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Genes Required for Developmental Signalling in *Myxococcus xanthus*: Three *asg* Loci

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***asg*-carrying strains of *Myxococcus xanthus* arose in a selection for mutants defective in cell-cell signalling during fruiting body development. All 15 *asg* mutations examined were found to lie in one of three genetic loci, *asgA*, *asgB*, or *asgC*. The loci were defined by linkage to different insertions of transposon Tn*5* and molecular cloning of *asgA*. *asg* mutants of all three types were deficient in the aggregation of cells into mounds of the sort that normally give rise to fruiting bodies. *asg* mutants were also deficient in spore formation; sporulation is normally one of the last steps in fruiting body development. Consistent with a requirement for cell-to-cell signalling, at 1 to 2 h *asg*$^+$-carrying cells release a material called A-factor that can rescue development of *asg* mutants. *asgA*, *asgB*, and *asgC* mutants released 5% or less of the *asg*$^+$ level of A-factor, as measured by bioassay. The experimental results are consistent with the hypothesis that a deficiency in A-factor production or release is the primary developmental defect in *asg* mutants and that aggregation and sporulation depend on A-factor. *asg* mutations at all three loci also changed the color and morphology of growing colonies, and failure to release A-factor may itself arise from a defect in growing cells.**

Myxococcus xanthus, although structurally a gram-negative bacterium, lives as a primitive multicellular organism (24). The rate of cell growth on a protein substrate such as casein increases with cell density, as if the cells fed cooperatively (25). *Myxococcus* cells move cooperatively; for example, certain classes of strains move only when cells are in close proximity (10). Starvation initiates a 24-h developmental process in which approximately 100,000 cells construct a fruiting body and form myxospores (7, 24, 28). Cooperative activities suggest passage of coordinating signals between cells.

The *asg* (group A signal) mutants of *M. xanthus* arose in a selection for signalling-defective mutants (9). *asg* mutants cannot develop fruiting bodies alone, but when mixed with wild-type cells they can form spores in the (mixed) fruiting bodies. This property suggests that *asg* mutants cannot produce or release an extracellular substance but can respond to its production by wild-type cells. The *asg* mutants were grouped together and distinguished from other groups on the basis of pairwise testing with other extracellularly complementable mutants. *asg* mutants (formerly *spoA* mutants) could not complement each other for development but could be complemented by mutants from other such groups; i.e., *bsg*, *csg*, and *dsg* (formerly the SpoB, SpoC, and SpoD groups, respectively). The extracellular complementation groups suggest that all *asg* mutants are defective in production of the same substance.

The original *asg* mutant isolates were found to be defective in sporulation, developmental lysis, and formation of compact aggregates (9, 13, 20). The strains also were shown to delay accumulation of the spore coat protein called protein S, which normally appears after 6 h of development (20). An analysis of the expression of 21 developmentally regulated genes in the original *asg* mutants indicated that development was blocked 1 to 2 h after the start of development but that the block could be lifted if the mutants were allowed to develop with wild-type cells (19). Since this rescue led to correctly timed expression of the earliest gene affected, it was proposed that the rescue event represented a normal intercellular signalling process. Rescue of blocked gene expression in an *asg* mutant was used to detect the putative signalling molecule called A-factor in medium conditioned by development of wild-type cells (19). Assayed in this way, A-factor is released from wild-type cells but not from *asg* mutant cells, beginning at 1 to 2 h of development (19).

Hagen et al. (9) identified 18 *asg* mutants, representing about one-third of all the extracellularly complementable mutants they isolated. The frequent occurrence of *asg* mutants suggested multiple *asg* loci. To test this suggestion, we searched for selectable genetic markers near *asg* mutations. We identified new insertions of transposon Tn*5* which, together with two previously identified insertions, show that *asg* mutations lie in three distinct genetic loci: *asgA*, *asgB*, and *asgC*. As a first step toward defining *asg* function, we wished to compare the three types of *asg* mutants. The linked Tn*5* markers have facilitated crosses that bring *asgA*, *asgB*, and *asgC* into the same genetic background to permit that comparison.

MATERIALS AND METHODS

Myxobacteria and phages. The strains used in this study are described in Table 1. The wild-type strain for the experiments reported here is DK1622. DK101 is a developmentally competent relative of DK1622 but is defective in social motility because of an *sglA1* mutation. The *asg* mutants are the SpoA mutants described by Hagen et al. (9) and were derived from DK101. Myxophages Mx4 *ts18 ts27 hrm* (6) and Mx8 *clp2* (21) have been previously described.

