Intercellular C-signaling in *Myxococcus xanthus* involves a branched signal transduction pathway

Lotte Søgaard-Andersen,¹ Frank J. Slack,^{1,2} Harvey Kimsey,³ and Dale Kaiser⁴

Department of Biochemistry, Stanford University School of Medicine, Stanford, California, 94305 USA

C-factor, the product of the *csgA* gene, is a cell-surface associated short-range intercellular signaling protein in *Myxococcus xanthus*. C-factor is required for at least four responses during starvation-induced fruiting body morphogenesis: rippling, aggregation, sporulation, and full expression of the *csgA* gene, all of which fail in a *csgA* mutant. To analyze the C-factor signaling pathway, eight Tn5 *lac* insertion mutants that began but failed to complete fruiting body aggregation were characterized. Seven of the insertions identified genes whose products function in the *csgA* signaling pathway. The seven mutants were differentially deficient in the C-factor responses, and could be divided into two classes on the basis of those differences. On one hand, the four mutants in class I were deficient in rippling and aggregation, but sporulated and produced C-factor at wild-type levels. The Tn5 *lac* insertions in the class I mutants mapped to the *frz* locus, which encodes a signal transduction system that controls the frequency of single cell reversals. On the other hand, mutants carrying any of the three closely linked class II Tn5 *lac* insertions had deficiencies in all four C-factor responses. Because the sporulation defect in the class II mutants is cell autonomous, the data suggest that the primary defect in these mutants is an inability to respond to the C-factor signal. All the data can be explained by a model in which the first part of the C-factor signaling pathway is common to all four C-factor-dependent responses. The genes identified by the class II insertions would function in the common part. Downstream of class II, the pathway branches. One branch includes the *frz* genes and leads to aggregation and rippling; the second branch leads to sporulation and controls the level of *csgA* gene expression. This model was confirmed in epistasis tests with characterized *frz* mutations, a *csgA* null mutation, and a class II mutation.

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Communication between cells by means of extracellular signals is important for morphogenesis and differentiation in multicellular organisms. In several systems extracellular signal molecules elicit two different responses. For example, in *Dictyostelium discoideum*, cAMP elicits two temporally separated responses, aggregation and sporulation (Gross 1994), and in *Drosophila melanogaster wingless* signaling results in simultaneous changes in gene expression and cell adhesiveness (Arias 1994). This raises the question of how the signal transduction pathways are structured to provide qualitatively different responses to one signal molecule.

Myxococcus xanthus cells undergo a multicellular developmental program in response to nutrient limitation (Shimkets 1990; Kim et al. 1992). Within 4 to 8 hr, the

cells begin to aggregate by gliding to foci where 10⁵ cells build a structure known as a fruiting body. Within a fruiting body, the rod-shaped cells differentiate into spherical, environmentally resistant spores. Aggregation and sporulation are temporally separated, and sporulation in wild-type cells does not occur until cell movements have led to the assembly of a structure with the external form of a fruiting body. The earliest phases of aggregation are often accompanied by ripple formation. Ripples consist of a series of equally spaced ridges of cells that move in a coordinated fashion like travelling waves on a surface (Shimkets and Kaiser 1982a). Rippling is not obligatory for the construction of fruiting bodies but generally accompanies this process (Shimkets 1990). Several different extracellular signals are required to coordinate fruiting body morphogenesis (Kaiser and Losick 1993).

One of these signals is the cell-surface associated protein C-factor, which is the product of the *csgA* gene (Hagen and Shimkets 1990; Kim and Kaiser 1990a,c; Shimkets and Rafiee 1990). In *csgA* mutants, the developmental process arrests early in the aggregation phase,

¹ These authors contributed equally to the work presented.
Present addresses: ²Department of Genetics, Harvard Medical School, Massachusetts General Hospital, Wellman 8, Boston, Massachusetts, 02114 USA; ³Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts, 02111 USA.
⁴Corresponding author.

ripples are not formed, and sporulation is severely impaired (Shimkets and Kaiser 1982a). *csgA* mutants also exhibit altered developmental gene expression as monitored by the expression of a panel of transcriptional fusions to the *lac* genes in Tn5 *lac*. Expression from Tn5 *lac* fusions that are normally expressed at 6 hr or later into development is abolished partially or completely in *csgA* mutants (Kroos and Kaiser 1987). One of the C-factor-dependent genes is *csgA* itself and full expression of *csgA* requires C-factor signaling (Kim and Kaiser 1991). Aggregation, sporulation, and developmental gene expression are rescued by codevelopment of *csgA* cells with wild-type cells (Shimkets et al. 1983; Kim and Kaiser 1990a) or by the addition of purified C-factor (Kim and Kaiser 1990a,c). Addition of C-factor to nonmotile cells induces sporulation in the absence of aggregation (Kim and Kaiser 1991). These observations suggest that C-factor can induce directly the two temporally separated morphogenetic processes aggregation and sporulation in fruiting body formation (Kim and Kaiser 1991).

Rescue of *csgA* cells by wild-type cells requires contact between the *csgA* and the *csgA*⁺ cells (Kim and Kaiser 1990a) and active motility in both cell types (Kroos et al. 1988; Kim and Kaiser 1990b), suggesting that cells are brought into the proper arrangements for efficient transmission of the C-signal by their own movements (Kim and Kaiser 1990d). This observation and the finding that a lower concentration of C-factor is required for aggregation than for sporulation (Kim and Kaiser 1991; Li et al. 1992) has led to the hypothesis that C-factor coordinates these two processes (Kim and Kaiser 1991; Li et al. 1992). This coordination would rely on two positive feedback loops: (1) increased *csgA* expression in response to C-factor signaling, and (2) increased C-factor signaling attributable to close and ordered packing of cells in the fruiting body resulting from the C-factor-induced aggregation (Kim and Kaiser 1990d, 1991; Sager and Kaiser 1993).

The molecular mechanism by which the C-signal is transmitted between cells is not yet known. Likewise, little is known about the intracellular transduction of the C-factor signal once it is received by a cell. The aim of this work is to begin to define the C-signal transduction pathway. Here, seven mutants that interrupt this pathway are described. On the basis of their properties and the properties of certain double mutants, we suggest that the signal transduction pathway has an upstream segment common to all four C-factor-dependent activities. Downstream of this common part, the pathway branches. One branch leads to the motility responses and a second branch to sporulation and to control of gene expression, including *csgA* itself.

Results

Isolation of Tn5 lac insertion mutants blocked in the aggregation phase

After 24 hr of normal development, *M. xanthus* cells have aggregated and constructed well-formed and steep-

sided fruiting bodies, whereas a *csgA* mutant has formed loose, irregular aggregates (Fig. 1A). To isolate mutations that may interrupt the earliest steps in the C-factor signal transduction pathway, mutants that arrested their development within the aggregation process (Agg[−] mutants) were sought beginning with a set of 42 Tn5 *lac* insertion mutants that all had a developmental defect. This set of mutants was retrieved originally from a collection of 10,000 Tn5 *lac* insertion mutants that were isolated in the fully motile strain DK1622, which serves as the standard or wild type for these studies.

Approximately half of the 42 developmental mutants were disabled in either adventurous (A) motility or in social (S) motility during vegetative growth (data not shown), affirming the strong connection between development and motility in *M. xanthus* (Shimkets 1990). Because *csgA* strains exhibit normal vegetative motility (i.e., are A⁺S⁺), mutants found to be disabled in A or S motility were not studied as part of this work. By monitoring the development of the remaining mutants on TPM starvation agar, the stage at which aggregation was altered in each of these mutants could be identified. Seven of the mutants exhibited a particular Agg[−] phenotype in which they initiated aggregation without completing it. The remaining mutants showed no aggregation, or completed aggregation but formed abnormal fruiting bodies, or completed their development more slowly than wild type (data not shown). The seven Agg[−] mutants and a previously isolated Tn5 *lac* insertion mutant, Ω 4491 in DK5285, which also exhibits an Agg[−] phenotype, were chosen for further study (Kroos et al. 1986, 1990; Kroos and Kaiser 1987). To construct an isogenic set of strains for detailed comparisons, the Tn5 *lac* insertions in the newly isolated Agg[−] mutants were crossed back into a wild-type strain, DK1622. In each case, the phenotype of the transductants was similar to the original mutant strain carrying the Tn5 *lac* insertion (data not shown), indicating that the Agg[−] phenotype was caused by the Tn5 *lac* insertion. All subsequent experiments were conducted with the backcrossed strains.

