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Developmental Cell Interactions of *Myxococcus xanthus*: Analysis of Mutants

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A set of developmental mutants have been examined that behave as if defective in cellular interactions necessary for the formation of myxospores during fruiting body development. Sporulation is rescued in these mutants if they are mixed with wild-type cells. Complementation experiments with whole cells divide the mutants into four groups (A, B, C, and D). Mutants of group A appear to be less responsive to starvation, a condition that normally initiates development. Mutants of group D respond to starvation but fail to synthesize myxobacterial hemagglutinin, a protein normally synthesized midway in development. Mutants of groups B and C respond to starvation and synthesize hemagglutinin, but they can be distinguished genetically. Group C mutations all map in a single cluster near insertion Ω1519 of transposon Tn*5*, which is distant from group B mutations. Thus, each group represents a different defect in development. All of the mutants are induced to sporulate by glycerol. Therefore, we argue that sporulation during fruiting body development depends on several prior interactions between cells.

Myxobacteria are unique among procaryotes in their ability to undergo sporulation coupled to primitive multicellular development—the formation of macroscopic structures called fruiting bodies (27). When *Myxococcus xanthus*, for example, is starved on a solid surface, more than 10^5 cells move toward a focus where they pile on top of one another, forming a haystack-like mound. Within this nascent fruiting body, rod-shaped vegetative cells differentiate into ovoid myxospores, which are resistant to dessication, heat, and radiation (32). The participation of many cells in the formation of a fruiting body suggests that there are coordinating interactions between the cells. Several low-molecular-weight substances have already been identified as intercellular signals: excreted adenosine which serves as a measure of cell density in *M. xanthus* (28), a low-molecular-weight lipid which speeds aggregation and can replace light in the maturation of fruiting bodies of *Stigmatella aurantiaca* (31), and four components of peptidoglycan (*N*-acetylglucosamine, *N*-acetylmuramic acid, D-alanine, and diaminopimelic acid) which induce organized cell movement in *M. xanthus* similar to that seen during the development of fruiting bodies (29, 30). Evidence for additional signaling substances in *M. xanthus* is presented here.

Hagen et al. (14) isolated a set of developmental mutants of *M. xanthus* that behave as if they are interaction defective. These mutants exhibit normal vegetative growth, but when they are placed under conditions that would induce the wild type to form fruiting bodies, they fail to sporulate. They can sporulate, however, if they are mixed with wild-type cells or with other mutants. Pairwise testing of 57 mutants divided them into four groups such that sporulation occurred upon challenging by starvation a mixture of two mutants belonging to different groups but not a mixture of two mutants belonging to the same group.

What is the primary defect in each of these mutants and what differentiates mutants in one group from those in another? To investigate these questions, we examined the developmental biochemistry and genetics of mutants representing the four groups. We wish to report that three of the groups can be distinguished by the stage in the fruiting process beyond which they fail to develop. A fourth distinction can be made on genetic grounds.

MATERIALS AND METHODS

Bacterial strains and phages. The strains of *M. xanthus* used in this study are listed in Table 1. The myxophages Mx1, Mx4, and Mx8 (6, 7, 25) and phage P1::Tn*5* (21) have been described previously.

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Buffers and media. The composition of CTT (15), clone fruiting (CF), and A1 (4) media have been described previously. Formulations of 0.1, 0.2, and 0.5% CTT are identical to CTT except that the concentration of Casitone is 0.1, 0.2, or 0.5% (wt/vol), respectively. Media supplemented with kanamycin sulfate (Sigma B grade) are labeled K, followed by a number indicating the final concentration in micrograms of kanamycin sulfate per ml. TM buffer is 10 mM Tris, pH 7.6–8 mM magnesium sulfate. TPM is TM with 1 mM potassium phosphate, pH 7.6, added. PM buffer is 10 mM potassium phosphate (pH 7.6)–8 mM magnesium sulfate.

Glycerol-induced myxosporulation. Each strain to be tested was grown in CTT to 5×10^8 cells per ml, and 1 μl was spotted on CTT agar supplemented with 0.75 M glycerol. After incubation for 1 day at 33°C, plates were heated for 1.5 h at 50°C. Vegetative cells are destroyed by this treatment, but glycerol-induced myxospores survive. Each spot was toothpicked onto a CTT plate to test for spores that had survived heating.

ppGpp accumulation. Cultures were grown in 0.5% CTT medium modified by the omission of potassium phosphate (23). After cultures were pelleted by centrifugation, they were resuspended in TM buffer supplemented with 30 to 40 μCi of $^{32}PO_4$ per ml. These suspensions were incubated at 33°C for 1 h. Nucleotides were then extracted and applied to polyethyleneimine chromatography plates pretreated as described previously (23). Ascending chromatograms were developed with 1.5 M KPO_4, pH 3.5 (9). Methods for determining positions of standards, autoradiography, and quantitation have been described previously (23).

Protease production. Strains, incubated at 33°C on CTT agar, were transferred by a single stab with a flat toothpick to casein agar plates. Casein agar is 10 mM Tris (pH 7.6)–1% agar–0.1% isoelectric casein (Difco)–20 mM $MgCl_2$. Casein precipitates in this mixture; proteases render the precipitate soluble. The area of the zone of solubilization of casein around each stab was recorded after 20 h. For each experiment with a particular strain, six to eight determinations of the activity were made. Plots of cleared area versus time were linear.

