Novel Developmental Genes, fruCD, of Myxococcus xanthus: Involvement of a Cell Division Protein in Multicellular Development
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Myxococcus xanthus is a gram-negative soil bacterium that undergoes multicellular development upon nutrient starvation. In the present study, two novel developmental genes, fruC and fruD, of M. xanthus were identified and characterized. The FruD protein has significant amino acid sequence similarity to the DivIVA proteins of many bacteria including Bacillus subtilis. Vegetative cells of the fruD mutant exhibited a filamentous phenotype. The fruC and fruD mutants displayed similar delayed-development phenotypes. The formation of tightly aggregated mounds by fruC and fruD mutants was slower than that by the wild-type strain. Spore formation by the fruC and fruD mutants initiated after 30 h poststarvation, whereas wild-type M. xanthus initiated spore formation after 18 h. The fruCD genes were constitutively expressed as an operon during vegetative growth and development. S1 mapping revealed that transcription initiation sites of the fruCD operon were located 114 (P1) and 55 bp (P2) upstream of the fruA initiation codon. Only the P1 promoter was active during vegetative growth, while both the P1 and P2 promoters were active during development. The FruD protein was produced as a cytoplasmic protein and formed an oligomer during vegetative growth and development.

Myxococcus xanthus, a gram-negative soil bacterium, is a model organism for studying mechanisms of multicellular morphogenesis and cell differentiation in prokaryotes, since the organism undergoes multicellular development upon nutrient starvation (7). During vegetative growth, M. xanthus cells grow in nutrient medium with a doubling time of approximately 4 h. Upon nutrient starvation on a solid surface, vegetative growth ceases and cells begin to gather into an aggregation center by gliding. Within 4 to 12 h poststarvation, the cells form mounds that are eventually converted into fruiting bodies. In the mounds, the motile, rod-shaped vegetative cells differentiate into nonmotile, refractile myxospores. Myxospores are resistant to sonication, heat, desiccation, and other stresses. When they are placed on a rich medium, they germinate to initiate vegetative growth. Many developmentally defective M. xanthus mutants were isolated with Tn5, a Tn5-derived transposon carrying the rep region of pSC101 (11). Among 855 independent Tn5 insertions, six were identified as developmental mutations. Three Tn5 insertions, Ω221, Ω328, and Ω530, were located within a single gene, lonD, essential for the development of M. xanthus. The lonD gene is homologous to the M. xanthus lonV and E. coli lon genes and is identical to the M. xanthus hsgA gene (12, 31). One Tn5 insertion, Ω786, was located within the upstream region of the fruA gene (23).

The fruA gene encodes a putative transcription factor essential for the development of M. xanthus. The amino acid sequence of the FruA protein contains a DNA-binding motif and has sequence similarity to response regulators of two-component His-Asp phosphorelay signal transduction systems (10, 23). The fruA gene was shown to play an important role in the C signal transduction pathway (10, 28). Genetic studies suggested that FruA may be activated by phosphorylation (10). Recently, we performed two-dimensional gel electrophoresis analysis to examine the effects of csgA and fruA mutations on the expression of M. xanthus developmental genes (16). While the expression of many developmental proteins was dependent on both fruA and csgA, several proteins including protein S and DoF were expressed in a fruA-dependent but csgA-independent manner. A model of the role of fruA in the C signal transduction pathway was proposed to explain these findings (10, 16). The gene encoding the DoF protein was cloned and characterized (14, 16). Regulation of fruA expression during vegetative growth and development was analyzed recently (15).

In this study, novel developmental genes fruC and fruD of M. xanthus were identified. The fruC and fruD genes are located 3 kb upstream of the fruA gene as an operon in the M. xanthus chromosome. The fruC and fruD mutants exhibited delayed-development phenotypes. The FruD protein exhibits significant amino acid sequence similarity to the DivIVA proteins of many bacteria.

MATERIALS AND METHODS

Microbial strains and plasmids. The bacterial strains used in this study were M. xanthus DZ51, sglA1 (17), Escherichia coli DH5α, supE44 ΔlacU169 (800 lacZΔM15) hisD17 recA1 gyrA96 thi-1 relA1 (2), and E. coli BL21(DE3) dcm ompT hsdS gal (29), Saccharomyces cerevisiae PJ69-4A MATa trp1-901 leu2-3,112
cells were counted.