Classification of the Agg[−] mutants according to their capacity to perform C-factor-dependent activities

Wild-type strains as well as strains carrying mutations in the A-signaling (*asg*), D-signaling (*dsg*), or B-signaling (*bsg*) pathways, are able to form ripples early in development when placed on low nutrient agar (Shimkets and Kaiser 1982a,b). *csgA* mutants are unable to ripple (Shimkets and Kaiser 1982a), presumably because C-factor is required to stimulate cell reversals required for rippling (Sager and Kaiser 1994). The ability of the Agg[−] mutants to ripple was tested on CF agar (see Materials and methods) and found to be abolished in all of them (data not shown). As rippling is considered a C-factor-dependent developmental event, these results suggest that the Tn5 *lac* mutations may disrupt C-signaling.

Rippling appears to require the activity of both the S and A motility systems (Shimkets and Kaiser 1982a). Likewise, C-factor signaling requires that the cells are

Figure 1. Aggregation phenotype of Tn5 *lac* insertion mutants. (A) Aggregation phenotype in *csgA*⁺ background. For comparison, the aggregation phenotypes of wild-type and *csgA* strains have been included. The *csgA* allele used throughout is *csgA*::Tn5-132ΩLS205. (B) Aggregation phenotypes of Tn5 *lac* insertion mutants in a *csgA* background. (C) Aggregation phenotype at low magnification in *csgA*⁺ and in *csgA*⁻ backgrounds; strains in the upper row carry the Tn5 *lac* insertions in a *csgA*⁺ background and those in the lower row in a *csgA*⁻ background. In all experiments A–C, 20 μl of a cell suspension with a calculated density of 1000 Klett units/ml in TPM buffer was placed on a TPM agar, and the cells allowed to develop for 24 hr. The Tn5 *lac* insertion present in a strain is indicated above each panel. Strains: (A) DK1622 (wild type), DK11016 (Ω7522), DK11064 (Ω7546), DK11018 (Ω7632), DK11019 (Ω7663), DK5208 (*csgA*), DK5285 (Ω4491), DK11063 (Ω7540), DK11020 (Ω7677), DK11015 (Ω7516); (B) DK11050 (Ω7522), DK11051 (Ω7546), DK11053 (Ω7632), DK11054 (Ω7663), DK5296 (Ω4491), DK11074 (Ω7540), DK11055 (Ω7677), DK11049 (Ω7516); (C) DK1622 (wild type), DK11016 (Ω7522), DK11064 (Ω7546), DK11018 (Ω7632), DK11019 (Ω7663), DK5208 (*csgA*), DK11050 (Ω7522, *csgA*), DK11051 (Ω7546, *csgA*), DK11053 (Ω7632, *csgA*), DK11054 (Ω7663, *csgA*). The complete genotype of these strains is given in Table 7. (A,B) Bar, 1 mm; (C) bar, 2 mm.

motile (Kim and Kaiser 1990b). To determine rigorously whether any of the Tn5 *lac* insertions disrupted a gene involved in A or S motility, each Tn5 *lac* insertion was crossed by Mx4 transduction into a representative S⁻ (DK1300 *sglG*) or A⁻ (DK1219 *cglC1*) host, and the effect on swarming motility assessed on 1/2CTT agar (see Materials and methods). Strains that carry both an A⁻ and an S⁻ mutation are nonswarming (Hodgkin and Kaiser 1979a,b) and grow as small, smooth-edged colonies. As judged by the structure of the swarming zone around vegetative colonies, none of the Agg⁻ Tn5 *lac* insertions generated a nonswarming (A⁻ S⁻) phenotype when crossed into either an A⁻ or S⁻ host (data not shown).

Thus, in a manner analogous to a *csgA* mutation, none of the mutations resulted in a defect in either of the A or S motility systems.

A screen for Agg⁻ mutants would be expected to identify additional *csgA* alleles. To test whether any of the Agg⁻ Tn5 *lac* insertions mapped to *csgA*, the Tn5 *lac* insertions (which confer Kan^r) were crossed into the strain DK5208 that carries the *csgA*::Tn5-132ΩLS205 allele (which confers Tet^r) by Mx4 transduction. Subsequently, the transductants were scored for the loss of Tet^r. In all cases, 100% of the resulting Kan^r transductants were still Tet^r (data not shown), indicating that the insertions do not map in or close to the *csgA* gene. We

Table 1. Sporulation frequencies of *Agg⁻ Tn5 lac* insertion mutants

<i>Agg⁻</i> class	Tn5 <i>lac</i> insertion	Strain	Percent sporulation ^a	Percent sporulation + DK1622 (wt) ^b
I		DK1622 (wt)	3.2	N.D.
		DK5208 (<i>csgA</i>)	<0.000006	0.8
		DK11017	1.2	N.D.
		DK11064	1.2	N.D.
		DK11019	1.5	N.D.
II		DK11020	1.9	N.D.
		DK5285	<0.000006	<0.000006
		DK11063	<0.000006	<0.000006
		DK11021	<0.000006	<0.000006
III		DK11016	<0.000006	<0.000006

^aPercent input cells (1.25×10^8) that formed spores, as judged by colonies that grew up on CTT agar.

^b 6.25×10^7 mutant cells (prepared as described in Materials and methods) were mixed with an equal number of wild-type cells at time zero. Percent values are the number of mutant spores after 3 days of incubation divided by the number of mutant cells at time zero. (N.D.) Not determined.

note that from a total of 10,000 insertions, no insertion in *csgA* has been identified, indicating that the Tn5 *lac* mutagenesis did not saturate the chromosome for “*csgA*-like” genes.

The *Agg⁻* mutants were examined for their ability to form spores after 72 hr of starvation in submerged culture (Kuner and Kaiser 1982; Kim and Kaiser 1990b; see Materials and methods) (Table 1). Approximately 3% of wild-type cells formed spores in this assay in agreement with previous observations (Kim and Kaiser 1990b). The four insertion mutants $\Omega 7522$, $\Omega 7546$, $\Omega 7632$, and $\Omega 7663$ (the Tn5 *lac* insertions are named by a four-digit number that refers to the site of transposon insertion) formed filamentous structures, much smaller and less condensed than wild-type fruiting bodies (Fig. 1A, showing aggregation on agar). Nevertheless, these mutants sporulated at the same level as the wild-type strain (Table 1), although, notably, the spores formed by these four mutants were not contained in the multicellular structures (data presented below). The mutants containing the insertions $\Omega 4491$, $\Omega 7540$, $\Omega 7677$, and $\Omega 7516$ sporulated at a very low level, the same as a *csgA* mutant (Table 1).

To investigate whether the sporulation defect in the mutants containing $\Omega 4491$, $\Omega 7540$, $\Omega 7677$, and $\Omega 7516$ were cell autonomous, wild-type cells were mixed with mutant cells to test if sporulation in the mutants could be rescued by extracellular complementation (Table 1). Sporulation in the four mutants was not rescued by codevelopment with an equal number of wild-type cells. In contrast, sporulation by a *csgA* mutant was rescued 100,000-fold by wild-type cells as observed previously (Kim and Kaiser 1990b). These four mutations being cell autonomous implies that their sporulation defect is not simply caused by the lack of production of an extracellular signal.

C-factor production by the *Agg⁻* mutants was measured in vivo by the ability of the mutant strains to rescue development of a *csgA* null mutant by extracellular

complementation (Table 2). As noted above, codevelopment with a motile *csgA⁺* strain, like DK1622, rescues sporulation efficiently in the *csgA* strain. The mutants containing insertions $\Omega 7522$, $\Omega 7546$, $\Omega 7632$, and $\Omega 7663$ all rescued sporulation of the *csgA* tester strain DK5208 efficiently, implying that the developmental block imposed by the Tn5 *lac* insertions is not in C-factor production or the ability to transmit the C-signal. The mutants with the insertions $\Omega 4491$, $\Omega 7540$, and $\Omega 7677$ rescued sporulation of the *csgA* strain ~10-fold less efficiently than wild-type cells, whereas the mutant carrying $\Omega 7516$ was unable to rescue sporulation of the *csgA* strain. As these four strains are fully motile, the results indicate that C-factor production is partially deficient in the strains carrying the insertions $\Omega 4491$,

Table 2. Sporulation of *csgA* mutant (DK5208) when codeveloped with *Agg⁻* mutants

<i>Agg⁻</i> class	Tn5 <i>lac</i> insertion in <i>Agg⁻</i> mutant	<i>Agg⁻</i> mutant codeveloped with DK5208	Percent sporulation of DK5208 ^a
I		none	<0.000006
		DK1622 (wt)	0.8
		DK11017	0.8
		DK11064	1.1
		DK11019	0.5
II		DK11020	0.6
		DK5285	0.08
		DK11063	0.06
		DK11021	0.08
III		DK11016	<0.000006

^aPercent input DK5208 cells (6.25×10^7) that formed spores in the presence of an equal number of the indicated potential donor cells, as judged by colonies that grew up on agar containing Tet.

Ω7540, and Ω7677 and completely deficient in the strain carrying Ω7516.

