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Evidence that the *Myxococcus xanthus frz* Genes Are Developmentally Regulated

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The frizzy (*frz*) mutants of *Myxococcus xanthus* are unable to form fruiting bodies. Instead of forming discrete mounds, these strains aggregate as filaments which have a circular and tangled appearance. Mutations leading to this phenotype have been mapped to five complementation groups, *frzA*, *frzB*, *frzCD*, *frzE*, and *frzF*. All have been found to be involved in the control of directional movement of the bacteria and, except for *frzB*, to be homologous to the chemotaxis genes of enteric bacteria. In this report we present a study of the regulation of expression of the first four genes of the *frz* gene cluster (*frzA*, *frzB*, *frzCD*, and *frzE*) by using Tn*5-lac* transcriptional fusions as reporters of gene expression. We found that these *frz* genes are developmentally regulated, with their transcription peaking at about the time of early mound formation (12 to 18 h). Analysis of FrzCD expression by enzyme-linked immunosorbent assay showed a 10-fold greater induction at 15 h of development over the level of vegetative cell expression. Northern blot hybridization analysis suggested that the *frz* genes were arranged as an operon. To test this hypothesis, double mutants were constructed which contained Tn*5-132* either upstream or downstream of the reporter Tn*5-lac*. The expression of the *frz* genes in the double mutants was consistent with the hypothesis that the first four genes (*frzA*, *frzB*, *frzCD*, and *frzE*) are organized as an operon with an internal promoter. Insertion mutations in *frzCD* lowered gene expression whether they were upstream or downstream of the reporter Tn*5-lac*, suggesting that the FrzCD protein regulates transcription of the entire operon from a promoter upstream of *frzA*. Evidence is presented suggesting that FrzE is required for induction of transcription as well. When *frz* mutations were placed in strains that were unable to aggregate (*tag*), the *frz* genes were expressed at an elevated level on fruiting agar; this high level of expression was maintained for several days. These results suggest that the *tag* gene products interact with the *frz* functions.

Myxococcus xanthus is a gram-negative, rod-shaped soil bacterium that moves by gliding motility. It exhibits a complex life cycle (36, 40). When grown vegetatively on a solid surface, cells travel in large groups referred to as swarms or hunting groups which feed on other microorganisms or organic material in their path. In the absence of nutrients, these swarms aggregate to form moundlike structures in which the cells differentiate into myxospores (34). These mounds of spores are called fruiting bodies. When nutrients are replenished, the spores germinate in numbers large enough to create a new hunting group.

We have been studying a group of mutants that exhibit an aberrant pattern of aggregation during development. These strains fail to form discrete mounds but, rather, assemble into complex multicellular filaments which have a circular and tangled appearance termed frizzy (Frz) (39). These strains, however, are capable of forming myxospores. Previous work in our laboratory has shown that the mutations causing the Frz phenotype are clustered on a 7.5-kilobase-pair piece of DNA (5). The mutations were separated into five complementation groups. Four of the complementation groups (*frzA*, *frzB*, *frzCD*, and *frzE*) were contiguous; the fifth one (*frzF*) was separated from the others by a 1.4-kilobase-pair segment of DNA. All mutations that led to the Frz phenotype were recessive. Mutant *frzD* strains showed a smooth-edged colony morphology typical of nonmotile strains. These mutations were dominant. The *frzD* lesions have recently been shown to lie within the *frzC* gene near the 3′ end. This gene is now called *frzCD* (7, 29).

Time-lapse videomicroscopy revealed another aspect of the phenotype of these mutants (6). The *frz* strains reverse their direction of movement much less frequently than does the wild-type strain, about once every 120 min, for the *frz* strains compared with about once every 7 min for the wild-type strain. In contrast, the *frzD* mutants reverse their direction of movement much more frequently than the wild-type strain does, about once every 2 min. The *frzD* mutants do not show a bias in the pattern of reversals, so that there is no net translocation of these cells, and thus they appear nonmotile. These two phenotypes, prolonged movement in one direction and frequent reversals with no bias in directional movement, are similar in some respects to the phenotypes of the chemotaxis mutants of enteric bacteria. The enteric bacteria move, with the aid of flagella, as smooth runs when rotation is counterclockwise or as tumbles when rotation is clockwise. Chemotaxis mutants either swim smoothly for an extended period of time or tumble continually (27).

Recent sequence information for the *frz* genes has shown that there are strong similarities between the *frz* gene products and those of the enteric bacterial chemotaxis genes (29). The FrzA protein exhibits 28.1% amino acid identity with the CheW protein of *Salmonella typhimurium*. The FrzCD protein contains a region of about 250 amino acids which is similar to the C-terminal portions of the methyl-accepting chemotaxis receptor proteins of the enteric bacteria. The FrzE protein shares sequence similarities with CheA and CheY proteins of *S. typhimurium*, and the FrzF protein shares similarities with the CheR protein (W. McCleary and D. Zusman, manuscript in preparation). These results lend

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strong support to the hypothesis that the *frz* genes of *M. xanthus* are involved in a signal transduction system similar to the chemotaxis system of enteric bacteria.

In this study we examined the genetic regulation of the *frz* gene cluster. The transposon Tn*5-lac*, which creates transcriptional fusions between upstream promoters and the *lacZ* gene of *Escherichia coli*, was used as a reporter of gene expression (25). β-Galactosidase levels were measured under various conditions and in several mutant backgrounds. The results indicated that *frz* expression is developmentally induced during mound formation. All four genes tested showed the same pattern of expression, suggesting coordinate regulation. Evidence is presented which suggests that the first four genes (*frzA*, *frzB*, *frzCD*, and *frzE*) are organized as an operon containing an internal promoter. The proteins transcribed from the internal promoter, FrzCD and FrzE, are hypothesized to act as transcriptional activators of an upstream promoter of the gene cluster. The pattern of regulation shows similarities to those of other two-component regulatory systems of sensory transduction in bacteria (24, 32).

MATERIALS AND METHODS

Bacterial strains. *M. xanthus* DZF1 was used as the parental wild-type strain (31). The *M. xanthus* strains used in this study are listed in Table 1. New strains were constructed by using either P1 transduction from *E. coli* as described previously (33) or by transduction with the myxophage Mx4 (10). Strain construction was verified by Southern hybridization (28).

Media and growth conditions. Casitone (Difco Laboratories, Detroit, Mich.)–yeast extract (CYE) (10) was used for vegetative growth of *M. xanthus*. Development was studied on clone fruiting (CF) agar (17). Some studies were done by using a motility agar, 1/2 CTT (19). Antibiotics were added to CYE at 75 mg of kanamycin sulfate per liter and 12.5 mg of oxytetracycline per liter. Antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo. Media components were obtained from Difco.

Development was induced by plating cells that were vegetatively grown in CYE broth at 32°C with aeration until the mid- or late log phase on CF agar. The cells were pelleted at 12,000 × *g* for 10 min and suspended at 4×10^9 cells per ml in TM buffer (0.01 M Tris hydrochloride, 0.008 M $MgSO_4$ [pH 7.6]). A total of 2.5×10^9 cells were spread onto a 100-mm-diameter CF agar plate and placed in a 34°C incubator once the plate had dried.

Scanning electron microscopy. Samples of 5 μl of cells, at 4×10^9 cells per ml in TM buffer, were applied to filters (pore size, 3 μm; Nuclepore Corp., Pleasanton, Calif.) resting on the surface of agar. Filters were removed at intervals and inverted over a solution of 2.5% glutaraldehyde for 8 to 16 h. After fixation, the filters were cut to fit the specimen holders. Samples were dehydrated in an ethanol series, dried to the critical point, and coated with platinum. Coated samples were viewed with a scanning electron microscope (DS-130; ISI) equipped for Polaroid photography.

Enzyme assays. Cells were harvested from plates in TM buffer by scrapping them off with a razor blade. Cells were frozen at −20°C and then broken by agitation in a Minibeadbeater (Biospec Products, Bartlesville, Okla.) by using zirconium beads (diameter, 0.10 to 0.15 mm). Cells and spores were broken after five 30-s pulses. Breakage was monitored microscopically. Samples were spun down for 5 min in a microfuge, and the supernatant fraction was used in the enzyme assays. The vegetative cells that were suspended in TM buffer before they were spread onto CF agar were the source of the zero time point. These cells were treated in the same manner as those that were scraped off the plate.

β-Galactosidase was assayed as described by Miller (30). Specific activity was expressed as nanomoles of product formed per minute per milligram of protein. Myxobacterial hemagglutinin was assayed as described by Cumsky and Zusman (12). Threonine deaminase was assayed as described by Shizuta and Tokushige (38), except that no isoleucine or AMP was added. Specific activity was expressed as nonomoles of product formed per hour per milligram of protein. Alkaline phosphatase was assayed as described by Kreuzer et al. (26) and expressed as nonomoles of product formed per minute per milligram of protein. The protein concentration was measured by the assay of Bradford (8) except for the experiment for which the results are presented in Fig. 7. In that case, BCA protein assay reagent (Pierce Chemical Co., Rockford, Ill.) was used. Bovine serum albumin was used as a standard in all cases.

