The DevT Protein Stimulates Synthesis of FruA, a Signal Transduction Protein Required for Fruiting Body Morphogenesis in *Myxococcus xanthus*

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Fruiting body formation in *Myxococcus xanthus* involves three morphologic stages—rippling, aggregation, and sporulation—all of which are induced by the cell surface-associated C-signal. We analyzed the function of the DevT protein, a novel component in the C-signal response pathway. A mutant carrying an in-frame deletion in the *devT* gene displays delayed aggregation and a cell autonomous sporulation defect, whereas it remains rippling proficient. To further define the function of DevT, the methylation pattern of FrzCD, a cytoplasmic methyl-accepting chemotaxis protein homologue, was examined in the ΔdevT mutant, and we found that DevT is required for methylation of FrzCD during development. Specifically, DevT was found to be required for the C-signal-dependent methylation of FrzCD. The ΔdevT mutant produced wild-type levels of C-signal. However, accumulation of the FruA response regulator protein, which is essential for the execution of the C-signal-dependent responses, was reduced in the ΔdevT mutant. The DevT protein was found to stimulate the developmentally activated transcription of the *fruA* gene. Epistasis analyses indicate that DevT acts independently of the A- and E-signals to stimulate *fruA* transcription. These findings suggest that the developmental defects of the ΔdevT mutant are associated with a lack of FruA to ensure a proper response to the C-signal during the aggregation and sporulation stages of development.

In response to starvation, *Myxoxoccus xanthus* cells initiate a developmental program in which multicellular morphogenesis and cell differentiation are highly coordinated (4, 32). The program results in the formation of multicellular fruiting bodies, within which the rod-shaped cells differentiate into spherical spores. Fruiting body formation depends on the starvation of cells at a high cell density on a solid surface and involves three morphologic stages: rippling, aggregation, and sporulation. Aggregation initiates 4 to 8 h after the initiation of starvation and culminates after 24 h with the formation of mound-shaped fruiting bodies, each of which is built from 10^5 cells. Sporulation initiates when the organized cell movements have resulted in the accretion of cells in the developing fruiting bodies. Rippling precedes and accompanies the earliest stages of aggregation (34). During rippling, cells accumulate in ridge-like structures that move coordinately and synchronously over the substrate like ripples on a water surface. Key questions in understanding fruiting body formation are how the correct temporal order of these morphologic stages is brought about and how the developmental program is propelled from one stage to the next.

At least five intercellular signals, known as the A-, B-, C-, D-, and E-signals, have important functions for the progression of the developmental program (2, 18). Mutants defective in the production of either of these signals are arrested in develop-ment at a particular stage. Examination of gene expression and fruiting body morphogenesis in these mutants have shown that the A- and B-signals are required early during development (7, 19, 23) and that the D- and E-signals are required for development after 3 to 5 h (1–3). The C-signal becomes important for development after ca. 6 h (19) and is the latest acting of the known signals.

The C-signal is encoded by the *csgA* gene (33) and induces rippling, aggregation, sporulation, and expression of genes that are induced after 6 h of starvation (15, 19, 21, 25, 34). The defects in aggregation, sporulation, and C-signal-dependent gene expression in *csgA* mutants are corrected by codevelopment with wild-type (wt) cells (9). *csgA* encodes two proteins (21), one with a size of 25 kDa, corresponding to full-length CsgA protein, and one with a size of 17 kDa, which is similar in size to the C-factor protein (16). Kaiser and coworkers have proposed that C-factor protein is identical to the C-signal based on the observation that addition of C-factor protein, which was purified from starving wt cells, to *csgA* cells rescues development of these cells (16). However, exogenous full-length CsgA protein also rescues development of *csgA* cells (24). Full-length CsgA protein shares homology to short chain alcohol dehydrogenases (24). The molecular difference between the full-length CsgA protein and the C-factor protein has yet to be elucidated. Thus, it remains to be resolved whether the *csgA* proteins act as enzymes to produce the C signal or whether one of the *csgA* proteins is the actual C signal.

Recent studies have revealed several components in the C-signal response pathway. The DNA-binding response regulator protein FruA is the earliest known component in the

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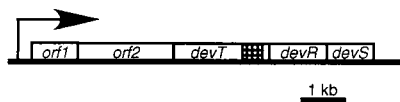† Present address: Kosan Biosciences, Hayward, CA 94545.