The aggregation phenotypes of the Tn5 *lac* insertion mutants differed from that of a *csgA* strain on TPM agar after 24 hr of starvation (Fig. 1A,C). Viewed at a low magnification as in Figure 1C, the mat of *csgA* mutant cells was featureless apart from a slight mottling. At the higher magnification of Figure 1A, the *csgA* mutant was observed to form small, rather loose aggregates. The mutants that carried insertions Ω7522, Ω7546, Ω7632, and Ω7663 formed small, dense, irregularly shaped aggregates (Fig. 1A) that tended to be organized in tangled filaments (Fig. 1C). Mutants with insertions Ω4491, Ω7540, and Ω7677 also resembled each other; they formed large, branched aggregates (Fig. 1A). The mutant with insertion Ω7516 formed small irregular aggregates.

From these aggregation and sporulation tests the mutants can be divided into three distinct phenotypic classes. Class I consists of the strains carrying the insertions Ω7522, Ω7546, Ω7632, and Ω7663. These mutants are unable to ripple, form small, dense, irregular aggregates that tend to be organized in tangled filaments, sporulate at wild-type levels, and produce wild-type levels of C-factor. Class II consists of the strains carrying insertions Ω4491, Ω7540, and Ω7677. These mutants are unable to ripple and they form extended, branched linear aggregates. They are unable to sporulate, and are cell autonomous with respect to sporulation (Table 1). They produce reduced amounts of C-factor (Table 2). Finally, the mutant carrying the insertion Ω7516 is the only member of class III. This mutant is unable to ripple, forms small, nonuniform aggregates, is unable to sporulate, is cell autonomous with respect to sporulation, and does not appear to produce C-factor.

Class I and class II insertions identify genes for components of the C-signal transduction pathway

Epistasis experiments can provide information about interactions between different genes, including the relative position of gene products in a regulatory pathway (Avery and Wasserman, 1992). To determine whether any of the Tn5 *lac* insertions affect genes in the C-factor signaling pathway, each of the Tn5 *lac* insertions was transduced into a *csgA* null mutant strain. Subsequently, the developmental phenotypes of the double mutants were examined and compared to those of the strains carrying single mutations.

Double mutant strains carrying a *csgA* null mutation in combination with any of the class I Tn5 *lac* insertions (Ω7522, Ω7546, Ω7632, or Ω7663), displayed the aggregation phenotype characteristic of the *csgA* mutant on TPM agar (Fig. 1B,C). In addition, the levels of sporulation in the double mutant strains in submerged culture were identical to that observed for the *csgA* mutant (Table 3). These results show epistasis of *csgA* over class I for aggregation and sporulation. They provide evidence that the genes identified by the class I insertions are in the same genetic pathway as *csgA*, and suggest that *csgA* acts upstream of the genes identified by the insertions.

Table 3. Effect of a *csgA* null mutation on the sporulation of *Agg*[−] mutants

Agg [−] class	Agg [−] insertion combined with <i>csgA</i> ::Tn5-132 ΩLS205	Strain	Percent sporulation ^a
I	none	DK5208	<0.000006
	Ω7522	DK11050	<0.000006
	Ω7546	DK11051	<0.000006
	Ω7632	DK11053	<0.000006
	Ω7663	DK11054	<0.000006
II	Ω4491	DK5296	<0.000006
	Ω7540	DK11074	<0.000006
	Ω7677	DK11055	<0.000006
III	Ω7516	DK11049	0.00006

^aPercent input cells (1.25×10^8) that formed spores, as judged by colonies that grew up on CTT agar.

Double mutant strains carrying a *csgA* null mutation in combination with any of the class II Tn5 *lac* insertions (Ω4491, Ω7540, or Ω7677) displayed the aggregation phenotype characteristic of the Tn5 *lac* insertion mutants forming branched linear aggregates (Fig. 1B) as observed previously for the Ω4491 insertion (Kroos and Kaiser 1987; Kroos et al. 1990). The sporulation levels of the double mutant strains under submerged culture conditions were at the same low levels as observed for both the *csgA* null strain and the Tn5 *lac* insertion strains (Table 3). The low level of sporulation in these strains precludes a test of epistasis based on spore levels. However, the aggregation data indicate that class II insertion mutations are epistatic to the *csgA* mutation with respect to aggregation. This epistasis is interpreted in the Discussion.

A strain containing both a *csgA* mutation and the class III insertion Ω7516 displayed an aggregation phenotype that differed from strains that carried either mutation singly (Fig. 1B). Moreover, the double mutant strain sporulated ≥ 10 -fold more than the strains that carry the constituent single mutations (Table 3; cf. Table 1). In this case no clear epistasis relationship can be deduced and it is not possible to conclude whether the gene identified by the insertion acts in the same regulatory pathway as *csgA*. Therefore, this mutation will not be considered further in this work.

The class I Tn5 lac insertions map to the frz locus

The class I mutants share characteristics with *frz* mutants; both are unable to ripple (Sager and Kaiser 1994), both aggregate improperly (Zusman 1982), whereas their ability to sporulate is not adversely affected (Zusman 1982). The *frz* genes code for a set of proteins that make up a signal transduction system that controls the frequency of single cell reversals (Blackhart and Zusman 1985b; McCleary et al. 1990; McBride et al. 1992). To test whether the Tn5 *lac* insertions in the *Agg*[−] strains

might be linked to, or inserted in the *frz* locus, two approaches were used. First, the linkage of the Tn5 *lac* insertions to the marker *frzE::Tn5(Tet^r)*Ω231 (Fig. 2A), in the strain DZF4041, was tested by transduction. Mx8 phage were grown on the strains containing the Tn5 *lac* insertions and transduced into DZF4041. In each of the class I insertions, close to 100% of the transductants had lost the *frzE::Tn5(Tet^r)*Ω231 marker, indicating that the class I insertions were linked closely to or within the *frz* locus. None of the class II insertions were linked to the *frzE* marker. In a second approach, the insertion points of the class I insertions were mapped physically using probes that either hybridized to the 5' end of the *lacZ* gene in Tn5 *lac* or to the *frz* locus. Ω7522 and Ω7546 had identical physical maps upstream of their Tn5 *lac* insertion points with both probes (Fig. 2A). The *frz* probe allowed for a detailed mapping of the insertion points, and demonstrated that the physical maps of Ω7522 and Ω7546 were consistent with insertions in *frzF* (Fig. 2A). Similar analyses demonstrated that the physical maps of Ω7632 and Ω7663 were compatible with insertions in *frzE* and *frzCD*, respectively (Fig. 2A).

The three class II Tn5 lac insertions are closely linked

Southern blot analyses with the *lacZ*-specific probe sug-

gested that insertions Ω7540 and Ω7677 were inserted in the same orientation and separated by 0.3 kb, as the physical maps of the DNA upstream of the insertions could be brought into register as shown (Fig. 2B). However, the physical map of the DNA upstream of insertion Ω4491 was different from that of Ω7540 and Ω7677 (data not shown; Fig. 2B). To analyze the physical relationship between these insertions, we again used two approaches. First, the linkage of Ω7540 and Ω7677 to Ω4491 was tested by transduction experiments using as a recipient the strain DK11075. This strain contains a derivative of Ω4491 in which the Kan^r gene has been replaced by a Tet^r element (see Materials and methods). Thus, the Ω7540 and Ω7677 insertions were transduced into DK11075. All of the resulting Kan^r transductants were found to be Tet^r, indicating that the Ω4491, Ω7540, and Ω7677 insertions are linked closely. Subsequently, Southern blot analyses with a probe specific for the region immediately upstream of Ω7540 cloned in plasmid pFS703 (see Materials and methods) showed that insertion Ω4491 changed the restriction fragment lengths in the neighborhood of Ω7540, and mapped Ω4491 0.7 kb downstream of Ω7540 in an orientation opposite to that of Ω7540 and Ω7677, as shown in Figure 2B. Ω4491 has been mapped previously to the *Ase* I fragment P on the physical map of the *M. xanthus* chromosome (Chen et al. 1991; He et al. 1994).

Figure 2. (A) Map of the *frz* locus and position of insertion mutations. The top line shows the *frz* locus with restriction sites used in the Southern blot analyses of the class I insertions; structural genes are indicated by open boxes. The insertion sites of the four class I mutation are marked by the labeled, filled-in lollipops. Open lollipops mark the insertion sites of the Tn5 elements that were used in the linkage and reconstruction experiments (cf. Table 7 for reference). In the second and third lines the regions deleted in the $\Delta frz('CD-F')$ and $\Delta frz(A-E')$ alleles are shown. (B) Physical map of the region containing the three class II insertions. Restriction sites used to map the insertions, indicated by filled-in lollipops, are shown. Ω4491 is inserted in an orientation opposite to that of Ω7540 and Ω7677. Distances are measured in kbp from the insertion site of Tn5 *lac* Ω7540; sites to the left and right of Ω7540 have a positive and negative value, respectively. The physical map of Ω4491 has been published previously (Kroos et al. 1986). The revised map differs slightly from that previously published, but the overall pattern remains unchanged. The double-ended arrow indicates the DNA fragment that was used as a probe to map the relative insertion points of the three class II insertions.