Northern blot analysis. Total cellular RNA was isolated from cells that were incubated on CF agar for 16 h by the

TABLE 1. Strains used in this study

Strain	Genotype	Phenotype (reference)
DZF1	Wild type	Wild type
DZF3264	*frzB*::Tn*5*Ω*3264*	Frz Kan^r (5)
DZF3373	*frzA*::Tn*5*Ω*214*	Frz Kan^r (5)
DZF3375	*frzC*::Tn*5*Ω*221*	Frz Kan^r (5)
DZF3380	*frzE*::Tn*5*Ω*229*	Frz Kan^r (5)
DZF3558	Δ*frzABCDE* Tn*5*-Ω*233*	Frz Kan^r (5)
DZF3590	*frzA*::Tn*S-lac*Ω*535*	Frz Kan^r
DZF3591	*frzC*::Tn*5-lac*Ω*536*	Frz Kan^r
DZF3592	*frzE*::Tn*5-lac*Ω*538*	Frz Kan^r
DZF3593	*frzF*::Tn*5-lac*Ω*540*	Frz Kan^r
DZF3576	*frzA*::Tn*5-lac*Ω*535*, *frzB*::Tn*5-132*Ω*3264*	Frz Kan^r Tet^r
DZF3577	*fraA*::Tn*5-lac*Ω*535*, *frzC*::Tn*5-132*Ω*220*	Pseudorevertant[a], Kan^r Tet^r
DZF3578	*frzA*::Tn*5-lac*Ω*535*, *frzE*::Tn*5-132*Ω*226*	Pseudorevertant[b], Kan^r Tet^r
DZF3579	*frzC*::Tn*5-lac*Ω*536*, *frzA*::Tn*5-132*Ω*214*	Frz Kan^r Tet^r
DZF3580	*frzC*::Tn*5-lac*Ω*536*, *frzB*::Tn*5-132*Ω*3264*	Pseudorevertant[a], Kan^r Tet^r
DZF3581	*frzC*::Tn*5-lac*Ω*536*, *frzE*::Tn*5-132*Ω*226*	Pseudorevertant[a], Kan^r Tet^r
DZF3582	*frzE*::Tn*5-lac*Ω*538*, *frzA*::Tn*5-132*Ω*214*	Frz Kan^r Tet^r
DZF3583	*frzE*::Tn*5-lac*Ω*538*, *frzB*::Tn*5-132*Ω*3264*	Frz Kan^r Tet^r
DZF3584	*frzE*::Tn*5-lac*Ω*538*, *frzC*::Tn*5-132*Ω*220*	Pseudorevertant[b], Kan^r Tet^r
DZF3585	*frzF*::Tn*5-lac*Ω*540*, *frzA*::Tn*5-132*Ω*214*	Pseudorevertant[a], Kan^r Tet^r
DZF3586	*frzF*::Tn*5-lac*Ω*540*, *frzB*::Tn*5-132*Ω*3264*	Frz Kan^r Tet^r
DZF3587	*frzF*::Tn*5-lac*Ω*540*, *frzC*::Tn*5-132*Ω*220*	Pseudorevertant[b], Kan^r Tet^r
DZF3588	*frzF*::Tn*5-lac*Ω*540*, *frzE*::Tn*5-132*Ω*226*	Pseudorevertant[b], Kan^r Tet^r
DZF3476	*frzA*::Tn*5-lac*Ω*353*, *tagE1552*	Frz Kan^r
DZF3502	*frzC*::Tn*5-lac*Ω*536*, *tagE1552*	*fru*$^-$ Kan^r
DZF3529	*frzE*::Tn*5-lac*Ω*538*, *tagE1552*	Frz Kan^r
DZF3536	*frzF*::Tn*5-lac*Ω*540*, *tagE1552*	Frz Kan^r

[a] The pseudoreversion was only partial.
[b] The pseudoreversion was complete. The fruiting bodies that formed were indistinguishable from the wild type.

method of Garriga and colleagues (14, 15). The RNA was denatured by using glyoxal and run on a 1.4% agarose gel as described by Maniatis et al. (28). The RNA was transferred to Nytran (Schleicher & Schuell, Inc., Keene, N.H.). Hybridizations were done at 60°C as described by Johnson et al. (21). Plasmids to be used as probes were made by using pSP64 and pSP65, which were purchased from Promega (Madison, Wis.), and were labeled according to the instructions of the manufacturer. Two probes were used; one contained the entire *frzA* gene and the other contained the 5′ portion of *frzCD* up to the *Sal*I site (see Fig. 2).

Immunological techniques. Antibodies to FrzCD were a gift from Mark McBride. The enzyme-linked immunosorbent assay studies were done in the laboratory of James Allison in our department by using anti-rabbit antibody conjugated to horseradish peroxidase. *O*-Phenylenediamine was used as chromogen, and the A_{415} was read as described by Ausubel et al. (2). Western blot (immunoblot) analysis was done as described by Ausubel et al. (2) by using ^{125}I-labeled protein A, which was purchased from Amersham Corp. (Arlington Heights, Ill.).

RESULTS

***frz* mutants continually encircle potential aggregation centers.** Strains with mutations in the *frz* genes failed to form fruiting bodies but instead aggregated into tangled filaments. To better understand strains with this phenotype, we examined the arrangement of cells by scanning electron microscopy. Samples were prepared by spotting the cells on Nuclepore filters resting on 1/2 CTT agar (which causes partial starvation and increased motility), as well as on CF agar, which normally induces fruiting body formation. On 1/2 CTT agar, both wild-type and *frz* cells appeared as ropelike aggregates, although the pattern was somewhat more exaggerated for *frz* mutants (Fig. 1A and C). Some of these ropelike aggregates tended to close up on themselves, forming rings or swirls. On CF agar, the *frz* mutants looked very different from wild-type strains. Whereas wild-type cells formed a compact mound (Fig. 1B), the Frz aggregates maintained the ropelike morphology observed on 1/2 CTT agar (Fig. 1C). At later times, spores were observed surrounded by rings of rods, forming a nest (Fig. 1D). This nestlike structure appeared to form from the ring or swirl structures which were also observed on 1/2 CTT agar (see arrows, Fig. 1C). The spores may have been deposited in the center of the nest on CF agar because they were nonmotile and more or less fell in; alternatively, they may have been pushed in by the encircling rods. Many fewer spores were observed on 1/2 CTT agar, so that nestlike spore structures were not present on this medium. The morphology of the *frz* mutants on fruiting agar suggested that although the cells cannot aggregate to form fruiting bodies, they still maintain some of the cell-cell association patterns typical of wild-type cells.

The *frz* gene cluster is developmentally regulated. Since the *frz* functions appear to be involved in directed cell movement of *M. xanthus* (6) and the Frz phenotype is most evident during development on fruiting agar, we reasoned that the *frz* genes might be involved in directing the movement of cells toward aggregation centers, one of the early steps in fruiting body formation. We therefore were interested in determining whether *frz* gene expression was developmentally induced and whether the timing of expression coincided with the time when cells were moving together to form mounds. We were unable to measure most of the gene products directly. However, gene expression could be monitored by assaying the level of β-galactosidase produced from strains containing a transcriptional fusion resulting from the insertion of the transposon Tn*5-lac* (25) into each of four *frz* genes, *frzA*, *frzCD*, *frzE*, and *frzF* (7). The positions of the Tn*5-lac* insertions are shown in Fig. 2. The direction of transcription for all four insertions was from *frzA* toward *frzF*.

Each of the four strains containing Tn*5-lac* (DZF3590 to DZF3593) was plated onto fruiting agar (CF agar) to induce development. Cells were harvested from the plates every 3 h for 48 h and disrupted in the Mini-beadbeater, and the level of β-galactosidase activity was measured (Fig. 3). In each case, some β-galactosidase activity was observed in vegetative cell extracts (zero time). The vegetative activity was found to increase slightly in late-log-phase cultures. During development there was a significant induction of activity that peaked at about 12 to 18 h into development. The level of β-galactosidase activity measured at the peak of expression was as much as 10-fold greater than that in extracts of vegetative cells growing in CYE or in cells harvested from the partial starvation medium (1/2 CTT) that was used to study motility (data no shown). The time of peak expression corresponded to the period when discrete mounds of cells became evident if wild-type cells were plated for development on the same medium. Following the time of peak expression, there was a significant decline in the specific activity of β-galactosidase. It is unlikely that this decline was due to cell lysis since the stage at which developmental cells become fragile has been shown to be later, just prior to spore formation (34). Under the conditions used for these experiments, it took an additional 24 to 36 h, once mounds were evident, for the cells to convert to myxospores. *frz* gene expression was not induced in liquid medium consisting of CF salts. These conditions did not support cell aggregation or sporulation. This pattern of induction of *frz* expression was similar to that of *mbhA*, which is also developmentally induced and requires a solid surface for expression (12).

