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NOTES

Effect of *dsp* Mutations on the Cell-to-Cell Transmission of CsgA in *Myxococcus xanthus*

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The *dsp* locus contains genes involved in the subunit synthesis and/or assembly of fibrils that radiate outward from the *Myxococcus xanthus* cell surface and attach to other cells. The *csgA* gene encodes an extracellular protein morphogen which is essential for fruiting body development. The question of whether fibrils are involved in the transmission of CsgA to adjacent cells was investigated in three ways. First, the *dsp* and *csgA* mutants were mixed in a ratio of 1:1 and allowed to develop; fruiting bodies containing spores derived from the *csgA* mutant were formed, suggesting efficient CsgA transfer. Second, the *csgA* mutation affected expression of many developmentally regulated genes differently from the way *dsp* affected their expression. Third, the expression of one developmentally regulated gene, which was partially expressed in *csgA* and *dsp* backgrounds, was almost completely inhibited in the presence of both mutations, suggesting that its promoter is regulated independently by two distinct stimuli, one that is *csgA* dependent and one that is *dsp* dependent. Together these results argue that fibrils are not necessary for cell-to-cell transmission or perception of CsgA, and their precise function remains unknown.

Myxococcus xanthus is a gram-negative soil bacterium that forms a multicellular fruiting body in response to nutritional stress (for reviews, see references 10 and 31). Intercellular communication is necessary to coordinate cell movement and differentiation involved in fruiting body morphogenesis. McVittie et al. (25) originally showed that certain pairs of developmental mutants could stimulate each other to develop. Hagen et al. (11) extended these findings by isolating four classes of such conditional sporulation mutants (*asg*, *bsg*, *csg*, and *dsg* mutants). Each mutant class arrested the developmental program at a different time as measured by the expression of developmentally regulated genes (21, 22). Biochemical, genetic, and immunological studies have shown that the *csgA* gene encodes an extracellular protein that has morphogenic properties (12, 17, 33, 35). CsgA is a novel type of developmental timer whose extracellular concentration rises steadily during fruiting body formation and which induces successive stages of the developmental pathway at successively higher concentrations (19, 24). Motility, or some function regulated by motility, appears to be essential for *csgA*-mediated cell-cell interactions. The nonmotile *mgl* mutants cease expressing developmental genes at about the same time as *csgA* mutants (20). Although they seem to produce CsgA, *mgl* cells fail to transmit the signal to adjacent cells (18).

Gliding motility is governed by two multigene systems known as A and S (14, 15). While fully motile cells (A^+ S^+) glide individually or in groups, cells containing an A system mutation (A^- S^+) display purely social behavior and glide in groups (16). Cells containing an S system mutation (A^+ S^-) glide primarily as individuals but can also move in groups (9, 16). With the exception of strains carrying *mglA* mutations, which eliminate motility, cells become nonmotile only when both the A and S systems are disrupted (14, 15). Since about two-thirds of the A^+ S^- mutants are defective in fruiting body formation (15, 30), even though they are still quite motile (16), it appears as if the S system encodes an essential developmental component apart from its ability to provide a means of movement. A candidate for this developmental component is the network of fibrils which extend about one cell length away from the cell surface and interact with adjacent cells (2, 9). In addition to their loss of motility, *mgl* mutants are deficient in production of fibrils (9). Development-specific proteins appear to be associated with fibrils (4, 8), and it is possible that fibrils are used to present specific proteins to adjacent cells. The *dsp* locus, a component of the S system, appears to encode the structural genes for the subunit synthesis, transport, and/or assembly of fibrils (1, 2, 9, 29).

In this work, the role of fibrils in the transmission of CsgA was investigated by using mutations in the *dsp* locus. The *dsp* mutations do not appear to interfere with CsgA transmission and appear to disrupt developmental gene expression at a point different from that of *csgA* mutations in the developmental program.