Growth on A1 medium and A1 medium lacking phenylalanine. Cells were grown in CTT medium to ~5 × 10^8/ml, washed three times with TPM buffer, and resuspended in TPM buffer at ~5 × 10^9/ml, and 10-μl aliquots were spotted on A1 agar medium or on A1 medium made up without phenylalanine. Growth was estimated visually by comparison of mutants with strain DK101.

pS determination. Protein S (pS) (17, 18) was measured in vegetative and developing cultures by two-dimensional immunodiffusion. CTT-grown cells, resuspended in CTT at 2.5×10^{10} cells per ml, were inoculated onto CF plates (100-mm diameter; 30 ml of CF) with a multipronged device that delivered 48 spots of 2 μl each. Each plate was printed twice so that 96 spots were applied (total, 5×10^9 cells). Plates were harvested in TM buffer after 0, 24, 32, 40, and 70 h of development at 33°C by the technique of Wireman and Dworkin (36). An ethanol-dry ice bath was used to freeze pelleted cells, and the frozen pellets were stored at −20°C. Sonic extracts of cells suspended in 25 mM Tris (pH 7.0)–10 mM EDTA were prepared by six 15-s bursts of an MSE sonicator at maximal setting (the sonic extract was prepared and kept at 0°C). The sonic extracts were clarified by centrifugation (12,000 × *g* for 20 min) at 4°C. The supernatant was assayed for protein content (22). pS was detected in two-dimensional immunodiffusion by using antibody (kindly provided by D. Zusman) that had been raised against purified pS. The precipitin reaction was allowed to proceed for 2 days at room temperature. After the plates were washed, lines of precipitate were first detected by their turbidity and subsequently by Coomassie blue stain.

Assay of myxobacterial hemagglutin (MBHA). Extracts of sonically disrupted developing cells (0, 24, or 48 h after initiation of development) were prepared as described above for determination of pS. Total protein was measured, and hemagglutinating activity was assessed with sheep erythrocytes (Flow Laboratories) as described previously by Cumsky and Zusman (10), but using a 1.5% suspension of erythrocytes.

Genetic methods. Insertions of Tn*5* into the *M. xanthus* genome near sporulation loci were obtained as described previously (21). Generalized transduction with phage Mx8 was carried out as described previously (15), using a multiplicity of 0.5 phage per cell, UV-irradiated phage, and anti-Mx8 serum on the plates to prevent reinfection. Kanamycin-resistant transductants were selected on CTTK30 plates incubated 5 to 7 days at 33°C. The sporulation phenotype of the transductants was determined after replica plating to CFK20 plates and incubation for 7 days at 33°C. The plates were then heated for 2.25 h at 50°C to kill surviving vegetative cells. The spores were germinated by overlayering the plate with enough concentrated Casitone in TPM soft agar to bring the final concentration of Casitone in the plate to 0.5%. After a further incubation of 3 days at 33°C, sporulation-proficient transductants showed dense growth. Genetic terms are specified in Table 1, footnote *a*.

Transmembrane rescue of sporulation. Each strain to be tested was grown in CTT and concentrated to 10^9 cells per ml. Ten microliters of the suspension was placed on a CF plate. After the spot had dried, a membrane filter was centered over the (bottom) spot. A second 10-μl spot (top) was then placed on the membrane so as to lie immediately above the bottom spot. After 7 days of incubation at 33°C, the presence of heat-resistant spores was determined as described above for glycerol-induced sporulation, except that the plates were heated for 2.25 h at 50°C. To estimate the extent of sporulation, material from above and below the membrane on each plate was sampled with a sterile toothpick in a standard manner and transferred to a CTT plate.

Membranes with 0.2-μm pores were polycarbonate (Nuclepore). They were 10 μm thick. Dialysis membranes were regenerated cellulose (Spectrapor). Both types of membranes were extensively washed and sterilized with ethanol and then dried on both sides on a PM agar plate before use.

Complementation for sporulation. Strains to be tested for complementation were mixed at equal density, spotted on PM agar plates to induce fruiting, and allowed to develop at 33°C for 7 days. The plates were then heated 2.25 h at 50°C to destroy vegetative cells and overlayered with soft agar containing enough Casitone and kanamycin to bring their final concentra-

TABLE 1. Bacterial strains[a]

