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EspC Is Involved in Controlling the Timing of Development in *Myxococcus xanthus*

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The *espC* null mutation caused accelerated aggregation and formation of tiny fruiting bodies surrounded by spores, which were also observed in the *espA* mutant and in CsgA-overproducing cells in *Myxococcus xanthus*. In addition, the *espC* mutant appeared to produce larger amounts of the complementary C-signal than the wild-type strain. These findings suggest that EspC is involved in controlling the timing of fruiting body development in *M. xanthus*.

Fruiting body development of *Myxococcus xanthus* consists of two major processes: (i) aggregation of 10^5 to 10^6 cells to form raised mounds and (ii) sporulation of individual cells in the mounds (4, 8). *M. xanthus* normally does not sporulate outside of mounds, suggesting that it has a mechanism that inhibits sporulation when the cells are outside of the mounds and induces sporulation only when the cells are in the mounds.

Previously, we described two genes that influence the timing of development and sporulation in *M. xanthus*: *espA* and *espB* (2). An *espA* null mutation triggered early development and formation of tiny fruiting bodies surrounded by spores. In contrast, the *espB* null mutation caused delayed and reduced sporulation. We were interested in identifying other genes that might be related to the EspAB pathway or might also be involved in regulating the timing of development. Since *espA espB* double mutants show the EspA phenotype, we hypothesized that mutations in functionally related genes in the pathway, when introduced into the *espB* strain, would also show the EspA phenotype in early development. We therefore mutagenized the *espB* strain, DZ4417, with the pMycoMar plasmid carrying the *magellan4* transposon, a derivative of the *mariner* transposon (10). Electroporants were plated on CF agar (12) containing kanamycin. Since pMycoMar cannot autonomously replicate in *M. xanthus*, only transformants carrying the transposon in the chromosome as a result of transposition should grow and form fruiting bodies on this medium. Among the approximately 50,000 colonies screened, nine colonies showed early development. Genomic DNA from each of the nine colonies was purified, and the site of the transposon insertion was determined as described previously (10). One of the mutants, DZ4422, had a transposon insertion in an unidentified open reading frame (ORF) encoding a histidine protein kinase that was similar to EspA. This ORF was therefore chosen for further study. Since the phenotype of the strain containing a mutation in this ORF was similar to that of the *espA* strain, this ORF was designated *espC* (for *e*arly *sp*orulation).

Characterization of the *espC* locus. To verify that the transposon insertion in *espC* was responsible for the EspA-like phenotype of DZ4422, we created a new mutant, DZ4586, which had an insertion of pBS102 in *espC*, as well as an in-frame deletion mutant, DZ4588, from which amino acids 43 to 797 were deleted using pBS113 (Fig. 1). When DZ4586 and DZ4588 were placed on CF agar (12), they showed the EspA phenotype (Fig. 2). While we were characterizing the *espC* locus, Youderian et al. deposited the DNA sequence of the *espC* gene in the GenBank DNA sequence database, as a sequence adjacent to the *aglR* and *aglS* A-motility genes (15). Figure 1 shows that the *espC* gene is 3.7 kb downstream of *aglS*. Sequence analysis showed three ORFs oriented in the same direction as *espC* in the region between *aglS* and *espC* (*orfA*, *orfB*, and *orfC* in Fig. 1). To test whether there is polarity between these ORFs and *espC*, we created plasmid insertion mutations in *orfA* (DZ4594) and *orfC* (DZ4595). However, the resulting mutants produced normal fruiting bodies on CF agar, suggesting that *orfA*, *orfB*, and *orfC* are transcribed independently from *espC*. Since the *espC* mutant displayed normal motility, it is unlikely that *espC* is related to *aglR* and *aglS*, which are required for A-motility. *orfD*, the ORF downstream

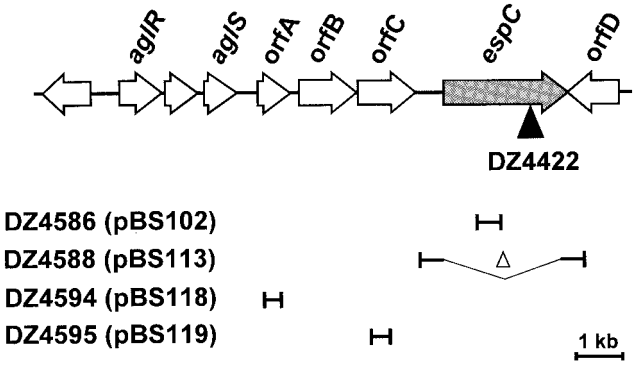

FIG. 1. Physical map of the *espC* region. The black arrowhead indicates the site of the *mariner* transposon insertion. The strains and sites of plasmid insertions are listed below. The site of the in-frame deletion in DZ4588 is indicated by the depressed bar.