To ensure that the β-galactosidase activity measured (Fig. 3) reflected an increase in transcription rather than an increase in stability of the LacZ protein, the stability of the enzyme was determined from extracts made from cells that were harvested during development. Vegetative or developmental cells were suspended in CF liquid to which rifampin (250 μg/ml) was added to inhibit the initiation of transcription. The cells were incubated at 34°C and harvested at various times, and the β-galactosidase activity was measured. The results (Fig. 4) showed that the β-galactosidase activity was relatively stable in all three samples (vegetative stage and at 17 and 28 h of development), with greater than 80% of the activity remaining after 1 h in the developmental cells and 90% of the activity remaining in the vegetative cells. The experiment was not extended beyond 1 h since the rifampin was toxic to the cells, so that by 1.5 h many cells were showing aberrant morphologies; any decline in activity beyond that time may have been more a reflection of cell death than stability of the protein. The data presented in Fig. 4 suggest that changes in enzyme activity reflect changes in the levels of transcription of the respective genes.

Since the level of expression of a reporter gene may differ from the level of the authentic gene products, we were interested in a more direct measurement of the FrzCD protein. In our laboratory we have recently raised antibodies to FrzCD (M. McBride and D. Zusman, manuscript in preparation), so that we were able to confirm the timing of expression of this gene using immunological techniques (Fig.

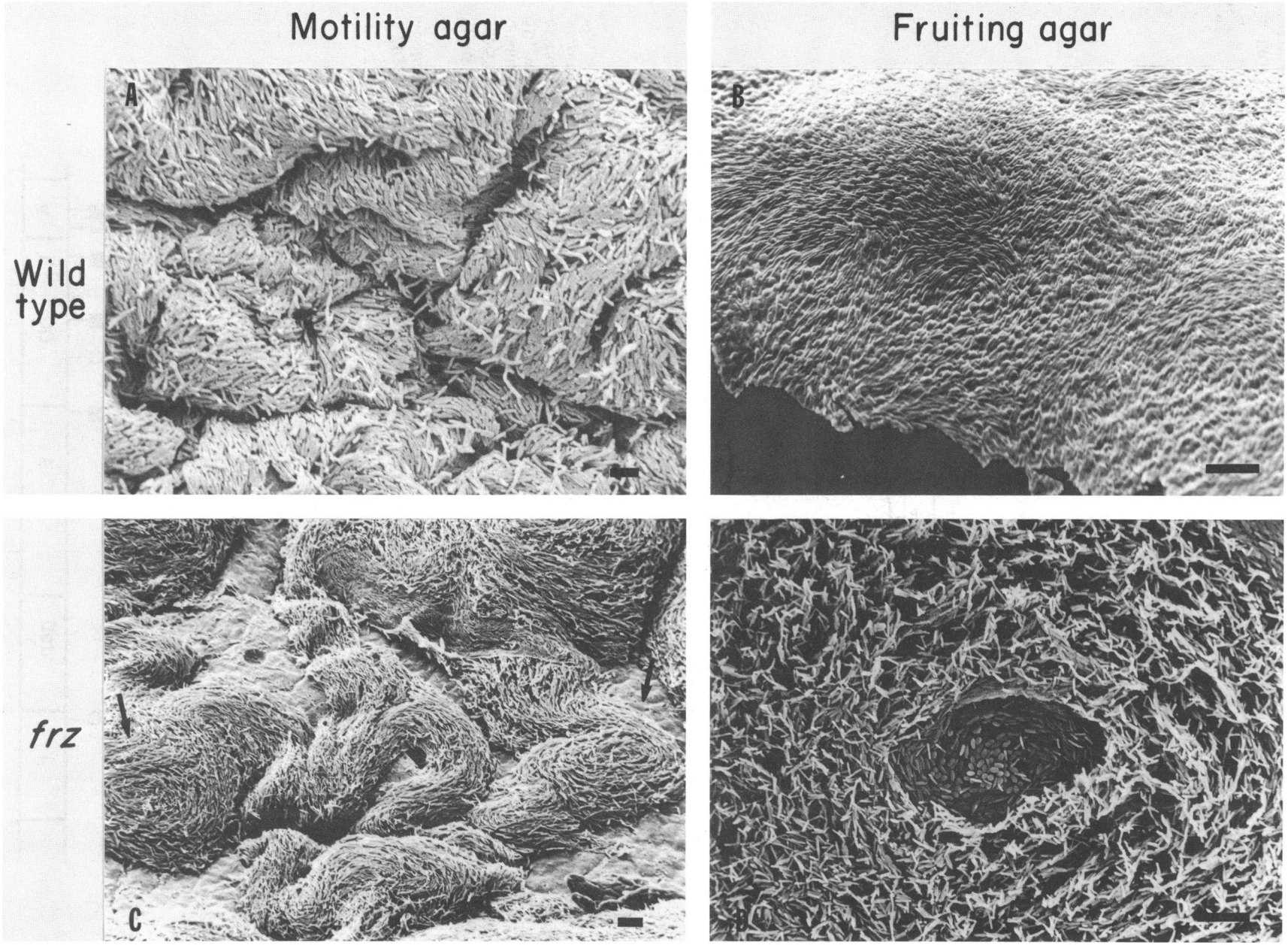

FIG. 1. Comparison of the morphology of *frz* and wild-type strains on motility and fruiting media by scanning electron microscopy. Samples were prepared as described in the text. (A) DZF1 (wild type) after 24 h on motility agar (1/2 CTT). Bar, 100 μm. (B) DZF1 after 18 h on fruiting agar (CF). Bar, 10 μm. (C) DZF3591 (*frzCD*) after 24 h on motility agar. The arrows indicate regions which we assumed were an earlier stage of what was observed at later times on fruiting agar. Bar, 5 μm. (D) DZF3591 after 24 h on fruiting agar (CF). Bar, 10 μm.

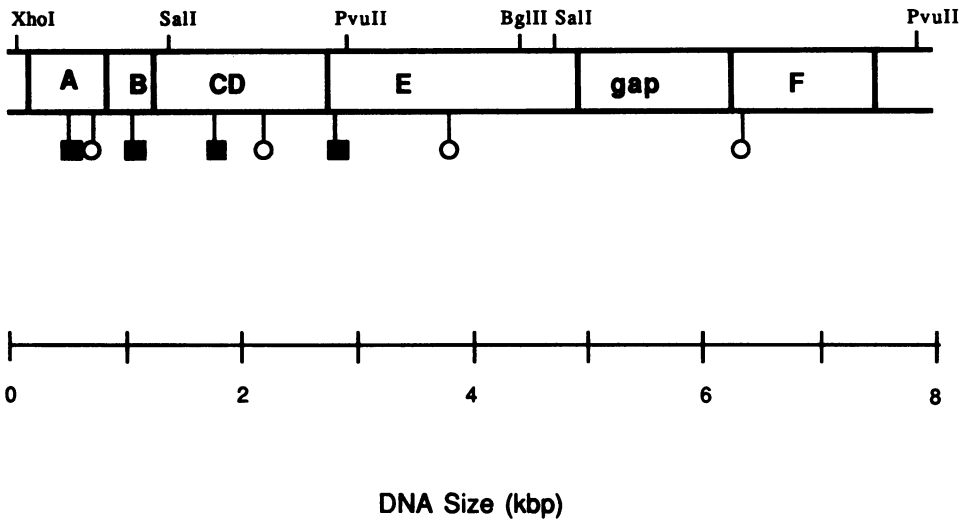

FIG. 2. Physical map of the *frz* gene cluster. Symbols: ○, Tn*5-lac*; ■, Tn*5-132*. The direction of transcription for all the Tn*5-lac* insertions was from left to right, as drawn previously (7). kbp, Kilobase pairs.

5). Western blot analysis showed that the antibody was specific to FrzCD. The relative amount of FrzCD was quantitated by enzyme-linked immunosorbent assay. Figure 5B, which is the average of three analyses of the same extracts, showed that the maximum amount of FrzCD was present between 12 and 15 h during development. Induction was about 10-fold over the vegetative level. The enzyme-linked immunosorbent assay data were remarkably similar to those obtained for the expression of *frzCD* based on β-galactosidase assays with the strain containing Tn*5-lac* in the *frzCD* gene (Fig. 3B). These results demonstrate that FrzCD is developmentally regulated and that the use of the *lacZ* reporter gene in the Tn*5-lac* insertion strains can yield an accurate representation of gene expression.