Strains and growth conditions. The bacterial strains used in this study are listed in Tables 1 and 2. *M. xanthus* cells were grown at 32°C in CTT broth or on CTT agar (13) supplemented, when necessary, with kanamycin sulfate (40 μg/ml), oxytetracycline (20 μg/ml), or trimethoprim (250 μg/ml). *Escherichia coli* was grown in L broth or on L agar supplemented, when necessary, with kanamycin sulfate (50 μg/ml), ampicillin (50 μg/ml), or chloramphenicol (12.5 μg/ml). The myxophage Mx4 *ts18 ts27* and its use for generalized trans-

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TABLE 1. Tn*5 lac* insertion strains of *M. xanthus*

Tn*5 lac* insertion[a]	*dsp*$^+$ derivative	*dsp-1693* derivative	*csgA*$^+$ derivative	*csgA205* derivative
ΩDK4400	LS701	LS700	DK4292	LS448
ΩDK4406	LS489	LS252	DK4294	LS251
ΩDK4411	LS729	LS728	DK5200	LS455
ΩDK4435	LS727	LS726	DK5204	LS248
ΩDK4455	LS721	LS720	DK5206	LS454
ΩDK4473	LS741	LS740	DK4473	LS450
ΩDK4492	LS743	LS742	DK4492	LS449
ΩDK4499	LS745	LS744	DK4499	LS446
ΩDK4500	LS713	LS247	DK4500	LS246
ΩDK4506	LS735	LS734	DK4506	LS451
ΩDK4514	LS711	LS704	DK4514	LS447
ΩDK4521	LS737	LS736	DK4521	LS456
ΩDK4531	LS751	LS750	DK4531	LS452
ΩLS234	LS715	LS722	LS234	LS235
ΩLS237	LS719	LS718	LS237	LS238

[a] The site of Tn*5 lac* insertion is designated by Ω, followed by a DK (D. Kaiser) number (18) or LS (L. Shimkets) number.

duction between *M. xanthus* strains has been described previously (5). The coliphage P1 *cam clr100* is a chloramphenicol-resistant (Cm^r), temperature-inducible variant of P1 (27).

Strain LS523 contains Tn*5-132*ΩLS205, which encodes tetracycline resistance (Tc^r), inserted in the *csgA* gene (7, 32). Liquid stocks of Mx4 *ts18 ts27* were prepared on LS523 cells as described by Rhie and Shimkets (26). The phage stocks were used to transduce strains containing Tn*5 lac* fused to developmentally regulated promoters to Tc^r. All the *csgA* Tn*5 lac* derivatives listed in Table 1 had a developmental phenotype similar to that of their parental strain, LS523. Strain LS302 contains *dsp-1693* linked to Tn*5-132* insertion ΩDK1407. Liquid Mx4 phage stocks were prepared on LS302 and used to infect strains containing developmentally regulated Tn*5 lac* fusions. Since ΩDK1407 was not inserted within a *dsp* gene, the Tc^r transductants displayed two phenotypes, Dsp$^-$ and Dsp$^+$. One of each was saved and tested for β-galactosidase production (Table 1).

In order to construct several of the strains used to examine developmental gene expression, it was necessary to develop a new selectable genetic marker. Tn*5* Tp is a transposable element derived from Tn*5* in which the gene encoding kanamycin resistance has been replaced with a gene encoding trimethoprim resistance (28). This transposon was allowed to transpose from pCHR66 to P1 for use as a suicide delivery system with *M. xanthus*. *E. coli* MC1061 (6) containing pCHR66 was infected with P1 *cam clr100* at 32°C with selection for Cm^r (20 μg/ml) and trimethoprim resistance (Tp^r) (20 μg/ml). Several hundred lysogens were pooled and thermally induced (27). The phage stock was used to infect SK388 (*lac* Str^r *sup polA1*; kindly furnished by S. Kushner) at a multiplicity of infection of 0.1 and 30°C with selection for Cm^r and Tp^r. Since pBR322-derived plasmids cannot replicate in *polA* mutants, the majority of the Tp^r strains contain Tn*5* Tp inserted in P1. A single P1 lysogen that mediates specialized transduction of Tn*5* Tp was thermally induced and used to replace Tn*5* or Tn*5-132* insertions with Tn*5* Tp. Transposon replacement by double homologous crossovers has been previously described for *M. xanthus* (3). Trimethoprim was dissolved in 80% ethanol at 15 mg/ml and added to molten CTT agar to a final concentration of 250 μg/ml. Phage stock (0.1 ml) was incubated with 10^8 log-phase recipient cells for 20 min at 32°C and plated on CTT containing trimethoprim. Tp^r transductants appeared after about 7 days at 32°C. Over 95% of the transductants were transposon replacements, as described previously for use of a similar replacement strategy to replace Tn*5* with Tn*5-132* (3).