FIG. 1. Physical map of the *dev* operon. White boxes indicate the genes. The hatched box within *devT* indicate the portion of the gene that was removed to construct the in-frame deletion mutation. The arrow indicate the direction of transcription of the *dev* operon.

pathway and is required for all four C-signal-dependent responses (5, 28). FruA synthesis is developmentally regulated at the transcriptional level, and FruA protein is not detectable until 3 h of starvation (5, 28). Since the C-signal is not required for FruA synthesis it has been suggested that C-signal transmission results in activation of FruA posttranslationally to interact with its downstream targets (5). The aspartate residue, which becomes phosphorylated in other response regulators, is conserved in FruA and is essential for FruA activity (5). Therefore, it has been hypothesized that C-signaling activates FruA by inducing phosphorylation of the protein (5). Downstream from FruA the C-signal response pathway branches, with one branch controlling rippling and aggregation. The proteins in the cytoplasmic Frz signal transduction system are components in this branch (36). The Frz proteins share homology with bacterial chemotaxis proteins and are essential for rippling and aggregation (29, 40) and control specific gliding motility parameters in response to the C-signal during development (11). The C-signal acts as a FruA-dependent input signal to the Frz system and induces methylation of the FrzCD protein, a methyl-accepting chemotaxis protein homologue, after 6 to 9 h of starvation (35). The second branch in the pathway controls sporulation. In this branch, FruA and the C-signal interact to induce transcription of the essential sporulation operon *devRS* after 6 to 9 h of development (5, 19).

Recently, it was shown that the *devRS* genes are part of the larger *dev* operon, which consists of five genes (B. Julien, A. Garza, and D. Kaiser, unpublished data) (Fig. 1). The *devRS* genes are the two distal genes in the *dev* operon. The *devT* gene

is part of this operon and is located immediately upstream from the *devRS* genes. *devT* is predicted to encode a protein with a size of ca. 58 kDa and which does not share homology to any known protein. Upstream from *devT* two additional reading frames, designated *orf1* and *orf2*, with unknown functions are located. An in-frame deletion in *devT* causes delayed aggregation and a reduction in the sporulation efficiency (Julien et al., unpublished). To investigate the function of DevT at the molecular level, we carried out a detailed characterization of the ΔdevT mutant. We show here that DevT stimulates FruA synthesis.

MATERIALS AND METHODS

Bacterial strains. All *M. xanthus* strains used in this work were derived from wt strain DK1622. The *M. xanthus* strains used in this work are listed in Table 1. DK11207 carries a 279-bp in-frame deletion in *devT*, corresponding to a deletion between codon 408 and codon 502 in *devT* (Julien et al., unpublished) (Fig. 1). Strains SA1300, SA1301, SA1303, and SA1304 were constructed by generalized transduction into DK11207 by using Mx8 *clp2 ts3* (38) propagated on DK11063, DK5208, DK9013, and JD300, respectively. DK11207/pSWU30 and SA1300/pEE244 were constructed by integration of the plasmids pSWU30 and pEE244, respectively, into the phage Mx8 *attB* site after electroporation (14). All strains constructed by generalized transductions or after electroporation of plasmids were tested by Southern blot analysis as described previously (30).

Growth, development, and measurement of β-galactosidase activity. *M. xanthus* cells were grown in CTT medium in liquid cultures or on CTT agar plates as described previously (10). Kanamycin or oxytetracycline was used for selective growth at concentrations of 40 or 10 μg/ml, respectively. Aggregation and rippling were monitored on CF agar (34) as described previously (36). Briefly, cells were grown to a density of 5×10^8 cells/ml in CTT, harvested and resuspended in TPM buffer (10 mM Tris-HCl [pH 7.5], 1 mM KH₂PO₄, 8 mM MgSO₄) at a calculated density of 5×10^9 cells/ml. Then, 20-μl aliquots of concentrated cells were spotted on CF agar and incubated at 32°C. Aggregation and rippling were monitored visually by using a Leica IMB/E inverted microscope or a Leica MZ8 stereomicroscope. Cells were photographed by using a Sony 3CCD color video camera. The levels of sporulation were determined after development for 72 and 120 h on CF agar. Unless otherwise mentioned, spore titers were determined as the number of sonication and heat-resistant CFU (36). To develop cells under submerged culture conditions, cells were treated as described for development on CF agar except that they were resuspended in MC7 buffer (10 mM morpholinepropanesulfonic acid [pH 7.0], 1 mM CaCl₂). Next, 25 μl of concentrated cells was mixed with 375 μl of MC7 buffer, transferred to a well with a diameter of 15 mm in a microtiter dish, and incubated in a humid chamber at 32°C for the