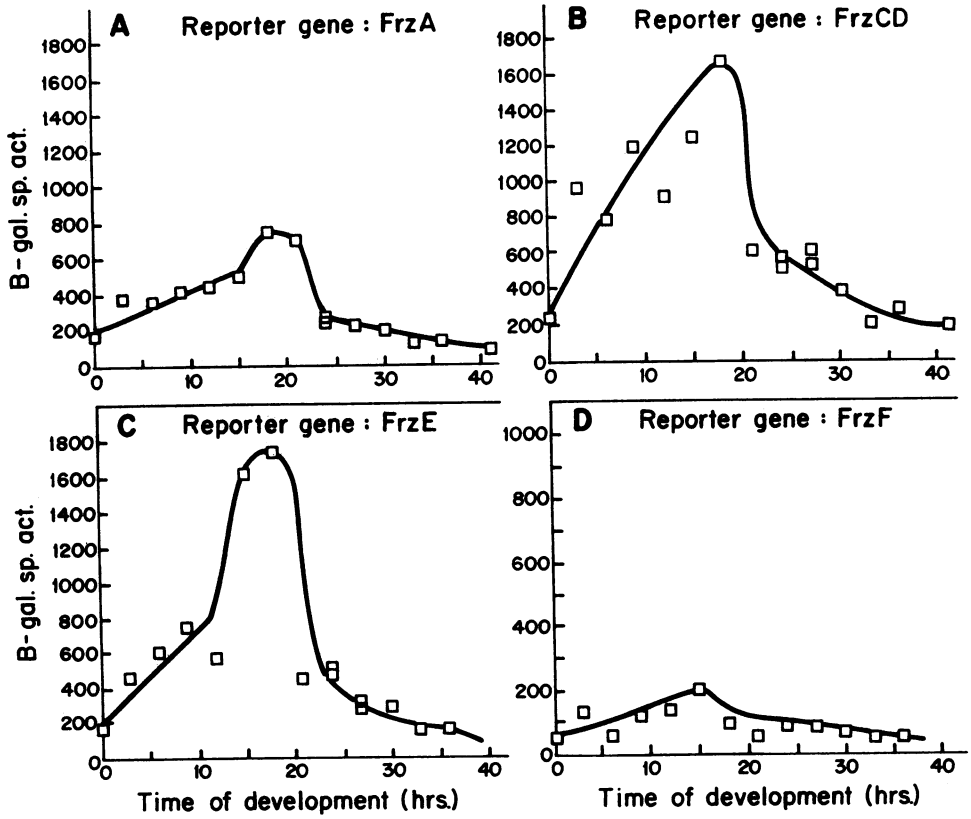

FIG. 3. Time course of induction of β-galactosidase (β-gal) from *frz*::Tn*5-lac* during development on CF agar. Strains were plated onto CF agar, and the level of β-galactosidase was measured at 3-h intervals. Cells were spread onto 100-mm-diameter plates. (A) DZF3590, *frzA*::Tn*5-lac*. (B) DZF3591, *frzC*::Tn*5-lac*. (C) DZF3592, *frzE*::Tn*5-lac*. (D) DZF3593, *frzF*::Tn*5-lac*.

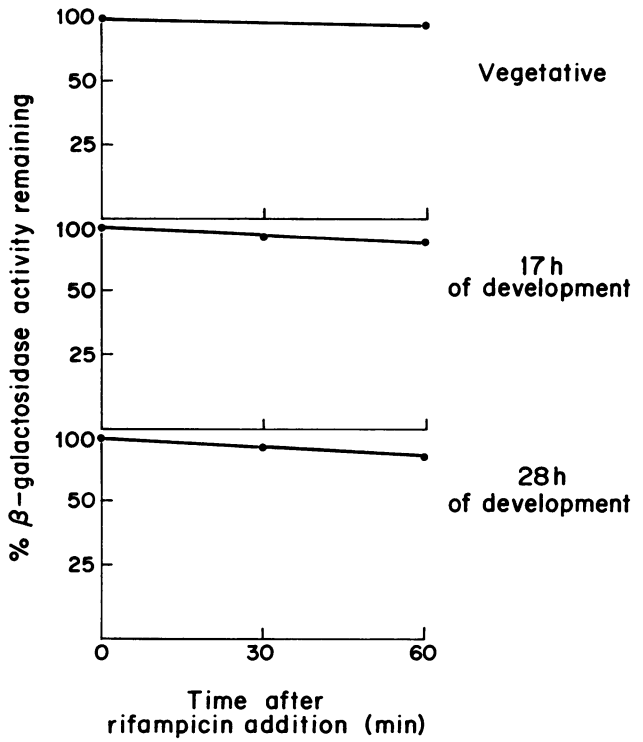

FIG. 4. Stability of β-galactosidase activity in strain DZF3591 following rifampin treatment. Cells were harvested from vegetative cultures and from cultures after 17 and 28 h of development and were suspended in CF liquid to which rifampin was added at 250 μg/ml. These cells were incubated at 34°C with aeration. Samples were taken at various times after the addition of rifampin and assayed for β-galactosidase activity; 100% activity was that measured immediately following the addition of rifampin and prior to incubation.

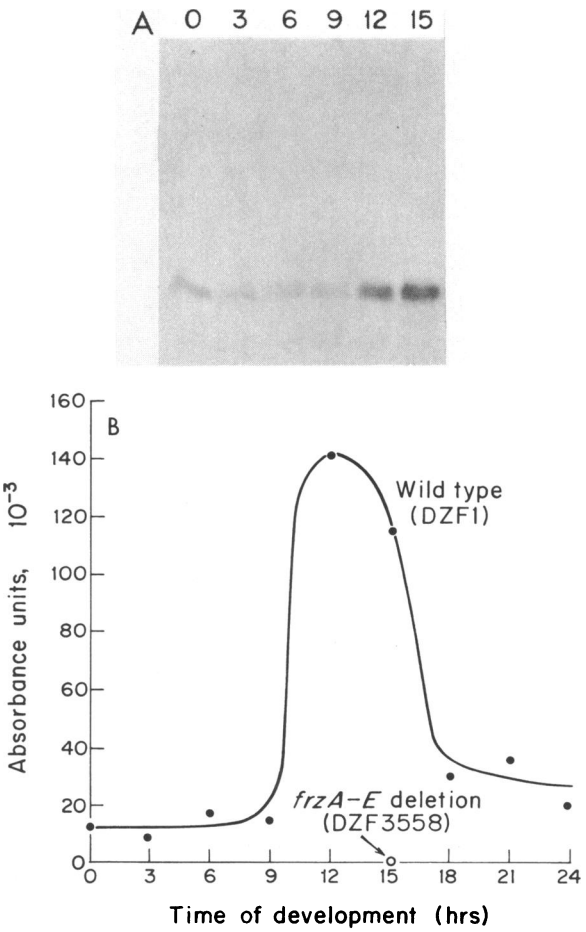

FIG. 5. (A) Western immunoblot analysis of FrzCD during development. Wild-type DZF1 was plated onto CF agar, and samples were collected every 3 h, as indicated by the numbers at the top of the gel. Equivalent amounts of protein were loaded onto the gel. Samples were run on a 10% polyacrylamide denaturing gel and probed with FrzCD antibody. (B) Enzyme-linked immunosorbent assay analysis of FrzCD during development. The relative amount of FrzCD protein present is expressed as absorbance units (10^{-3}). The curve is the average of three analyses. A strain containing a deletion from *frzA* through *frzE* (DZF3558) was used as a control.

The timing of the developmental program of the *frz* mutants is similar to that of the wild type. Three developmental markers, myxobacterial hemagglutinin (12), threonine deaminase, and alkaline phosphatase (R. Weinberg and D. Zusman, manuscript in preparation), were assayed in the *frz*::Tn*5-lac* mutants to correlate *frz* expression with the developmental program and to determine whether the *frz* mutations disrupted this program. The activities of the first two of these markers normally peak from 12 to 15 h into development, at about the time of mound formation. The third is specific to maturing spores (Weinberg and Zusman, in preparation). The times of expression of these marker enzymes were nearly identical in wild-type and *frz* strains (data not shown). Protein S, a major spore protein (20), was also produced, and it had its characteristic developmental kinetics (data not shown). Thus, the *frz* mutations did not appear to interrupt the timing of the developmental program of gene expression, although the cells failed to aggregate into raised mounds. Furthermore, because some of the markers we measured, alkaline phosphatase and protein S, were expressed at later times in development than the *frz* genes were, the decline in expression observed for *frz* was most likely real and not an artifact of cell fragility.

