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Developmental Bypass Suppression of *Myxococcus xanthus csgA* Mutations

HO GUN RHIE AND LAWRENCE J. SHIMKETS*

Department of Microbiology, University of Georgia, Athens, Georgia 30602

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The *csgA* mutations of *Myxococcus xanthus* (formerly known as *spoC*) inhibit sporulation as well as rippling, which involves ridges of cells moving in waves. Sporulating revertants of CsgA cells were isolated by direct selection, since spores are much more resistant to heat and ultrasonic treatment than are vegetative cells. The revertants fell into seven groups on the basis of phenotype and the chromosomal location of the suppressor alleles. Group 1 contained one allele that was a back mutation of the original *csgA* mutation. Group 2 contained two linked alleles that were unlinked to the *csgA* locus and restored fruiting-body formation, sporulation, and rippling. Group 3 revertants regained the ability to sporulate in fruiting bodies but not the ability to ripple. Revertants in groups 4 to 7 were able to sporulate but unable to form fruiting bodies or ripples. The suppressors were all found to be bypass suppressors even though they were not selected as such in most cases. The *csgA* mutation prevented expression of several developmentally regulated promoters, each fused to a *lacZ* reporter gene and assayed by β-galactosidase production. In four of five suppressor groups (groups 4 to 7), expression of each of these *csgA*-dependent fusions was restored, which suggests that bypass suppression restores developmental gene expression near the point at which expression is disrupted in CsgA mutants. Bypass suppression did not restore production of C factor, and morphological manifestations of development such as rippling and fruiting-body formation were usually abnormal. One interpretation of these results is that C factor has multiple functions and few suppressors can compensate for all of them.

The developmental pathway is a series of requisite, dependent steps that mediate the transition between cells or cellular communities of widely different structure or function. The myxobacteria have one of the most striking developmental pathways among the procaryotes; it contains many elements that are functionally analogous to development in eucaryotic organisms, including directed cell movement, multicellular morphogenesis, cellular differentiation, and regulated gene expression (11, 20, 27). In a rich medium, cells grow exponentially and divide by transverse binary fission as do other bacteria. But when nutrients become limiting, multicellular development is initiated on a solid surface, and tens of thousands of cells move toward an aggregation center, where they assemble a fruiting body with a species-specific shape. Within the fruiting body, rod-shaped vegetative cells differentiate into round, optically refractile, dormant myxospores which are resistant to heat, desiccation, UV light, and ultrasonic treatment (32). One species, *Myxococcus xanthus*, has a well-developed genetic system and a wide variety of developmental mutants. The CsgA mutants of this species appear to be defective in producing an intercellular signal that is required for sporulation (14). They are also defective in a type of multicellular movement, known as rippling, which can be observed during both vegetative growth and development. Ripples are spatially separated ridges of cells that move synchronously in a processive manner (30). The *csgA* mutations map to a region of the chromosome (25) that has been isolated by recombinant DNA techniques and examined in some detail (28, 29).

Suppression has emerged as a powerful genetic approach in the analysis of development (6), and a specific type of suppression was used to isolate developmental pathway mutants. Suppressor mutations compensate for the effect of the original mutation and restore the wild-type phenotype without restoring the original nucleotide sequence. Three types of suppression have been characterized in *Escherichia coli*: intragenic, informational, and indirect (Table 1). Intragenic suppression occurs when a second mutation within the gene carrying the first mutation introduces a compensatory alteration that restores a functional gene product. Informational suppression functions at the translational level; one example involves an altered anticodon of a suppressor tRNA which inserts the wild-type amino acid at the mutant codon. Unlike the other forms of suppression, indirect suppression bypasses the effect of the original mutation without restoring a functional gene product. Three types of indirect suppressors have been identified: overproduction, interaction, and bypass. Overproduction suppression compensates for a mutation that reduces the specific activity of an enzyme by increasing the cellular level of the mutant protein. Interaction suppression is usually allele specific and can occur when two or more proteins physically interact with each other. Mutations that alter one component of the protein complex to make it noninteractive may be suppressed by mutations that alter the second component in such a way as to restore the interaction. Bypass suppression occurs when a second gene product restores wild-type function by either substituting for the mutant gene product or restoring the pathway in a different manner. It is not allele specific and is the only form of suppression expected for null mutations generated by transposon insertion. Hence, a selection scheme in which the starting mutation is a null mutation would be expected to yield primarily bypass suppressors. While back mutation can also correct the phenotype of cells containing a mutation caused by transposon insertion, a bypass suppressor allele can be distinguished from a back mutation because it will map distal to the original mutation (Table 1).

This work grew out of an effort to isolate mutations whose temporal position in the *M. xanthus* developmental pathway would be well defined and guaranteed by the selection

* Corresponding author.

TABLE 1. Comparison of the different types of genetic suppression with back mutation

Type	Suppression of[a]:		Location[b]
	Point mutations	Transposon insertion null mutations	
Back mutation	+	+	+
Intragenic suppression	+	−	+
Informational suppression	+	−	−
Indirect suppression			
Overproduction	+	−	+ or −
Interaction	+	−	−
Bypass	+	+	−

[a] +, Suppressor restores the wild-type phenotype.
[b] Location of the suppressor mutation relative to the original mutation. +, Suppressor mutation is located in the same locus as the original mutation.

scheme used. Bypass suppression was used to select mutations in genes that affect the developmental pathway downstream from *csgA*. Thus, the location of the *csgA* mutation creates the proximal boundary for suppressor isolation in the pathway, and bypass mutants that reenter the developmental pathway will presumably do so at this or a more distal step. CsgA mutants produce few spores, and there is a strong selection for spore survival that may be exploited to isolate sporulating revertants (32). Mapping of the mutations was accomplished by transposon tagging (24) and transduction (13). Developmental gene expression was examined with a series of *lacZ* fusions to developmentally regulated promoters. The results demonstrate that four of five groups of bypass suppressor mutations restored developmental gene expression near the block imposed by *csgA* without restoring the wild-type phenotype.