A model for the C-factor signaling pathway

The sporulation and aggregation data suggest that the genes identified by the class I and II insertions act in the *csgA* pathway. On the one hand, the class I mutations resulted in a defect in the rippling and aggregation part of the C-factor response, leaving sporulation and C-factor production intact. On the other hand, the class II mutants had defects in all four C-factor responses including C-factor production. The class II mutants are cell autonomous, thus suggesting that the primary defect in the class II mutants is on the reception side of the C-factor signal rather than in the production of C-factor. The data also suggest that the class I insertions identify genes that code for components that are only involved in the motility responses to C-factor, whereas the class II insertions identify genes that code for components that are involved in all the C-factor responses. All these observations can be explained by a model for the C-signaling pathway in which the first part is common to all C-factor-dependent responses and is the target of the class II insertions. Downstream of class II the pathway branches; one branch leads to the motility responses and includes the Frz proteins, and the second branch leads to the sporulation and C-factor production response (Fig. 6, below).

Testing for a branch in the C-factor signaling pathway

Several specific predictions from the model presented above could be tested in reconstruction experiments with several characterized *frz* loss-of-function alleles and a *frz* gain-of-function allele in combination with either a *csgA* null allele or a class II insertion. First, the epistasis relationship between *frz* loss-of-function alleles and *csgA* was tested; the prediction is that a *csgA* null mutation should be epistatic to the *frz* loss-of-function alleles (Avery and Wasserman 1992). The *frz* loss-of-function alleles were transduced into the fully motile wild-type strain DK1622 and the isogenic strain DK5208, which contains the *csgA* null allele. The *frz* loss-of-function alleles, which included two insertion mutations (*frzCD*::Tn5 *lac* Ω536 and *frzE*::Tn5 Ω226) and two

deletions (Δfrz (*CD-F*) and Δfrz (*A-E'*) (Fig. 2A), have on their own an aggregation defect similar to that caused by the class I insertions, as expected (Fig. 3). They conferred an inability to ripple (data not shown) as previously observed with the *frzCD*::Tn5 *lac* Ω536 allele (Sager and Kaiser 1994), and they did not result in a sporulation defect (Table 4). Also, with these *frz* mutants it was observed that the spores formed were not contained in aggregates (Fig. 4). Moreover, the *frz* mutants rescued sporulation of a *csgA* null mutant in codevelopment assays as efficiently as the wild-type strain (Table 5). The *csgA* mutation proved to be epistatic to the *frz* loss-of-function alleles, when the *csgA*, *frz* double mutants were tested for their ability to sporulate (Table 4) and aggregate (see Fig. 3). Because the functional and genetic characteristics of the single and double mutant strains containing *frz* loss-of-function alleles are identical to those observed with the class I mutants, this reasserts the mapping of the class I insertions to the *frz* locus.

The availability of the *frz* allele *frzCD*::Tn5 Ω224 (from here on denoted *frzD*; Blackhart and Zusman 1985a) provided a critical tool for testing the model for a branched C-signaling pathway. The *frzD* allele contains Tn5 inserted near the carboxyl end of *frzCD* (McBride et al. 1989; see Fig. 2A). Based on two criteria *frzD* behaves as a gain-of-function allele: (1) Its phenotype is distinct from and opposite to that caused by the *frz* loss-of-function mutations with respect to single cell reversal frequency [increased frequency for gain-of-function versus decreased frequency for loss-of-function alleles (McCleary et al. 1990; Blackhart and Zusman 1985b)]. (2) The *frzD* allele is dominant to the wild-type allele in a merodiploid strain (Blackhart and Zusman 1985a,b; McBride et al. 1989). If *frz* is involved only in the motility-related branch of the C-factor pathway as depicted in Figure 6, then a *frz* gain-of-function allele would be predicted to be epistatic to a *csgA* null mutation with respect to the aggregation phenotype and but not with respect to the sporulation phenotype. To test this prediction, DK1622 and DK5208 derivatives that contain the *frzD* allele were constructed. The DK1622 derivative that carried the *frzD* allele had the characteristic non-

Figure 3. Aggregation patterns of *frz* and *csgA*[−], *frz* double mutants. For each strain, 20 μl of a cell suspension with a calculated cell density of 500 Klett units/ml in TPM buffer was placed on a TPM agar plate, and the cells allowed to develop for 24 hr. The relevant *frz* allele is abbreviated above each column; the full names of the alleles used are from left to right *frzCD*::Tn5 *lac* Ω536, *frzE*::Tn5 Ω226, Δfrz (*A-E'*)::Kan^r, Δfrz (*CD-F*)::Kan^r, and *frzCD*::Tn5 *lac* Ω224. The top row shows the aggregation phenotype caused by the respective *frz* mutation in a *csgA*⁺ background; the bottom row shows the aggregation phenotype in a *csgA*[−] background. The strains used are (from top, left to bottom, right): DK1622, DK8505, DK9711, DK9032, DK9033, DK8506, DK5208, DK9037, DK9048, DK9034, DK9035, DK9036. Bar, 1 mm.

Table 4. Sporulation of *frz* mutants in *csgA*⁺ and *csgA*[−] backgrounds

<i>frz</i> allele ^a	Percent sporulation ^b in <i>csgA</i> ⁺ background	Percent sporulation ^b in <i>csgA</i> [−] background ^c
Wild type	1.3	<0.000006
<i>frzCD::Tn5 lac</i> Ω536	0.6	<0.000006
<i>frzE::Tn5</i> Ω226	1.3	<0.000006
$\Delta frz(A-E)::Kan^r$	1.0	<0.000006
$\Delta frz'(CD-F)::Kan^r$	1.9	<0.000006
<i>frzCD::Tn5</i> Ω224	3.2	<0.000006

^aAll *frz* mutations used are loss-of-function alleles except for *frzCD::Tn5* Ω224, which is a gain-of-function allele. This allele is denoted *frzD* in the text and in Figs. 3 and 4.

^bPercent of input cells (1.25×10^8) that formed spores, as judged by colonies that grew up on CTT agar.

^c*csgA* allele is *csgA::Tn5-132*ΩLS205.

spreading, relatively smooth cell mat of *frzD* strains (see Fig. 3; Blackhart and Zusman 1985a); it was unable to form aggregates on either TPM agar plates (see Fig. 3) or in submerged culture (Fig. 4). It did not form ripples (Sager and Kaiser 1994; data not shown). It sporulated at wild-type levels (Table 4), and produced wild-type levels of C-factor based on its ability to rescue development of a *csgA* mutant (Table 5). Most important, the *csgA*, *frzD* double mutant had the aggregation phenotype characteristic of the *frzD* single mutation strain (see Fig. 3), whereas it had the sporulation phenotype characteristic of the *csgA* single mutation strain (Table 4). Therefore, these data support a branched C-signal transduction pathway in which the Frz proteins are involved in the motility response to C-signaling and not in the sporulation response.

The model for the branched C-signaling pathway also

Figure 4. Aggregation and spore formation in *frz* mutants in submerged culture. For each strain, 400 μ l of a cell suspension with a calculated density of 62.5 Klett was placed in a 15-mm microtiter well and then incubated for 72 hr. The *frz* allele present in a strain is abbreviated above each panel; the full names of the alleles used are $\Delta frz'(CD-F)::Kan^r$ and *frzCD::Tn5* Ω224. After 72 hr of starvation, the wild-type strain (DK1622) has formed dark spore-filled fruiting bodies, relatively few spores (the small dark dots) are visible outside of the fruiting bodies. The two *frz* strains fail to aggregate and the spores are visible as tiny, refractile black dots. The strains used are (from left to right): DK1622, DK9033, DK8506. Bar, 100 μ m.

Table 5. Sporulation of a *csgA* mutant (DK5208) when codeveloped with *frz* mutants

<i>frz</i> allele ^a	Strain codeveloped with DK5208	Percent sporulation of DK5208 ^b
—	none	<0.000006
Wild type	DK1622	1.0
<i>frzCD::Tn5 lac</i> Ω536	DK8505	0.3
<i>frzE::Tn5</i> Ω226	DK9711	0.8
$\Delta frz(A-E)::Kan^r$	DK9032	0.7
$\Delta frz'(CD-F)::Kan^r$	DK9033	0.6
<i>frzCD::Tn5</i> Ω224	DK8506	1.0

^aAll *frz* mutations used are loss-of-function alleles except for *frzCD::Tn5* Ω224, which is a gain-of-function allele. This allele is denoted *frzD* in the text and in Figs. 3 and 4.