Is the *frz* gene cluster an operon? Since the *frz* genes are contiguous on the chromosome, are transcribed in the same orientation (7), appear to be coordinately regulated, and have very little noncoding region between genes (29), it seemed plausible that the genes were organized as an operon. We reasoned that if the genes were organized as an operon, then transposon insertions in the upstream genes should be polar and diminish transcription of downstream genes. To test this hypothesis, RNA was isolated from cells that were at 16 h of development. These cells were of strains containing Tn*5* in *frzA* (DZF3373), *frzB* (DZF3264), *frzCD* (DZF3375), or *frzE* (DZF3380), as well as wild-type strain DZF1. Northern blots were probed with one of two riboprobes; one contained the *frzA* gene and the other contained the 5′ region of *frzCD* up to the *Sal*I site (Fig. 2). This region of *frzCD* was chosen as a probe since it did not share sequence identity with the chemoreceptors. We were concerned that *M. xanthus* might encode other proteins that shared the conserved receptor domain since other bacteria are known to encode several receptor proteins. The specificity of these probes was checked by genomic Southern blotting analysis. Both probes hybridized only to their cognate restriction fragments (data not shown). The results of the Northern blot hybridizations are shown in Fig. 6. The hybridization bands were broad and diffuse, indicating that the mRNA was very unstable. (Control experiments done by

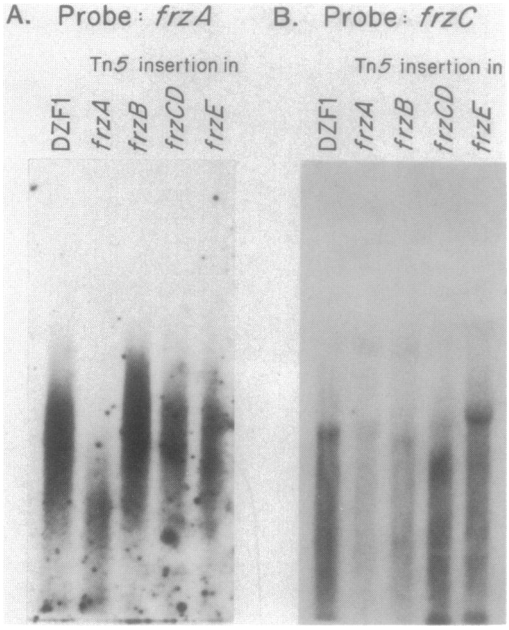

FIG. 6. Northern blot analysis of RNA isolated from cells after 16 h of development. RNA from wild-type DZF1, as well as strains with Tn*5* in each of the *frz* genes (*frzA* in DZF3373, *frzB* in DZF3264, *frzCD* in DZF3375, and *frzE* in DZF3380), were probed with the region encompassing the entire *frzA* gene (A) or the 5′ end of *frzCD* up to the *Sal*I site (B).

the same Northern blot procedure for the *mbhA* mRNA gave very sharp and discrete bands. This indicates that the smearing is not an artifact of the procedure.) Nevertheless, analysis of the relative abundance of the broad bands gave some indication of the relative abundance of the mRNA and, therefore, of gene transcription.

Since *frzA* is the most upstream gene in the cluster, if the *frz* genes were part of an operon, then the level of *frzA* transcript would be expected to be the same in all strains except that containing the Tn*5* in the gene itself. The results that were obtained were not totally consistent with this prediction (Fig. 6A). The levels obtained in DZF1 and *frzB*::Tn*5* were equivalent; however, those in *frzCD*::Tn*5* and *frzE*::Tn*5* appeared to be lowered. This result could not be explained by polar effects and suggested that these two proteins, FrzCD and FrzE, might be necessary for maximal transcription of *frzA*. The results obtained with the *frzCD* probe were more straightforward. Insertions in the two upstream genes, *frzA* and *frzB*, lowered the level of transcription of *frzCD*, while that in the downstream gene, *frzE*, did not (Fig. 6B). The Tn*5* insertion in *frzCD* was downstream of the *Sal*I site; therefore, *frzCD* transcription could still be measured in this strain. The results obtained with the *frzCD* probe suggested that the genes were organized as an operon.

The pattern obtained on the Northern blots, a smear instead of a band, suggested that the RNA was unstable and not amenable to further analysis. We therefore used the reporter genes described above to test our hypothesis. Double mutants were constructed with the transposon Tn*5-132* either upstream or downstream of the reporter Tn*5-lac*. (Tn*5-132* is similar to Tn*5*, except that it contains the tetracycline resistance gene in place of the kanamycin resistance gene [3]). The genotypes of the double mutants were confirmed by Southern blot analysis (data not shown). If the

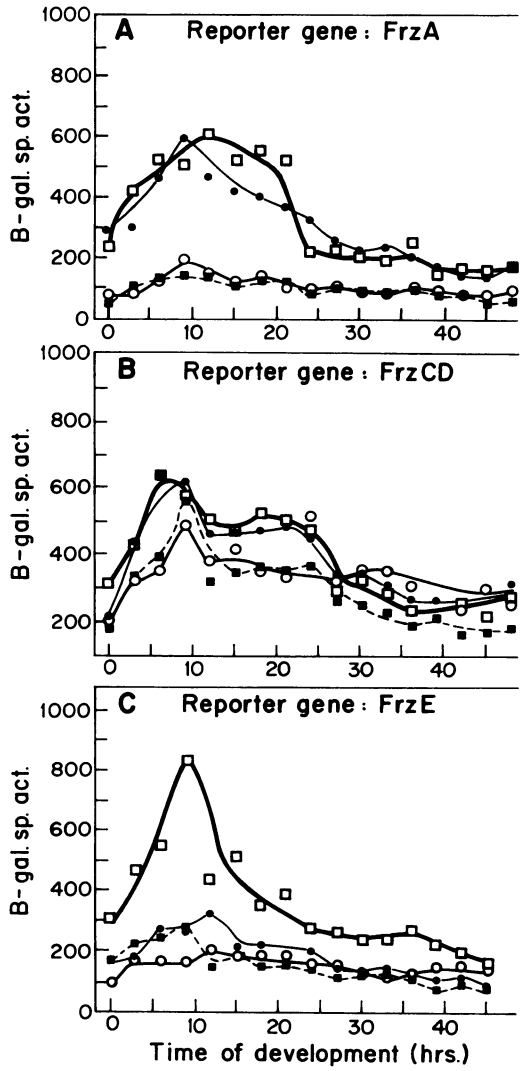

FIG. 7. Effect of the presence of a second transposon in *frz* on the expression of β-galactosidase from *frz*::Tn*5-lac*. Tn*5-132* was inserted either upstream or downstream of the reporter Tn*5-lac*, and the level of expression was measured during development on CF agar. Time points were taken at 3-h intervals. Cells were spread onto 150-mm-diameter plates. (A) Reporter gene *frzA*. Symbols: □, DZF3590, *frzA*::Tn*5-lac*; ●, DZF3576, *frzA*::Tn*5-lac frzB*::Tn*5-132*; ■, DZF3577, *frzA*::Tn*5-lac frzCD*::Tn*5-132*; ○, DZF3578, *frzA*::Tn*5-lac frzE*::Tn*5-132*. (B) Reporter gene *frzCD*. Symbols: □, DZF3591, *frzCD*::Tn*5-lac*; ●, DZF3579, *frzCD*::Tn*5-lac frzA*::Tn*5-132*; ■, DZF3580, *frzCD*::Tn*5-lac frzB*::Tn*5-132*; ○, DZF3581, *frzCD*::Tn*5-lac frzE*::Tn*5-132*. (C) Reporter gene *frzE*. Symbols: □, DZF3592, *frzE*::Tn*5-lac*; ●, DZF3582, *frzE*::Tn*5-lac frzA*::Tn*5-132*; ■, DZF3583, *frzE*::Tn*5-lac frzB*::Tn*5-132*; ○, DZF3584, *frzE*::Tn*5-lac frzCD*::Tn*5-132*. The bold line is used to delineate the expression of β-galactosidase in the control strains which did not have Tn*5-132*.

genes were arranged as an operon, then insertional mutations upstream of the reporter Tn*5-lac* should have been polar and should have lowered the level of β-galactosidase activity, while those downstream should have had no effect. Figure 7 shows the level of β-galactosidase activity measured in extracts of the double mutants during the course of development on CF agar. (It should be noted, when comparing these data with those presented in Fig. 3, that the conditions used in the two experiments were slightly different. For the experiments reported in Fig. 7, cells were

spread onto larger plates [diameter, 150 mm], which were more convenient for preparing developmental cells. This resulted in less synchronous development; however, the pattern of gene expression was still evident.) In the case of *frzA* expression (Fig. 7A), the results obtained by using Tn*5-lac* expression paralleled those obtained by Northern blot analysis. The Tn*5-132* insertion in *frzB* (DZF3576) had no effect, yet those in *frzCD* (DZF3577) and *frzE* (DZF3578) greatly reduced the level of β-galactosidase activity from that of Tn*5-lac* in *frzA*. Figure 7B shows that *frzCD* expression was not affected by the upstream Tn*5-132* insertions in *frzA* (DZF3579) or *frzB* (DZF3580), nor was it affected by the downstream insertion in *frzE* (DZF3581). This result did not parallel that obtained by Northern blot analysis. The reasons for this are addressed below. Figure 7C shows that *frzE* expression was lowered by all Tn*5-132* insertions upstream of *frzE*. These results support the general hypothesis that there is one operon for *frzA*, *frzB*, *frzCD*, and *frzE* but indicate several complexities, which are addressed below.