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nation, sonication-resistant refractile spores were treated at 50 °C for 2 h, spread on a counting chamber under a microscope. For germi-

TM buffer, and sonicated to disrupt vegetative cells. Sonication-resistant refrac-

tile spores were counted in a counting chamber under a microscope. For germi-

FIG. 1. (A) Gene organization of the fruACD region of M. xanthus. Top horizontal line, restriction map. Circles and triangles, locations of Km r and Tc r insertion mutations, respectively; open symbols, insertion mutations promoting proficient development and those producing deficient development, respectively; stippled bar, location of ΔfruCD1 mutation; arrows below the lines, fruACD genes and open reading frames deduced from the nucleotide sequence. The lower part of diagram shows an expanded view of the fruC r region and the inserts of plasmids used for complementation experiment and promoter activity analysis. Bent arrows, P1 and P2 transcription initiation sites. The indicated DNA segments were synthesized by PCR and inserted into pTCl or pSI1403attP. Nucleotide A of the fruC ATG initiation codon is indicated as +1. (B) FruC amino acid sequence deduced from the nucleotide sequence. Numbers on the right indicate residues from the N-terminal end. (C) Alignment of amino acid sequences of M. xanthus FruD and various bacterial DivIVA proteins. White lettering on a black background, identical amino acid residues.

Plasmid vector pUC19 (34) was used for cloning. pET11 Km-GST (23) was

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ured 3-200 gal4 gal80 LYS2::GAL1-HER3 GAL2-DE2 met+::GAL7-lacZ (19) was also used.

Plasmid vector pUC19 (34) was used for cloning. pET11 Km-GST (23) was used for overexpression of the gat-gst gene. pSI1403attP (14) was used for construction of lacZ transcriptional fusions. pGADT7 and pGBK7 (Clontech) were used for yeast two-hybrid system experiments. pMF1 (23) is a pUC19 derivative carrying the fruACD region.

growth conditions. M. xanthus cells were grown at 30 °C in Casitone-yeast extract (CYE) medium (4) or on CYE agar (CYE medium containing 1% agar). Kanamycin sulfate (40 μg/ml) or oxytetracycline (6.25 μg/ml) were used for the selection of kanamycin-resistant (Kmr) and tetracycline-resistant (Tc r) M. xanthus cells, respectively.

E. coli cells were grown at 37 °C in Luria-Bertani medium (21). Ampicillin (100 μg/ml), kanamycin sulfate (50 μg/ml), or tetracycline (12.5 μg/ml) was used when required. Yeast cells were grown at 30 °C in yeast-peptone-dextrose medium or synthetic dextrose (SD) medium (27).

Photographs of vegetative M. xanthus cells were taken during a phase-contrast microscope. To estimate average cell sizes, the lengths of more than 200 cells were measured.

development and sporulation. Development of M. xanthus cells was induced on clone fruiting (CF) agar (13, 17). M. xanthus cells were grown to late log phase in CYE medium, washed once with TM buffer (10 mM Tris-HCl [pH 7.6], 8 mM MgSO4), and resuspended in TM buffer at a density of 2 × 108 cells/ml. Aliquots (10 μl) of cell suspension were spotted on CF agar and incubated at 30 °C. The spots were photographed after dissection under a microscope.

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The number of spores was measured as described by Jain and Inouye (18).

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S1 nuclease mapping. The S1 nuclease mapping procedure was adapted from the method of Berk and Sharp (3). First, the 5′ ends of a 2,993-bp KpnI-Mael fragment of pMFA02 DNA were labeled with [γ-32P]ATP (3,000 Ci/mmol) by using Ready-To-Go T4 polynucleotide kinase (Amersham Pharmacia Biotech). The resulting labeled fragment was digested with KpnI. The 384-bp ApaLI-Mael fragment was hybridized with 40 μg of total RNA from vegetative cells or developing cells at 6, 12, and 24 h poststarvation and treated with 40 U of S1 nuclease at 37°C for 30 min. Then, protected DNA fragments were analyzed on 6% polyacrylamide gels containing 8 M urea.