MATERIALS AND METHODS

Bacterial strains. The strains of *M. xanthus* used in this study are listed in Table 2; all are in the yellow phase of the yellow-tan phase variation. Strains DK4293 through DK5204 were constructed by L. Kroos and A. Kuspa (23). Plasmids pJDK8 in DZF3361 and pJDK10 in DZF3427 were kindly furnished by J. Downard (8). Vegetative cultures were grown in CTT broth at 32°C (16). To induce development and rippling, colonies from CTT agar plates were transferred to CF agar plates (14) and incubated at 32°C. Fruiting bodies and ripples were observed under a Wild dissecting stereomicroscope, and myxospores were examined under a phase-contrast microscope.

Bacteriophage growth and transduction. Stocks of the generalized transducing phages Mx4 and Mx8 were prepared on donor cells in a modified CT broth (20 g of Casitone [Difco Laboratories, Detroit, Mich.] liter of tap water^{-1}). *M. xanthus* was grown in CT broth at 32°C until it reached a cell density of 50 Klett units with a red filter (2.5×10^8 cells ml^{-1}). For strains with S motility, phage was added to a multiplicity of infection of 0.2; for S$^-$ mutants, the multiplicity of infection was 0.01. The culture was then shaken at 32°C for 12 to 24 h until the cells lysed. Several drops of chloroform were added, and the cell debris was removed by centrifugation at 27,000 × *g* for 13 min. The supernatant was stored over chloroform at 4°C.

The kanamycin resistance marker of Tn*5 lac* was used as a selectable marker for transducing adjacent chromosomal genes to other *M. xanthus* strains. A phage stock in CT was irradiated for 30 s under a 260-nm germicidal UV lamp at

TABLE 2. Myxobacterial strains

Strain	Genotype	Suppressor group	Reference or derivation
DK101	*sglA1*		16
DK1622	Wild type		30
DK4293	ΩDK4401		23
DK4294	ΩDK4406		23
DK4499	ΩDK4499		23
DK4500	ΩDK4500		23
DK4531	ΩDK4531		23
DK5204	ΩDK4435		23
LS202	*csgA741*		28
LS203	*csgA653*		28
LS204	*csgA731*		28
LS205	*csgA205*		28
LS224	*ops*ΩpJDK8		Mx8(DZF3361) × DK1622→Km^r
LS226	*tps*ΩpJDK10		Mx8(DZF3427) × DK1622→Km^r
LS234	ΩLS234		P1 Tn*5 lac* × DK1622→Km^r, Lac$^+$
LS500	*csgA741 csp-500*	5	UV on LS202
LS501	*csgA741 csp-501*	4	UV on LS202
LS502	*csgA741 csp-502*	3	Spontaneous derivative of LS202
LS503	*csgA741 csp-503*	4	Spontaneous derivative of LS202
LS504	*csgA741 csp-504*	6	Spontaneous derivative of LS202
LS505	*csgA741 csp-505*	4	Spontaneous derivative of LS202
LS506	*csgA653 csp-506*	6	UV on LS203
LS507	*csgA653 csp-507*	4	UV on LS203
LS508	*csgA653 csp-508*	3	UV on LS203
LS512	*csgA731 csp-512*	7	UV on LS203
LS513	*csgA205 csp-513*	6	Spontaneous derivative of LS205
LS515	*csgA*$^+$	1	UV on LS205
LS520	*csgA*$^+$	1	P1 Tn*5-132* × LS515→Tet^r Km^s
LS523	*csgA205*		P1 Tn*5-132* × LS205→Tet^r Km^s
LS529	*csgA205 csp-529*	4	UV on LS523
LS530	*csgA205 csp-530*	4	UV on LS523
LS531	*csgA205 csp-531*	6	UV on LS523
LS532	*csgA205 csp-532*	3	UV on LS523
LS535	*csgA741 csp-501*	4	Mx4 (pool of LS501 Tn*5 lac*) × LS202→Km^r Spo$^+$
LS536	*csgA205 csp-529*	4	Mx4 (pool of LS529 Tn*5 lac*) × LS523→Km^r Spo$^+$
LS537	*csgA205 csp-532*	3	Mx4 (pool of LS532 Tn*5 lac*) × LS523→Km^r Spo$^+$
LS539	*csgA*$^+$	1	Mx4 (pool of LS520 Tn*5 lac*) × LS202→Km^r Spo$^+$
LS540	*csgA741 csp-500*	5	Mx4 (pool of LS500 Tn*5 lac*) × LS523→Km^r Spo$^+$
LS559	*csgA205 csp-559*	2	P1 Tn*5 lac* × LS523→Km^r Spo$^+$
LS560	*csgA741 csp-560*	2	P1 Tn*5 lac* × LS202→Km^r Spo$^+$
LS561	*csgA205 csp-531*	6	Mx4 (pool of LS531 Tn*5 lac*) × LS523→Km^r Spo$^+$
LS562	*csgA741 csp-560*	2	P1 Tn*5 lac* Tc^r × LS560→Tc^r Spo$^+$

2,000 μW cm^{-2}. The irradiated phage stock was mixed with log-phase recipient cells at a multiplicity of infection of 4.0 (17). After 30 min of adsorption at room temperature, the mixture was plated on a CTT agar plate containing 20 μg of kanamycin sulfate ml^{-1}. The plate was overlaid to 40 μg of kanamycin sulfate ml^{-1} 12 to 24 h later. In cases where the recipient already contained a Tn*5* or a Tn*5 lac* insertion in the chromosome, the transposons were replaced with Tn*5-132* or Tn*5 lac* Tc^r (kindly furnished by B. Laue and R. Gill), which encode tetracycline resistance, by homologous recombination with a specialized P1 transducing phage (4). To do replacement of Tn*5* and Tn*5 lac* in S^+ strains, 10^8 cells were mixed with P1 Tn*5-132* and P1 Tn*5 lac* Tc^r at a multiplicity of infection of 5. After 20 min of adsorption at room temperature, the mixture was plated on a CTT agar plate containing 8 μg of oxytetracycline ml^{-1} and overlaid to 20 μg of oxytetracycline ml^{-1} 12 h later. Plates were incubated for 3 additional days at 32°C until single colonies were visible.

