. J. Bacteriol. **84**:250–257.
9. **Forst, S., J. Delgado, and M. Inouye.** 1989. Phosphorylation of OmpR by the osmosensor EnvZ modulates expression of the *ompF* and *ompC* genes in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **86**:6052–6056.
10. **Hamood, A. N., and B. H. Iglewski.** 1990. Expression of the *Pseudomonas aeruginosa toxA* positive regulatory gene (*regA*) in *Escherichia coli*. J. Bacteriol. **172**:589–594.
11. **Hess, J. F., R. B. Bourret, and M. I. Simon.** 1988. Histidine phosphorylation and phosphoryl group transfer in bacterial chemotaxis. Nature (London) **336**:139–143.
12. **Hess, J. F., K. Oosawa, N. Kaplan, and M. I. Simon.** 1988. Phosphorylation of three proteins in the signaling pathway of bacterial chemotaxis. Cell **53**:79–87.
13. **Hess, J. F., K. Oosawa, P. Matsumura, and M. I. Simon.** 1987. Protein phosphorylation is involved in bacterial chemotaxis. Proc. Natl. Acad. Sci. USA **84**:7609–7613.
14. **Jin, S., R. Roitsch, R. G. Ankenbauer, M. P. Gordon, and E. W. Nester.** 1990. The VirA protein of *Agrobacterium tumefaciens* is autophosphorylated and is essential for *vir* gene regulation. J. Bacteriol. **172**:525–530.
15. **Keener, J., and S. Kustu.** 1988. Protein kinase and phosphoprotein phosphatase activities of nitrogen regulatory proteins NTRB and NTRC of enteric bacteria: roles of the conserved amino-terminal domain of NTRC. Proc. Natl. Acad. Sci. USA **85**:4976–4980.
16. **Koshland, D. E., Jr.** 1981. Biochemistry of sensing and adaptation in a simple bacterial system. Annu. Rev. Biochem. **50**:765–782.
17. **Koshland, D. E., Jr., D. A. Sanders, and R. M. Weis.** 1988.

Roles of methylation and phosphorylation in the bacterial sensing system. Cold Spring Harbor Symp. Quant. Biol. **53**:11–17.
18. **Liu, J., and J. S. Parkinson.** 1989. Role of CheW protein in coupling membrane receptors to the intracellular signaling system of bacterial chemotaxis. Proc. Natl. Acad. Sci. USA **86**:8703–8707.
19. **Lupas, A., and J. Stock.** 1989. Phosphorylation of an N-terminal regulatory domain activates the CheB methylesterase in bacterial chemotaxis. J. Biol. Chem. **264**:17337–17342.
20. **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.
21. **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.
22. **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.
23. **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.
24. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 433. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Parkinson, J. S.** 1988. Protein phosphorylation in bacterial chemotaxis. Cell **53**:1–2.
26. **Parkinson, J. S., S. R. Parker, P. B. Talbert, and S. E. Houts.** 1983. Interactions between chemotaxis genes and flagellar genes in *Escherichia coli*. J. Bacteriol. **155**:265–274.
27. **Radford, S. E., E. D. Laue, R. N. Perham, S. R. Martin, and E. Appella.** 1989. Conformational flexibility and folding of synthetic peptides representing an interdomain segment of polypeptide chain in the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*. J. Biol. Chem. **264**:767–775.
28. **Ravid, S., P. Matsumura, and M. Eisenbach.** 1986. Restoration of flagellar clockwise rotation in bacterial envelopes by insertion of the chemotaxis protein CheY. Proc. Natl. Acad. Sci. USA **83**:7156–7161.
29. **Ronson, C. W., B. T. Nixon, and F. M. Ausubel.** 1987. Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell **49**:579–581.
30. **Rosenberg, E.** 1984. Myxobacteria: development and cell interactions. Springer-Verlag, New York.
31. **Salinovich, O., and R. Montelaro.** 1986. Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by SDS-PAGE. Anal. Biochem. **156**:341–347.
32. **Sanders, D. A., B. L. Gillece-Castro, A. M. Stock, A. L. Burlingame, and D. E. Koshland, Jr.** 1989. Identification of the site of phosphorylation of the chemotaxis response regulator protein, CheY. J. Biol. Chem. **264**:21770–21778.
33. **Sanders, D. A., B. Mendez, and D. E. Koshland, Jr.** 1989. Role of the CheW protein in bacterial chemotaxis: overexpression is equivalent to absence. J. Bacteriol. **171**:6271–6278.
34. **Stewart, R. C., and F. W. Dahlquist.** 1987. Molecular components of bacterial chemotaxis. Chem. Rev. **87**:997–1025.
35. **Stock, A., T. Chen, D. Welsh, and J. Stock.** 1988. CheA protein, a central regulator of bacterial chemotaxis, belongs to a family of proteins that control gene expression in response to changing environmental conditions. Proc. Natl. Acad. Sci. USA **85**:1403–1407.
36. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53**:450–490.
37. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled extensive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
38. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153**:3–11.
39. **Weinberg, R. A., and D. R. Zusman.** 1989. Evidence that the *Myxococcus xanthus frz* genes are developmentally regulated. J. Bacteriol. **171**:6174–6186.
40. **Wente, S. R., M. Villalba, V. L. Schramm, and O. M. Rosen.** 1990. Mn^{2+}-binding properties of a recombinant protein-tyrosine kinase derived from the human insulin receptor. Proc. Natl. Acad. Sci. USA **87**:2805–2809.
41. **Wylie, D., A. Stock, C.-Y. Wong, and J. Stock.** 1988. Sensory transduction in bacterial chemotaxis involves phosphotransfer between Che proteins. Biochem. Biophys. Res. Commun. **151**: 891–896.
42. **Zusman, D. R.** 1982. Frizzy mutants: a new class of aggregation-defective developmental mutants of *Myxococcus xanthus*. J. Bacteriol. **150**:1430–1437.
43. **Zusman, D. R.** 1984. Cell-cell interaction and development in *Myxococcus xanthus*. Q. Rev. Biol. **59**:119–138.