Growth and development. Cells were grown in CTT liquid at 32°C with vigorous shaking, and stocks were maintained on CTT agar or CTT agar supplemented with 40 μg of kanamycin sulfate per ml (10). To initiate development on agar, exponentially growing cells were sedimented (6,000 × *g*, 10 min, 4°C), washed in TPM buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM KH_2PO_4, 8 mM Mg_2SO_4), sedi-

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TABLE 1. Myxobacterial strains

Strain	Genotype	Derivation	Source or reference
DK101	*sglA1*	Spontaneous mutation of strain FB	10
DK412 (5062)[a]	*asgA412 sglA1*	UV irradiation of DK101	9
DK418 (5063)	*asgA418 sglA1*	UV irradiation of DK101	9[b]
DK471 (5065)	*asgA471 sglA1*	UV irradiation of DK101	9
DK473 (5066)	*asgA473 sglA1*	UV irradiation of DK101	9
DK476 (4564)	*asgA476 sglA1*	UV irradiation of DK101	9
DK480 (4312)	*asgB480 sglA1*	UV irradiation of DK101	9
DK495 (5067)	*asgA495 sglA1*	ICR191[c] of DK101	9
DK516 (5068)	*asgA516 sglA1*	Ethyl methanesulfonate treatment of DK101	9
DK739 (5069)	*asgA739 sglA1*	ICR191 of DK101	9
DK752 (5070)	*asgA752 sglA1*	ICR191 of DK101	9[b]
DK753 (5071)	*asgA753 sglA1*	ICR191 of DK101	9
DK756 (5072)	*asgA756 sglA1*	ICR191 of DK101	9
DK762 (5073)	*asgA762 sglA1*	UV irradiation of DK101	9[b]
DK767 (4566)	*asgC767 sglA1*	UV irradiation of DK101	9[b]
DK777 (5074)	*asgA777 sglA1*	UV irradiation of DK101	9[b]
DK1218	*cglB2*	DK1622 (Mx8) × DK321 → Sgl$^+$[d]	11
DK1622	Wild type	Transduction of DK1217 → Agl$^+$	14
DK4312	Tn*5 lac-4411 sglA1 asgB*	DK4411 (Mx8) × DK480 → Km^r Asg$^-$	19
DK4324	Tn*5 lac-4521 sglA1 asgB*	DK4521 (Mx8) × DK480 → Km^r Asg$^-$	19
DK4396	Tn*5 lac-4411 sglA1 asgB*	DK4312 (Mx8) × DK101 → Km^r Asg$^-$	This work
DK4397	Tn*5 lac-4411 sglA1*	DK4312 (Mx8) × DK101 → Km^r Asg$^+$	This work
DK4398	Tn*5 lac-4411 asgB*	DK4312 (Mx8) × DK1622 → Km^r Asg$^-$	19
DK4399	Tn*5 lac-4411*	DK4312 (Mx8) × DK1622 → Km^r Asg$^+$	19
DK4411	Tn*5 lac-4411*	P1::Tn*5 lac* × DK1622 → Km^r	16
DK4521	Tn*5 lac-4521*	P1::Tn*5 lac* × DK1622 → Km^r	16
DK4560	Tn*5*-4560 *sglA1*	Tn*5* pool (Mx8) × DK476 → Km^r Asg$^+$	This work
DK4561	Tn*5*-4561 *sglA1*	Tn*5* pool (Mx8) × DK767 → Km^r Asg$^+$	This work
DK4564	Tn*5*-4560 *sglA476 asgA*	DK4560 (Mx4) × DK476 → Km^r Asg$^-$	This work
DK4566	Tn*5*-4561 *sglA1 asgC*	DK4561 (Mx4) × DK767 → Km^r Asg$^-$	This work
DK5054	Tn*5*-4560 *sglA1*	DK4564 (Mx8) × DK101 → Km^r Asg$^+$	This work
DK5055	Tn*5*-4560 *sglA1 asgA476*	DK4564 (Mx8) × DK101 → Km^r Asg$^-$	This work
DK5056	Tn*5*-4560	DK4564 (Mx8) × DK1622 → Km^r Asg$^+$	This work
DK5057	Tn*5*-4560 *asgA476*	DK4564 (Mx8) × DK1622 → Km^r Asg$^-$	This work
DK5058	Tn*5*-4561 *sglA1*	DK4566 (Mx8) × DK101 → Km^r Asg$^+$	This work
DK5059	Tn*5*-4561 *sglA1 asgC*	DK4566 (Mx8) × DK101 → Km^r Asg$^-$	This work
DK5060	Tn*5*-4561	DK4566 (Mx8) × DK1622 → Km^r Asg$^+$	This work
DK5061	Tn*5*-4561 *asgC*	DK4566 (Mx8) × DK1622 → Km^r Asg$^-$	This work
DK5090	Tn*5*-5090 *sglA1*	Tn*5* pool (Mx4) × DK476 → Km^r Asg$^+$	This work

[a] The numbers in parentheses refer to DK strains which were constructed by introduction of a Tn*5* or Tn*5 lac* marker which is linked to the *asg* mutation of that strain. These strains retain the *asg* mutation.

[b] These strains are described but not referred to by strain numbers by Hagen et al. (9).

[c] ICR191 is a frameshift mutagen.