Selection of revertants. CsgA revertants were found spontaneously or were induced by UV light or transposon insertion. For spontaneous reversion, vegetative cells growing exponentially in CTT broth were suspended in TM buffer (10 mM Tris [pH 7.6], 1 mM $MgSO_4$) and spread evenly at a density of 10^7 cells cm^{-2} on CF agar plates. After incubation for 5 days at 32°C, plates were incubated for 2 h at 50°C, which kills vegetative cells but not myxospores (14). Heat-treated cells were suspended in TM buffer and subjected to sonic oscillation with 60 μW cm^{-2} for 60 s (Heat System Ultrasonics). For UV-induced reversion, exponentially growing vegetative cells were resuspended in TM buffer at a density of 10^9 cells ml^{-1} and irradiated at 1,500 μW cm^{-2} for 9 to 10 s under a germicidal UV lamp, which killed 99 to 99.9% of the cells. The irradiated cells were diluted 1:3 in CTT broth and grown for 24 h at 32°C in the dark. The cells were concentrated in CTT broth to 500 Klett units and 0.2 ml was spread on a CF agar plate. The plates were incubated for 5 days at 32°C and then heat treated as described above for the spontaneous revertants. The sonicated cell suspensions were plated on CTT agar, and 5 days later individual colonies were transferred to CF agar. Strains that were capable of forming myxospores were saved for further study. On the average, sporulating CsgA revertants arose spontaneously at a frequency of 1.1×10^{-7} but have been isolated from cultures irradiated with UV light at a 100-fold-higher frequency.

Transposon-induced suppressors were isolated by infecting CsgA mutants with P1 Tn*5 lac* as previously described (21). Transposon insertions suppressing the *csgA* mutation were isolated at a frequency of 2×10^{-4}. Transductants were transferred to CF agar and tested for sporulation.

Genetic mapping. Genetic mapping was done by transposon tagging. Tn*5 lac* insertions were isolated near the suppressor loci and used as selectable markers to determine linkage with other relevant loci. Independent chromosomal insertions of Tn*5 lac* were isolated by infecting a sporulating revertant with P1 Tn*5 lac* and selecting for kanamycin resistance. Several pools of 200 Tn*5 lac* insertions were prepared on each revertant. A stock of the generalized transducing phage Mx4 was grown on each pool and used to transduce the parent CsgA mutant to kanamycin resistance. Transductants that formed spores on CF agar plates contained a Tn*5 lac* insertion adjacent to the suppressor allele. The alternate approach of transducing the revertants with pools prepared on wild-type cells (24) and screening the transductants for loss of sporulation was not effective because the frequency of Tn*5 lac* insertion into genes that are required for sporulation was much higher than the frequency of insertion near a particular suppressor locus.

An Mx4 phage stock was grown on a strain with a wild-type suppressor allele linked to Tn*5 lac* and used to transduce the original revertant strains to kanamycin resistance. The transductants were tested for sporulation on CF agar. When a Tn*5 lac* insertion is linked to the wild-type suppressor allele, some of the kanamycin-resistant transductants do not sporulate, and their frequency measures the genetic linkage between Tn*5 lac* and the suppressor locus, since cotransduction frequency between two markers is proportional to the physical distance between them (31). If the two suppressor loci are separated by more than 62 kilobase pairs, the amount of DNA packaged by Mx4, all transductants will sporulate (13, 19).

Determination of spore number. Vegetative cells growing exponentially in CTT broth were resuspended in TM buffer and spread uniformly at a density of 10^7 cells cm^{-2} on CF agar plates. After incubation for 5 days at 32°C, cells were scraped off the surface of the CF agar plate and subjected to sonic oscillation at 60 μW cm^{-2} (Heat System Ultrasonics) for 10 s to disrupt cell clumps. The myxospores were counted in a Petroff-Hausser counting chamber under a phase-contrast microscope.

Expression of β-galactosidase. Tn*5 lac* insertions in the *M. xanthus* chromosome which result in developmentally regulated β-galactosidase production were isolated as described by Kroos and Kaiser (21). Mx4 was grown on such strains and used to transduce $CsgA^+$, CsgA, and CsgA suppressor cells to kanamycin resistance. Plasmid pJDK8 was used to create a *lacZ* fusion with the promoter for protein S1, and pJDK10 was used to create a *lacZ* fusion with the promoter for protein S2 (8). For rapid screening of β-galactosidase activity during development, transductants were transferred from CTT agar to CF agar plates. After 4 days on CF agar at 32°C, the plates were sprayed with a saturated aqueous solution of the β-galactosidase substrate 4-methylumbelliferyl-β-D-galactoside, which generates a hydrolysis product that fluoresces under long-wavelength UV light (33).

RESULTS

Isolation of suppressors. On a low-nutrient medium known as CF agar, wild-type *M. xanthus* cells form fruiting bodies containing dormant spores. Myxospores may be selectively isolated from a mixed population of spores and vegetative cells by subjecting the population to heat and sonic stress which destroys only vegetative cells (14). When plated on CTT agar, the spores will germinate and form colonies of growing cells. On CF agar, the average viable wild-type spore density was 4.4×10^6 spores cm^{-2}. In contrast, CsgA mutants carry out residual sporulation at a frequency of 4.5 spores cm^{-2}, and about 12% of these spores have permanently regained the ability to sporulate at high efficiency (data not shown).