Strain	Description	Derivation	Reference or source
DK101	Spo$^+$	From *M. xanthus* FB	*b*
DK412	SpoA, *spo-412*	UV on DK101	*c*
DK423	SpoB, *spo-423*	UV on DK101	*c*
DK428	SpoB, *spo-428*	UV on DK101	*c*
DK429	SpoD, *spo-429*	UV on DK101	*c*
DK433	SpoB, *spo-433*	UV on DK101	*c*
DK439	SpoD, *spo-439*	UV on DK101	*c*
DK440	SpoB, *spo-440*	UV on DK101	*c*
DK454	SpoB, *spo-454*	UV on DK101	*c*
DK460	SpoB, *spo-460*	UV on DK101	*c*
DK468	SpoB, *spo-468*	UV on DK101	*c*
DK471	SpoA, *spo-471*	UV on DK101	*c*
DK473	SpoA, *spo-473*	UV on DK101	*c*
DK474	SpoB, *spo-474*	UV on DK101	*c*
DK476	SpoA, *spo-476*	UV on DK101	*c*
DK478	SpoB, *spo-478*	UV on DK101	*c*
DK480	SpoA, *spo-480*	UV on DK101	*c*
DK495	SpoA, *spo-495*	ICR on DK101	*c*
DK516-22	SpoA, *spo-51622*	EMS on DK101	C. Manoil
DK527	SpoB, *spo-527*	Spontaneous from DK101	Manoil and Kaiser (23)
DK631	SpoB, *spo-631*	NTG on DK101	*c*
DK653	SpoC, *spo-653*	EMS on DK101	*c*
DK731	SpoC, *spo-731*	ICR on DK101	*c*
DK739	SpoA, *spo-739*	ICR on DK101	*c*
DK741	SpoC, *spo-741*	ICR on DK101	*c*
DK751	SpoB, *spo-751*	ICR on DK101	*c*
DK753	SpoA, *spo-753*	ICR on DK101	*c*
DK756	SpoA, *spo-756*	ICR on DK101	*c*
DK929	Spo$^+$, Ω929	Mx8 (pool Tn*5* in DK101) × DK439 → Km^r[Spo$^+$]	*d,e*
DK1519	Spo$^+$, Ω1519	Mx8 (pool Tn*5* in DK101) × DK731 → Km^r[Spo$^+$]	*d,e*
DK1526	SpoC, *spo-741*, Ω1519	Mx8 (DK1519) × DK741 → Km^r[Spo$^-$]	*e*
DK1529	SpoC, *spo-653*, Ω1519	Mx8 (DK1519) × DK653 → Km^r[Spo$^-$]	*e*
DK1535	SpoC, *spo-731*, Ω1519	Mx8 (DK1519) × DK731 → Km^r[Spo$^-$]	*e*
DK1548	SpoC, *spo-653*, Ω1519	Mx8 (DK1529) × DK101 → Km^r[Spo$^-$]	*e*
DK1550	SpoC, *spo-653*, Ω1519	Mx8 (DK1529) × DK101 → Km^r[Spo$^-$]	*e*
DK1556	SpoC, *spo-731*, Ω1519	Mx8 (DK1535) × DK101 → Km^r[Spo$^-$]	*e*
DK1558	SpoC, *spo-731*, Ω1519	Mx8 (DK1535) × DK101 → Km^r[Spo$^-$]	*e*
DK1867	Spo$^+$, Ω1867	Mx8 (pool Tn*5* in DK101) × DK429 Km^r[Spo$^+$]	*d,e*
DK2447	Spo$^+$, Ω1519	Mx8 (DK1519) × DK101 → Km^r[Spo$^+$]	*e*
DK2448	SpoA, *spo-480*, Ω1519	Mx8 (DK1519) × DK480 → Km^r[Spo$^-$]	*e*
DK2450	SpoD, *spo-439*, Ω1519	Mx8 (DK1519) × DK439 → Km^r[Spo$^-$]	*e*
DK2453	SpoB, *spo-751*, Ω1519	Mx8 (DK1519) × DK751 → Km^r[Spo$^-$]	*e*
DK3223	SpoD, *spo-439*, Ω1867	Mx8 (DK1867) × DK439 → Km^r[Spo$^-$]	*e*
DK3224	SpoD, *spo-429*, Ω1867	Mx8 (DK1867) × DK429 → Km^r[Spo$^-$]	*e*

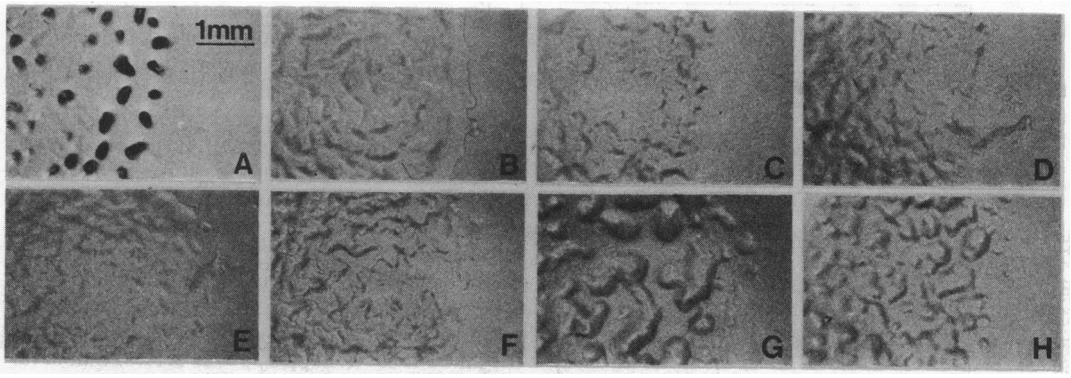

FIG. 1. Cultures were grown in CTT medium to 5×10^8 cells per ml. Drops of 10 μl were placed on CF agar, incubated at 33°C, and photographed after 3 days. (A) DK101; (B) DK480, a mutant of group A; (C) DK468, a mutant of group B; (D) DK751, a mutant of group B; (E) DK731, a mutant of group C; (F) DK741, a mutant of group C; (G) DK429, a mutant of group D; (H) DK439, a mutant of group D.

tions to 0.5% and 25 μg/ml, respectively. This medium allows spores of the Spo$^-$ recombinant strains, which are Km^r, to germinate and to form colonies, whereas spores of the tester strains, which are Km^s, fail to grow. The tester strains were: DK101 (Spo$^+$), DK480 and DK756 (SpoA), DK468 (SpoB), DK731 (SpoC), and DK429 and DK439 (SpoD).