[d] Strain constructions are described in an abbreviated form. For DK4560, an Mx8 phage stock which was grown on a pool of insertions in DK1622 was used to infect DK476, and transductants were screened for the wild-type Asg$^+$ phenotype. Km^r before another phenotype designation indicates that selection for Km^r was applied before screening.

mented again, and suspended in TPM buffer to a calculated density of 1,000 Klett units or approximately 5×10^9 cells per ml, and 20-μl portions were spotted on TPM agar plates containing 1.5% Bacto-Agar (Difco Laboratories) in TPM buffer. The plates were incubated at 32°C for 3 days. TPM agar is also referred to as starvation agar in the text.

For development on an amino acid-free solid surface, cells were washed, suspended in MC7 buffer (10 mM morpholinepropanesulfonic acid [MOPS; pH 7.0], 1 mM $CaCl_2$), and spotted on MC7 agarose plates (1% electrophoresis grade agarose [J. T. Baker Chemical Co.] in MC7 buffer). MC7 buffer was chosen to allow comparison with development in submerged culture. Submerged culture development was performed by a modified procedure of Kuner and Kaiser (18). Cells were prepared as for development on MC7 agarose, and 25 μl of the washed cell suspension was added to 375 μl of MC7 buffer in wells of 24-well tissue culture plates (Falcon; Becton Dickinson Labware). The plates were incubated in a humid chamber at 32°C for 3 days. Under these conditions, wild-type cells settle to form a thin mat on the bottom of a well and develop with timing similar to that seen on TPM agar. Although *asg* mutants have a defect in cell-cell cohesion and fall out of buffer suspensions more slowly than do wild-type strains (see Results), in practice, *asg* mutant cells can sediment through the short buffer height of a submerged culture well (1 to 2 mm) and form a mat within a few hours.

Production of heat-resistant and sonication-resistant spores was assayed as follows. For TPM agar development, one spot of cells was scraped from the agar with a triangular inoculating loop and suspended in 1 ml of MC7. The suspension was incubated for 2.25 h in a water bath at 50°C and then sonicated for a total of 45 s in three pulses of 15 s each at full power with a microtip-equipped sonicator (Heat Systems-Ultrasonics) to disrupt fruiting bodies and break nonspore cells. Tenfold dilutions of the resulting suspensions were made in MC7, and viable spores were measured by determining the number of CFU on CTT agar. (The resulting

suspensions were agitated vigorously in a bath sonicator just before dilution and plating to disrupt clumps of spores.) For spore assays of submerged culture development, 0.6 ml of MC7 was added to each well, and the tissue culture plate was incubated for 2.25 h with its lower, outside surface immersed in a water bath at 50°C. Sonication was performed by placing the sonic probe in each well of the tissue culture plate. Sonic treatment and determination of the number of viable spores were as described above.

For extracellular complementation of development, *asg* mutant cells were prepared as described above for development and mixed in a 1:1 ratio with wild-type cells, and the cell mixture was placed in tissue culture wells for submerged culture or spotted on TPM agar. After 3 days of incubation, viable spores were determined by CFU assay as described above. Colonies deriving from *asg* mutant spores were distinguished from wild-type colonies by their morphology and color (see below and Results). The number of viable spores produced by the mutant or the wild-type cells was doubled for direct comparison with controls containing twice the number of wild-type cells alone.

Genetic methods. Transduction with Mx8 or Mx4 was performed as described by Sodergren and Kaiser (30), except that when phage Mx4 was used its stocks were propagated on bacteria that were growing on 1/2CTT agar (TPM buffer containing 0.5% Casitone [Difco] and 1.5% Bacto-Agar [Difco]). Plates were overlaid to a final kanamycin sulfate concentration of 70 μg/ml about 18 h after plating to select kanamycin-resistant (Km^r) transductants. These were picked to CTT agar containing 40 μg of kanamycin sulfate per ml. The capacity to form fruiting bodies was scored by transferring approximately 10^8 cells from growth plates to TPM agar. These plates were examined for darkened fruiting bodies after incubation at 32°C for 3 days.

The colony color of *asg* mutants was sometimes used for visual selection or scoring. Wild-type *M. xanthus* displays phase variation in colony color between tan and yellow (5). In contrast, colonies of 12 of the 18 original *asg* mutants were either tan or faintly yellow and did not show variation to bright yellow colonies (9, 20). It was noticed early that when these tan, sporulation-deficient strains were transduced to asg^+ the resulting sporulation-proficient transductants all formed bright yellow colonies. Conversely, when *asg* mutations were transduced into yellow-tan, sporulation-proficient (wild-type) strains the color phenotype (stable tan, stable faint yellow, or phase-variable yellow-tan) of the particular mutant donor was cotransduced 100% with the *asg* mutation. With the exception of DK412 and DK418, the transductions described in Results yielded only tan or faintly yellow *asg* transductants. Strains DK412 and DK418 carry *asgA* and yet give yellow colonies. *asgC767* showed a variation on the general theme. When this mutation was transduced into DK101 or DK1622, the expected percentage of *asg* transductants was obtained, and in DK101, *asgC767* produced stable tan colonies. However, in DK1622, *asgC767* transductants were initially tan, but they gave rise to bright yellow variants when streaked out and transferred. For comparison with the other *asg* mutants, a tan phase variant of *asgC767* in the DK1622 background (DK5061) was used.