Eighteen independent spontaneous, UV-induced, or transposon-induced *csgA* suppressor mutations have been isolated and grouped according to phenotype and the chromosomal location of the mutation (Fig. 1 and Table 3). Those in groups 1 and 2 have regained their ability to form fruiting bodies, myxospores, and ripples and are indistinguishable from wild-type cells. Group 3 includes strains that form fruiting bodies containing myxospores but fail to ripple. Suppressors in groups 4 to 7 sporulate on CF agar without forming fruiting bodies or ripples. The group 5 strain has the

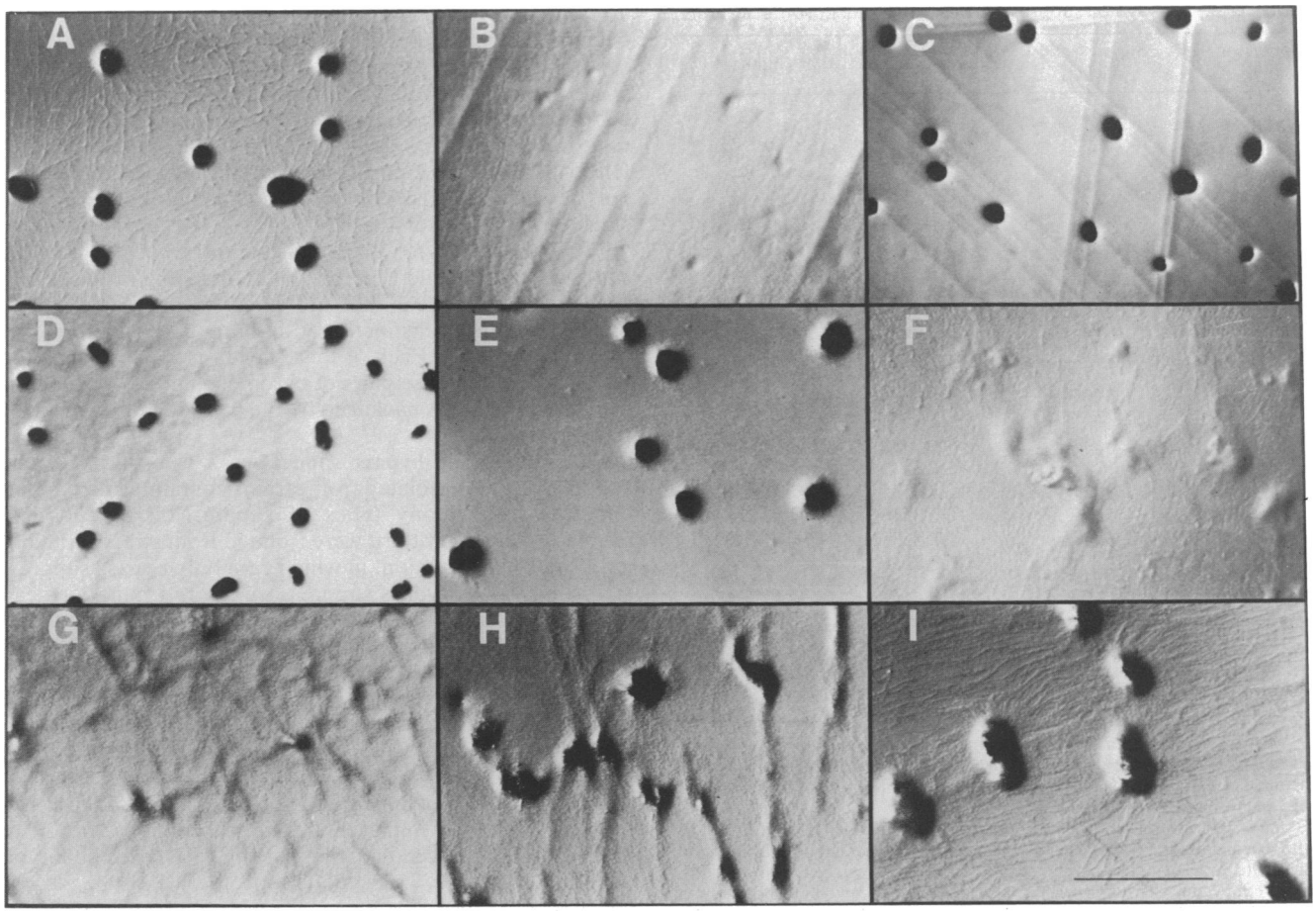

FIG. 1. Developmental phenotype of CsgA$^+$, CsgA, and CsgA suppressors. (A) DK1622, CsgA$^+$; (B) LS523, CsgA; (C) LS520, group 1; (D) LS561, group 2; (E) LS532, group 3; (F) LS501, group 4; (G) LS500, group 5; (H) LS531, group 6; (I) LS512, group 7. Bar, 310 μm.

unique property of forming spores on CTT agar at low temperature (15°C). There was a marked difference between groups with regard to the extent of sporulation. Strains in groups 1, 2, and 3 produced wild-type spore levels, whereas the others formed fewer spores (Table 3).

TABLE 3. Characteristics of the classes of CsgA suppressors

Strain	No. of alleles	Developmental phenotype			
		Spores on CTT agar	Spores (% of wild type) on CF agar[a]	Fruiting bodies	Ripples
CsgA		−	10^{-4}	−	−
Wild type		−	100	+	+
Group 1	1	−	86 ± 3	+	+
Group 2	2	−	103 ± 17	+	+
Group 3	3	−	116 ± 2	+	−
Group 4	6	−	1 ± 0.4	−	−
Group 5	1	+	10 ± 0.1	−	−
Group 6	4	−	36 ± 0.1	−	−
Group 7	1	−	2 ± 0.1	−	−

[a] Vegetative cells grown in CTT broth to 5×10^8 cells ml^{-1} were centrifuged (12,000 × *g* for 10 min), suspended in TM buffer, spread uniformly onto the surface of CF agar at a density of 10^7 cells cm^{-2}, and incubated at 32°C for 4 days. Cells were harvested in TM buffer and dispersed by sonication (10 s at 60 μW cm^{-1}), and direct counts of myxospores were done in a Petroff-Hausser chamber under a phase-contrast microscope. Each value represents the average of two independent determinations.