^b 6.25×10^7 *csgA* mutant cells (prepared as described in Materials and methods) were mixed with an equal number of potential donor cells. Percent values are the number of *csgA* mutant spores after 3 days of incubation divided by the number of *csgA* mutant cells at time zero, as judged by colonies that grew up on agar containing Tet.

predicts that a class II mutation should be epistatic to *frz* loss-of-function mutations with respect to both aggregation and sporulation, but epistatic to a *frz* gain-of-function mutation only with respect to sporulation. To test these predictions, double mutants containing the class II insertion Ω7540, and the *frz* loss-of-function allele *frzE::Tn5*(Tet^r) Ω231, or the *frz* gain-of-function allele *frzCD::Tn5*(Tet^r) Ω224 (see Fig. 2A) were constructed. The double mutant strain carrying both Ω7540 and the *frzE* loss-of-function allele had the aggregation (Fig. 5) and the sporulation phenotypes (Table 6) characteristic of the Ω7540 mutation. However, the strain carrying Ω7540 and the *frzD* gain-of-function allele had the aggregation phenotype characteristic of the *frzD* allele (Fig. 5) and the sporulation phenotype characteristic of the Ω7540 insertion (Table 6). These results support the location of the class II genes upstream of the *frz* genes and the position of a bifurcation downstream of the class II genes.

Expression of C-factor-dependent genes in *frz* mutants

Mutations in *csgA* decrease the level of expression of certain developmentally regulated genes, known for that reason as C-factor-dependent genes (Kroos and Kaiser 1987). As monitored by transcriptional fusions to *lacZ*, such genes are expressed at 6 hr or later into development of wild type, whereas in *csgA* mutants their expression is either decreased or abolished. If the C-factor signal transduction pathway is branched at the position shown in Figure 6, and if as indicated in that model *frz* function is required for the motility responses but not for gene expression, then *frz* mutants should retain normal levels of C-factor-dependent gene products.

To test this prediction, the *frz* loss-of-function allele

Figure 5. Aggregation of *frzE* or *frzD* mutants with or without Tn5 *lac* Ω7540. 20 μ l of a cell suspension with a calculated density of 1000 Klett units in TPM buffer was placed on a TPM agar plate, and the cells allowed to develop for 24 hr. The *frz* alleles used are abbreviated above each column; the full names of the alleles used are *frzE*::Tn5(Tet^r)Ω231, and *frzCD*::Tn5(Tet^r)Ω224. The top row shows the aggregation phenotype in a strain that contains Tn5 *lac* Ω4499 (Kroos et al. 1986); the bottom row shows the aggregation phenotype associated with the same *frz* alleles in a strain that also contains Tn5 *lac* Ω7540. Tn5 *lac* Ω4499 has no known effect on sporulation or aggregation (Kroos et al. 1986) but is present in the strains to control for the presence of a Tn5 *lac* element. The strains used are (from top left to bottom right): DK4499, DK9038, DK9043, DK11063, DK9049, and DK9050. Bar, 1 mm.

frzE::Tn5(Tet^r) Ω231 and the *frz* gain-of-function allele *frzCD*::Tn5(Tet^r) Ω224 were transduced into strains containing C-factor-dependent fusions, and β -galactosidase expressed from the fusions was measured. Five C-factor-dependent transcriptional fusion reporters (Ω4401, Ω4403, Ω4414, Ω4435, and Ω4499) were analyzed, which have

Table 6. Effect of Tn5 *lac* Ω7540 on the sporulation of *frz* mutants

<i>frz</i> allele ^a	Percent sporulation in strain not carrying Tn5 <i>lac</i> Ω7540 ^{b,c}	Percent sporulation in strain carrying Tn5 <i>lac</i> Ω7540 ^c
Wild type	3.8	<0.000006
<i>frzE</i> ::Tn5(Tet ^r)Ω231	2.2	<0.000006
<i>frzCD</i> ::Tn5(Tet ^r) Ω224	4.0	<0.000006

^a*frzE*::Tn5(Tet^r) Ω231 and *frzCD*::Tn5(Tet^r) Ω224 are loss-of-function and gain-of-function alleles, respectively; *frzCD*::Tn5(Tet^r) Ω224 is denoted *frzD* in the text and in Fig. 5.

^bThe strains that do not contain Ω7540 contain Tn5 *lac* Ω4499. Tn5 *lac* Ω4499 is a developmentally expressed Tn5 *lac* insertion with no known effect on aggregation or sporulation (Kroos et al. 1986).

^cPercent input cells (1.25×10^8) that formed spores, as judged by colonies that grew up on CTT agar.

Figure 6. Model for the C-factor signal transduction pathway. The cell-surface associated C-factor on one cell interacts with a sensor on (or in) a second cell, leading to the activation of the intracellular signal transduction pathway. The first part of the pathway is shared by all C-factor-dependent activities and defined by the class II insertions. Farther downstream, the pathway branches: One branch leads to the motility response to C-factor, and the second branch leads to the sporulation and *csgA* expression part of the response. The Frz/class I proteins are components in the branch leading to the motility response. For simplicity, the C-factor is shown as the signal interacting directly with the sensor. The molecular nature of the C-factor signal and C-factor sensor is unknown. The reciprocal stimulation of both cells as shown in the diagram is not a prerequisite for activation of the pathway as evidenced by the extracellular complementation of *csgA* cells by wild-type cells. The model does not exclude other components and branchpoints in the pathway.

expression times ranging from 6 hr (Ω4499) to 25 hr (Ω4401). The five Tn5 *lac* fusions provide a test for correct timing of C-factor-dependent gene expression as well as its level. Specific activities of β -galactosidase expressed by each fusion in both loss-of-function and gain-of-function *frz* mutants are shown in Figure 7. For comparison, β -galactosidase expression in wild-type and *csgA* cells is included. These measurements show similar expression times of the fusions in the *frz* mutants and in the wild-type strain throughout the 6- to 25-hr interval; in particular, there was no indication of an expression delay in the *frz* mutant background. Importantly, the level of β -galactosidase expression is not decreased by either the *frz* loss- or gain-of-function alleles; the fusion genes Ω4499, Ω4414, Ω4403, and Ω4435 may even be expressed at a higher level in both types of *frz* mutants. These data support a bifurcation in the C-signal transduction pathway, past which one branch includes *frz* function (and motility responses), whereas the other branch leads to expression of at least five genes and regulation of *csgA* expression.

Discussion

Based on the results presented, a model for the C-signal transduction pathway is proposed (Fig. 6). The signaling event in this pathway is the interaction between C-factor signal from one cell acting on the C-signal sensor belonging to a second cell. (It should be understood that the nature and location of the C-signal sensor on or in the signaled cell are not being specified by the model.) This interaction activates the signaling pathway in the signaled cell. Once received by a cell, the signal is trans-

Figure 7. Developmental expression of β -galactosidase from Tn5 *lac* insertions. Specific activity of β -galactosidase is expressed from five C-factor-dependent Tn5 *lac* fusions. β -galactosidase expressed by the Tn5 *lac* fusions in wild-type cells ($\square$), *csgA* cells ($\circ$), *frzE::Tn5* (Tet^r) Ω 231 cells (Δ), and *frzCD::Tn5*(Tet^r) Ω 224 cells ($\blacktriangle$) was determined as described in Materials and methods. Specific activity of β -galactosidase is given as nanomoles of orthonitrophenyl phosphate per minute per milligram of protein. The averages of two determinations from each of two cultures are given. Strains are listed in the order wild-type cells, *csgA* cells, *frzE* cells, and *frzCD* cells. (Strains containing Ω 4499) DK4499, DK5246, DK9038, DK9043; (strains containing Ω 4414) DK5279, DK5287, DK9039, DK9044; (strains containing Ω 4403) DK4368, DK5270, DK9040, DK9045; (strains containing Ω 4435) DK5204, DK5253, DK9042, DK9047; (strains containing Ω 4401) DK4293, DK5229, DK9041, DK9046.

duced through the class II gene product that is essential to all the C-factor responses. Downstream of the class II gene product, the pathway contains a branchpoint: one branch leads to the motility responses to C-factor (rippling and aggregation), whereas the second branch leads to sporulation and control of gene expression, including the *csgA* gene. In this model, the Frz proteins are components in the motility response branch.