To isolate Tn*5* insertions near putative *asg* loci, 513 independent insertions (isolated by K. Mayo) in asg^+-carrying strain DK101 were examined by the method of Kuner and Kaiser (17). Mx8 phage stocks grown on 13 pools of Tn*5* insertion strains (34 to 58 insertion strains per pool) kindly provided by K. Mayo were used to transduce DK476 and DK767 to Km^r. We screened 240 transductants derived from each pool for colony color and development on TPM agar. Single-colony isolates of developmentally competent transductants were retested for development. Linkage of Tn*5* insertions to *asg* mutations was confirmed by additional transductions with Mx4 into the parental *asg* mutants.

Cloning the *asgA* locus. Six additional pools of Tn*5* insertion strains (2,980 new Tn*5* insertions; half of which were provided by H. Kaplan) were screened for Tn*5* linked to *asgA*. Since each pool contained about 500 insertions, only one unique Tn*5* insertion linked to *asgA* was expected for each pool (17, 30), so only one Tn*5* insertion was characterized from each pool. The cotransduction frequencies observed for these new Tn*5* insertion markers and either $asgA476^+$ or $asgA516^+$ were 5, 8, 9, 27, 89, and 89%. Tetracycline resistance (Tc^r) was substituted for Km^r in the two 89% linked markers (Ω5090 and Ω5091) by the method of Avery and Kaiser (3). Crosses between the Km^r version of Tn*5* at Ω5090 and the Tc^r version of Ω5091 and vice versa yielded no recombinants in over 200 transductants tested. Subsequent cloning experiments revealed that these two insertions are about 200 base pairs apart. The DNA flanking Ω5090 in its Tc^r form was cloned by the method of R. Gill (8).

Plasmid DNA was introduced into *Myxococcus* isolates by electroporation by a procedure worked out by J. Rodriguez (manuscript in preparation). Briefly, exponentially growing cells were washed twice at 4°C in electroporation buffer (5 mM potassium phosphate [pH 7.6], 272 mM sucrose, 15% [vol/vol] glycerol) and suspended to about 5×10^9 cells per ml in the same buffer. Five micrograms of DNA was added to 0.6 ml of cell suspension in 5 μl of TE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA), and this mixture was subjected to an electrical field of 6,250 V/cm in a Gene Pulser (Bio-Rad) with a capacitance setting of 25 μF. The treated suspension was immediately diluted in 1 ml of CTT broth and then plated on CTT agar containing 40 μg of kanamycin sulfate per ml. Within 18 h the plates were overlaid to a final concentration of 70 μg of kanamycin sulfate per ml. Colonies appeared after 4 days of incubation at 32°C.

Pili. Cells which had pili at one end or the other were scored by electron microscopy of negatively uranyl acetate-stained cells (10). The cells were grown in CTT liquid medium.

Cohesion assay. Cell-cell cohesion was measured by the method of Shimkets (26) with minor changes. Exponentially growing cells were sedimented (6,000 × *g*, 10 min, 4°C), washed in MC7 buffer, sedimented, and suspended to approximately 5×10^8 cells per ml (100 Klett units) in MC7 buffer with $MgCl_2$ added to 1 mM. The suspension was left without agitation at room temperature. The turbidity of the upper part of the suspension was measured at various times in a Klett-Summerson colorimeter equipped with a red filter. A cell-free wash fluid containing a factor able to restore cohesion to *asg* mutants was prepared as described for A-factor in the next paragraph. For use in the cohesion assay, these supernatants were lyophilized, suspended in 0.1 volume with distilled water, and dialyzed against two changes of 40 volumes of MC7 buffer at 4°C. Congo red binding was determined as described by Arnold and Shimkets (1), with 5, 10, and 25 μg of Congo red per ml.

A-factor production and assay. A-factor was harvested from developing cells as described by Kuspa et al. (19). Briefly, cells growing exponentially in CTT liquid were washed in 0.5 volume of cold (4°C) MC7 buffer and suspended in room temperature MC7 buffer at about 5×10^9 cells per ml. This cell suspension was incubated at 32°C with

vigorous shaking (300 rpm), and after 2 h a cell-free supernatant was harvested by centrifugation at 10,000 × *g* for 15 min. This material is called crude A-factor.