Mapping and genetic characterization of suppressors. Mapping studies require a selectable marker at or near the suppressor locus. Because Tn*5 lac* can transpose to many different sites within the *M. xanthus* genome, it has been used to locate the suppressor alleles by transposon tagging. This use requires a method to identify a strain containing a Tn*5 lac* insertion linked to a particular locus among hundreds of other strains with a Tn*5 lac* insertion located elsewhere in the genome. To do this, a library of independent chromosomal Tn*5 lac* insertions was obtained by infection of a revertant with the suicide vector PlΩTn*5 lac*. Each kanamycin-resistant transductant was formed by an independent transposition of Tn*5 lac* into the chromosome. To identify a Tn*5 lac* insertion near the suppressor locus, the generalized transducing phage Mx4 was grown on a pool of 200 independent strains and used to transduce a CsgA mutant to kanamycin resistance. Transductants were then tested for sporulation proficiency, which occurred upon replacement of the wild-type suppressor allele with the mutant suppressor allele jointly transferred with the Tn*5 lac* insertion. Linked transposons were found for six of the seven suppressor groups.

To determine the genetic linkage between a suppressor allele and a particular Tn*5 lac* insertion, the wild-type suppressor allele and its linked Tn*5 lac* were transduced into the revertants. In cases in which the transductants have lost the ability to sporulate, the suppressor allele in the recipient

TABLE 4. Linkage of suppressor loci with transposon insertions

Suppressor group	Suppressor allele	Linked Tn*5 lac*	Linkage (%)
1[a]	LS520	ΩLS539	73
2[b]	LS559	ΩLS559	100
		ΩLS560	>99
	LS560	ΩLS559	>99
		ΩLS560	100
3[c,d]	LS532	ΩLS537	>99
	LS502		
	LS508		
4[c]	LS501	ΩLS535	78
		ΩLS536	81
	LS503	ΩLS535	78
		ΩLS536	80
	LS505	ΩLS535	85
		ΩLS536	79
	LS507	ΩLS535	69
		ΩLS536	76
	LS529	ΩLS535	88
		ΩLS536	81
	LS530	ΩLS535	90
		ΩLS536	76
5[c]	LS500	ΩLS540	33
6[c,d]	LS531	ΩLS561	>99
	LS504		
	LS506		
	LS513		
7	LS512		

[a] Mx4 was grown on a strain containing the wild-type suppressor and its linked Tn*5 lac*. The phage lysate was used to transduce other suppressors to kanamycin resistance. Linkage indicates percent cotransduction of the wild-type suppressor allele with Tn*5 lac*.

[b] Mx4 was grown on LS559 containing ΩLS559 or LS560 containing ΩLS560, and the lysate was used to transduce a strain containing ΩLS560tc or ΩLS559tc to kanamycin resistance. All kanamycin-resistant transductants were tetracycline sensitive, which indicates that the two transposons are closely linked.

[c] Mx4 was grown on a suppressor containing a linked Tn*5 lac* and used to transduce CsgA mutants to kanamycin resistance.

[d] All transductants gained the ability to sporulate, which indicates tight linkage between the transposon and the suppressor, but maintained the unique phenotype of the original suppressor. Assignment of other alleles to these groups is based on the similarity of phenotype.

maps near the donor wild-type allele. The frequency with which nonsporulating transductants are detected is proportional to the physical distance between the transposon and the suppressor allele (31). Alternately, the Tn*5 lac* insertion linked to the suppressor allele was transduced into the *csgA* background. In this case, sporulating transductants are indicative of linkage between a Tn*5 lac* insertion and a suppressor locus, and the frequency of transduction is proportional to the physical distance.

The suppressor strains and their linked Tn*5 lac* insertions are listed in Table 4. The lone group 1 allele was formed by excision of Tn*5-132* from the *csgA* gene which regenerated a functional *csgA* gene and restored the wild-type phenotype. This was demonstrated in two ways. First, the group 1 allele was cloned and shown to have the same restriction map as *csgA* (data not shown). Second, ΩLS539, a Tn*5 lac* insertion isolated near the group 1 allele, maps to the same location as the *csgA* gene (data not shown). None of the other suppressor alleles are linked with the *csgA* gene. The two group 2 suppressor alleles are Tn*5 lac* insertions that completely restored the wild-type phenotype, and they map to the same genetic locus. One group 3 allele was found to be so tightly linked to ΩLS537 that it has not yet been possible to position ΩLS537 next to the wild-type allele to map the other alleles. Therefore, LS502 and LS508 have been assigned to group 3 because their phenotypes are identical to that of LS532. All six group 4 alleles were found to be linked with ΩLS535 and ΩLS536 and to be tightly clustered in a region estimated to be less than 4 kilobase pairs, as determined by cotransduction frequency. The lone group 5 allele is linked with ΩLS540. The four group 6 suppressors have identical phenotypes, which is the primary basis for their clustering in a single group. One of these, LS531, is so tightly linked to ΩLS561 that it has not been possible to isolate the transposon next to the wild-type allele for mapping studies. The single group 7 allele has not been mapped and has a phenotype that is distinctively different from those of the other suppressors. Each of these seven groups is separated by at least 62 kilobase pairs of chromosomal DNA, which is the amount of DNA packaged by the transducing phage Mx4 (19).