Promoter activity during vegetative growth and development. To separately assess two promoter activity, the P1 promoter region (positions 97 to 215 upstream from the fruC initiation codon) and the P2 promoter region (positions 50 to 128 upstream from the fruC initiation codon) were synthesized by PCR with appropriate primers containing BamHI sites at their 5′ ends and then cloned into the BamHI site of pSI1403attP, giving pFL1 and pFL2, respectively (Fig. 1A). The orientations and sequences of inserted fragments were confirmed by DNA sequencing. pFL1 and pFL2 were integrated into the Mx8 attB site of the M. xanthus DZF1 chromosome to generate MxFL1 and MxFL2, respectively. β-Galactosidase specific activity was determined during vegetative growth and development as described previously (14). Units of β-galactosidase specific activity were nanomoles of o-nitrophenol produced per minute per milligram of protein.

Preparation of antisense against FruD. To purify the FruD protein fused to glutathione-S-transferase (GST), a gfruD-fruD fusion gene under the control of a T7 promoter was constructed. The fruD coding sequence was amplified by PCR and cloned into the NdeI-BamHI site of pET11 Km-GST (23) to generate pET11 Km-GST-fruD. E. coli BL2(DE3) cells harboring pET11 Km-GST-fruD were grown at 37°C in Luria-Bertani medium to an A590 of 0.5, then isopropyl-β-D-thiogalactopyranoside was added at a final concentration of 1 mM, and the culture was incubated for another 2 h. Induced cells from 200 ml of culture were harvested and suspended in phosphate-buffered saline (PBS; 137 mM NaCl, 8.1 mM Na2HPO4, 2.7 mM KCl, 1.5 mM KH2PO4 [pH 7.4]) at a density of 2 × 1010 cells/ml and then passed through a French pressure cell. The cell lysate was centrifuged at 10,000 × g at 4°C for 10 min. The pellet containing the GST-FruD inclusion body was denatured with 10 ml of PBS containing 3 M urea. The solubilized crude extract was refolded by dialysis against PBS. After dialysis, approximately 10% of the GST-FruD fusion protein was present in the soluble fraction. The soluble fraction containing the refolded GST-FruD fusion protein was loaded on a column of glutathione-Sepharose 4B (Amersham Pharmacia Biotech) equilibrated with PBS. The GST-FruD fusion protein was eluted with elution buffer (50 mM Tris-HCl [pH 8.0], 10 mM reduced glutathione). The purified GST-FruD fusion protein was used to immunize a rabbit.

Western blot analysis. Cell proteins were separated by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked with PBS containing 3% skim milk, reacted with a 1,000-fold dilution of rabbit anti-GST-FruD antiserum, and then reacted with a 100-fold dilution of a biotin-labeled goat anti-rabbit immunoglobulin G antibody. The FruD protein was detected by immunoblotting with an ABC-POD (R) kit (Wako, Osaka, Japan).

FruD production during vegetative growth and development of M. xanthus. Vegetative cells and developing cells at 24 and 48 h poststarvation were dissolved in solubilizing buffer (2% SDS, 0.08 M Tris-HCl [pH 6.8], 10% glycerol, 0.1 M β-mercaptoethanol) and boiled for 5 min. Each sample was analyzed by SDS–15% PAGE. Then, the FruD protein was detected by Western blot analysis using anti-GST-FruD antiserum.

Subcellular fractionation of M. xanthus cells. M. xanthus vegetative cells were lysed by EDTA-lysozyme and fractionated to periplasm, cytoplasm, and total membrane fractions as described previously (24). Each fraction was analyzed by SDS–15% PAGE. Then, localization of the FruD protein was determined by Western blot analysis using anti-GST-FruD antiserum.

Gel filtration chromatography. M. xanthus vegetative cells and developing cells at 6 h poststarvation were harvested, washed once with 0.1 M phosphate buffer (pH 6.8), resuspended in the same buffer, disrupted by sonication, and centrifuged at 100,000 × g for 30 min at 4°C. The supernatant fraction was subjected to TSK-Gel G2000 (Tosoh, Tokyo, Japan) gel filtration chromatography (0.1 M phosphate buffer [pH 6.8]; flow rate, 0.1 ml/min). Each fraction was analyzed by SDS–15% PAGE. The FruD protein was detected by Western blot analysis using anti-GST-FruD antiserum.