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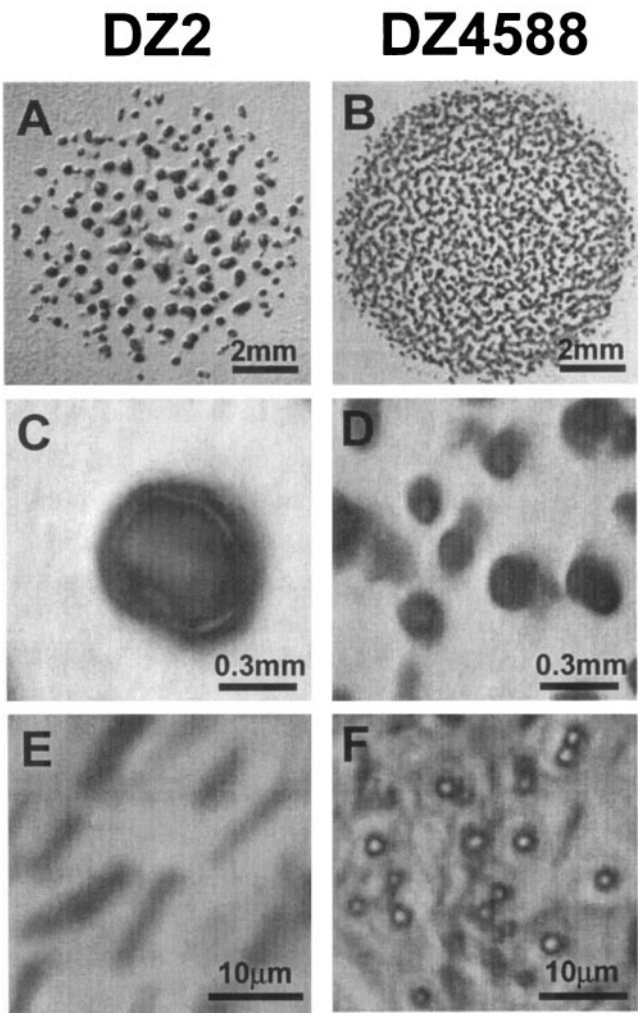

FIG. 2. Developmental phenotype of the *espC* mutant. Cells (20-μl spots containing 5×10^9 cells ml^{-1}) of *M. xanthus* strains DZ2 (A, C, and E) and DZ4588, the *espC* mutant (B, D, and F), were placed on CF agar plates and incubated at 32°C for 5 days. Panels A and B show the density of fruiting bodies in each spot, panels C and D show the relative size of individual fruiting bodies, and panels E and F show individual cells or spores outside of fruiting bodies.

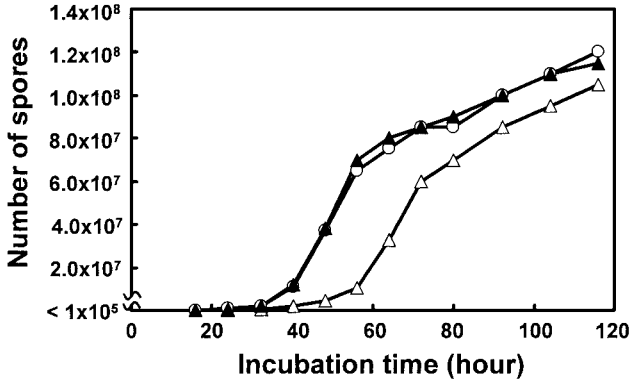

FIG. 3. Timing of sporulation of the *espC* mutant. *M. xanthus* DZ2 (Δ; wild type), DZ4227 (○; *espA*), and DZ4588 (▲; *espC*) were spotted on CF agar plates and incubated at 32°C. Cells were harvested at different time points, and the numbers of sonication-resistant refractile spores were counted.

of the *espC* gene, is oriented convergently with *espC*, suggesting that the *espC* gene is monocistronic.

Reverse transcription-PCR experiments showed that the level of *espC* mRNA increased under developmental conditions but that no *espC* mRNA was detected under the vegetative conditions (data not shown). This indicates that expression of *espC* is developmentally regulated, which is similar to what occurs with *espA*.

Similarity of EspC to other proteins. Sequence analysis using SMART (11) predicted that EspC contains 842 amino acids with the following domain structure: a MASE1 domain (from amino acids 25 to 300), a PAS domain (from amino acids 307 to 380), a PAC domain (from amino acids 382 to 424), a His kinase A domain (from amino acids 439 to 510), a histidine kinase-like ATPase domain (from amino acids 550 to 663), and a signal receiver domain (from amino acids 690 to 804). MASE1 is a predicted integral membrane sensory domain found in histidine kinases, diguanylate cyclases, and other bacterial signaling proteins (1). PAS/PAC domains are involved in many signaling proteins, where they are used as a sensor domain for energy or oxygen stress (14). The His kinase A domain and the histidine kinase-like ATPase domains constitute the histidine protein kinase found in many sensory kinases of two-component systems (9). This domain (from amino acids 439 to 663) was most similar to corresponding domains from MokA (6) (50% identity) and EspA (2) (49% identity) from *M. xanthus*.