A downstream location of the class II genes relative to the C-factor/sensor interaction in this pathway is implied by the cell autonomy of the class II mutants. Nevertheless, the class II mutations are found to be epistatic to a *csgA* null allele with respect to aggregation (Fig. 1B), as if class II preceded C-factor. This paradox can be explained by the C-factor-dependent induction of *csgA* expression (Kim and Kaiser 1991). Such induction is observed from the outside in: Addition of purified C-factor to *csgA* cells increases *csgA* expression as monitored by a *csgA-lacZ* fusion (Kim and Kaiser 1991). This induction implies a pathway leading from C-factor outside the cell to an increase of *csgA* gene expression within that cell. Because the class II mutants produce ~10-fold less C-factor than wild-type cells (Table 2), the class II gene products are likely components in the pathway that elevates *csgA* expression, which would position the class II gene product upstream of *csgA* expression in a C-factor-producing cell. This combination of observations, the cell autonomy of class II mutants, the increase in *csgA* gene expression when C-factor is added, and the epistasis of class II insertions over *csgA* suggest that the class II genes act downstream of signal transmission between two cells but upstream of *csgA* expression in a cell that is producing C-factor. Ordering of genes based on genetic epistasis experiments in a signaling pathway that increases production of the signal molecule is a special situation, because closing the loop in such a pathway places every gene in that pathway upstream as well as downstream of every other gene in the same pathway. In that sense, all the components (C-factor, sensor, class II gene products, *csgA* expression) in the lower branch in

Figure 6 are all upstream as well as downstream of each other.

A branchpoint in the C-signaling pathway is implied by the characteristics of *frz* mutants — both those isolated by Zusman and co-workers (Table 7) and those newly isolated in this work as class I. Under development-inducing conditions, mutants with *frz* loss-of-function alleles sporulate and produce C-factor at normal levels, yet they are aggregation deficient. That the sporulation of *frz* mutants remains C-factor dependent is shown by the low level of sporulation of *csgA*, *frz* double mutants. The model in Figure 6 predicts that developmental expression of C-dependent genes would be normal in *frz* mutants. In line with this prediction, the level of expression of five different C-factor-dependent genes suffered no decrement as a consequence of either *frz* loss-of-function or *frz* gain-of-function mutations. Results of Kashefi and Hartzell (1995) and Weinberg and Zusman (1989) using mutations in different *frz* genes, different strain backgrounds and different reporter genes also fit this prediction. Finally, a *frz* gain-of-function allele is epistatic to a *csgA* mutation or a class II insertion mutation with respect to aggregation but *not* with respect to sporulation. All these data are consistent with the branchpoint downstream of the class II components shown in Figure 6, which shows how the Frz proteins would be required for the C-factor-dependent motility functions but not for the C-factor-dependent sporulation and gene expression, including the expression level of *csgA*. Components specific to the lower branch in the pathway of Figure 6 have yet to be identified. The absence of such mutants in the group examined here is consistent with that model and the use of a screen for mutants with an aggregation defect. Mutations in genes specific to the lower branch may have little or no effect on aggregation. Other screening criteria may have to be applied to identify components in this branch.

M. xanthus and *Dictyostelium discoideum* share several developmental features although they are widely separated evolutionarily (Kaiser 1993). In *D. discoideum*

Table 7. *Myxococcus xanthus* strains used

Strain	Relevant genotype	Reference or source
DK1219	<i>cglC1</i>	Hodgkin and Kaiser (1979b)
DK1300	<i>sglG</i>	Hodgkin and Kaiser (1979b)
DK1622	wt	Kaiser (1979)
DK4293	Tn5 <i>lac</i> Ω4401	Kroos et al. (1986)
DK4368	Tn5 <i>lac</i> Ω4403	Kroos et al. (1986)
DK4499	Tn5 <i>lac</i> Ω4499	Kroos et al. (1986)
DK5204	Tn5 <i>lac</i> Ω4435	Kroos et al. (1986)
DK5208	<i>csgA::Tn5-132</i> ΩLS205	Shimkets and Asher (1988); Kroos and Kaiser (1987)
DK5229	Tn5 <i>lac</i> Ω4401, <i>csgA::Tn5-132</i> ΩLS205	Kroos and Kaiser (1987)
DK5246	Tn5 <i>lac</i> Ω4499, <i>csgA::Tn5-132</i> ΩLS205	Kroos and Kaiser (1987)
DK5253	Tn5 <i>lac</i> Ω4435, <i>csgA::Tn5-232</i> ΩLS205	Kroos and Kaiser (1987)
DK5270	Tn5 <i>lac</i> Ω4403, <i>csgA::Tn5-132</i> ΩLS205	Kroos and Kaiser (1987)
DK5279	Tn5 <i>lac</i> Ω4414	Kroos and Kaiser (1987)
DK5285	Tn5 <i>lac</i> Ω4491	Kroos et al. (1990)
DK5287	Tn5 <i>lac</i> Ω4414, <i>csgA::Tn5-132</i> ΩLS205	Kroos and Kaiser (1987)
DK5296	<i>csgA::Tn5-132</i> ΩLS205, Tn5 <i>lac</i> Ω4491	Kroos and Kaiser (1987)
DK7516	Tn5 <i>lac</i> Ω7516	P1::Tn5 <i>lac</i> → DK1622 ^a
DK7522	Tn5 <i>lac</i> Ω7522	P1::Tn5 <i>lac</i> → DK1622 ^a
DK7540	Tn5 <i>lac</i> Ω7540	P1::Tn5 <i>lac</i> → DK1622 ^a
DK7546	Tn5 <i>lac</i> Ω7546	P1::Tn5 <i>lac</i> → DK1622 ^a
DK7632	Tn5 <i>lac</i> Ω7632	P1::Tn5 <i>lac</i> → DK1622 ^a
DK7663	Tn5 <i>lac</i> Ω7663	P1::Tn5 <i>lac</i> → DK1622 ^a
DK7677	Tn5 <i>lac</i> Ω7677	P1::Tn5 <i>lac</i> → DK1622 ^a
DK8505	<i>frzCD::Tn5 lac</i> Ω536	Sager and Kaiser (1994)
DK8506	<i>frzCD::Tn5</i> Ω224	Sager and Kaiser (1994)
DK9032	<i>Δfrz(A'-E')::Kan^r</i>	Mx8[DZF3558] → DK1622 ^a
DK9033	<i>Δfrz('CD-F)::Kan^r</i>	Mx8[DZF4059] → DK1622 ^a
DK9034	<i>csgA::Tn5-132</i> ΩLS205, <i>Δfrz(A-E')::Kan^r</i>	Mx8[DZF3558] → DK5208 ^a
DK9035	<i>csgA::Tn5-132</i> ΩLS205, <i>Δfrz('CD-F)::Kan^r</i>	Mx8[DZF4059] → DK5208 ^a
DK9036	<i>csgA::Tn5-132</i> ΩLS205, <i>frzCD::Tn5</i> Ω224	Mx8[DZF3460] → DK5208 ^a
DK9037	<i>csgA::Tn5-132</i> ΩLS205, <i>frzCD::Tn5 lac</i> Ω536	Mx8[DZF3591] → DK5208 ^a
DK9038	Tn5 <i>lac</i> Ω4499, <i>frzE::Tn5(Tet^r)</i> Ω231	Mx8[DZF4041] → DK4499 ^a
DK9039	Tn5 <i>lac</i> Ω4414, <i>frzE::Tn5(Tet^r)</i> Ω231	Mx8[DZF4041] → DK5279 ^a
DK9040	Tn5 <i>lac</i> Ω4403, <i>frzE::Tn5(Tet^r)</i> Ω231	Mx8[DZF4041] → DK4368 ^a
DK9041	Tn5 <i>lac</i> Ω4401, <i>frzE::Tn5(Tet^r)</i> Ω231	Mx8[DZF4041] → DK4293 ^a
DK9042	Tn5 <i>lac</i> Ω4435, <i>frzE::Tn5(Tet^r)</i> Ω231	Mx8[DZF4041] → DK5204 ^a
DK9043	Tn5 <i>lac</i> Ω4499, <i>frzCD::Tn5(Tet^r)</i> Ω224	Mx8[DZF4042] → DK4499 ^a
DK9044	Tn5 <i>lac</i> Ω4414, <i>frzCD::Tn5(Tet^r)</i> Ω224	Mx8[DZF4042] → DK5279 ^a
DK9045	Tn5 <i>lac</i> Ω4403, <i>frzCD::Tn5(Tet^r)</i> Ω224	Mx8[DZF4042] → DK4368 ^a
DK9046	Tn5 <i>lac</i> Ω4401, <i>frzCD::Tn5(Tet^r)</i> Ω224	Mx8[DZF4042] → DK4293 ^a
DK9047	Tn5 <i>lac</i> Ω4435, <i>frzCD::Tn5(Tet^r)</i> Ω224	Mx8[DZF4042] → DK5204 ^a
DK9048	<i>csgA::Tn5-132</i> ΩLS205, <i>frzE::Tn5</i> Ω226	Mx8[DK9711] → DK5208 ^a
DK9049	Tn5 <i>lac</i> Ω7540, <i>frzE::Tn5(Tet^r)</i> Ω231	Mx8[DZF4041] → DK11063 ^a
DK9050	Tn5 <i>lac</i> Ω7540, <i>frzCD::Tn5(Tet^r)</i> Ω224	Mx8[DZF4042] → DK11063 ^a
DK9711	<i>frzE::Tn5</i> Ω226	A. Spormann (Stanford University, Palo Alto, California)
DK11015	Tn5 <i>lac</i> Ω7516	Mx4[DK7516] → DK1622 ^a
DK11016	Tn5 <i>lac</i> Ω7522	Mx4[DK7522] → DK1622 ^a
DK11018	Tn5 <i>lac</i> Ω7632	Mx4[DK7632] → DK1622 ^a
DK11019	Tn5 <i>lac</i> Ω7663	Mx4[DK7663] → DK1622 ^a
DK11020	Tn5 <i>lac</i> Ω7677	Mx4[DK7677] → DK1622 ^a
DK11049	<i>csgA::Tn5-132</i> ΩLS205, Tn5 <i>lac</i> Ω7516	Mx4 [DK7516] → DK5208 ^a
DK11050	<i>csgA::Tn5-132</i> ΩLS205, Tn5 <i>lac</i> Ω7522	Mx4[DK7522] → DK5208 ^a
DK11051	<i>csgA::Tn5-132</i> ΩLS205, Tn5 <i>lac</i> Ω7546	Mx4[DK7546] → DK5208 ^a
DK11053	<i>csgA::Tn5-132</i> ΩLS205, Tn5 <i>lac</i> Ω7632	Mx4[DK7632] → DK5208 ^a
DK11054	<i>csgA::Tn5-132</i> ΩLS205, Tn5 <i>lac</i> Ω7663	Mx4[DK7663] → DK5208 ^a
DK11055	<i>csgA::Tn5-132</i> ΩLS205, Tn5 <i>lac</i> Ω7677	Mx4[DK7677] → DK5208 ^a
DK11063	Tn5 <i>lac</i> Ω7540	Mx4[DK7540] → DK1622 ^a
DK11064	Tn5 <i>lac</i> Ω7546	Mx4[DK7546] → DK1622 ^a
DK11074	<i>csgA::Tn5-132</i> ΩLS205, Tn5 <i>lac</i> Ω7540	Mx4[DK7540] → DK5208 ^a
DK11075	Tn5 <i>lac</i> (Tet ^r) Ω4491	P1::Tn5-132 → DK5285
DZF3460	<i>sglA1</i> , <i>frzCD::Tn5</i> Ω224	McCleary et al. (1990)