RESULTS

Fruiting body formation and sporulation. CF medium (14) is a dilute nutrient for *M. xanthus* that supports limited growth before cells plated on it exhaust its amino acids. When a drop of a suspension of growing cells is placed on CF agar, development occurs over a period of 2 to 3 days. The time course of development of strain DK101 serves as a base for comparison of mutant strains derived from it. Within about 1 day after DK101 has been placed on CF agar, loose translucent ridges and mounds of rod-shaped cells arise at many sites within the perimeter of the original drop of cell suspension (17, 26; our own observations). The mounds gain sharper definition as the spaces between them are depleted of cells. By the end of the second day, the mounds have turned dark and opaque because the rod-shaped vegetative cells within them have shortened into spherical, thick-coated spores. Figure 1A illustrates DK101 at 3 days. Most of the aggregation and mounding is complete before sporulation occurs. Significant numbers of heat-resistant myxospores do not appear under these conditions until about 35 h after plating (Fig. 2).

Mutants representative of each of the four groups make fewer than 0.01% of the number of heat-resistant myxospores produced by the wild type (Table 2). Cultures of representative mutants on CF agar were examined daily, and photographs taken on day 3 are presented in Fig. 1. Although their fruiting-competent parent had formed mature, spore-containing fruiting bodies by this time, none of the mutants developed beyond formation of irregular, loose, translucent mounds without spores. When a mutant was mixed with wild-type cells, there was a 100- to 10,000-fold increase in the number of spores (Table 2).

Starvation, a solid surface, and high cell density are normally required for fruiting body formation and the associated sporulation (28). However, these three requirements and the whole of multicellular morphogenesis can be bypassed by the addition of glycerol (12). Glycerol induces *M. xanthus* cells growing in liquid

[a] The kanamycin-resistant phenotype is designated Km^r. Spo$^+$, SpoA, SpoB, SpoC, and SpoD are used to designate the sporulation-proficient (Spo$^+$) and sporulation-deficient phenotypes of groups A, B, C, and D, respectively. The genotypes are *spo*$^+$ and *spo*$^-$, with the various alleles indicated by number. Ω1519, for example, designates a particular site in *M. xanthus*, site number 1519, at which Tn*5* has inserted. Mx8 (DK998) × DK101 → Km^r[Spo$^-$] indicates that Mx8 grown on DK988 was used as a donor in transduction with DK101. Transductants were selected on kanamycin and then screened for the Spo$^-$ phenotype. EMS, Ethyl methane sulfonate; ICR, ICR-191; NTG, nitrosoguanidine.

[b] Reference 16.

[c] Reference 14.

[d] "Pool Tn*5* in DK101" indicates a library of Tn*5* insertions derived in strain DK101 as described previously by Kuner and Kaiser (21).

[e] This work.

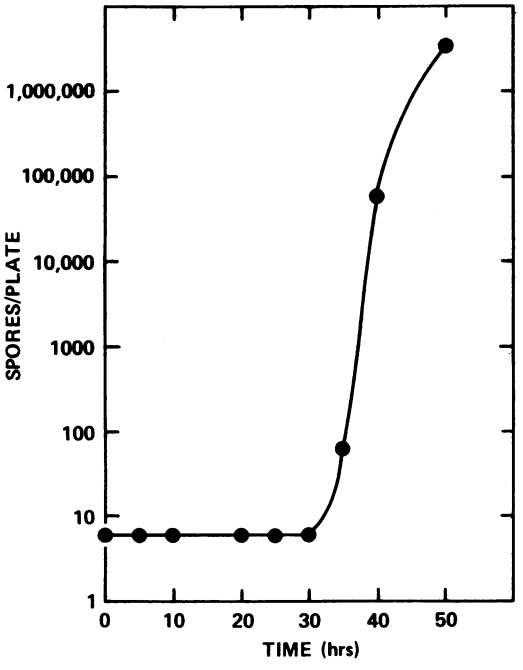

FIG. 2. Kinetics of sporulation. DK101 cells at a density of 2.5×10^{10} cells per ml were distributed in 16 drops of 2 μl each on a series of polycarbonate filters, each laid on a CF agar plate. A set of plates were incubated at 33°C for each time indicated and then heated to 50°C for 2.25 h to destroy vegetative cells. The filters were aseptically removed from treated plates, transferred to 3 ml of TM buffer, and sonicated to release and disperse the spores. Titers of the resulting spore suspensions were determined on CTT plates.

culture with excess nutrients to form spores without fruiting bodies. The glycerol-induced spores lack parts of the coat present on fruiting body myxospores (18), but they are the same shape and are heat resistant (32). All of the mutants listed in Table 3, including members of all four groups, were induced by 0.75 M glycerol to form spores that survived heating to 50°C and that were capable of germinating on CTT medium. On the basis of reconstruction experiments (data not shown), these positive responses indicated that the efficiency of glycerol-induced sporulation of the mutants was at least 10% the efficiency of wild type. Retention of the capacity for glycerol-induced sporulation argues that the primary defects in these mutants are not in the conversion of rod-shaped cells to spherical and resistant spores, but rather in earlier developmental processes upon which sporulation is dependent.

Biochemical correlates of fruiting. Guanosine tetra- and pentaphosphate accumulate in cultures of *M. xanthus* that are developing fruiting bodies in response to starvation, whether the limitation is for carbon and energy sources or for an amino acid (24). This accumulation, possibly related to a stringent response (8), may be one of the earliest events in fruiting body development. Whether or not development is initiated by a stringent response, accumulation of the stringent nucleotides is one measure of the capacity of the cells to sense starvation. Cultures growing in rich medium (CTT broth) were transferred to buffer lacking any source of nutrient and containing $^{32}PO_4$. After 1 h of starvation, the parental strain DK101 had synthesized ppGpp, and the amount of ^{32}P in ppGpp was 0.17 of that in its precursor, guanosine triphosphate (pppG). All of the mutants listed in Table 3 had ratios at least one-half this value (data not shown). Typical prestarvation ppGpp/pppG ratios in strains related to DK101 are <0.01 (24), and relaxed mutants of *Escherichia coli*, for comparison, accumulate no detectable ppGpp (8). Thus, all of the mutant groups appear able to respond to total starvation.