Phenotype of the *espC* null mutant. The *espC* null mutant produced many tiny fruiting bodies under starvation conditions, which is similar to what occurs in the *espA* mutant (Fig. 2B). In addition, the *espC* mutation caused sporulation outside of fruiting bodies, which is also similar to the *espA* mutation (Fig. 2F). The *espC* mutation also caused earlier sporulation initiation than in the wild-type strain DZ2 (Fig. 3). The sporulation timing of the *espC* mutant, DZ4588, was very similar to that of the *espA* mutant, DZ4227. *espC* was epistatic to *espB*, *pktA5*, or *pktB8*, similar to what occurs in the *espA* mutant (2, 3). When 10^8 cells of each double mutant, DZ4590 (*espB espC*), DZ4591 (*pktB8 espC*), and DZ4592 (*pktA5 espC*), were spotted onto CF plates, all of them showed an EspC-like phenotype. Sporulation defects were also rescued. As shown in Table 1, the sporulation efficiencies of DZ4225 (*espB*), DZ4282 (*pktB8*), and DZ4308 (*pktA5*) were increased to near wild-type level in the presence of the *espC* mutation in the double mutants. These results suggest that EspC is involved in controlling the timing of fruiting body development in *M. xanthus.*

Extracellular complementation of the *csgA* mutation by the *espA* and *espC* mutants. Aggregation-independent early sporulation is also observed when the CsgA protein is overproduced in the cell (7). The CsgA protein is proposed to be a signal for C-signaling in *M. xanthus*, and the *csgA* null mutant is defective in fruiting body development (5, 13). Since the effects of the *espA* and *espC* null mutations mimic the overproduction of the CsgA protein, we tested whether the *espA* and *espC* mutants overproduce the extracellular signal molecules that rescue the developmental defects of the *csgA* mutant. When 5×10^7 cells

TABLE 1. Effect of the *espC* mutation on sporulation frequency

Strain	Genotype	No. of spores[a]	% of wild-type spores
DZ2	Wild type	9.5×10^7	100
DZ4588	*espC113*	1.1×10^8	110
DZ4215	*espA497*	1.0×10^8	105
DZ4589	*espA497 espC113*	8.5×10^7	89
DZ4225	*espB582*	1.5×10^7	16
DZ4590	*espB582 espC113*	8.5×10^7	89
DZ4308	*pktA5-571*	1.0×10^7	11
DZ4592	*pktA5-571 espC113*	9.5×10^7	100
DZ4282	*pktB8-586*	1.3×10^7	14
DZ4591	*pktB8-586 espC113*	9.0×10^7	95

[a] Number of sonication-resistant refractile spores per spot after 5 days of incubation at 32°C.

of the tetracycline-resistant *csgA* mutant (DZ4655) were mixed with 5×10^7 cells of the *espC* mutant (DZ4588) and placed as 20-μl spots on CF plates, the mixed cells produced 1.2×10^5 tetracycline-resistant spores per spot in 5 days (Table 2). Under the same conditions, the wild-type strain (DZ2) and the *espA* mutant (DZ4227) stimulated the *csgA* mutant to produce only 5.1×10^3 and 1.1×10^3 tetracycline-resistant spores, respectively. Thus, the *espC* mutant appeared to produce more complementing factor than the wild-type strain, stimulating the *csgA* mutant to produce 24-fold more spores than the wild-type strain, while the *espA* mutant appeared to produce less complementing factor than the wild type. This suggests that, although the developmental phenotypes of the *espC* and *espA* mutants are very similar, the functions of EspC and EspA may be different. We speculate that EspC may function in controlling C-signal generation. It is not known at this point whether the *espC* mutant overproduces CsgA, as observed in the CsgA-overproducing strain. It is also not clear how the *espA* mutant, which produces smaller amounts of the complementary C-signal than the wild-type strain, shows a developmental phenotype similar to the CsgA-overproducing strain and the *espC* mutant. However, since all of the *espA* and *espC* mutants and the CsgA-overproducing strain show similar altered timing in fruiting body development, further studies of EspA and EspC will help us to understand the timing mechanism of the fruiting body development in *M. xanthus.*

TABLE 2. Extracellular complementation of the *csgA* mutation by the *espA* and *espC* mutants

Strains	Genotype[b]	No. of spores[a]
DZ4655	*csgA* (Tet^r)	<50
DZ4655 + DZ2	*csgA* (Tet^r) + wt	5.1×10^3
DZ4655 + DZ4227	*csgA* (Tet^r) + *espA*	1.1×10^3
DZ4655 + DZ4588	*csgA* (Tet^r) + *espC*	1.2×10^5

[a] Number of tetracycline-resistant viable spores per spot on CF plates after 5 days of incubation.
[b] wt; wild type.

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