Extracellular complementation and developmentally regulated gene expression. The *csgA* mutants fail to form spore-filled fruiting bodies because they are unable to produce a 17.7-kDa extracellular polypeptide (12, 17, 19, 24, 32, 35). To determine whether fibrils are involved in presenting CsgA to adjacent cells, the ability of *dsp* cells to serve as a CsgA donor was tested by using the extracellular complementation assay. In this assay, the two mutant cell lines were tagged with different antibiotic resistance markers, mixed at a 1:1 ratio, and allowed to develop. Neither strain was able to form fruiting bodies or sporulate by themselves (Fig. 1). The *csgA* strain formed <0.01% of the wild-type levels of spores, while the *dsp* strain formed 1% of the wild-type spore levels (not shown). Mixture of these two mutants restored fruiting body morphogenesis (Fig. 1). The spore number produced by the mixture of mutants increased dramatically to about 63% of the wild-type levels, and about 98% of the spores were derived from the *csgA* parent (not shown). These results suggest that the CsgA donor does not need to contain fibrils to produce or secrete CsgA. Since *dsp* cells are not stimulated to sporulate, even in response to wild-type cells (30), fibrils could be required for CsgA perception.

A second and independent way of determining whether fibrils are necessary for CsgA presentation or perception involves identifying the point where the two mutations

TABLE 2. *M. xanthus* strains and their derivations

Strain	Genotype[a]	Derivation, source, or reference
DK1622	Wild type	34
DK3470	*dsp-1693* Tn*5*ΩDK1407	30
LS302	*dsp-1693* Tn*5-132*ΩDK1407	J. Arnold and L. Shimkets
LS322	Tn*5-132*ΩDK1407	J. Arnold and L. Shimkets
LS450	*csgA205*::Tn*5-132*ΩLS205 Tn*5 lac*ΩDK4473	Mx4(LS523) × DK4473→Tc^r
LS491	*dsp-1693* Tn*5* Tp ΩDK1407	P1::Tn*5* Tp × DK3470→Tp^r Km^s S$^-$
LS523	*csgA205*::Tn*5-132*ΩLS205	32
LS912	*csgA205*::Tn*5* Tp ΩLS205 Tn*5 lac*ΩDK4473	P1::Tn*5* Tp × LS450→Tp^r Km^r
LS914	*dsp-1693* Tn*5* Tp ΩDK1407 *csgA205*::Tn*5-132*ΩLS205 Tn*5 lac*ΩDK4473	Mx4(LS491) × LS450→Tp^r Tc^r Km^r S$^-$

[a] The site of Tn*5 lac* insertion is designated by Ω, followed by a DK (D. Kaiser) number or LS (L. Shimkets) number. The :: designation refers to transposon insertion within the designated gene or bacteriophage. Thus, ΩLS205 causes the mutation *csgA205*. Where the :: designation is not used, the gene containing the insertion has not been assigned a genetic name. For example, ΩDK1407 is linked to the *dsp* locus but does not cause a *dsp* mutation; the gene containing ΩDK1407 is currently unknown. The genotypes of many other strains are listed in Table 1.

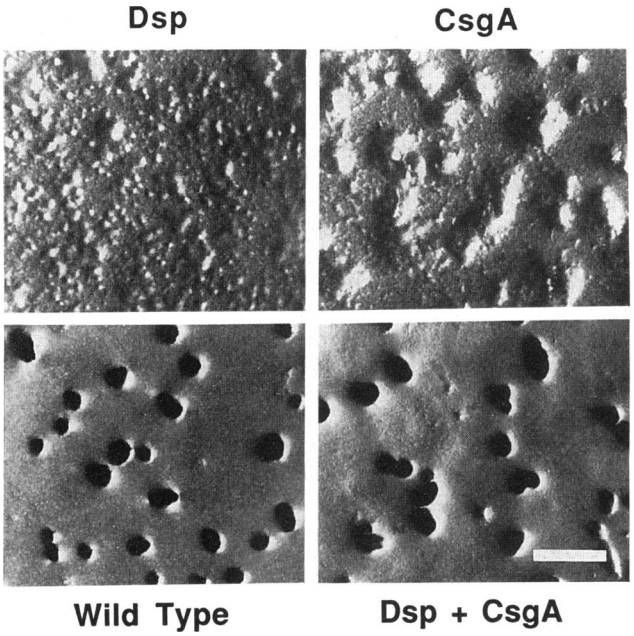

FIG. 1. Extracellular complementation between *dsp* and *csgA* mutants. Exponentially growing cells were suspended to 1,000 Klett units in TM buffer and placed on CF agar in 10-μl spots containing a 1:1 cell ratio. Plates were incubated at 32°C for 4 days and photographed. Bar, 500 μm.