A-factor was assayed by measuring the restored expression of an *asg*-dependent gene in an *asg* mutant strain. The *asg* mutant strain used in the assay, DK4324, contained a developmentally regulated gene which is normally expressed in wild-type cells early and was transcriptionally fused to *lacZ* by insertion of Tn*5 lac* (19). Expression of this gene, and consequently of β-galactosidase, depends on *asg*$^+$ function or, in an *asg* mutant, on exogenous A-factor. In practice, exponentially growing test cells, DK4324, were washed by suspension of growing cells in 1 volume of MC7 buffer at room temperature. After 10 min, the cells were sedimented and suspended to a calculated density of 5×10^9 cells per ml (1,000 Klett units) in MC7, and 25 μl of the concentrated suspension was distributed to each well of a 24-well tissue culture plate. Each well was then brought to 0.4 ml (final volume) with MC7 buffer or crude A-factor diluted in MC7 buffer. These tissue culture plates were incubated for 20 h at 32°C in a humid chamber. β-galactosidase activity was determined by hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside. A-factor units were defined as the amount of factor required to produce 1 U of β-galactosidase (1 nmol of *o*-nitrophenyl-β-D-galactopyranoside per min for an entire test well containing 1.25×10^8 cells) above the background (19). To compare the activities of different extracts, appropriate dilutions of each extract were assayed and the linear portion of an activity-versus-volume curve was used to estimate A-factor activity.

RESULTS

Three *asg* loci. Hagen et al. (9) identified 18 mutants belonging to synergism group A, the *asg* mutants. Previous crosses had shown that Tn*5 lac* insertion Ω4411 is linked to *asg-480* (the *asg* mutation in DK480) but not to *asg-476* (19). Recently, K. A. Mayo and D. Kaiser (Mol. Gen. Genet., in press) isolated a Tn*5* insertion, Ω4678, tightly linked to *asg-480* and loosely linked to Tn*5 lac* at Ω4411. Fourteen other *asg* mutations in the Hagen collection were found to be too remote from this insertion to be cotransduced with it (Mayo and Kaiser, in press). Therefore, *asg-480* defines one of several *asg* loci.

To locate the other *asg* loci, selectable genetic markers near other *asg* mutations were needed. Accordingly, 513 independent and random insertions of Tn*5* in wild-type *Myxococcus* strains were screened for linkage to mutations *asg-476* and *asg-767*, two *asg* mutations not linked to Ω4678. Km^r (the marker carried by Tn*5*) transductants were screened for cotransduction of *asg*$^+$, i.e., for acquisition of the ability to develop normally and of yellow colony color. The *asg* mutants are tan (9, 20) and cannot undergo normal development, while their *asg*$^+$-carrying parents are yellow (capable of phase variation) and developmentally competent. Experience showed that *asg* mutations (versus *asg*$^+$) always cosegregated with tan (versus yellow) colony color. Thus, the finding of yellow colonies on a kanamycin plate indicated successful transduction of *asg*$^+$. When yellow colonies were found in a particular cross, many colonies were picked from that cross without bias for yellow or tan color to test developmental competence. Developmentally competent transductants were found for both strains DK476 and DK767, which carry the original *asg-476* and *asg-767* mutations. The suspected linkage of these new Tn*5* insertions to the *asg* mutations was tested by transduction of those insertions from developmentally competent transductants back into the parental *asg* mutants. Thus, Tn*5* Ω4560 was demonstrated to be linked to *asg-476* and Tn*5* Ω4561 was found to be linked to *asg-767*.

TABLE 2. Cotransduction tests of *asg* mutations with Tn*5* Ω4560, Tn*5* Ω4561, and Tn*5* Ω5090

Recipient strain	No. of asg$^+$-carrying transductants/total no. of Km^r transductants tested for Asg phenotype (% cotransduction) with the following transductional donor		
	DK4560 (Tn*5* Ω4560)	DK4561 (Tn*5* Ω4561)	DK5090 (Tn*5* Ω5090)
DK412	50/359 (14)	0/104 (<1)	
DK418	54/406 (13)	0/120 (<0.8)	
DK471	59/360 (16)	0/110 (<0.9)	
DK473	49/299 (16)	0/120 (<0.8)	
DK476	118/823 (13)	0/120 (<0.8)	692/778 (89)
DK495	40/237 (17)	0/120 (<0.8)	
DK516	29/228 (13)	0/120 (<0.8)	714/801 (89)
DK739	46/240 (19)	0/120 (<0.8)	
DK752	47/240 (19)	0/118 (<0.8)	
DK753	44/240 (18)	0/120 (<0.8)	
DK756	49/345 (14)	0/120 (<0.8)	
DK762	37/240 (15)	0/115 (<0.9)	
DK777	37/239 (16)	0/120 (<0.8)	
DK480	0/120 (<0.8)	0/119 (<0.8)	
DK767	0/480 (<0.2)	527/800 (66)	