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[b]	Source or reference
Strains[a]		
DK1622	wt	13
DK5208	*csgA*::Tn*5-132* ΩLS205	19
DK11207	Δ*devT*	B. Julien et al., unpublished data
DK9013	*asgB480* Tn*5-132* Ω4679	5
DK11063	*fruA*::Tn*5 lac*Ω7540	36
DK11207/pSWU30	Δ*devT attB*::pSWU30	This study
JD300	*esg*::Tn*5* Ω258	39
SA1300	Δ*devT fruA*::Tn*5 lac*Ω7540	This study
SA1301	Δ*devT csgA*::Tn*5-132* ΩLS205	This study
SA1303	Δ*devT asgB480* Tn*5-132* Ω4679	This study
SA1304	Δ*devT esg*::Tn*5* Ω258	This study
DK11063/pEE244	*fruA*::Tn*5 lac*Ω7540 *attB*::pEE244	5
SA1300/pEE244	Δ*devT fruA*::Tn*5 lac*Ω7540 *attB*::pEE244	This study
Plasmids		
pSWU30	Tet[r]; derivative of pBGS18 containing the Mx8 *attP* region	37; S. Wu, unpublished data
pEE244	Tet[r]; SWU30 derivative containing the entire *fruA*	5

[a] All *M. xanthus* strains are derivatives of DK1622.
[b] Tet[r], tetracycline resistance.

indicated periods of time. To measure specific activities of β-galactosidase during development, cells were induced to develop on CF agar as described above. Cells were harvested at the indicated time points and treated as described previously (36). Quantification of the specific activities of β-galactosidase was performed as described previously (20).

Immunoblot analysis. Cells were exposed to starvation as described above. At the indicated times, cells were harvested. Cells were pelleted by centrifugation and immediately frozen and stored at −80°C until all samples were collected. Cells were lysed in 100 μl of sodium dodecyl sulfate (SDS) lysis buffer (30) containing protease inhibitors as described previously (27), boiled for 5 min, and loaded on Tris-glycine–SDS–12.5% polyacrylamide gels (prepared from a 29:1 acrylamide-bisacrylamide stock solution) for detection of FruA or on 11.56% acrylamide–0.08% bisacrylamide Tris-glycine-SDS gels for detection of FrzCD. For CsgA immunoblots, cells were lysed in 100 μl of Tris-Tricine-SDS lysis buffer (31) containing protease inhibitors as described previously (27), boiled for 5 min, and loaded onto Tris-Tricine–SDS–10% polyacrylamide gels (31) (prepared from a 40:2 acrylamide-bisacrylamide stock solution). Subsequently, proteins were transferred to Immobilon P membranes (Millipore) with a semidry blotting apparatus (Pharmacia). Immunoblots were prepared by standard procedures (30). The blots were probed with rabbit anti-FruA serum (28), rabbit anti-CsgA serum (21) or rabbit anti-FrzCD serum (27), followed by treatment with peroxidase-conjugated goat anti-rabbit immunoglobulin G (Boehringer-Mannheim). The blots were developed with Renaissance Plus chemiluminescence reagent (NEN Life Science Products). Protein concentrations were determined by using the Bio-Rad protein assay according to the manufacturer's suggestions, with bovine immunoglobulin G as a standard.

Purification of MalE-CsgA protein and FrzCD methylation assay. The MalE-CsgA protein was purified from *Escherichia coli* as described previously (24). Protein concentrations were determined by using the Bio-Rad protein assay. The C-factor activity of the protein was determined in the C-factor bioassay (17). The specific activity of the protein was 65 C-factor U/mg of protein. One C-factor unit is the amount of protein that restores wt fruiting body formation and sporulation to 2.5×10^5 *csgA* cells (17). The effect of the fusion protein on FrzCD methylation was analyzed as described previously (35). Briefly, 2.5×10^8 of the relevant cells were developed as described for submerged culture development except that the cells were developed in A50 buffer (10 mM morpholinepropanesulfonic acid, 1 mM $CaCl_2$, 4 mM $MgCl_2$, 50 mM NaCl; pH 7.2). After 6 h of starvation, overlying buffer was removed and replaced with prewarmed A50; cells were harvested by gently scraping them off the bottom of the well, and then they were transferred to a 12-ml polypropylene tube. Cells were shaken vigorously to break clumps, the fusion protein was added to give a final volume of 400 μl per 2.5×10^8 cells, and the cells were transferred to a 32°C water bath and incubated for 30 min with shaking. Subsequently, cells were harvested and FrzCD immunoblots prepared as described above to determine the level of FrzCD methylation.