Classification as bypass suppressors. Two-thirds of the suppressors were isolated from strains containing *csgA* point mutations, and many types of suppression are possible (Table 1). The other third were isolated from strains containing a *csgA* null mutation, in which case only bypass suppression is possible. Bypass suppressors of *csgA* mutations can be distinguished from other types of suppressors in that suppression does not require a CsgA gene product. LS523 is a suitable host for this experiment because it has a Tn*5-132* insertion, ΩLS205tc, in the *csgA* gene (28), which provides a tetracycline resistance marker and a null mutation in the *csgA* gene. Mx4 was grown on LS559, which has the *csgA* gene containing Tn*5-132* ΩLS205tc and the suppressor allele caused by the insertion of Tn*5 lac* ΩLS559. Southern hybridization has shown that this strain still contains ΩLS205tc in the *csgA* locus even though it has the wild-type phenotype (data not shown). Transduction of the wild-type strain, DK1622, to tetracycline resistance did not result in kanamycin-resistant transductants; conversely, transduction of DK1622 to kanamycin resistance did not result in tetracycline-resistant colonies, which suggests that the *csgA* gene is unlinked with the suppressor locus (Table 5). Furthermore, the tetracycline-resistant transductants from wild-type recipients have the typical CsgA phenotype, which again indicates that ΩLS205tc is inserted in the *csgA* gene. When the phage stock was used to transduce strains containing *csgA* point mutations, LS202 and LS204, to kanamycin resistance, all transductants obtained the ability to sporulate but did not obtain the tetracycline-resistant *csgA* allele, which indicates that the suppressor locus suppresses all *csgA* mutations. These results are indicative of bypass suppression. This suppressor mutation is caused by a transposon insertion. Wild-type strains containing this insertion also sporulated, which indicates that this gene is not essential for sporulation. Similar results were obtained with LS560, which contains ΩLS560, the other Tn*5 lac* insertion that causes this phenotype (not shown).

In the case of the third suppressor group, Mx4 was grown on LS537, which contains the *csgA* gene containing ΩLS205tc and the suppressor linked to Tn*5 lac* ΩLS537, and used to transduce LS202 and LS204 to kanamycin resistance. All of the transductants acquired the ability to sporulate but did not acquire the tetracycline-resistant *csgA* allele. When the same phage stock was used to transduce DK1622 to tetracycline resistance, none of the transductants obtained kanamycin resistance. The results again suggest bypass suppression. The phenotypes of the original suppressor and the suppressor containing Tn*5 lac* ΩLS537 are identical, and it seems improbable that this insertion is the cause of the

TABLE 5. Classification of suppressors as bypass suppressors[a]

Donor	Recipient	No. of: Km^r transductants analyzed	No. of: Tc^r transductants analyzed	No. of: Km^r Tc^r transductants	No. of: Sporulating transductants	Cotransduction of Tn*5 lac* and sporulation (%)
LS559	LS202	44		0	44	>98
	LS204	32		0	32	>98
	LS523	150		150	150	>99
	DK1622	54		0	54	
	DK1622		54	0	0	
	LS562	60		0	60	
LS537	LS202	42		0	42	>98
	LS204	43		0	43	>98
	LS523	354		354	354	>99
	DK1622	112		0	112	
	DK1622		28	0	0	
LS535	LS202	85		0	58	68
	LS204	72		0	46	64
	LS523	60		60	44	70
	DK1622	89		0	89	
LS540	LS202	36		36	12	33
	LS523	34		34	11	32
	DK1622	64		0	64	
LS561	LS202	44		0	44	>98
	LS204	60		0	60	>98
	LS523	92		92	92	>99
	DK1622	44		0	44	
	DK1622		18	0	0	

[a] Mx4 was grown on a strain containing the suppressor allele and its linked Tn*5 lac* and was used to transduce recipients to kanamycin or tetracycline resistance. Transductants were screened for tetracycline and kanamycin resistance and sporulation.

suppression. Rather, it appears that recombination between the suppressor allele and the insertion is infrequent.

In the case of the fourth suppressor group, Mx4 was grown on LS535, which has a suppressor locus located near ΩLS535, and a point mutation in the *csgA* locus. Transduction of the suppressor from this strain to LS523, which contains the *csgA* allele caused by ΩLS205tc, occurred 73% of the time, which suggests bypass suppression. The suppressor was also transferred to two other CsgA mutants, LS202 and LS204, at a similar frequency without concomitant transfer of the *csgA* allele, which would have resulted in tetracycline-sensitive transductants. Transduction of the mutant suppressor allele into wild-type cells did not interrupt sporulation.

For the fifth suppressor group, Mx4 was grown on LS540, which contains a suppressor allele linked to Tn*5 lac* ΩLS540 and a *csgA* point mutation, and used to transduce LS523 to kanamycin resistance. All of the transductants remained resistant to tetracycline, and 32% of them acquired the ability to sporulate, demonstrating bypass suppression. The suppressor allele was also transferred to another CsgA point mutant, LS202, at a similar frequency and did not interrupt sporulation of wild-type cells.

In the case of the sixth suppressor group, Mx4 was grown on LS561, which has the *csg* allele ΩLS205tc and the suppressor linked to Tn*5 lac* ΩLS561. Transduction of the suppressor from this strain to LS202 and LS204 occurred without transfer of the tetracycline-resistant *csgA* allele. Transduction of DK1622 to tetracycline resistance resulted in transfer of the *csgA* locus only. The suppressor appears to be tightly linked to Tn*5 lac* ΩLS561.

Therefore, in each case the suppressors turned out to be bypass suppressors even though they were not always selected as such.

Expression of CsgA-dependent *lacZ* fusions in the suppressors. The results presented above offer a strong argument that a suppressor mutation restores the developmental pathway at some point at or distal to the *csgA* block. The point at which the suppressors restore development may be determined with biochemical or genetic markers that monitor the progress of a cell during development. The markers chosen for this experiment are developmentally regulated promoters fused to the *lacZ* gene. The fusions appear not to interrupt the developmental pathway in and of themselves but are expressed during development in a precise manner (8, 22). If development is restored close to the point at which it is disrupted by the *csgA* mutation, then there should be no interruption in the expression of these developmental markers. If development is restored at some point much further down the developmental pathway, then intermediate steps in the pathway may be skipped, and certain developmentally regulated promoters may not be expressed.