M. xanthus will grow on a minimal medium called A1 that contains six amino acids (4). All of the mutant strains listed in Table 3 grew as well as DK101 on A1 medium, showing that the mutants are not auxotrophs. *M. xanthus* synthesizes phenylalanine slowly, and the withdrawal of phenylalanine from A1 medium reduces the growth rate in liquid culture about twofold (4). DK101 grows on solid A1 medium, but when phenylalanine is eliminated, growth stops and fruiting bodies form (4, 24). Apparently, phenylalanine limitation, resulting from the absence of exogenous phenylalanine, is sufficient to induce development under conditions of high cell density on a solid surface (28). Almost all mutants of groups B, C, and D, like DK101, stopped vegetative growth when placed on minimal medium from which phenylalanine has been omitted (Table 3). They did not form fruiting bodies, of course, but the arrest of growth indicates a response to phenylalanine limitation. Mutants of group A, with one exception, continued vegetative growth on A1 medium lacking phenylalanine (Table 3). It is as if A mutants had lost the normal capacity to sense or to respond to phenylalanine limitation.

Production of two development-specific proteins has been examined. pS, which has a molecular weight of 23,000, accumulates in very large amounts in developing populations of wild-type cells (18). All four groups of mutants are still capable of synthesizing pS, though mutants of group A delayed the onset of its production (Table 3). MBHA, a protein with lectin activity, accumulates during the aggregation phase of *M. xanthus* development (10, 11). Strain DK101 developing on CF medium accumulated MBHA

TABLE 2. Rescue of sporulation in sporulation-defective mutants by wild type[a]

Condition	No. of Km^r spores				
	Wild type (DK2447)	Group A (DK2448)	Group B (DK2453)	Group C (DK1535)	Group D (DK2450)
Alone	3.7×10^7	<6	3×10^3	6	8
Mixed with (Km^s) wild type	2.9×10^7	5.7×10^2	2.6×10^7	3.3×10^3	3.8×10^3

[a] The five kanamycin-resistant strains listed across the top were plated on CF medium alone (line 1) or mixed in equal proportions with their wild-type parent DK101 (line 2), which is kanamycin sensitive. After 6 days at 33°C, the plates were heated, and the number of Km^r survivors (spores) was determined by transfer of a sample to kanamycin-containing CTT plates.

in the soluble fraction to high levels at 24 h, levels which fell by 48 h, as reported by Cumsky and Zusman (10). Among the mutants, all of the C strains and all of the B strains, except DK433, accumulated MBHA (Table 3). However, none of the 10 A strains or either D strain produced significant amounts of MBHA.

Strains of group A and of group D also have

TABLE 3. Biochemical changes in fruiting

Strain	Group	Growth arrested by Phe starvation[a]	Protease activity[b]	pS first detected (h)[c]	MBHA (U/mg of protein)[d]		
					24 h	48 h	Phenotype[e]
DK101	Wild type	+	1.00	24	941	444	+
DK412	A	+	0.48	48	0 (125)	0 (133)	−
DK471	A	−	0.49	48	0 (61)	0 (133)	−
DK473	A	−	0.59	72	0 (80)	0 (125)	−
DK476	A	−	0.46	35	0 (200)	N	−
DK480	A	−	0.57	48	0 (83)	0 (95)	−
DK495	A	N[f]	0.64	48	0 (83)	0 (105)	−
DK516-22	A	−	0.38	48	0 (91)	0 (95)	−
DK739	A	N	0.54	48	0 (56)	0 (100)	−
DK753	A	−	0.48	72	0 (100)	0 (154)	−
DK756	A	−	0.87	48	0 (100)	0 (105)	−
DK468	B	+	1.20	24	400	174	+
DK751	B	−	0.99	24	1,000	258	+
DK433	B	+	0.48	N	0 (63)	0 (111)	−
DK474	B	+	1.03	N	250	364	+
DK478	B	+	0.82	N	191	211	+
DK631	B	N	N	N	485	258	+
DK731	C	+	0.79	24	1,391	727	+
DK741	C	+	1.15	24	800	842	+
DK653	C	−	0.92	N	210	0 (80)	+
DK429	D	+	0.50	24	0 (70)	0 (526)	−
DK439	D	N	0.86	24	0 (111)	0 (132)	−

[a] +, No growth or formation of aggregates on A1 medium agarose from which phenylalanine was omitted; −, cells grew vegetatively on this medium. Phe, Phenylalanine.

[b] Area of zone of casein solubilization (see text) normalized to the zone obtained with the parental strain DK101 (1.00).

[c] pS was detected by two-dimensional immunodiffusion as described in the text.

[d] Soluble MBHA was assayed as described in the text. Activity is expressed in units per milligram of protein. A unit is defined as the reciprocal of the dilution endpoint. A zero indicates that no hemagglutination was observed at any dilution tested, and the highest activity that could have escaped detection is given in parentheses. Activity at 0 h was zero for all strains.

[e] +, MBHA activity > 25% of that of DK101 at 24 or 48 h. This column summarizes the preceding two columns.

[f] N, Not tested.

somewhat lower average levels of extracellular protease, measured by their capacity to hydrolyze casein, than wild type or the other mutants, although there is considerable strain-to-strain variation within a group (Table 3). The average values are 0.55 for group A, 0.68 for group D, 0.90 for group B, and 0.95 for group C, all relative to 1 for the *spo*$^+$ parent DK101. A role for protease(s) in *Myxococcus* development is not known.