RESULTS

Phenotype of a ΔdevT mutant. To analyze the role of the DevT protein in rippling and aggregation, the isogenic strains DK1622 (wt) and DK11207 (ΔdevT) were exposed to starvation on CF starvation agar. DK11207 carries an in-frame deletion in the *devT* gene (see Materials and Methods) (Fig. 1). After 24 h of starvation, wt cells had aggregated to form translucent mounds, and cells were rippling between the mounds (Fig. 2). After 48 h, rippling was no longer observed and the mounds had darkened. In agreement with the observation of Julien et al. (unpublished data), the ΔdevT cells were delayed in aggregation and translucent mounds were not detectable until after 48 to 72 h (Fig. 2). The mounds formed by the ΔdevT cells remained translucent until at least 120 h (Fig. 2). Interestingly, the ΔdevT cells were rippling after 24 h, and rippling was still observed even after 120 h (Fig. 2). Thus, DevT is important for timely aggregation.

The effect of the ΔdevT mutation on sporulation was assessed after starvation of DK1622 and DK11207 on CF agar for 72 and 120 h. After 72 and 120 h, 6 and 10% of wt cells, respectively, had differentiated into spores. In agreement with the observation of Julien et al. (unpublished data), we ob-
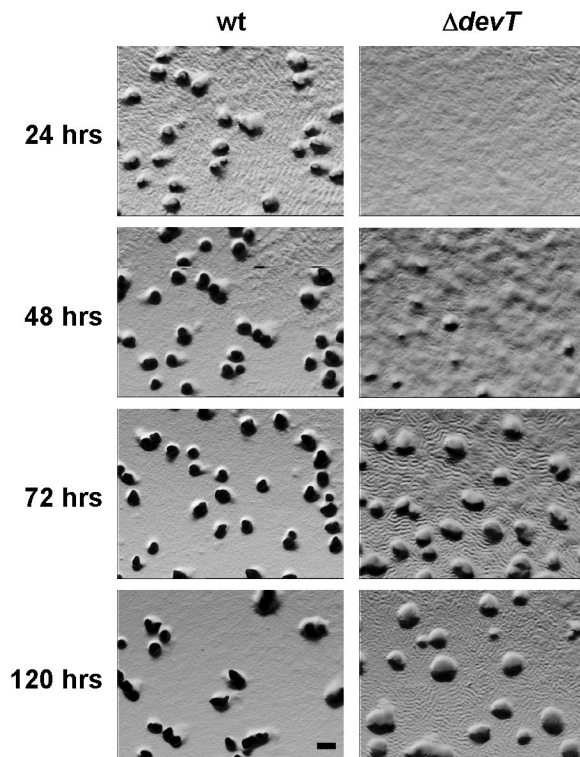

FIG. 2. Rippling and aggregation phenotypes of wt and ΔdevT mutant strains. DK1622 (wt) and DK11207 (ΔdevT) cells were exposed to starvation on CF agar for the indicated period of times. Bar, 0.1 mm. Cells were viewed with a Leica MZ8 stereomicroscope.

served that the ΔdevT mutation caused a sporulation defect and, after 72 and 120 h, the sporulation efficiencies of the ΔdevT cells were 10^5- and 10^4-fold lower, respectively, than that of wt cells. This sporulation efficiency was calculated from the number of heat- and sonication-resistant, germinating spores (see Materials and Methods). To distinguish whether the ΔdevT mutation interfered with spore formation or spore germination, ΔdevT cells that had been starved for 72 and 120 h were examined microscopically. This analysis showed that the number of spores formed by the ΔdevT cells is similar to the number of heat- and sonication-resistant, germinating spores. Thus, the DevT protein is important for spore formation.

To investigate whether the sporulation defect in the ΔdevT mutant was cell autonomous, ΔdevT cells were codeveloped with an equal number of wt cells, and then the numbers of spores formed by the ΔdevT cells were measured after 72 and 120 h of starvation. To distinguish spores formed by the ΔdevT cells from those formed by wt cells, the ΔdevT derivative DK11207/pSWU30, which carries the plasmid pSWU30 integrated at the phage Mx8 *attB* site, was used instead of DK11207. pSWU30 confers resistance to oxytetracycline and, thus, colonies formed from the ΔdevT spores could be identified on the basis of their resistance to oxytetracycline. The sporulation defect in the ΔdevT mutant remained unaffected by codevelopment with wt cells (data not shown). Thus, in the ΔdevT mutant rippling is observed for an extended period of time, aggregation is delayed, and the sporulation efficiency is
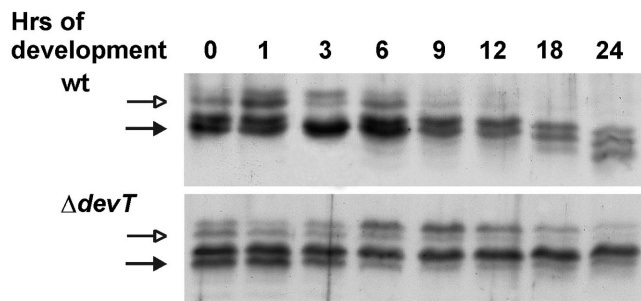
FIG. 3. Immunoblot analyses of the FrzCD methylation patterns in wt and Δ*devT* cells. Total cell lysates were prepared from cells after exposure to starvation in submerged culture for the indicated periods of time and reacted with anti-FrzCD antibody. (Top) Proteins isolated from DK1622 (wt) cells; (bottom) proteins isolated from DK11207 (Δ*devT*) cells. The two upper bands represent nonmethylated FrzCD (indicated by the open arrows), and the four lower bands represent methylated FrzCD (indicated by the solid arrows). Protein from 5×10^7 cells was added to each lane.