Tn*5* Ω4560 and Tn*5* Ω4561 were transduced from wild-type strains into 15 *asg* strains to determine which other mutations are linked to these two Tn*5* markers. Of the 15 *asg* mutations tested, 13 were linked to Tn*5* at Ω4560 (Table 2). The cotransduction frequency of *asg*$^+$ with Tn*5* Ω4560 was 13 to 19% of the Km^r transductants for these 13 *asg* mutations. Since the cotransduction frequencies are similar, these 13 mutations linked to Tn*5* Ω4560 define a locus we call *asgA*. *Asg-480* was not linked to Tn*5* Ω4560 or Tn*5* Ω4561 (Table 2), confirming results showing that this mutation is not linked to 15 *asg* mutations in the Hagen collection (Mayo and Kaiser, in press). Therefore, *asg-480* defines a second locus, *asgB*, which is linked to Tn*5 lac* Ω4411. *asg-767* is the only mutation among the 15 tested that was linked to Tn*5* Ω4561 and was cotransduced with Tn*5* Ω4561 in 66% of the Km^r transductants of DK767, the strain containing *asg-767* (Table 2). Cotransduction of *asg*$^+$ with Tn*5* Ω4561 was not detected when any of the other 14 *asg* mutants were used as recipients. Thus, *asg-767* defines a third distinct locus, *asgC*.

Linkage of Ω4560 and Ω4561 to the *asgA* and *asgC* mutations was confirmed by transfer of *asg* mutant alleles into wild-type genetic backgrounds by cotransduction with the appropriate Tn*5* (Table 3). In these experiments, *asg* mutants containing a linked Tn*5* insertion were used as transductional donors in crosses with the developmentally competent (*asg*$^+$carrying) strains DK101 and DK1622 as recipients. Cotransduction of Tn*5* Ω4560 and *asgA* was obtained for each of the *asgA* alleles at about the same frequency as cotransduction of the *asgA*$^+$ alleles (compare Tables 2 and 3). *asgC767* was cotransduced with Tn*5* Ω4561 in about 60% of the transductants analyzed (Table 3), which also agrees roughly with the frequency of *asgC*$^+$ and Tn*5* Ω4561 cotransduction (Table 2). Table 3 also shows that *asgB480* cotransduced with Tn*5 lac* Ω4411 into DK1622 and DK101 at an average frequency of 26%, which agrees with the frequency previously reported for *asgB*$^+$ and Tn*5 lac* Ω4411 (19). In sum, all of the cotransduction data agree that Tn*5* Ω4560, Tn*5 lac* Ω4411, and Tn*5* Ω4561 are linked to three distinct *asg* loci: *asgA*, *asgB*, and *asgC*.

TABLE 3. Introduction of *asg* mutations into *asg*$^+$-carrying strains

Transductional donor (genotype)	No. of *asg*-carrying transductants/total no. of Km^r transductants tested for Asg phenotype (% cotransduction) with the following transductional recipient:	
	DK1622	DK101
DK5063 (*asgA418* Tn*5* Ω4560)	6/62 (10)	
DK5065 (*asgA471* Tn*5* Ω4560)	12/139 (9)	
DK5066 (*asgA473* Tn*5* Ω4560)	10/91 (11)	
DK4564 (*asgA476* Tn*5* Ω4560)	14/182 (8)	23/200 (12)
DK5067 (*asgA495* Tn*5* Ω4560)	5/63 (8)	
DK5068 (*asgA516* Tn*5* Ω4560)	5/39 (13)	
DK5069 (*asgA739* Tn*5* Ω4560)	10/96 (10)	
DK5070 (*asgA752* Tn*5* Ω4560)	16/101 (16)	
DK5071 (*asgA753* Tn*5* Ω4560)	18/146 (12)	
DK5072 (*asgA756* Tn*5* Ω4560)	8/38 (21)	
DK5073 (*asgA762* Tn*5* Ω4560)	9/58 (16)	
DK5074 (*asgA777* Tn*5* Ω4560)	6/56 (11)	
DK4312 (*asgB480* Tn*5* *lac* Ω4411)	102/374 (27)	30/129 (23)
DK4566 (*asgC767* Tn*5* Ω4561)	153/249 (61)	249/422 (59)

If all of the *asgA* alleles occupy a locus that is linked to an insertion of Tn*5*, then it should be possible to clone the *asgA* locus and use Tn*5* to identify the proper piece of DNA (Materials and Methods). For this purpose, another Tn*5* insertion (Ω5090) which is more tightly linked (89% cotransduction with *asgA*) was isolated (Materials and Methods; Table 2). The DNA from the left and right sides of Tn*5* Ω5090 was cloned into two plasmids, pAK100 and pAK104, in *Escherichia coli* (Fig. 1). Each plasmid was used to transform strains containing alleles of *asgA* by electroporation as described in Materials and Methods. We suspect that electroporation of a plasmid which is unable to replicate in *M. xanthus* leads to plasmid integration and formation of a tandem duplication of the cloned DNA segment, as demonstrated for plasmids introduced with bacteriophage P1 (29, 31). Electroporation of plasmid pAK100, which contains 14-kilobases of *Myxococcus* DNA, generated Km^r, fruiting-proficient transformants of all 13 *asgA* mutant strains, while pAK104 generated none (Table 4). Therefore, all of the *asgA* mutations are on the same side of Tn*5* Ω5090 and within 14 kilobases of it.