Double *frz* mutations can lead to pseudoreversion. The construction of double mutants lacking two *frz* functions revealed another interesting phenomenon: The introduction of a second *frz* mutation sometimes caused pseudoreversion of the Frz phenotype. Some of the double mutants more closely resembled the wild-type phenotype on CF agar than the Frz phenotype (Table 1 and Fig. 8C). In addition, partial pseudoreversion phenotypes were observed in some strains; these strains showed evidence of fruiting bodies as well as frizzy filaments (Fig. 8B). Southern blot hybridization confirmed that these strains were haploid for the *frz* gene cluster. Thus, the phenotype was not the result of the formation of partial diploids in *frz*. Although we do not know the reason for the pseudoreversion, a similar phenomenon has been described for some flagella and chemotaxis double mutants of *S. typhimurium* and has proved useful in deciphering protein interactions (35). One possible explanation of the pseudoreversion we observed may be that there was residual expression of downstream genes from promoters reading outward from the transposon; however, no evidence for outward promoters from Tn*5* have been observed (3). The pseudoreversion effects may also have been due to balancing of the individual activities of the remaining Frz functions, leading to more normal patterns of movement. Alternatively, the existence of two mutations might allow other proteins whose normal function is similar but not identical to that of the Frz proteins to act in their place.

Interactions of *frz* and *tag* aggregation functions. Since the *frz* genes were developmentally regulated, as indicated in the experiments described above, we were interested in studying their interactions with other developmentally regulated aggregation functions. The *frz* mutants were originally isolated as a subclass of mutants that were unable to aggregate properly but were still competent to sporulate. Another member of this class was a group of mutants called *tag* (temperature dependent for aggregation). These mutants appeared to be the wild type at 28°C, but at 34°C they were unable to form raised aggregates. The phenotype of these mutants suggested that they might be blocked at an earlier step in aggregation than the *frz* mutants were. This idea was strengthened when it was found that the *tag* genes were expressed earlier in development than the *frz* genes were. These data were obtained by using Tn*5-lac* as a reporter, as has been done for the *frz* genes (K. O'Connor and D. Zusman, manuscript in preparation).

If a strain had mutations in both the *tag* and *frz* genes, the *tag* phenotype should be epistatic to the *frz* phenotype if the *tag* mutants were blocked at an earlier step in aggregation and if these two steps were linearly dependent (that is, completion of the *tag* step would be required for the *frz* functions to act). To test this hypothesis, *tag* mutations were transduced into *frz* mutants. The results obtained did not support our hypothesis. The *frz tag* double mutants showed two phenotypes, depending on the specific alleles involved. One was similar to Frz, while the other was a novel phenotype which did not appear to be Frz, Tag, or the wild type (Fig. 9). The second class was defective in aggregation; mutants in this class formed amorphous mounds and never sporulated. This novel phenotype was obtained whenever a *tagC* mutation was introduced into any of the *frz* mutants and also resulted from the introduction of a *tagE* lesion in certain *frz* mutants. Even though the Tag phenotype is temperature dependent, the phenotype of the double mutants did not appear to be affected by temperature; the strains looked identical at 28 and 34°C. These results suggested that although our original hypothesis was incorrect, there did seem to be some interaction between the two systems since the double mutations caused a much more severe change in phenotype (that is, the inability to sporulate) than did either mutation alone.

A number of developmental markers were assayed in the double mutants with the amorphous mound phenotype to determine the stage in development at which they were arrested. They did not express myxobacterial hemagglutinin or alkaline phosphatase. Furthermore, although these strains were unable to form spores on CF agar, they were capable of sporulating when induced with glycerol (13).

A *tagE* mutation prolongs *frz* expression. During the course of these experiments it was noted that a particular *tagE* lesion (*tagE1552*), when present in a *frzCD* background, conferred a nonsporulating phenotype on the cells (DZF3502). However, when this *tagE1552* mutation was present in the *frzA* (DZF3476), *frzE* (DZF3529), or *frzF* (DZF3536) background, the Frz phenotype was retained. Since these strains were constructed by using the *frz*:: Tn*5-lac* strains, we were able to assay for *frz* gene expression in the double mutants by measuring β-galactosidase activity during development, as described above. Figure 10 shows that at 34°C, all four strains exhibited sustained expression of the β-galactosidase reporter gene, lasting several days as opposed to hours (compare Fig. 3 and Fig. 10A). In addition, the nonsporulating *frzCD tagE* (DZF3502) mutant exhibited a significantly higher level of *frzCD* gene expression, nearly 4 times greater than that seen in the absence of *tagE1552* (compare Fig. 10A with Fig. 3). At 28°C the *tagE1552* mutation had no effect on the expression of *frzA* or *frzCD* (Fig. 10B). Thus, although the phenotype of the *frz tagE* double mutants showed no temperature effect, gene expression did. These results suggest that a developmental function that is absent from *tagE1552* mutants modulates *frz* expression and may be particularly important in turning off transcription. The increase in expression in the nonsporulating strain has been observed in Western immunoblots as well (McBride and Zusman, in preparation).

DISCUSSION

Role of *frz* genes in developmental aggregation. The myxobacteria are unique among procaryotes in their ability to build a multicellular structure, the fruiting body. The studies presented here address how the cells undergo the first stage of this process, directed movement to an aggregation center. Previous work from our laboratory has shown that the Frz

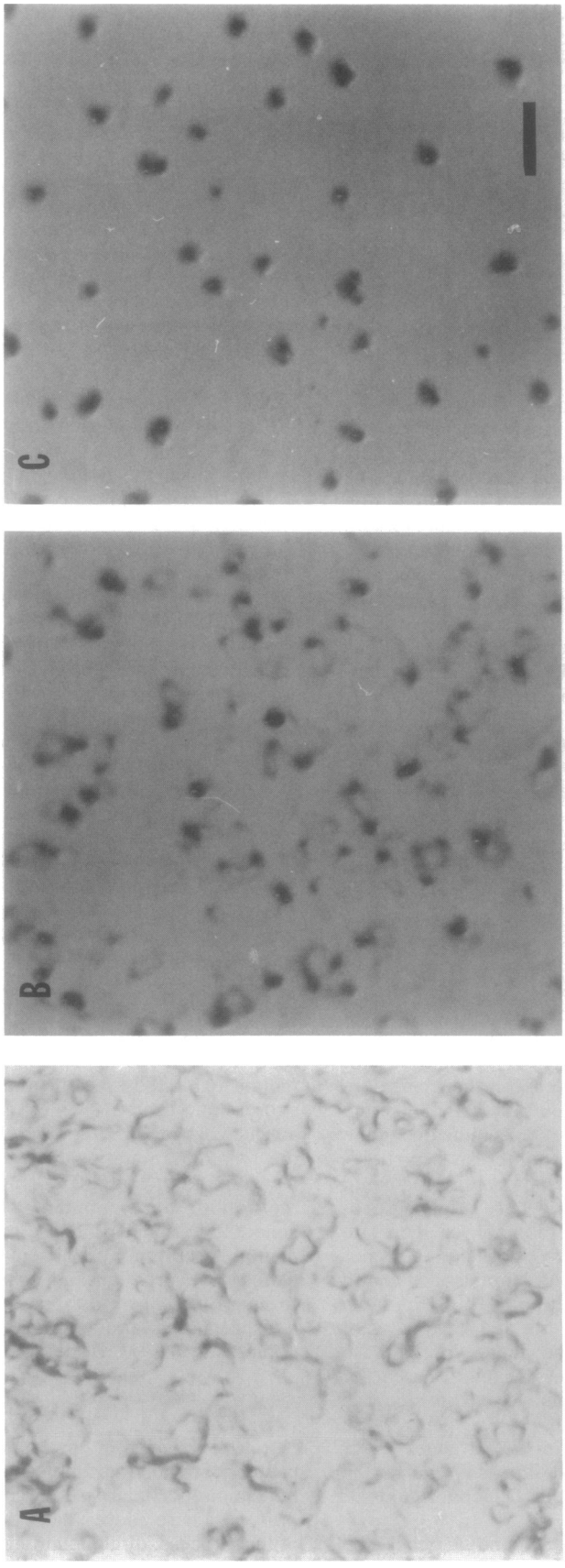

FIG. 8. Examples of the different phenotypes observed in the *frz-frz* double mutants. Cells were plated onto CF agar and incubated for 48 h at 34°C. (A) DZF3579, *frzCD*::Tn*5-lac frzA*::Tn*5-132*, retained the Frz phenotype. (B) DZF3577, *frzA*::Tn*5-lac frzCD*::Tn*5-132*, showed a partial pseudorevertant phenotype; there was still evidence of swirling. (C) DZF3578, *frzA*::Tn*5-lac frzE*::Tn*5-132*, was a complete pseudorevertant that was indistinguishable from the wild type. Bar, 0.058 cm.