 Yeast two-hybrid system. The fruD coding sequence was cloned into the NotI-BamHI sites of pGAD7, containing the GAL4 activation domain, and pGBKT7, containing the GAL4 DNA-binding domain, to generate pGAD7-fruD and pGBKT7-fruD, respectively. Yeast transformation and two-hybrid selection were carried out with host strain PJ69-4A (19). β-Galactosidase activity from a reporter gene was assayed as described by Miller (21).

Nucleotide sequence accession number. The nucleotide sequence data for fruCD reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB100269.

RESULTS

Two novel genes required for multicellular development in M. xanthus. Previously, we cloned and characterized the fruA gene, encoding a putative transcription factor required for multicellular development of M. xanthus (23). To investigate the possible presence of another developmental gene(s) in this region, the 4.4-kb fruA upstream region was sequenced (Fig. 1A). DNA sequence analysis revealed that five novel open reading frames exist in this region. orf269 encodes a hypothetical protein conserved in several bacteria, and orf169 encodes a DnaJ family protein. Six novel Km1 insertion mutations (Ω9 through Ω14) as well as one deletion mutation (Δ1) in this region were constructed in an E. coli plasmid. All seven mutations could be introduced into the M. xanthus chromosome by double-homologous recombination, indicating that no genes essential for vegetative growth are present in this region. M. xanthus cells carrying Km1 Ω10, Ω11, Ω12, and Ω13 insertions and the Δ1 deletion exhibited delayed-development phenotypes, while those carrying Km1 Ω9 and Ω14 insertions were development proficient. These results indicate that the two novel genes, designated fruC and fruD, are developmental genes (Fig. 1A). The fruC and fruD genes are located 3 and 3.5 kb upstream of the fruA gene, respectively, and are oriented oppositely to fruA.

To analyze the upstream regulatory region of the fruC gene, fruC segments with various lengths of upstream regions were integrated into the Mx8 attB site of the M. xanthus fruC::Km Ω110 chromosome, generating MxCC1 to MxCC3 (Fig. 1A). MxCC1 exhibited development similar to that of the wild type, whereas MxCC2 and MxCC3 exhibited delayed-development phenotypes. The results of complementation experiments indicate that a DNA segment up to 497 bp upstream of the fruC initiation codon is required for fruC expression.

Amino acid sequences of the fruC and fruD products. The fruC gene encodes a protein of 134 amino acids with a calculated molecular weight of 14,564 (Fig. 1B). No known proteins in databases have significant amino acid sequence similarity to the FruC protein. Computer analysis suggests that the FruC protein is a cytoplasmic protein. The fruD gene encodes a protein of 175 amino acids with a calculated molecular weight of 20,390 (Fig. 1C). The deduced amino acid sequence of the FruD protein has up to 45% identity with those of DivIva proteins of many bacteria including Geobacter metallireducens, Clostridium perfringens, Bacillus halodurans, Bacillus subtilis, and Listeria innocua. Computer analysis suggests that the FruD protein is a cytoplasmic protein and that the central region of the FruD protein has an α-helical coiled-coil structure. B. subtilis DivIva is also a cytoplasmic protein containing an α-helical coiled-coil structure (5, 9). As expected from the high G+C content of M. xanthus genomic DNA, M. xanthus genes have very high G+C content at the third-codon positions. The codon usages in the fruC and fruD genes were similar to those in other M. xanthus genes, and the G+C contents at the third-codon positions in the fruC and fruD genes were 91 and 90%, respectively.
Characterization of the fruCD mutants. M. xanthus fruC::Km and fruD::Km, and ΔfruCD1 cells grew at the same rates as wild-type DZF1 cells in CYE medium. B. subtilis divIV4 mutants produced filamentous cells, together with a few minicells (5, 8, 25). In M. xanthus fruCD1 vegetative cultures, filamentous cells formed more frequently than in DZF1 cultures, while no minicells were found (data not shown). The average lengths of M. xanthus fruD::Km, ΔfruCD1 vegetative cells were greater than that of DZF1 cells (6.3 ± 1.6 μm), while that of fruC::Km vegetative cells (5.6 ± 1.5 μm) was similar to that of DZF1 cells. These results suggest that the M. xanthus fruD mutant exhibits some impairment of cell division.