Table 7. (Continued)

Strain	Relevant genotype	Reference or source
DZF3558	<i>sglA1</i> , Δ <i>frz(A-E')</i> ::Kan ^r	Blackhart and Zusman (1985a)
DZF3591	<i>sglA1</i> , <i>frzCD</i> ::Tn5 <i>lac</i> Ω 536	Weinberg and Zusman (1989)
DZF4041	<i>sglA1</i> , <i>frzE</i> ::Tn5[Tet ^r] Ω 231	D. Zusman (University of California, Berkeley)
DZF4042	<i>sglA1</i> , <i>frzCD</i> ::Tn5[Tet ^r] Ω 224	D. Zusman
DZF4059	<i>sglA1</i> , Δ <i>frz('CD-F')</i> ::Kan ^r	McBride et al. (1992)

^aMx4 and Mx8 are abbreviations for Mx4*ts18ts27*hrm and Mx8*clp2ts3*, respectively. Phage were grown on the indicated strains and transduced ($\rightarrow$) to the indicated recipient strain with selection for the antibiotic resistance marker present at the Tn5 element.

different responses to extracellular cAMP (chemotaxis, early aggregation, and cell differentiation) are mediated by at least four different cAMP receptors (Devreotes 1994). In light of the parallels, what of the possibility that the C-factor signal might be processed through different sensors? Although this possibility cannot be ruled out entirely by the available data, the block imposed by the class II mutations on all C-factor-dependent activities—rippling and aggregation, which are early events, as well as sporulation, which is a late event—leads to the simple view embodied in Figure 6 that the C-factor signal is received by a single sensor that is common to all four responses.

Although aggregation normally precedes sporulation in fruiting body development, and many aggregation-defective mutants (such as *asgA*, *asgB*, and *asgC*) are sporulation defective, other published data suggest that sporulation does not lie at the end of a linearly dependent aggregation pathway. For example, several mutants with severe aggregation defects have been isolated that sporulate at high levels (Morrison and Zusman 1979; Rhie and Shimkets 1989). Likewise, addition of purified C-factor to nonmotile cells (*mglA* or A^-S^-) restores sporulation without restoring the capacity to aggregate (Kim and Kaiser 1991). This uncoupling of aggregation and sporulation has led previously to the suggestion that the two processes result from two independent parallel pathways (Morrison and Zusman 1979; Rhie and Shimkets 1989). However, the data presented here together with the lines of evidence listed below imply that the two processes are not independent and suggest that the uncoupling of the two processes in several mutants might be understood within the context of a branched C-factor signaling pathway, like Figure 6. Starvation induces both aggregation and sporulation (Manoil and Kaiser 1980), A-signaling precedes and is required for both aggregation and sporulation, and C-signaling is required for and occurs concomitant with both aggregation and sporulation (Kaiser and Losick 1993). Mutations that interfere with starvation recognition (Manoil 1978), A-signaling or C-signaling prevent both aggregation and sporulation (Kaiser and Losick 1993). These observations imply that aggregation and sporulation proceed through a common pathway that depends on proper starvation recognition, A-signaling and C-signaling. The data presented in this report demonstrate that uncoupling of aggregation and sporulation can occur downstream of the C-factor signaling event (i.e., the common developmental pathway has a

bifurcation downstream of this event). The normal confinement of spores to fruiting bodies or to their immediate neighborhood, however, implies that the two branches in the C-factor signaling pathway of Figure 6 are cross-connected, thus coordinating the two processes during this stage of fruiting body formation. Although it is premature to draw specific cross-connections, some deductions can be made. The uncoupling of aggregation and sporulation in the *frz* mutants suggests that in wild-type cells sporulation may be inhibited either directly or indirectly by an element in the aggregation pathway. As normal aggregation proceeds, the inhibition would be alleviated, and sporulation would then follow. In this framework, sporulation by the *frz* mutants can be explained by an absence from the start of the putative sporulation inhibitor. Interestingly, a higher concentration of C-factor is required to initiate sporulation than to initiate aggregation (Kim and Kaiser 1991; Li et al. 1992). Thus one candidate for the trigger that relieves the inhibition of sporulation is an elevation of the level of C-factor signaling.

How does C-factor induce rippling and aggregation? The *frz* genes encode homologs of the proteins involved in the chemotactic response of enteric bacteria (McBride et al. 1989; McCleary and Zusman 1990; McCleary et al. 1990), and are involved in controlling the single cell reversal frequency (Blackhart and Zusman 1985b; McCleary et al. 1990; McBride et al. 1992). It has been hypothesized that the Frz proteins constitute a signal transduction system that regulates the single cell reversal frequency in response to environmental cues (McBride et al. 1992; Shi et al. 1993). The FrzCD protein contains a region homologous to the carboxy-terminal portion of the methyl-accepting chemotaxis proteins, or MCPs (McBride et al. 1989). FrzCD undergoes methylation and demethylation in response to various stimuli (McCleary et al. 1990; McBride et al. 1992; McBride and Zusman 1993; Shi et al. 1993) and displays a reduced level of methylation in a *csgA* mutant compared with a wild-type strain during development (McBride and Zusman 1993). FrzCD is a cytoplasmic protein that lacks homology to the amino-terminal extracellular domain and the two membrane spanning segments of the MCPs (McBride et al. 1989, 1992), thus arguing against the idea that FrzCD is a cell-surface exposed C-factor sensor. Rather FrzCD and other Frz proteins would act downstream of the sensor and the class II component in a branch of the signaling pathway devoted to control of cell movement

as shown in the model of Figure 6. Evidence that C-factor signaling increases FrzCD methylation in a class II dependent manner is reported separately (Søgaard-Andersen and Kaiser 1996). These results support the location of class II between the signaling event and the Frz proteins. Moreover, they suggest that C-factor acts as a cell-bound signal, which is transduced by the Frz signal transduction system to regulate the frequency of cell reversals during aggregation and rippling. Sager and Kaiser (1994) showed that the Frz proteins are necessary for the propagation of ripples. Furthermore they provided evidence that addition of partially purified C-factor changes the reversal frequency of starving *M. xanthus* cells located outside fruiting bodies. Both effects are consistent with the pathway shown in Figure 6.