Hagen et al. (14) reported that group A mutants, in contrast to the others, are tan in color and fail to give rise to yellow colonies typical of tan-yellow phase variation (5). Careful examination of two group A strains, DK753 and DK756, revealed that slightly yellow variants did arise, but were only slightly more yellow than their parents. These variant colonies were not mixtures of a few fully yellow cells among many tan cells, because when the slightly yellow colonies were streaked out, all of the resulting colonies were slightly yellow. In acetone extracts, their yellow pigment had no more than one-third the absorbance of yellow variants of DK101. These results suggest that group A strains do vary in phase but that they produce less yellow pigment or that they produce a pigment having a lower molar extinction coefficient than the wild type so that phase variation is masked.

In other tests, representatives of the four groups of mutants were found to be indistinguishable from wild type with respect to sensitivity to phages Mx1 and Mx8 (all strains are sensitive), sensitivity to phage Mx4 wild type (all strains are resistant), light induction of pigment production (all strains are inducible), and sensitivity to myxococcal antibiotic TA (35) (all strains are sensitive).

Transmembrane rescue. Does the rescue of mutant cells by wild-type cells require contact between the cells or can it result from transfer through the medium? To investigate this question, a membrane filter having pores too small for the passage of cells was placed between the populations of interacting cells. DK101, which is approximately 0.5 μm in diameter, can penetrate Millipore filters having average pore diameters of 0.9 or 0.45 μm, but not of 0.2 μm (Sodergren and Kaiser, submitted for publication). Consequently, filters having 0.2-μm-diameter pores were chosen for study.

Mutants of all four groups were rescued by wild-type cells on the opposite side of the membrane filter in a procedure described above. Mutants were rescued both above (mutant/wild type) and below (wild type/mutant) the wild-type cells. The wild-type cells sporulated in both arrangements, but sporulation was not observed when the same mutant was placed on both sides of the filter. These two controls show that the technique used for the sampling of spores is qualitatively valid. Although DK101 formed spores within 3 days, positive responses from the mutants were not obtained until 5 days. Reconstruction indicated that a 2-day delay corresponded to an approximately 100-fold decrease in sporulation from wild-type (DK101) levels. Several mutants of each group were tested, and rescue by DK101 has been observed with six strains of group A (DK473, 480, 495, 516-22, 739, 756), three of group B (DK468, 631, 751), all three strains of group C (DK653, 731, 741), and both strains of group D (DK429 and DK439).

Although there is rescue of sporulation when any pair of mutants from different groups are brought very close together by mixing them, seldom was there rescue when a membrane filter separated two mutant strains from each other. However, strain DK429 (group D) did rescue sporulation of strain DK756 (group A) and strain DK468 (group B) in the arrangements A/D and B/D. Under these conditions, DK756 and DK468 formed spores, but DK429 did not.

Since representatives of all four groups of mutants can be rescued by the wild type across a membrane filter, it was possible to explore the properties of the material transferred from wild-type to mutant cells by changing the filter. Three different patterns of response to reducing the average pore size of the filter are evident in Table 4. Group A strains sporulated when separated from wild type by a filter with 0.2-μm pores but not when separated by any of the dialysis membranes. Both the group B and the group D strains sporulated when separated from the wild type by a membrane with 0.2-μm pores or a dialysis membrane with large pores. The group C strains were rescued across all four membrane types. Although the technique precluded estimation of the absolute number of spores formed, approximately the same amount of material was sampled in each test so that comparisons would be valid. The results imply that members of different rescue groups differ from each other either in the size, amount, or binding properties of the material required from wild-type cells to rescue their sporulation.

Genetic characterization. Are mutants in the same group genetically related to each other? The sporulation phenotypes in *M. xanthus* do not lend themselves to selection based on the growth of colonies because sporulation occurs in large populations of cells undergoing development of fruiting bodies. Nevertheless, *spo* genes can be mapped by using nearby insertions of the transposon Tn*5* (21). Tn*5* specifies an aminoglycoside phosphotransferase which renders individual cells that carry it resistant to the antibiotic kanamycin (3). Starting from a library of

TABLE 4. Rescue of sporulation through membranes

Type of membrane interposed between wild type[a] and mutant	Rescue of sporulation in mutant:[c]			
	DK473 (A)	DK468 (B)	DK731 (C)	DK439 (D)
0.2-μm polycarbonate	+	+	+	+
50,000 NMWCO[b] dialysis	−	+	+	+
10,000–12,000 NMWCO dialysis	−	−	+	−
3,500 NMWCO dialysis	−	−	+	−

[a] Wild type is strain DK101.
[b] NMWCO, Nominal molecular weight cutoff for a globular protein.
[c] Sporulation was assayed by heat resistance, and the physical arrangement is described in the text. Controls in which mutant cells were added instead of wild type so that mutant cells were above and below the membrane were uniformly negative.

1,000 independent insertions of Tn*5* into *M. xanthus*, Kuner and Kaiser (21) showed how to find those that are linked to a particular *spo* mutation.