FIG. 4. Immunoblot analyses of the FrzCD methylation patterns in *csgA* and Δ*devT csgA* cells in response to exogenous MalE-CsgA protein. Total cell lysates were prepared from cells that had been starved for 6 h in submerged culture, brought into suspension, and exposed to the indicated amounts of MalE-CsgA fusion protein for 30 min while in suspension. Subsequently, proteins were reacted with anti-FrzCD antibody. (Left panel) Proteins isolated from DK5208 (*csgA*) cells; (right panel) proteins isolated from SA1301(Δ*devT csgA*) cells. Protein from 5×10^7 cells were added to each lane. The two upper bands represent nonmethylated FrzCD (indicated by the open arrows), and the three lower bands represent methylated FrzCD (indicated by the solid arrows). One C-factor unit is the amount of protein that restores wt fruiting body formation and sporulation to 2.5×10^8 *csgA* cells (17).

reduced. The Δ*devT* mutation being cell autonomous implies that the developmental defects are not caused by the lack of an intercellular signal.

The DevT protein acts upstream of the Frz signal transduction. The FrzCD protein shows modulated methylation during development (26). Thus, the pattern of methylation of FrzCD may serve as a molecular marker to determine the time of action of components required for fruiting body formation (6). To further define the role of DevT and to determine whether DevT acts upstream or downstream of the events that lead to FrzCD methylation, the FrzCD methylation pattern was analyzed in DK1622 (wt) and in DK11207 (Δ*devT*) cells during starvation.

As previously reported (26), nonmethylated and methylated FrzCD is present during the first 6 h of development in wt cells (Fig. 3). From 9 h on, the ratio between nonmethylated FrzCD and methylated FrzCD decreased in wt cells and, after 18 h, FrzCD was only present in the methylated form. In the Δ*devT* cells the pattern of FrzCD methylation was similar to that in wt cells until 6 h (Fig. 3). However, the ratio between nonmethylated FrzCD and methylated FrzCD did not decrease significantly in the Δ*devT* cells until 18 to 24 h. Moreover, the nonmethylated form of FrzCD was still present after 24 h. The different patterns of FrzCD methylation in wt and Δ*devT* cells suggest that DevT acts upstream from the events leading to methylation of FrzCD during development and is required either for the production of input signals to the Frz system or for the reception of the signals.

The DevT protein is required for C-signal-dependent methylation of FrzCD. C-signal induces the methylation of FrzCD after 6 h of starvation (37). To investigate whether DevT is required for the C-signal-dependent FrzCD methylation, we analyzed C-signal-dependent methylation of FrzCD in a Δ*devT* mutant by using a previously described experimental setup (35). Specifically, *csgA* cells (DK5208) and Δ*devT csgA* cells (SA1301) were starved at a high cell density on a solid surface. At 6 h the cells were gently harvested and then exposed to various amounts of a MalE-CsgA fusion protein for 30 min while in suspension (see Materials and Methods). The MalE-
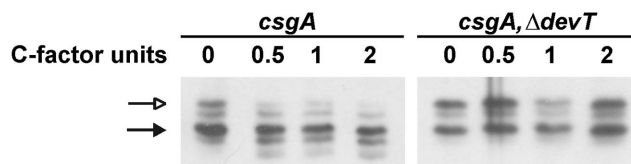
CsgA protein has C-signal activity and rescues aggregation and sporulation in *csgA* cells (24) and induces FrzCD methylation in this assay (35). In this assay, cells are exposed to the MalE-CsgA protein while in suspension and under these conditions they do not aggregate (35). Therefore, any effect of the fusion protein on FrzCD methylation is not secondary to a primary effect on aggregation. In response to the addition of 0.5, 1.0, and 2.0 C-factor units of MalE-CsgA protein to the *csgA* cells, the level of FrzCD methylation increased (Fig. 4). However, in the Δ*devT csgA* cells the same amounts of MalE-CsgA protein did not have any effect on FrzCD methylation (Fig. 4). Thus, DevT is required for the C-signal to act as an input signal to the Frz system.