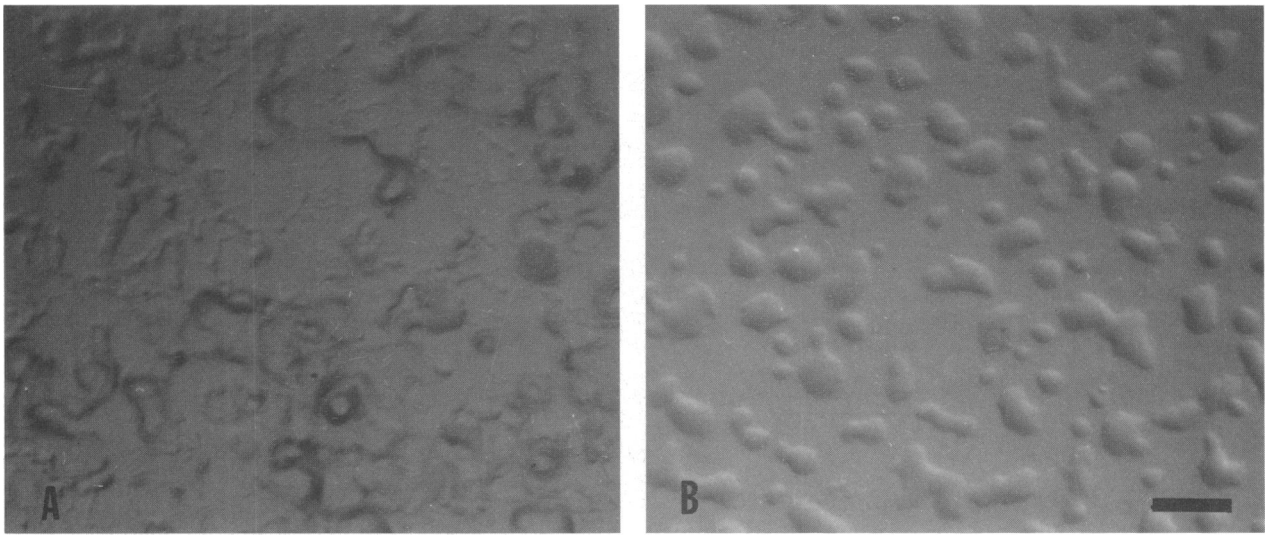

FIG. 9. Phenotypes of *frz tagE* double mutants. Cells were plated onto CF agar and incubated at 34°C. Photographs were taken 48 h after plating. (A) DZF3476, *frzA*::Tn*5-lac tagE1552*. (B) DZF3502, *frzCD*::Tn*5-lac tagE1552*, nonsporulating strain. Bar, 0.071 cm.

proteins are essential for proper directed movement (6). Strains that are mutant in these functions exhibit abnormal patterns of reversal of movement at the single-cell level and form aberrant aggregates at the multicellular level. Sequence analysis of the *frz* genes revealed that most of the *frz* protein products share large regions of sequence identity with enteric chemotaxis proteins (29). In this report we have demonstrated that the *frz* genes are expressed developmentally, with the peak activity coinciding with the time of mound formation. This is a period when one can see directed cell movement. The *frz* gene products may function as a sensory transduction system for developmental taxis. In this regard, it has been demonstrated from work by Kaiser and colleagues (17) that diffusible signals are exchanged between cells during development. Developmental-specific chemotaxis systems have been described in *Caulobacter crescentus* (11), *Rhizobium meliloti* (4), and *Dictyostelium discoideum* (37).

Regulation of *frz* expression. The expression of the *frz* genes studied (*frzA*, *frzCD*, *frzE*, and *frzF*) was found to be coordinate during development. One explanation for this pattern of expression was that the genes were organized as an operon. This seemed likely since all the genes were contiguous on the chromosome and transcribed in the same orientation. Furthermore, DNA sequence analysis has revealed that there were very short noncoding regions between genes: 32 nucleotides between *frzA* and *frzB*, 5 nucleotides between *frzB* and *frzCD*, and 33 nucleotides between *frzCD* and a potential start site of *frzE* (29). To test this hypothesis, we introduced a transposon into various *frz* genes and measured the effect of this mutation on the transcription of other genes in the cluster either directly by Northern blot analysis or by measuring expression of a reporter gene (*lacZ*) to see whether the transposons had polar effects on the transcription of downstream genes, which would have been indicative of cotranscription. These studies were confined to the first four genes of the cluster, *frzA*, *frzB*, *frzCD*, and *frzE*, because the low level of expression of β-galactosidase from *frzF*::Tn*5-lac* made it difficult to interpret the results.

One of the problems of working on a system in which the protein products cannot be measured directly is that one is never certain whether the transcription patterns observed actually reflect synthesis of the gene product. We were able to get around this problem for one of the gene products, FrzCD. The results obtained by looking at the FrzCD gene product directly paralleled those obtained by the more indirect methods with the reporter gene. These findings suggest that the expression of β-galactosidase for the *frz*::Tn*5-lac* constructs accurately reflects transcription of the *frz* genes. Furthermore, the results of the Northern blot analyses and the reporter gene studies were also compatible.

Several lines of evidence suggest that, in fact, the *frz* genes are organized as an operon. (i) Northern blot analysis with the *frzCD* probe (Fig. 6B) indicated that *frzCD* is cotranscribed with *frzA* and *frzB*. Transposon insertions in these two upstream genes diminished the level of *frzCD* transcript. (ii) The *frzE*::Tn*5-lac* data (Fig. 7C) imply that *frzE* is cotranscribed with the upstream genes *frzA*, *frzB*, and *frzCD* since insertions in these three upstream genes decreased the level of β-galactosidase synthesized from *frzE*::Tn*5-lac*. However, other data suggest that it is not a simple operon. Both Northern blot analysis (Fig. 6A) and *frzA*::Tn*5-lac* data (Fig. 7A) indicate that the proteins FrzCD and FrzE are required for maximal transcription of *frzA*. In both instances these downstream transposon insertions lowered the level of transcription of *frzA*. Perhaps the least straightforward results were those obtained in the experiments involving *frzCD* transcription. Northern blot analysis (Fig. 6B) indicated that transposons in *frzA* and *frzB* were polar on *frzCD* transcription, while the data on *frzCD*::Tn*5-lac* expression showed no polar effects by *frzA*::Tn*5* and *frzB*::Tn*5* (Fig. 7B). These results could be reconciled if a second promoter that was not dependent on the FrzCD protein for maximal induction were present just upstream of *frzCD*. If this were the case, then the transcription measured by Northern blot analysis (Fig. 6B) would be from the *frzA* promoter since the FrzCD protein would be present in the wild-type as well as in the *frzA*::Tn*5* and *frzB*::Tn*5* strains. The *frzCD*::Tn*5-lac* strain, on the other hand, would report transcription from the *frzCD* promoter (Fig. 7B). Since Tn*5-lac* disrupted the *frzCD* gene, no *frzCD* gene product would be present for the induction of the *frzA* promoter. This gene organization would also ex-

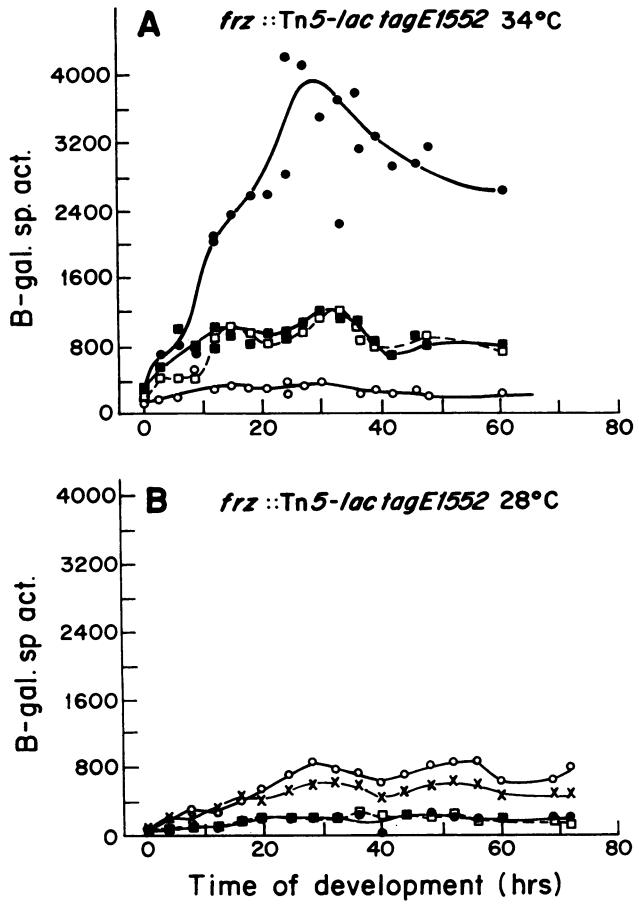

FIG. 10. Effect of the *tagE1552* mutation on *frz*::Tn*5-lac* expression. The conditions were as described in the legend to Fig. 2. (A) CF agar plates were incubated at 34°C. Symbols: ●, DZF3502, *frzCD*::Tn*5-lac tagE1552*, nonsporulating strain; ■, DZF3529, *frzE*::Tn*5-lac tagE1552*; □, DZF3476, *frzA*::Tn*5-lac tagE1552*; ○, DZF3536, *frzF*::Tn*5-lac tagE1552*. (B) CF agar plates were incubated at 28°C. Symbols: ○, DZF3502, *frzCD*::Tn*5-lac tagE1552*, nonsporulating strain; ×, DZF3591 *frzCD*::Tn*5-lac*, control; ●, DZF3476, *frzA*::Tn*5-lac tagE1552*; □, DZF3590, *frzA*::Tn*5-lac*, control. β-gal, β-Galactosidase.