An insertion of Tn*5*, Ω1519, was found near *spo-731*, the mutation carried by the SpoC mutant DK731. When a *spo*$^+$ Ω1519 strain (DK1519) was crossed to each of the mutants of group C (Table 5), 77, 76, and 79% of the kanamycin-resistant transductants became Spo$^+$, showing that the three *spo* mutations are linked to Ω1519 to approximately the same degree. Crosses between pairs of mutants were performed to determine whether the three mutation sites lie on the same or on opposite sides of the insertion point of Ω1519. Donor strains for the three-factor crosses were kanamycin-resistant, sporulation-defective strains DK1535, DK1526, and DK1529, which carry *spo-731*, *spo-741*, and *spo-653*, respectively. Mx8 lysates grown on strains DK1535, DK1526, and DK1529 were each used to transduce the parental strains DK731, DK741, and DK653 to kanamycin resistance. It is evident from the data shown in Table 5 that recombination between the three *spo* mutations can occur but is rare. Therefore, the three independent group C mutations are at different but closely linked sites, forming a single cluster near Ω1519. Clustering of group C *spo* mutations on the same side of Ω1519 has subsequently been confirmed by cloning (L. R. Shimkets, R. Gill, and D. Kaiser, Proc. Natl. Acad. Sci. U.S.A., in press).

Transduction of kanamycin resistance from a *spo*$^+$ Ω1519 donor did not render any of a set of representative A, B, or D group *spo* mutants *spo*$^+$ (Table 5, lower part). Since the DNA capacity of Mx8 for transduction is about 50 kilobases (25) and since the cluster of group C *spo* mutations is very close to Ω1519, the cluster is many kilobases away from any of the other mutations tested.

Two Tn*5* insertions, Ω1867 and Ω929, were found linked to type D mutations *spo-429* and *spo-439*. Transduction of kanamycin resistance from a *spo*$^+$ Ω1867 donor rendered more than half of the transductants *spo*$^+$ (Table 6). However, no Spo$^+$ were found among the Km^r transductants of any recipient tested belonging to group A, B, or C (Table 6).

Introduction of *spo* mutations into wild-type genetic backgrounds by transduction. Group C and group D *spo* mutations were transduced into fruiting-component *spo*$^+$ strains to test whether the original Spo$^-$ phenotypes were the result of a single mutation or of multiple mutations. Three different *spo*$^+$ strains were employed: DK101, DK1050, and DK1622. DK101 is the original *spo*$^+$ parent of all of the *spo* mutants investigated here; it sporulates well, but its fruiting bodies are poorly formed due to a defect in S-motility, *sglA1* (16). DK1050 grows dispersed, because it carries a temperature-sensitive defect in S-motility (David Morandi, unpublished data), but its fruiting bodies are well formed. DK1622 is fully motile (A$^+$S$^+$), grows nondispersed in liquid culture, and fruits well. Of the three strains, DK1622 most closely resembles fresh soil isolates of *M. xanthus*. Each of these strains was transduced to kanamycin resistance from *spo*

TABLE 5. Mapping the mutations in group C strains[a]

Recipient		Fraction of Spo$^+$ transductants with donor:			
Group	Strain	*spo*$^+$	*spo-731*	*spo-741*	*spo-653*
C	DK731	37/48 (77%)	0/1,217	1/161	2/90
C	DK741	129/169 (76%)	0/866	0/261	1/140
C	DK653	289/366 (79%)	6/1,378	1/438	N
A	DK480	0/69			
A	DK756	0/420			
B	DK468	0/129			
B	DK631	0/84			
B	DK423	0/391			
B	DK428	0/408			
B	DK460	0/275			
B	DK527	0/57			
B	DK440	0/423			
B	DK454	0/250			
D	DK429	0/39			

[a] Mx8 stocks grown on each donor strain carrying Ω1519 were used to transduce kanamycin resistance to the recipient strains. The scores are fractions in which the denominator is the total number of kanamycin-resistant transductants examined and the numerator is the number among them which are Spo$^+$. N, Not tested.

TABLE 6. Mapping mutations in group D strains[a]

Recipient (*spo*⁻)		Fraction of Spo⁺ transductants with donor (*spo*⁺):	
Group	Strain	Ω929	Ω1867
D	DK429	3/132 (2%)	95/109 (87%)
D	DK439	4/140 (3%)	67/110 (61%)
A	DK480	0/250	
A	DK756	0/225	
B	DK468	0/233	
B	DK751	0/261	
B	DK440	0/531	
B	DK454	0/516	
C	DK653	0/116	

[a] Mx8 grown on DK929 or DK1867 was used to transduce *spo*⁻ strains to kanamycin resistance. The transductants were transferred to CFK15 plates, and the colonies which germinated after heating and overlaying with nutrient agar were scored as Spo⁺. The scores are fractions in which the denominator is the total number of kanamycin-resistant transductants examined and the numerator is the number of Spo⁺ Kmʳ.

donors, and kanamycin-resistant transductants were screened on CF agar for their Spo phenotype. Sporulation-defective recombinants were found (Table 7). In general, the frequency of cotransduction of *spo* mutations with Tn*5* was comparable to the frequency of cotransduction of their *spo*⁺ alleles.

The resulting Spo⁻ transductants could sporulate when mixed with corresponding Spo⁺ parental strains (data not shown). Thus, the sporulation defect and the capacity to be rescued behave as if associated with the same mutation. Pairs of Spo⁻ recombinants for the *spo-653* and *spo-731* alleles of group C were mixed with complementation tester strains of all four groups. These strains complemented (sporulated with) group A, B, and D strains, but not group C strains. Thus, group C specificity accompanied the *spo* allele in transduction.