To test whether the fruiting-proficient transformants carried duplications of *asgA*, as expected, selection for Km^r was relaxed, allowing duplication-carrying strains to segregate. Transformants DK5102 (from DK476) and DK5103 (from DK516) were grown without selection for 20 generations and then plated, and colonies were screened for sensitivity to kanamycin (Km^s) and the capacity to form

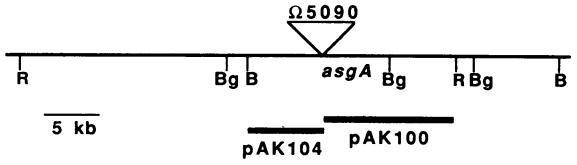

FIG. 1. The *asgA* region. The locations of Tn*5* Ω5090, various restriction sites (R, *Eco*RI; B, *Bam*HI; Bg, *Bgl*II), and the approximate location of the *asgA* locus are shown. The locations of restriction sites were determined by Southern analysis of Tn*5* Ω5090-containing strains and with additional plasmid clones which are not described. kb, Kilobases.

TABLE 4. Identification of the *asgA*$^+$-carrying clone

Recipient strain	% of *asgA*$^+$-carrying transformants (total no. of Km^r transformants) with the following plasmid:	
	pAK100	pAK104
DK412	92 (104)	
DK418	97 (93)	
DK471	97 (98)	
DK473	97 (93)	
DK476	96 (94)	<8 (12)
DK495	96 (102)	
DK516	96 (180)	<0.7 (150)
DK739	96 (103)	
DK752	97 (95)	
DK753	94 (106)	
DK756	91 (92)	
DK762	97 (160)	
DK777	99 (100)	

[a]CsCl-purified plasmid DNA (5 μg) was used to electroporate 3 × 10^9 cells (see Materials and Methods), and 100 to 300 transformants were obtained per μg of DNA.

fruiting bodies. One of four DK5102 Km^s segregants and two of six DK5103 Km^s segregants were fruiting deficient, indicating that the *asgA* mutant alleles were present in the original transformants and that they are recessive to *asgA*$^+$.

What are the functions of *asgA*, *asgB*, and *asgC*? Previous studies which had used *asg-480* to represent the entire *asg* group could not have resolved this issue (9, 20). Furthermore, the *asg* mutants were derived by mutagenesis of strain DK101, which contains a mutation (*sglA1*) that decreases their motility, and the mutant phenotypes were examined in the original mutagenized background because linked markers that would have permitted out-crossing were not available. This left ambiguity as to which aspects of the described phenotype were caused by the *asg* mutations and which were caused by *sglA1* or other mutations which happened to be present in the mutant isolates. To remove these ambiguities, a set of isogenic strains was constructed (Table 3) which contained each of the *asg* mutations in an otherwise DK1622 genetic background. We expect that the *asg* mutations in these strains have been purified away from *sglA1* and from >99% of the mutagenized genome of the original mutant isolates. (The DNA capacity of transducing myxophage Mx8 is <1% of the genome of *M. xanthus*.) These strains permit a close comparison of the effects of *asg* mutations within the same locus and those of *asg* mutations in different loci.

Aggregation. During development of wild-type *M. xanthus* on starvation agar, cells aggregated into nearly symmetric translucent mounds by 14 h (Fig. 2A). Translucent mounds matured as their constituent cells sporulated, becoming darkened fruiting bodies by 72 h. In submerged culture (18), the sequence of events in fruiting body development was similar to that on agar, but development was more rapid; aggregates usually formed 2 to 4 h sooner in submerged cultures, and fruiting bodies darkened by 24 h.

Figure 2 illustrates the development of *asg* mutants. None of the *asgA* mutants were able to complete aggregation (Fig. 2B). They did form some loose aggregates at the edges of their cell spots on agar, but none of those loose aggregates darkened, even after 6 days of incubation. No aggregation of *asgA* mutant cells was apparent in submerged culture (Fig. 2B); the cell mat remained flat and featureless.

asgB mutant cells did form aggregates on starvation agar, but they were slower to form and were structurally less