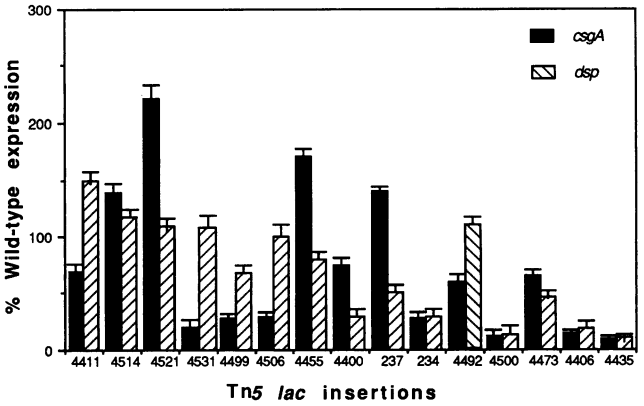

FIG. 2. Effect of *dsp* and *csgA* mutations on developmentally regulated β-galactosidase expression from Tn*5* *lac* transcriptional gene fusions. Percentages of peak β-galactosidase specific activities in the *csgA* background relative to that of otherwise isogenic *csgA*$^+$ derivatives and in the *dsp* background relative to that of otherwise isogenic *dsp*$^+$ derivatives are shown. See Table 1 for strain names. The Tn*5* *lac* insertions are ordered from left to right on the *x* axis according to the approximate time that expression is initiated. The times of expression listed below are the averages of three determinations made on CF agar and in submerged culture in this work and made on TPM agar previously (22). The times of expression of the fusions are as follows: ΩDK4411, 2 h after initiation of development; ΩDK4514, 4 h; ΩDK4521, 4 h; ΩDK4531, 4 h; ΩDK4499, 6 h; ΩDK4506, 7 h; ΩDK4455, 8 h; ΩDK4400, 9 h; LS237, 10 h; LS234, 10 h; ΩDK4492, 11 h; ΩDK4500, 14 h; ΩDK4473, 16 h; ΩDK4406, 17 h; and ΩDK4435, 22 h. Results are the average peak β-galactosidase activities from three experiments, and the ranges are indicated.

adversely affect development. If fibrils are involved in CsgA transmission and/or reception, the *csgA* and *dsp* genes would belong to the same signal transduction pathway and their mutations would be expected to inhibit developmental gene expression in similar ways. In terms of their morphological phenotypes, *csgA* and *dsp* cells are blocked at similar places; both fail to ripple, aggregate, or sporulate (24, 30, 34). However, the time when the developmental program begins to deviate from that of the wild type can be more precisely determined through the use of development-specific markers that are induced at well-defined places in the pathway (22). The developmental markers we used consisted of *lacZ* transcriptional fusions to promoters expressed at different developmental times (21, 22). Since the *dsp* locus has not been well characterized, initial experiments were conducted with two *dsp* alleles to determine whether they behaved similarly in this assay. The *dsp-1693* allele and the *dsp-1694* allele were transduced into a set of four *lacZ* fusions and assayed for β-galactosidase at intervals during development. The effects of each allele were examined with respect to the time expression was initiated, the time of maximal specific activity, and maximal specific activity. Allelic differences were not obvious, and the experiment was continued with allele *dsp-1693*.

The expression of a number of *lacZ* fusions in the *csgA* background was compared with the expression of these fusions in otherwise isogenic *dsp* and wild-type backgrounds. The *csgA-205* allele was transduced into the same set of Tn*5* *lac* fusion strains, and the resulting set of isogenic strains (Table 1) was assayed for β-galactosidase during development. When β-galactosidase specific activity was plotted as a function of developmental time, *dsp* mutations were observed to affect expression of different developmentally regulated genes in four ways: expression was abolished (ΩDK4500), reduced (ΩLS237), increased (ΩDK4411), or not significantly altered (ΩDK4506). The *csgA* mutation also affected developmental gene expression in these four ways, although it did not affect each gene the same way as *dsp* mutations. For example, *csgA* reduces expression of Ω4531, whereas *dsp-1693* does not significantly alter expression.