DevT is not required for accumulation of the *csgA* proteins. The previous analyses showed that DevT acts between the C-signaling event and the Frz system. To determine whether DevT is required for the accumulation of the two *csgA* encoded proteins during development, quantitative CsgA immunoblots were performed. Total protein was isolated from DK1622 (wt) and DK11207 (Δ*devT*) cells that had been starved for different periods of time on CF agar, separated by SDS-polyacrylamide gel electrophoresis, and reacted with anti-CsgA serum (see Materials and Methods). The accumulation profiles of the two *csgA*-encoded proteins were similar in wt and Δ*devT* cells (Fig. 5): the 25-kDa *csgA* protein was detected in vegetatively growing cells and reached a maximum after 24 h. Low levels of the 17-kDa *csgA* protein were detected in vegetatively growing cells and reached a maximum after 24 h. Thus, the DevT protein is not required for accumulation of the *csgA* proteins during development.

DevT stimulates transcription of the *fruA* gene. FruA protein is required for the C-signal to act as an input signal to the Frz system (35). To determine whether DevT is required for accumulation of FruA during development, we carried out quantitative FruA immunoblot analyzes of total protein isolated from developing DK1622 (wt) and DK11207 (Δ*devT*) cells with anti-FruA serum. In wt cells FruA protein was detected by 6 h and reached a maximum after 24 h (Fig. 6A). In the Δ*devT* mutant, the level of FruA protein was lower than in wt cells at all time points (Fig. 6A), and at 24 h the level of FruA protein was approximately fivefold lower in the Δ*devT*
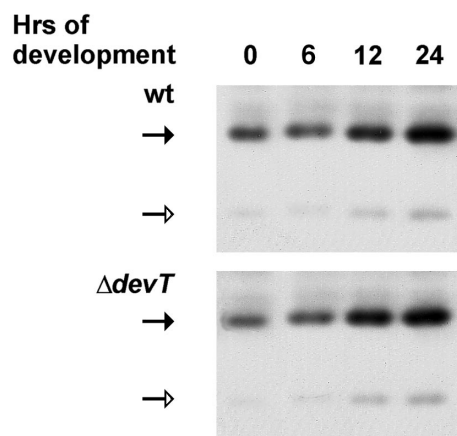
FIG. 5. Immunoblot analyses of the accumulation of the *csgA* proteins in wt and Δ*devT* cells. Total cell lysates were prepared from cells after exposure to starvation on CF agar for the indicated periods of time and reacted with anti-CsgA serum. (Top) Proteins isolated from DK1622 (wt) cells; (bottom) proteins isolated from DK11207 (Δ*devT*) cells. Solid and open arrows indicate the 25-kDa full-length *csgA* protein and the 17-kDa *csgA* protein, which is similar in size to the C-factor protein, respectively. A total of 7.5 μg of total protein was added per lane.

cells than in wt cells. Thus, DevT stimulates accumulation of FruA during development.

To examine whether DevT is required for the developmentally regulated transcription of the *fruA* gene, transcription of *fruA* was measured by using the transcriptional *fruA*::Tn5 *lac*Ω7540 fusion. Tn5 *lac*Ω7540 is inserted 167 bp downstream from the transcriptional start site of *fruA*, and the *lac* genes are transcribed from the *fruA* promoter (5). Tn5 *lac*Ω7540 has a polar effect on the downstream *fruA* gene, and a strain carrying Tn5 *lac*Ω7540 does not synthesize FruA protein (E. Ellehauge, unpublished data). To measure *fruA* transcription in a Δ*devT* strain, Tn5 *lac* Ω7540 was introduced into the Δ*devT* strain DK11207 by Mx8-dependent generalized transduction (see Materials and Methods), yielding SA1300. Subsequently, the plasmid pEE244, which carries the entire *fruA* gene including the promoter, was introduced into SA1300 giving rise to SA1300/pEE244. pEE244 carries the Mx8 *attP* site and integrates by site-specific recombination at *attB* on the *M. xanthus* chromosome. Likewise, pEE244 was introduced into DK11063, which carries Tn5 *lac*Ω7540, giving rise to DK11063/pEE244.