To examine the effects of fruC and fruD mutations on fruiting body formation, the processes of development in M. xanthus fruC::Km, fruD::Km, ΔfruCD1, and wild-type DZF1 strains were compared. Vegetative cells of each strain were concentrated, spotted on CF agar plates, and incubated at 30°C. The morphological changes during the development of each strain were photographed (Fig. 2A). The time courses for fruC, fruD, and ΔfruCD1 mutant aggregation were slower than that measured for DZF1. Although the developmental time courses for the three mutants were slower than that for DZF1, the final fruiting body morphologies of the three mutants were similar to that of DZF1. When the fruC, fruD, and ΔfruCD1 mutants were spotted on TM buffer-agar plates, similar delays in development were observed.

Sporulation of the fruCD mutants. To investigate the effects of the fruCD mutations on spor formation, the processes of sporulation for M. xanthus fruC::Km, fruD::Km, ΔfruCD1, and wild-type DZF1 strains were compared. Vegetative cells of each strain were concentrated and spotted on CF agar plates. Each spot was scraped off the agar surface during development, and the refractile spores were counted (Fig. 2B). In DZF1, sporulation formation started 18 h poststarvation. The number of DZF1 spores increased steadily until 30 h, when the number reached a maximal value. In contrast, the number of spores in the three mutants increased steadily until 48 h, when the number reached a maximal value. The rates of spor formation for the three mutants were similar to that for the wild type. The number of spores produced by the three mutants after 96 h were approximately 60% of the number produced by DZF1 (Fig. 2B). Over 75% of myxospores obtained from the fruiting bodies of fruC, fruD, and ΔfruCD1 mutants and the wild-type DZF1 strain aged for 96 h germinated and formed colonies on a CYE plate after 5 days (data not shown).

Expression of the fruCD genes during vegetative growth and development of M. xanthus. The expression of the fruCD genes was examined by RT-PCR analysis (Fig. 3). Total RNAs were prepared from DZF1 vegetative cells and developing cells at 6, 12, and 24 h poststarvation and treated with RT. The synthesized cDNA was used as a template for PCR with primers within the fruCD gene. The expected 133-bp RT-PCR product was amplified from total RNAs of every stage when treated by RT (Fig. 3, lanes 5 to 8), while it was not detectable in the absence of RT treatment (Fig. 3, lanes 1 to 4). Another PCR was performed with forward and reverse primers within the fruC and fruD genes, respectively. An RT-PCR product was also amplified in this experiment (data not shown). These results indicate that the fruCD genes are expressed as an operon during vegetative growth and development.

Determination of the transcription initiation sites in the fruCD operon. To determine the transcription initiation site of the fruCD operon, S1 nuclease mapping analysis was performed with total RNAs prepared from vegetative cells and developing cells at 6, 12, and 24 h after starvation of M. xanthus DZF1. For RNAs from vegetative cells, one transcription initiation site (P1) was detected 114 bp upstream of the initiation codon of the fruC gene (Fig. 4A, lane V). For RNAs from developing cells, P1 and P2 transcription initiation sites were detected 114 and 55 bp, respectively, upstream of the initiation codon of the fruC gene (Fig. 4A and B, lanes D6, D12, and

FIG. 2. (A) Morphogenesis during the development of M. xanthus DZF1 (wild type) and fruCD1 mutants. Vegetative cells of each strain were spotted on CF agar plates. The spots were photographed through a dissecting microscope at the indicated times. (B) Sporulation of M. xanthus DZF1 (wild type) and fruCD1 mutants. Vegetative cells of each strain were spotted on TM buffer-agar plates. The spots were scraped off the agar surface and sonicated. The refractile spores were counted in a counting chamber in triplicate. Open circles, DZF1 (wild type); solid squares, fruC::Km; solid triangles, fruD::Km; solid diamonds, ΔfruCD1 mutant.
D24), while transcription from P1 decreased during development. These results indicate that the P1 promoter is active during vegetative growth and that activity decreases during development, while the P2 promoter is specific for development. From the sequence ladders shown in Fig. 4A and B, P1 and P2 transcription initiation sites mapped to the A and G residues, respectively, 114 and 55 bp upstream of the fruC initiation codon. Based on these results, the /H1100235 and /H1100210 regions for the two promoters of the fruCD operon are assigned as shown in Fig. 4C.