The time at which developmentally regulated gene expression was initiated for each *lacZ* fusion was estimated by harvesting cells at time intervals around the initiation time. The time of expression reported in the legend to Fig. 2 is the average of three determinations under different developmental conditions, i.e., CF agar (11) and submerged culture (23) in this work and TPM agar in previous work (22). The time at which each marker was induced in wild-type cells varied slightly under different developmental conditions. For the sake of this comparison between *dsp* and *csgA*, the maximum level of mutant expression is plotted as a percentage of maximum wild-type expression for *csgA* and *dsp* cells (Fig. 2). The earliest *lacZ* fusion whose expression is drastically reduced in *csgA* cells is ΩDK4531, which is expressed about 4 h into the developmental program; expression of this fusion is normal in *dsp* cells. The earliest time *dsp* cells affect developmental gene expression is about 9 h into the developmental program, as seen for ΩDK4400. Expression of this fusion is only slightly reduced in *csgA* cells. Large differences in the relative levels of gene expression between *dsp* and *csgA* were observed for about half of the *lacZ* fusions examined, especially those expressed during the 4- to 11-h period of development, suggesting that *csgA* and *dsp* inhibit the developmental pathway in different ways.

The ΩDK4473 fusion was the only fusion found to be partially expressed in the *dsp* and *csgA* backgrounds. Expression of ΩDK4473 was reduced to 55 to 58% of wild-type

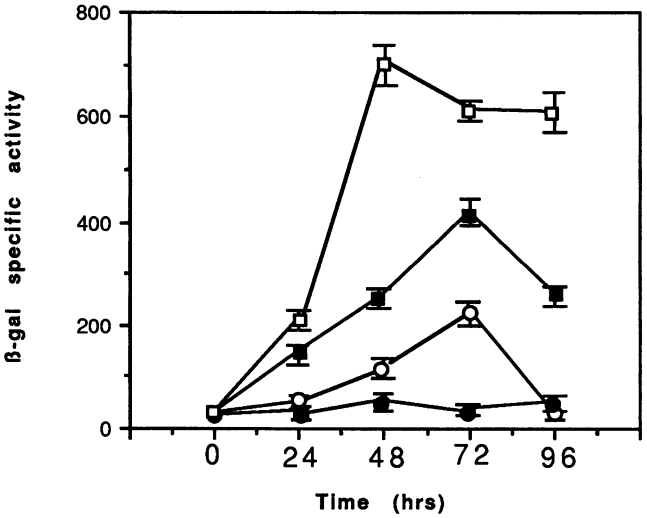

FIG. 3. Developmentally regulated ΩDK4473 expression in the following genetic backgrounds: *csgA*$^+$ *dsp*$^+$ (DK4473; open squares), *csgA* (LS450; closed squares), *dsp* (LS491; open circles), and *csgA dsp* (LS914; closed circles). Cells were plated on CF agar for development and harvested at various times for assay of β-galactosidase (β-gal) specific activity (given as nanomoles of *o*-nitrophenol per minute per milligram of protein). The data shown are the averages of two experiments with error bars indicating the range.

levels in *csgA* cells and 30 to 32% of wild-type levels in *dsp* cells (Fig. 3). The *csgA dsp* cells decreased expression of ΩDK4473 additively to 13% of wild-type levels, suggesting that expression is independently regulated by both a *dsp*-dependent stimulus and a *csgA* stimulus.

Conclusions. It is clear from this study that the cell surface fibrils are not necessary for CsgA production, secretion, or perception. First, *dsp* mutants, which lack fibrils, effectively transmit CsgA to *csgA* mutants, thereby restoring their ability to sporulate. Second, *csgA* and *dsp* affect 4- to 11-h developmental gene expression in different ways. *csgA* but not *dsp* reduced expression of ΩDK4531 and ΩDK4506, while *dsp* but not *csgA* reduced expression of ΩLS237 and ΩDK4400. Furthermore, the effect of these mutations on expression of ΩDK4473 was additive, suggesting that expression of this gene fusion is regulated independently by *dsp*-dependent and *csgA*-dependent stimuli. The precise developmental function(s) of the fibrils remains unknown.

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