Expression of the *fruA*::Tn5 *lac*Ω7540 fusion was low at the onset of starvation in DK11063/pEE244; expression increased after 3 h and reached a maximum after 24 h of starvation (Fig. 6B). In SA1300/pEE244 the level of expression of the *fruA*::Tn5 *lac*Ω7540 fusion was strongly reduced compared to the level in DK11063/pEE244 at all time points (Fig. 6B), and at 24 h the specific activity of β-galactosidase in SA1300/pEE244 was approximately fivefold lower than in DK11063/pEE244. Thus, DevT stimulates the developmentally regulated activation of *fruA* transcription.

The intercellular A and E signals are required for transcriptional activation of the *fruA* gene (5, 28). To determine whether DevT acts in the A- or in the E-signaling pathway to promote *fruA* transcription, we performed epistasis experi-
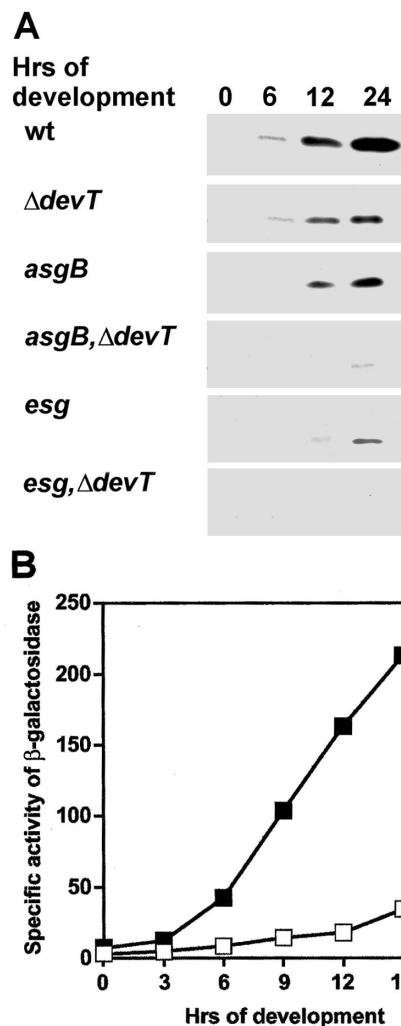

FIG. 6. Synthesis of the FruA protein during development. (A) Immunoblot analyses of the accumulation of FruA protein. Total cell lysates were prepared from cells after exposure to starvation on CF agar for the indicated periods of time and reacted with anti-FruA serum. Top panel, proteins isolated from DK1622 (wt) cells; second panel, proteins isolated from DK11207 (Δ*devT*) cells; third panel, proteins isolated from DK9013 (*asgB*) cells; fourth panel, proteins isolated from SA1303 (*asgB* Δ*devT*) cells; fifth panel, proteins isolated from JD300 (*esg*) cells; bottom panel, proteins isolated from SA1304 (*esg* Δ*devT*) cells. A total of 5.0 μg of total protein was added per lane. (B) Specific activity of β-galactosidase expressed from the *fruA*::Tn5 *lac*Ω7540 transcriptional fusion. Specific activities of β-galactosidase were determined in cells after exposure to starvation on CF agar for the indicated periods of time. Symbols: ■, DK11063/pEE244 (*fruA*::Tn5 *lac*Ω7540 *attB*::pEE244); □, SA1300/pEE244 (Δ*devT fruA*::Tn5 *lac*Ω7540 *attB*::pEE244). The specific activities of β-galactosidase are given in nanomoles of o-nitrophenyl phosphate per minute per milligram. Each value is the average of two determinations from two independent experiments.

ments in which the accumulation of FruA protein was analyzed in *asgB*, *esg*, and Δ*devT* single mutants and compared to the accumulation in *asgB* Δ*devT* and *esg* Δ*devT* double mutants. A strain carrying an *asgB* mutation is unable to synthesize the A-signal (22), and a strain carrying an *esg* mutation is unable to synthesize the E signal (2). As shown in Fig. 6A, the FruA

accumulation profile in the *asgB* mutant was similar to that observed in the Δ*devT* mutant. In the *asgB* Δ*devT* double mutant, however, the FruA accumulation profile was different from those observed in the strains carrying either of the single mutations and FruA was not detected until after 24 h of starvation. In the *esg* mutant, FruA accumulated at low levels and accumulation was not detected until 12 and 24 h. In the *esg* Δ*devT* double mutant, accumulation of the FruA protein was not detected. These data suggest that DevT acts independently of the A-signaling and E-signaling pathways to promote *fruA* transcription.