To demonstrate the in vitro activity of the P1 and P2 promoters, P1-lacZ and P2-lacZ transcriptional fusion genes were constructed and integrated into the Mx8 attB site of the M. xanthus chromosome, giving MxFL1 and MxFL2, respectively. MxFL1 exhibited 19 and 15 U of β-galactosidase activity during vegetative growth and 12 h poststarvation, respectively. MxFL2 exhibited 14 and 21 U of β-galactosidase activity during vegetative growth and 12 h poststarvation, respectively. These results indicate that P1 and P2 display promoter activity predominantly during vegetative growth and during development, respectively.

**FruD protein production during vegetative growth and development.** To examine FruD production during vegetative growth and development, total protein from vegetative cells and developing cells at 24 and 48 h after starvation of M. xanthus DZF1 and fruC::Km and fruD::Km mutants was separated by SDS-PAGE and subjected to Western blot analysis using anti-GST-FruD antiserum (Fig. 5A). In DZF1 cells, a protein band at 22 kDa was detected during vegetative growth and development; the density of the 22-kDa band decreased during development (Fig. 5A, lanes 1 to 3). The 22-kDa band was absent in the fruD::Km mutants during vegetative growth and development (Fig. 5A, lanes 7 to 9). These results indicate that the 22-kDa protein is the FruD protein. The estimated molecular mass of the FruD protein is in good agreement with the value of 20.4 kDa calculated from the DNA sequence. Since anti-GST-FruD antiserum was used in the present experiment, a protein band at 28 kDa is likely to be the GST protein of M. xanthus. FruD production in the fruC::Km mutant was similar to that in DZF1 (Fig. 5A, lanes 4 to 6), suggesting that the insertion mutation in the fruC gene does not affect FruD production.

**Localization of the FruD protein.** To examine the localization of the FruD protein, vegetative cells of M. xanthus DZF1 were disrupted and separated into periplasmic, cytoplasmic,
...were subjected to gel filtration chromatography, each eluate fraction was analyzed by SDS-PAGE followed by Western blot analysis using anti-GST-FruD antiserum (Fig. 6A). The FruD protein was detected as the 22-kDa band only in the cytoplasmic fraction (Fig. 5B, lane 12). These results indicate that the fruD gene is expressed as a cytoplasmic protein. The localization of the FruD protein in the cytoplasm is consistent with the prediction of computer analysis described above.

**Oligomer formation of the FruD protein.** Oligomer formation of the B. subtilis DivIVA protein has been reported (22). To examine whether the FruD protein exists as a monomer or an oligomer under native conditions, soluble fractions from M. xanthus vegetative cells and developing cells at 6 h poststarvation were subjected to gel filtration chromatography. After TSK-Gel G2000 gel filtration chromatography, each eluate fraction was analyzed by SDS-PAGE followed by Western blot analysis using anti-GST-FruD antiserum (Fig. 6A). The FruD proteins from both vegetative cells and developing cells were eluted at positions corresponding to an oligomer. From the elution patterns of molecular weight markers, the molecular mass of the native FruD oligomer was estimated to be approximately 100 kDa. Since the monomer size of the FruD protein was estimated to be 20.4 kDa as described above, it is likely that the FruD protein exists as an oligomer in vegetative and developing cells of M. xanthus.

The yeast two-hybrid system was used to ascertain the FruD-FruD interaction. The fruD gene was cloned into two-hybrid system vectors pGADT7 and pGBD7. Yeast cells containing pGAD7-frud and pGBK7-frud formed colonies on SD medium lacking tryptophan, leucine, histidine, and adenine at 30°C after 1 week (Fig. 6B), indicative of a FruD-FruD interaction. A similar result was obtained when the FruD-FruD interaction was measured by a β-galactosidase assay (Fig. 6B). These findings are consistent with the results of gel filtration chromatography demonstrating that the FruD protein exists as an oligomer.