DISCUSSION

The sporulation-defective mutants examined here are a distinct class of fruiting-defective mutants characterized by their capacity to be rescued by added wild-type cells (14). Wild type can rescue representatives of all four groups through filters having a pore diameter of 0.2 μm, and in the case of mutants of groups B, C, and D, through modified dialysis membrane with pores that block globular proteins larger than 50,000 molecular weight. Although it is not yet possible to exclude the passage of very fine cell extensions through the pores of dialysis membrane, that possibility seems remote, and diffusible materials are a more likely explanation. In fact, soluble components of *Myxococcus* peptidoglycan do rescue group C mutants (30).

Mutants of all four groups can be induced to sporulate by addition of 0.75 M glycerol. Glycerol-induced sporulation bypasses the need for high cell density, starvation, and a solid surface, which are conditions normally required for the formation of myxospores during fruiting body formation (28). Glycerol-induced myxospores resemble fruiting body myxospores in many ways. Changes in cell shape during sporulation are similar. Both types of myxospores resist temperature extremes, desiccation, UV light, and physical breakage (32). The coats of both types contain α-1,3-glucan as well as large amounts of galactosamine (20, 34), and lipopolysaccharide is undetectable in both (33). There are clear differences between the two kinds of spores, but many of them may be consequences of the fact that fruiting body myxospores arise from starved cells, whereas glycerol myxospores arise from rapidly growing cells. Differences of this type include excess membrane in glycerol spores that folds and involutes (2, 37), more ribosomes in glycerol spores (37), and a higher capacity for amino acid oxidation in glycerol spores (1, 13). There are two differences not obviously explained by starvation: fruiting body spores have a thicker coat including pS, whereas glycerol spores have a thin coat and lack pS (18). On the basis of the similarities, we have suggested that glycerol activates the normal fruiting body sporulation steps that cause the change in shape and the acquisition of resistance proper-

TABLE 7. Introduction of *spo* mutations into fruiting-proficient strains

Donor			Cotransduction frequency of *spo* mutations with Tn*5* into recipient:[a]		
Group	Tn*5*	Strain	DK101	DK1050	DK1622
C	Ω1519	DK1535	366/429 (85%)	126/136 (93%)	26/34 (76%)
C	Ω1519	DK1526	42/49 (86%)	64/84 (76%)	31/39 (80%)
C	Ω1519	DK1529	70/95 (74%)	4/22 (18%)	6/11 (54%)
D	Ω1867	DK3224	110/121 (91%)		
D	Ω1867	DK3223	31/45 (69%)		

[a] Cotransduction frequency is presented as a fraction in which the denominator is the total number of kanamycin-resistant transductants examined and the numerator is the number of Spo⁻ kanamycin-resistant transductants.

ties (19). According to that view, the retention of glycerol inducibility implies that the mutants retain the capacity for the differentiation of myxospores but are blocked in steps upon which the induction of myxospores during fruiting body formation depends. What might those steps be?

Mutants belonging to group A may be defective in one of the first steps in development. Although they accumulate guanosine-3′-diphosphate 5′-diphosphate when starved for carbon and amino acids and although they arrest vegetative growth and begin to aggregate under these conditions, they fail to respond to withdrawal of phenylalanine from the medium. *M. xanthus* synthesizes phenylalanine slowly, and elimination of phenylalanine from solid A1 medium induces the wild type to stop growing and to form fruiting bodies (4, 24). However, phenylalanine starvation on a solid medium fails to induce growth arrest in mutants of group A. Instead, they continue to grow vegetatively. When starved for carbon and amino acids, mutants in group A delay (relative to the wild type) the production of pS, a protein whose synthesis marks the early stages of fruiting body formation (18). Both their failure to respond to phenylalanine limitation and their delayed response to more intense starvation support the view that group A mutants have a reduced sensitivity to starvation.

Mutants of groups B, C, and D, by contrast, do arrest vegetative growth when limited for phenylalanine, do accumulate normal levels of guanosine-3′-diphosphate 5′-diphosphate, and do make pS at the normal time. Thus, they appear able to sense starvation and to start development, but they fail to complete aggregation (Fig. 1) and they fail to sporulate. Synthesis of MBHA, which may be a lectin involved in cell aggregation (10, 11), occurs at the middle of normal fruiting body development. Mutants of groups B and C accumulate MBHA, but mutants of groups A and D do not.

The genetic analysis shows a tendency for mutations in mutants belonging to the same group to be clustered together on the linkage map, although few mutations have been mapped to date. The three members of group C, although they have arisen by independent mutations, all have mutations mapping close to each other and on the same side of the Ω1519 insertion of Tn*5* into *M. xanthus*. Recently, it has been shown that these mutants all belong to the same genetic complementation group (Shimkets et al., in press). These three sites appear to lie within a single gene or transcription unit. Both mutations of group D are linked to insertion Ω1867 of Tn*5*. The C mutations are not cotransduced with the Tn*5* insertion linked to group D, and the D mutations are not linked to Ω1519. Neither group A nor group B mutations are linked to the insertions of Tn*5* near group C or D mutations. Therefore, the C mutations and the D mutations represent separate and group-specific loci.

The biochemical and genetic tests presented here reveal that the four groups of mutants, which were initially distinguished from each other by their pattern of intercellular complementation, represent different kinds of developmental defects. If it is true, as argued above, that all four groups of mutants retain the intrinsic cellular capacity to form myxospores, yet fail to form them when challenged by starvation, then myxosporulation could lie at the end of a dependent developmental pathway. Each group of mutants would fail to progress beyond its own characteristic stage along the pathway: group A very early, group D early, groups B and C late. Since the rescue of mutants by wild-type cells appears to be mediated by the passage of substances from one cell to another, it is possible that these mutants are defective in the synthesis or release of substances that normally would pass from cell to cell to coordinate and organize the multicellular process of fruiting.

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