Tn*5 lac* is a derivative of the transposable element Tn*5*, which contains a promoterless *lacZ* gene in the left end of the inverted repeat IS50L (21). When Tn*5 lac* inserts in the proper orientation next to a functional promoter, β-galactosidase is expressed and can be used as a marker for expression of that promoter fusion. Gene fusions can also be constructed in vitro by ligating an *M. xanthus* gene fragment to a *lacZ* gene that lacks a promoter and integrating the hybrid gene into the chromosome (8). Eight *lac* fusions whose expression is dependent on the CsgA gene product and one fusion whose expression is not dependent on the CsgA product (*tps*) were transduced into CsgA$^+$, CsgA, and CsgA suppressor backgrounds. Relative levels of β-galactosidase activity were observed by spraying a petri plate containing developing bacterial colonies with the substrate 4-methylumbelliferyl-β-D-galactoside, which produces a blue fluorescent product when hydrolyzed by the enzyme (33). Representatives from groups 4 to 7 were able to express all of the fusions at levels similar to or higher than that of the wild type (Table 6), which suggests that the point at which developmental gene expression is restored in CsgA mutants containing these suppressors is close to the point at which it is arrested in CsgA mutants, within the resolution of these markers. The group 3 strain was unable to express β-galactosidase from ΩLS234 and ΩDK4500, which are expressed at 10 and 14 h of development, respectively, but did express all other fusions, including ΩDK4531 and ΩDK4406, which are expressed at 7 and 11 h, respectively. One explanation for this result is that ΩLS234 and ΩDK4500 lie on a branch of the developmental pathway different from that on which ΩDK4531 and ΩDK4406 lie.

Extracellular complementation. CsgA mutants sporulate when mixed with developing wild-type cells or with sporulation mutants of another genotype, as if they fail to produce but can respond to an essential intercellular signal, the C signal (14). One possible biochemical mechanism for suppression would be the invention of a novel method to synthesize the C signal that is independent of the *csgA* gene. This was examined by quantitating the spores produced by CsgA cells in the presence and absence of a representative of each suppressor group. To permit identification of myxospores produced by CsgA (LS205) cells in mixture with wild-type (DK1622) or CsgA suppressor cells, the CsgA mutants were marked genetically with kanamycin resistance. Cultures of these strains were mixed and allowed to develop, and the resulting myxospores were analyzed for

TABLE 6. Expression of developmentally regulated promoters during development of CsgA$^+$, CsgA, and CsgA suppressor strains

Strain	Fluorescence with given promoter fusion[a]								
	tps	ΩLS234	ΩDK4500	ΩDK4531	ΩDK4499	ΩDK4435	ΩDK4401	ΩDK4406	*ops*
DK1622, wild type	+++	+++	+++	+	++	+++	++	++	++
LS523, CsgA	+++	−	−	−	+	−	+	−	−
LS532, group 3	+++	−	−	+	++	+++	+	++	+
LS501, group 4	+++	++	+++	+	+++	+++	+	++	+
LS500, group 5	+++	+++	+++	+	+++	+++	+	+++	+++
LS531, group 6	+++	++	+++	+	++	+++	+	++	+
LS512, group 7	+++	++	+++	++	+++	+++	+	+++	+++

[a] Relative amount of fluorescence observed when colonies grown on CF agar plates at 32°C were sprayed with 4-methylumbelliferyl-β-D-galactoside. Times of expression of the fusions can be found in reference 22 for ΩDK4500 (14 h), ΩDK4531 (7 h), ΩDK4499 (6 h), ΩDK4435 (25 h), ΩDK4401 (30 h), and ΩDK4406 (11 h), and in reference 8 for *tps* (5 h) and *ops* (18 h). The time of expression of ΩLS234 is 10 h (unpublished data).

kanamycin resistance (Table 7). The positive control mixture, DK1622 plus LS205, gave approximately equal numbers of kanamycin-sensitive and -resistant colonies and demonstrates how efficient extracellular complementation can be. The negative control mixture, containing only CsgA cells (LS523 plus LS205), produced few colonies. In the mixtures of suppressors and LS205, most of the myxospores arose from suppressor cells. The group 2 suppressor, which was the most efficient in extracellular complementation, was still 10-fold less efficient than the wild type. It therefore appears that suppression is not due to the production of C factor.

DISCUSSION

CsgA mutants, which are unable to produce an intercellular signal but are able to respond to it, are easily identified by their phenotype, which allows them to be extracellularly complemented for sporulation. However, mutations in other related steps of the C-signaling process, such as perception of the signal, transduction of this information through the membrane, regulation of gene expression, or initiation of sporulation, are not likely to be extracellularly complemented. Other strategies need to be adopted to isolate and identify such mutants. Mutations in such genes are likely to disrupt developmental gene expression at a similar point to that for *csgA*. Therefore, we attempted to isolate mutations in such genes by selecting for mutations that bypass the *csgA* mutation and restore the developmental pathway downstream. Seventeen alleles constituting six distinct groups of bypass suppressors were found. Restoration of the pathway of gene expression was observed in most cases. In only one of the groups was it obvious that intermediate steps had been skipped. Therefore, it is likely that this mutant collection defines a narrow portion of the developmental pathway near the block imposed by CsgA. Whether these mutations are in genes responsible for other steps in C signaling remains to be determined.