**DISCUSSION**

In this study, two novel developmental genes, fruC and fruD, of M. xanthus were analyzed. The fruCD genes were constitutively expressed as an operon during vegetative growth and development. From S1 nuclease mapping analysis, two distinct promoters (P1 and P2) for the fruCD operon were identified. The P1 promoter was active during vegetative growth and became weaker during development, while the P2 promoter was specific for development, suggesting that fruCD transcription is differentially regulated during vegetative growth and development of M. xanthus. In vivo activity of the P1 and P2 promoters was demonstrated by P1-lacZ and P2-lacZ transcriptional fusions, although their β-galactosidase activity was not so high. Complementation experiments involving the fruC mutation revealed that a fruC region further upstream is required for fruCD expression. These results suggest the presence of a cis regulatory element(s) at the region of fruC promoters further upstream, since such regulatory elements are reportedly in many M. xanthus genes, including dofA and fruA (14, 15). Lack of the regulatory element(s) may explain the observed β-galactosidase activity from the P2 promoter during vegetative growth.
Several *M. xanthus* genes which are expressed by two distinct promoters have been reported. The *M. xanthus* sigD gene, encoding a stationary-phase sigma factor, has two distinct promoters (33). Expression of the sigD gene is differently regulated by the two promoters during vegetative growth and development: the downstream of promoter is specific for development. The ΔsigD mutant exhibited growth defects during the late log phase and stationary phase, with reduced cell viability. The deletion mutant displayed a delayed-development phenotype, yielding fewer spores than the wild type.

The FruD protein displays significant amino acid sequence similarity to the DivIVA proteins of many bacteria such as *B. subtilis*. The DivIVA protein is known to be a functional homologue of the *E. coli* MinE protein, although the amino acid sequences of the DivIVA proteins exhibit no similarity to that of the MinE protein (5, 8). The *E. coli* MinE protein determines the division site, which is at the middle of the cell, by controlling the topological specificity of MinCD division inhibitors. The *B. subtilis* DivIVA protein also sequesters MinCD division inhibitors at the cell poles (5, 8). Vegetative cells of the *M. xanthus* fruD mutant exhibited a filamentous phenotype similarly to that the *B. subtilis* divIVA mutant. Therefore, it is likely that the *M. xanthus* FruD protein is involved in cell division, although the exact function of the FruD protein in cell division is not clear.

A second function of the *B. subtilis* DivIVA protein in sporulation has been reported (30). In sporulating cells, the *B. subtilis* DivIVA protein participates in chromosome segregation. It interacts with the chromosome segregation machinery to help position the oriC region of the chromosome at the cell pole in preparation for asymmetric division. The developmental processes of *M. xanthus* differ from those of *B. subtilis* in many points. Upon nutritional starvation on a solid surface, *M. xanthus* cells aggregate to form mounds, within which cells are converted into myxospores. In contrast, upon nutritional starvation in a liquid, *B. subtilis* cells undergo asymmetric cell division leading to the formation of endospores. It is of great interest that a cell division gene may also participate in bacterial development in both *M. xanthus* and *B. subtilis*.

The *fruC*, *fruD*, and Δ*fruCD* mutants displayed similar delayed-development phenotypes. The start of spor formation in the three mutants was delayed by 12 h in comparison to that for the wild type, while the rates of spor formation in *fruC* and *fruD* mutants were similar to that in the wild type. These results suggest that the FruC and FruD proteins may play similar roles at an aggregation stage during development in *M. xanthus*. It is possible that the retardation of spor formation in *fruC* and *fruD* mutants is due to delayed aggregation. The recently described genes *espAB* may control the timing of spor formation in coordination with aggregation (6). Sporulation of the *espA* mutant occurred faster than that of the wild type, while the *espB* mutant sporulated more slowly than the wild type. The EspA protein has significant amino acid sequence similarity to a sensor protein of a two-component signal transduction system and is considered to function as an inhibitor that delays sporulation until developmental aggregation is complete, while the EspB protein may antagonize EspA function. The phenotype of the *espB* mutant exhibits some similarity to those of the *fruC*, *fruD*, and Δ*fruCD* mutants.

Homooligomer formation of the *B. subtilis* DivIVA protein has been reported (22). Since some *B. subtilis* DivIVA mutants failed to form normal DivIVA oligomers, DivIVA oligomer formation may be crucial for its activity. In the present study, the size of the native form of the *M. xanthus* FruD protein was estimated to be approximately 100 kDa. If one postulates that the FruD protein forms homooligomers, the number of FruD monomers may be four or five. This value is significantly lower than the reported value of 10 to 12 for the *B. subtilis* DivIVA oligomer. Further studies are necessary to understand the function of the FruC and FruD proteins in the development of *M. xanthus*.

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**REFERENCES**