DISCUSSION

devT is part of the *dev* operon, which is transcribed after 6 to 9 h of development in a C-signal- and FruA-dependent manner (5, 19; Julien et al., unpublished). A Δ*devT* mutant displays delayed aggregation and a cell autonomous sporulation defect, whereas it remains rippling proficient. Using the pattern of FrzCD methylation as a molecular marker to further define the function of DevT, it was found that DevT is required for methylation of FrzCD during development. This deficiency was further narrowed down to an inability of the Δ*devT* mutant to respond properly to the C-signal, suggesting that DevT acts between the C-signaling event on the cell surface and the cytoplasmic Frz signal transduction system. The Δ*devT* mutant produced normal levels of the *csgA* proteins, indicating that the defect in methylation of FrzCD is associated with a failure to respond properly to the C-signal rather than in the production of the signal. Consistent with this observation, DevT was found to be required for normal levels of accumulation of the FruA protein, which is indispensable for the C-signal to act as an input signal to the Frz signal transduction system (35). Specifically, DevT was found to stimulate *fruA* transcription. This effect of the Δ*devT* mutation is not caused by a polar effect on the downstream *devRS* genes in the *dev* operon, because a *devR devS* double mutant accumulates wt levels of FruA protein (5). Nor is this effect of the Δ*devT* mutation on *fruA* expression an indirect effect caused by blocking sporulation because other mutants that are deficient in sporulation have normal levels of FruA accumulation (5).

In addition to DevT, the early acting intercellular A- and E-signals are required for transcriptional activation of the *fruA* gene during development (5, 28). Interestingly, epistasis analyses in which the accumulation of FruA in Δ*devT*, *asgB*, and *esg* single mutants was compared to the accumulation of FruA in Δ*devT asgB* and Δ*devT esg* double mutants strongly indicate that DevT acts independently of these two signaling pathways to promote *fruA* transcription. Thus, regulation of the *fruA* promoter is highly complex. Since DevT does not share significant sequence homology with known proteins (Julien et al., unpublished), the molecular mechanism underlying DevT-stimulated transcription of *fruA* remains unknown. Likewise, it remains to be shown whether the effect of DevT on *fruA* transcription is direct or indirect.

The inability of the C-signal to induce FrzCD methylation shows that the C-signal response pathway does not function normally in the Δ*devT* mutant. Consistently, the Δ*devT* mutant accumulates reduced amounts of FruA. FruA is a central component in the C-signal response pathway and is required for

rippling, aggregation, sporulation, and C-signal-dependent gene expression (5, 35, 36). Downstream from FruA the C-signal response pathway branches, with one branch leading to rippling and aggregation and the other to gene expression and sporulation. The observation that a defect in *devT* causes delayed aggregation and reduced sporulation and does not interfere with rippling indicates that the defect caused by the *devT* mutation precedes the separation between aggregation and sporulation in the response pathway. The lack of FruA could explain the differential defects in the C-signal-dependent response, i.e., sufficient amounts of FruA are synthesized to allow rippling while insufficient amounts are synthesized to allow timely aggregation and sporulation. It should be emphasized that we cannot rule out that the DevT protein may have functions in aggregation and sporulation other than stimulating FruA synthesis. Further experimentation will address this issue.

The ordered execution of the four C-signal-dependent responses relies on an ordered increase in the level of C signaling during development in combination with distinct activity thresholds (8, 15, 21, 25). Thus, low levels of C-signaling bring about rippling, intermediate levels bring about aggregation as well as the expression of early C-signal-dependent genes, and high levels result in sporulation and expression of late C-signal-dependent genes. The level of C-signaling is predicted to increase during development. There are two contributions to this increase. First, the two *csgA* proteins accumulate during development (16, 21). The proteins encoded by the *act* operon are involved in controlling the level and timing of *csgA* transcription (8). Second, C-signaling results in aggregation and, therefore, a higher cell density. Because C-signaling depends on direct cell-cell interactions (16), the prediction is that aggregation per se results in increased levels of C-signaling. The data described here provide evidence for an additional positive feedback loop in the C-signal response pathway. Activated FruA induces DevT accumulation by stimulating transcription of the *dev* operon (5, 19), and DevT is required for *fruA* transcription. The loop is closed by the C-signal-dependent activation of FruA (5). This positive feedback loop may constitute a regulatory device that ensures a burst in FruA and Dev protein synthesis once a certain level of C-signaling is reached. In this context, it is interesting that the *dev* operon is primarily expressed in cells within developing fruiting bodies (12). Therefore, the level of C-signaling that sets this positive feedback loop in motion may only be reached in cells within the fruiting bodies. Once the positive FruA/DevT feedback loop is initiated, it may ensure that the developmental program goes to completion.

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