TABLE 7. Sporulation in mixtures of CsgA$^+$ and CsgA suppressor cells[a]

Strain	Myxospores cm^{-2} recovered from CF agar plates and germinated on:		% of myxospores from LS205
	CTT	CTT+Km	
DK1622 + LS205	8.8×10^5	4.0×10^5	45.0
LS523 + LS205	2.0	0	0
LS561 + LS205	1.8×10^6	7.0×10^4	4.0
LS532 + LS205	1.5×10^5	1.6×10^3	1.1
LS501 + LS205	1.6×10^4	$<1.0 \times 10^2$[b]	<0.6
LS500 + LS205	1.6×10^4	$<1.0 \times 10^2$[b]	<0.6
LS531 + LS205	2.2×10^4	4.4×10^2	2.0
LS512 + LS205	2.4×10^4	4.0×10^2	1.7

[a] Vegetative cultures, grown separately in CTT broth to 5×10^8 cells ml^{-1}, were harvested by centrifugation and suspended in TM buffer. Mixtures, made by combining strains at equal ratios, were spread uniformly on the surfaces of CF agar plates and incubated at 32°C for 4 days. Plates were heated at 50°C for 2 h to kill vegetative cells. Cells were resuspended in TM buffer and sonicated to disperse clumps of spores (5 s at 60 μW cm^{-1}). The myxospores were diluted in TM buffer and plated in CTT soft agar on CTT agar and CTT-kanamycin (30 μg ml^{-1}) (CTT+Km) plates to allow the spores to germinate. The resulting colonies were counted after incubation at 32°C for 4 days.

[b] No colonies observed; value is limit of detection.

Four mechanisms of bypass suppression have been discovered in other organisms which warrant closer examination in *M. xanthus*. First, bypass suppression may be due to production of a particular biochemical by a novel mechanism. For example, *Saccharomyces cerevisiae* Cho1 mutants are unable to synthesize phosphatidylserine, a precursor for the major membrane lipids phosphatidylethanolamine and phosphatidylcholine, and grow only if exogenous ethanolamine or choline is provided (3). Suppressors of the *cho1* mutation, altered at the *eam1* gene, produce abnormally high levels of phytosphingosine, which is apparently degraded to ethanolamine (1). Thus, the Eam1 mutants synthesize ethanolamine by a novel pathway that does not have phosphatidylserine as an intermediate (2). By analogy, bypass suppressors of *csgA* mutations might have devised an alternate method for synthesizing the C signal. This was tested by examining the ability of the suppressor strains to extracellularly complement CsgA mutants, and it was found that the suppressor strains are not efficient inducers of CsgA sporulation. Therefore, it is unlikely that the suppressors produce the C signal by an alternate mechanism.

Second, bypass suppression may be due to activation of an alternate pathway for completion of a complex process. Strains carrying *recB* and *recC* mutations in *E. coli* K-12 carry out conjugational recombination at 0.3 to 2% of the wild-type level. The fact that residual recombination proficiency remains suggests that an alternative minor pathway of recombination, independent of the *recB* and *recC* products, may be operative in *E. coli* (7). Rec$^+$ suppressors of *recB recC*$^+$, *recB*$^+$ *recC*, and *recB recC* have been isolated; unlike the parent, the suppressors have a high level of ATP-independent DNase activity which seems to participate in the pathway of recombination (5). In *M. xanthus*, myxospores may be induced artificially with glycerol (10) or naturally by starvation (9). The two types of spores differ in ultrastructure (18), which suggests that the pathways for making these spores have at least some different steps.

Indeed, glycerol sporulation is relatively rapid and is not dependent on the cell interactions that are required before fruiting-body sporulation is initiated (14). Furthermore, only 1 of 14 different Tn*5 lac* fusions to developmentally regulated promoters activated by starvation from the Kroos and Kaiser collection was also expressed during glycerol induction (L. Kroos, Ph.D. dissertation, Stanford University, Stanford, Calif., 1986). Since these same *lacZ* fusions are expressed during sporulation of the suppressed stains, and since development of the suppressed strains is still subject to many of the conditions required for fruiting-body development, it is likely that suppression works by restoring the starvation-induced pathway.

Third, bypass suppression may be due to mutations affecting transcriptional regulation. In the L-arabinose operon in *E. coli*, L-arabinose binds to the repressor protein and converts the repressor into an activator whose binding at the initiator site is necessary for transcription. L-Arabinose-utilizing revertants were isolated from a mutant containing a deletion that covers all known point mutations in the *araC* gene for the multifunctional regulatory protein. The revertants contained the original deletion plus a second mutation mapping to the promoter region. They have a *cis*-dominant constitutive phenotype characteristic of such transcriptional mutants (12). By analogy, constitutive CsgA-dependent gene expression might bypass the *csgA* mutation.

A fourth type of bypass suppression may occur at the level of membrane receptors and transmembrane signaling processes. Response of *E. coli* to a chemoattractant is determined by a series of steps that constitute a signaling pathway (15). Periplasmic proteins bind the attractant and transmit this information to a transmembrane receptor, which in turn signals the flagellum. When the chemoattractant is abundant, the transmembrane receptor is methylated, the flagella rotate counterclockwise, and the cells swim smoothly. One class of transmembrane receptor mutants is always overmethylated and biased toward constant swimming even in the absence of an attractant (26). This type of mutation has essentially bypassed the function of the periplasmic binding proteins. By analogy, similar mutations in the receptor for the CsgA signal would falsely indicate the presence of the C signal. This possibility has not been examined.

The various mechanisms for CsgA bypass suppression require further investigation. At the heart of this problem is an interesting paradox. The suppressor strains appear to have separated the morphological aspects of development from the temporal pathway of gene expression. Group 3 appears to bypass one or more steps in the pathway of gene expression yet forms fruiting bodies containing spores. On the other hand, several suppressors (groups 4 to 7) appear to restore the normal pattern of gene expression without restoring fruiting-body formation. Only one suppressor group, group 2, is able to ripple. This result suggests that the developmental pathway has branches that occur at a morphological level in addition to those that occur at the genetic level. One way this might occur is if C factor is multifunctional and has different roles in sporulation, rippling, aggregation, and regulation of gene expression. A bypass suppressor that compensates for one of these roles may be physically or temporally constrained from compensating for another. There is evidence that CsgA does have different roles in rippling and sporulation. Although both behaviors depend on CsgA, they are regulated by very different environmental and genetic factors (30). Furthermore, certain *csgA* mutations disrupt sporulation but not rippling (28). Therefore, it is possible that CsgA is a multifunctional protein.

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