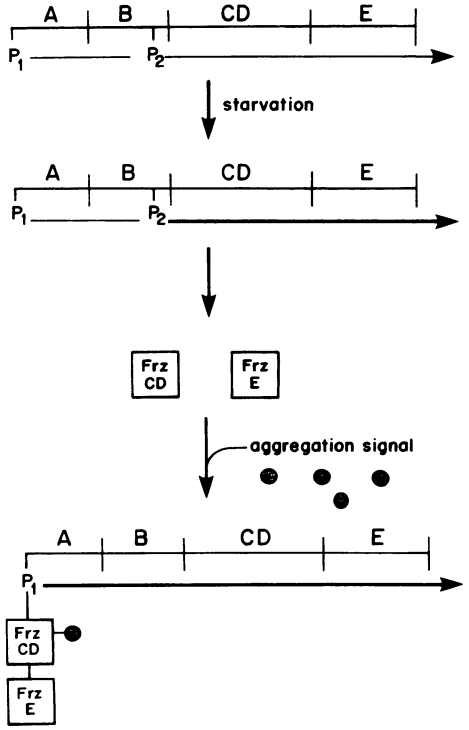

FIG. 11. Model of regulation of the *frz* gene cluster. During vegetative growth there was weak transcription from both P_1, the promoter upstream of *frzA*, and P_2, the promoter upstream of *frzCD*. At the onset of starvation, transcription from P_2 was increased, leading to the synthesis of FrzCD and FrzE. These two proteins then caused increased transcription from P_1. In order for FrzCD to act as a stimulator of transcription, it may have to bind to some effector molecule. Transcription from P_1 may preclude transcription from P_2.

plain genetic mapping data in which full complementation of *frzCD* and *frzE* was only achieved when the two genes were contiguous (5). The most plausible gene organization of these four *frz* genes is that presented in Fig. 11. All four genes compose an operon which has two promoters (one upstream of *frzA* and the other upstream of *frzCD*), and expression from the upstream promoter appears to require both the FrzCD and FrzE proteins.

How is expression of these genes regulated during development? As described in the legend to Fig. 11, we hypothesize that during vegetative growth there is a low level of expression from one or both promoters or perhaps a vegetative upstream promoter. Upon starvation or induction of the developmental cycle, transcription from promoter P_2 increases, allowing sufficient synthesis of both FrzCD and FrzE proteins. These proteins may then interact to initiate the regulatory cascade described above. We suggest that the FrzCD protein may have to bind some signal in order to activate transcription from promoter P_1 because it is known that during development signals are exchanged between cells and FrzCD is homologous to the methyl-accepting receptor proteins of other bacteria (29). This model implies that transcription from P_2 is induced prior to that from P_1. We have no direct evidence that this is the case, but the time differential may be too small to have been evident in the experiments described here. The promoter region for *frzCD* must lie within the coding region of *frzB*, since there are only 5 nucleotides of noncoding region between the two genes. This hypothesis is consistent with an unusually high AT bias identified near the terminus of the *frzB* gene (29). This arrangement suggests that transcription through *frzB* from the promoter upstream of *frzA* (P_1) may preclude use of the *frzCD* promoter (P_2).

At a later stage of development, when aggregation into mounds was completed, *frz* gene expression from all promoters appeared to be very low. We hypothesize that this may occur, in part, because the aggregation signal is no longer present in the cell. The FrzCD protein lacking the aggregation signal may no longer promote transcription from the *frzA* promoter. Results of the studies involving the *tag* mutants suggest that the *tag* gene products may be involved in down regulating transcription of *frz* and may provide us with a means of understanding what is turning off gene expression. The increased level of *frzCD* expression in the nonsporulating mutant suggests that the process of sporulation may be important in modulating expression. If our hypothesis is correct and the Frz proteins are involved in directing cell movement, then it seems logical that when cells differentiate into nonmotile myxospores, expression of the *frz* genes should be turned off. Closer examination of the

tag-frz mutants that did sporulate revealed that the aggregates formed were not identical to those of the *frz* mutants. Instead of the spores being deposited in the center, they were deposited on the periphery of the aggregate. This observation may suggest that the relative position of the cells may also be important in modulating gene expression.

It should be emphasized that the model presented here is only a hypothesis and that many questions about the *frz* genes remain. It does serve as an outline from which to address in greater detail the regulation of expression of the *frz* genes. For instance, we do not know the actual promoters for any of the *frz* genes. We were unable to recognize any known promoter region in the sequence. It is possible that additional promoters are located upstream of *frzE* since strains with Tn*5* insertions in *frzCD* continued to express the *frzE* gene, although at a lower level, and the expression did not appear to be developmentally regulated. It is possible that FrzE alone is required for the use of P_1 since all mutations we looked at in *frzCD* were polar and should have lowered the level of *frzE* transcription. However, the *frzE*::Tn*5-lac* data seem to argue against this, since in this instance P_1 was used. Further experiments must be done to clarify this point.

Similarities between the regulation of *frz* and other regulatory systems. The model proposed here is consistent with some aspects of the regulation of motility genes in other systems, such as *C. crescentus* (9, 11), *Bacillus subtilis* (18), and the enteric bacteria (23, 35). In these species, a hierarchy of gene expression exists that reflects the morphogenesis of the motility apparatus; proteins that are used early are expressed first and are required for the expression of later genes. In a number of instances, regulation appeared to be at the level of transcription. In the case of the Frz proteins, FrzCD senses some environmental parameter since it shares sequence identity with the chemotaxis receptor proteins. This would place it at the start of a signal transduction cascade and would be analogous to the hierarchical regulation described above.

The gene organization we propose for *frz* is very similar to what has been described for the *glnA* operon of *S. typhimurium* (24). In both instances the two proteins that regulate the operon lie downstream of the regulated gene and can be transcribed from an internal promoter. Transcription of *glnA* requires NtrB and NtrC. The gene order of this operon is p *glnA* p *ntrB ntrC*. In both instances transcription is turned on in response to changes in environmental conditions; in the case of *glnA* it is nitrogen limitation, and in the case of *frz* we think it is starvation (development). It is also of interest that NtrB and NtrC are members of a family of two-component regulatory systems. The cell uses these systems to monitor changes in the environment and to promote transcription of appropriate genes that allow the cell to adapt to these changes (22). The two chemotaxis proteins with which FrzE shares sequence similarity, CheA and CheY (McCleary and Zusman, in preparation), are also members of this family of signal transducers (32), extending the analogy even further.

A second observation that agrees with the model proposed here is the following. Besides sharing sequence identity with the methyl-accepting chemoreceptors at the C-terminal end, the N-terminal portion of FrzCD shows 32% amino acid identity (over 79 residues) to a region of *B. subtilis* RpoD (sigma 43) (29). Although this region of RpoD has been implicated in the binding of sigma factor to DNA (16), it is not yet known whether this region of FrzCD would actually bind to DNA. It is possible that interaction of FrzCD with FrzE or some aggregation signal is necessary for FrzCD to bind the DNA. In this regard, it is worth noting that the motility genes, including the chemotaxis genes, of both *B. subtilis* (18) and *E. coli* (1) are transcribed by minor sigma factors.

In conclusion, the Frz proteins appear to be necessary for proper fruiting body formation. The genes are maximally transcribed during the time of mound formation. The expression of these genes appears to be autoregulated, with the FrzCD and FrzE proteins acting as positive regulators of transcription, although other factors must be involved in the developmental regulation. The role of the Frz proteins may be to direct the cells to aggregation centers and initiate the mound-building process. We do not know what signals are involved in directing these movements. It seems reasonable that some signal(s) must be responsible for this movement, even though none has been identified. The sequence homology of the *frz* proteins to the chemotaxis proteins makes them likely transducers of such signals. It is also apparent that other developmental systems such as Tag must act in conjunction with Frz in directing proper fruiting body formation. We are currently trying to resolve some of the questions raised here.

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