Recently, an amino acid sequence similarity between C-factor and the family of short chain alcohol dehydrogenases was pointed out (Baker 1994; Lee and Skimkets 1994). Therefore, the question arises whether C-factor is the signal molecule that activates the C-signaling pathway in a responding cell, or C-factor acts as an enzyme that produces a signal molecule that activates the pathway, or whether the putative enzymatic activity of C-factor modifies a cell-surface component and then that modification serves as the signal that is transmitted to the interior of the cell. C-factor signaling apparently requires cell-cell contact (Kim and Kaiser 1990a). All three modes of action are compatible both with this requirement as well as with the model in Figure 6. Further experiments are required to distinguish between these possibilities.

A loss-of-function mutation in the gene encoding an extracellular signal often results in a phenotype that is similar to that conferred by mutations in genes coding for components in the downstream signaling pathway. For example, mutations in genes of the *Drosophila wingless* signaling pathway have phenotypes very similar to that of a *wingless* mutation (Noordermeer et al. 1994; Siegfried et al. 1994). In another case, Delta was discovered as the signal for Notch among mutants with the same developmental block as a Notch mutant (Artavanis-Tsakonas and Simpson 1992). The same logic was applied to identify genes involved in C-factor signal transduction in *M. xanthus*. A collection of developmental mutants was screened for those that displayed abnormal aggregation, one of the earliest defects in a *csgA* mutant. The versatility of this screening procedure is that potentially it allows one to identify the earliest acting components in the C-factor signal transduction pathway. The fact that 7 of 8 Tn5 *lac* insertion mutants found using this strategy have Tn5 *lac* inserted in genes in the *csgA* pathway demonstrates the feasibility of this approach to map signaling pathways in *M. xanthus*.

Materials and methods

Strains

M. xanthus strains used are listed in Table 7.

General methods

M. xanthus and *Escherichia coli* were grown as described (Thöny-Meyer and Kaiser 1993). Generalized transductions with myxophage Mx4ts18ts27hrm and Mx8clp2ts3 were done as described (Avery and Kaiser 1983; Stephens and Kaiser 1987), respectively. All strains constructed by generalized transduction had at least two isolates analyzed by Southern blot analysis to check that the transferred marker had integrated at the correct position and had the correct structure. When required for a genetic construction, the *aphII* gene on Tn5 *lac* that confers Kan^r was replaced with the gene conferring Tet^r from Tn5-132 as described (Avery and Kaiser 1983).

Aggregation, sporulation, extracellular complementation, rippling, and motility assays

The aggregation phenotypes were monitored on TPM [10 mM Tris-HCl (pH 7.5), 1 mM KH₂PO₄, 8 mM MgSO₄] 1.5 % agar plates. Briefly, cells were grown to a density of Klett 100 (5 × 10⁸ cells/ml) in CTT medium, harvested, and resuspended in TPM buffer at a calculated density of 1000 Klett units. Twenty-microliter aliquots of the cell suspension were spotted on TPM agar plates and incubated at 32°C. Aggregation was followed visually with an inverted microscope (Leitz Labovet, Wetzlar, Germany) equipped with a long-working-distance condensor and phase contrast objectives, or with a Leica Orthoplan microscope in bright field without a condensor and with a ×1 objective. Starvation-induced sporulation was measured in submerged culture (Kuner and Kaiser 1982). Briefly, cells were grown to a density of 100 Klett units in CTT medium, harvested, and resuspended in MC7 [10 mM morpholinepropanesulfonic acid, (pH 7.0), 1 mM CaCl₂] to a calculated density of 1000 Klett units. Twenty five-microliter aliquots of these suspensions were mixed with 375 µl of MC7, transferred to a microtiter well with a diameter of 15 mm, and incubated in a humid chamber for 72 hr at 32°C. Development was followed visually as described above. Spore titers were determined by transferring the cells from a well to a microfuge tube containing 400 µl of MC7, followed by sonication for 15 sec at 30% output capacity with a standard microtip, 5 min of sonication in a cup horn with ice water cooling, and incubation at 50°C for 2 hr. After serial dilution of the samples, 10-µl aliquots were plated on CTT agar with or without antibiotics as indicated. Spores were counted as colonies after 3–5 days of incubation at 32°C to allow germination and growth to occur. If no colonies were observed after 5 days of incubation at 32°C, a 100-µl aliquot of cells harvested from a well and treated as described was plated on CTT agar with or without antibiotics as indicated to obtain a more precise estimate of the sporulation frequency. To check for extracellular C-factor complementation, 12.5-µl aliquots of cells in MC7 at a density of 1000 Klett units were mixed, transferred to 375 µl of MC7, placed in a microtiter well, and incubated at 32°C for 72 hr as described. Spore titers were then measured as described. The ability of strains to form ripples was observed on CF agar plates (Shimkets and Kaiser 1982a). Cells prepared as in the sporulation assay were spotted in 10-µl aliquots on CF agar plates and incubated at 32°C. The plates were scanned visually over the next 48 hr. Motility was tested by spotting 10-µl aliquots of cell suspensions at a calculated density of 1000 Klett units in TPM buffer on 1/2CTT agar (Hodgkin and Kaiser 1979b) and incubating at 32°C. Motility mutants were detected by their failure to spread (swarm) to the same degree as the fully motile strain DK1622.

Tn5 lac mutagenesis

Tn5 lac was introduced into the fully motile DK1622 by P1-mediated transduction (Kroos and Kaiser 1984). Kan^r transductants were picked to fresh CTT + Kan plates. Approximately 10,000 transductants were examined for increased *lacZ* expression during development over a 3-day period of fruiting body development after regrowth on CTT plates. A loopfull of vegetatively growing cells was transferred from the CTT agar to TPM agar plates containing 40 µg/ml of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and to CTT agar plates containing 20 µg/ml X-gal. Levels of β-galactosidase activity were estimated by comparing the intensity of indigo blue dye deposited by developing cells versus vegetatively growing cells. Aggregation, sporulation, and motility defects were detected visually in a dissecting microscope. Altogether, 273 mutants displaying increased *lacZ* expression during development, developmental defects, or motility defects were retained. Among the 273 mutants, 42 displayed abnormal development and were analyzed in more detail in this report.

Physical mapping of Tn5 lac insertions

Chromosomal DNA was isolated as described (Avery and Kaiser 1983) and independent digests prepared with the indicated restriction enzymes (Fig. 2). Southern blotting followed published procedures (Sambrook et al. 1989). A probe specific for the 5' end of *lacZ* was isolated from pLRK31 (Kroos et al. 1986) that contains the 3.4-kbp *Bam*HI–*Eco*RI fragment of the left-side of *Tn5 lac* including the 5' end of *lacZ*. The restriction fragment containing the entire *frz* locus isolated from pBB12 (Blackhart and Zusman 1985a) was used as the *frz* specific probe. A probe specific for the region immediately upstream of Ω7540 was isolated from pFS703, obtained as follows. Chromosomal DNA from DK11063 that contains Ω7540 was digested with *Sal*II and DNA fragments with sizes of 10–15 kbp isolated from an agarose gel (*Sal*II has one restriction site in *Tn5 lac*, ~9 kbp from the left-most end of *Tn5 lac* and one site 3.3-kbp upstream of Ω7540). These *Sal*II fragments were ligated into the *Sal*II site in pBluescript II SK⁺ (Stratagene) and transformed into *E. coli* JM106 [e14[−](McrA[−]), *endA1*, *hsdR17*, *gyrA96*, *thi-1*, *supE44*, *relA1*, Δ(*lac-proAB*)] (Yanisch-Perron et al. 1985) with selection for Ap^r and Kan^r. The plasmid containing the correct *Sal*II fragment was denoted pFS700. Subsequently, the *Bam*HI fragment containing the entire left-most end of *Tn5 lac*, except for 54 bp of the left IS50 sequence, was deleted from pFS700 generating pFS703 that contains 54 bp of the left end of IS50 and 3.3 kbp of *M. xanthus* DNA upstream of Ω7540. To confirm that pFS700 and pFS703 contained the DNA upstream of Ω7540, the *M. xanthus* DNA from either plasmid was shown by Southern blotting to hybridize to the same restriction fragments from DK11063 as a *lacZ* specific probe.

Measurement of β-galactosidase activity

Cells were induced to develop under submerged conditions as described in the sporulation assay. At the indicated times, cells from a well were harvested into 0.4 ml of MC7 and stored at −20°C until all were collected. Samples were sonicated 15 sec with a standard microtip (Heat Systems-Ultrasonics) and then 5 min in a cup horn (Tekmar) with ice-water cooling. Otherwise quantitation of β-galactosidase specific activity was performed as described (Kroos et al. 1986) except that β-mercaptoethanol was omitted from the Z-buffer and that samples were not sedimented before they were assayed.

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Intercellular C-signaling in *Myxococcus xanthus* involves a branched signal transduction pathway.

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