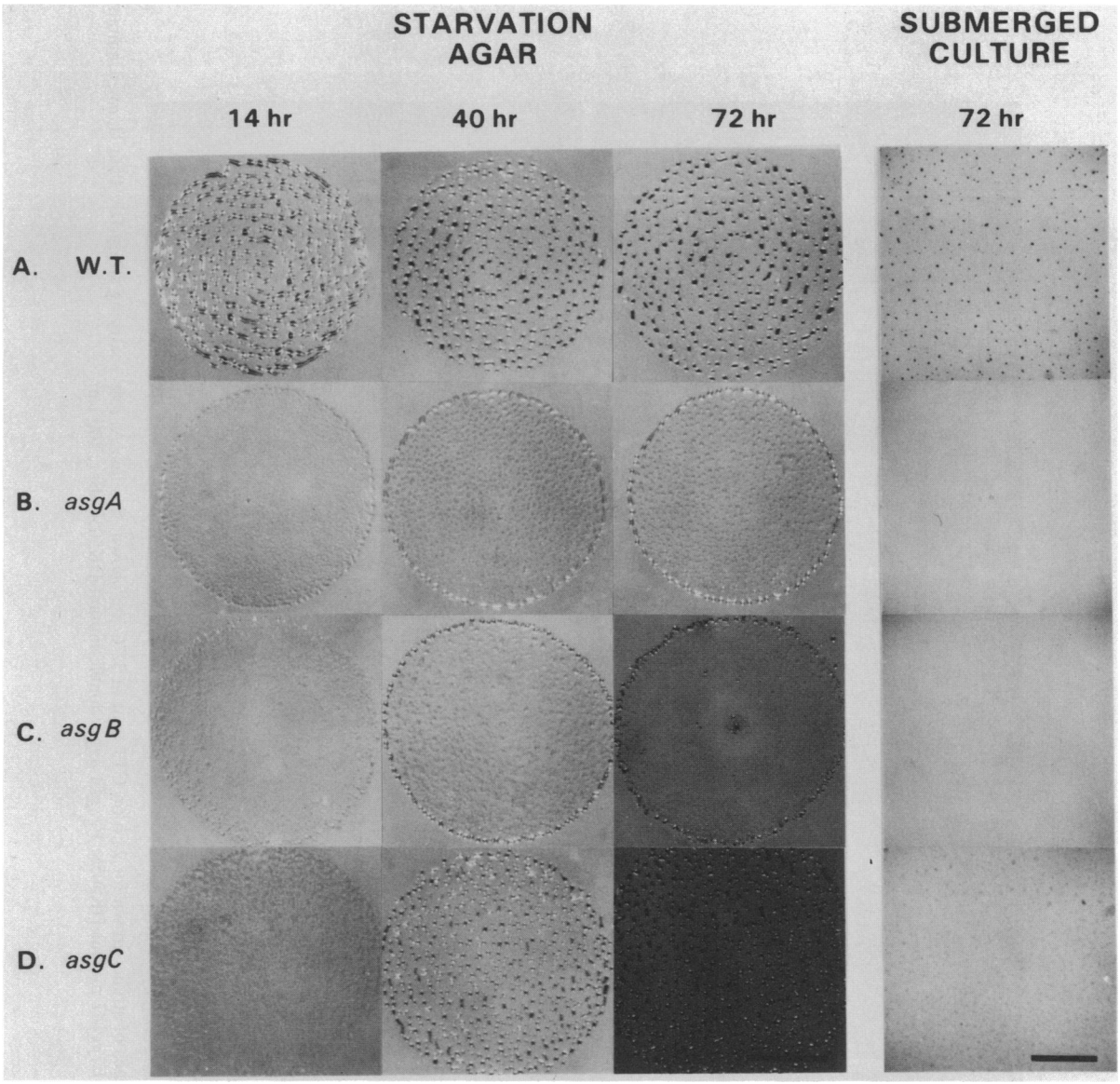

FIG. 2. Developmental morphology of *asg* mutants. The development of DK5060 (A), DK5057 (B), DK4398 (C), or DK5061 (D) cells incubated at 32°C for various times on starvation agar or in submerged culture is shown. On agar, cells were inoculated onto starvation (TPM) agar as 20-μl spots at a density of 5×10^9 cells per ml. For submerged culture, 1.25×10^8 cells were inoculated into a 1.7-cm-diameter well containing starvation (MC7) buffer. Bars, 2.5 mm. W.T., wild type.

compact with less-well-defined borders than in the wild type (Fig. 2C). These structures did eventually darken somewhat, but they never became like wild-type fruiting bodies. In submerged culture, the *asgB* mutant underwent no detectable morphological development.

asgC mutant cells formed fruiting bodies on starvation agar at 72 h that looked normal (Fig. 2D). However, the timing of their aggregation was delayed. The *asgC* mutant first formed translucent mounds at least 12 h after mounds were observed for the wild type (compare Fig. 2A and D). As with the other *asg* mutants, the developmental defect of the *asgC* mutant was more striking in submerged culture than on agar. In submerged cultures, the *asgC* mutant displayed limited aggregation, but the limited aggregates never darkened or became sharply defined (Fig. 2D). Evidently, none of the three types of *asg* mutants aggregate normally. *asgA*, *asgB*, and *asgC* mutants differed in the timing and extent of residual aggregation.

Sporulation. The capacities of the three types of *asg* mutants to form myxospores were compared after development on starvation agar or in submerged culture. Viable spores formed under these conditions were assayed by determining the number of CFU in a sample of cells in which nonspore cells were destroyed by heat and sonication (Materials and Methods). The results are shown in Table 5. By and large, the sporulation level paralleled the extent of morphological development as described in the previous section. On starvation agar, the *asgA* mutant produced less than 10 spores per spot of cells (10 is the limit of detection for these experiments). Six other *asgA* alleles were tested with the same result (data not shown). On agar, the *asgB* mutant produced 43% and the *asgC* mutant produced 100% of the

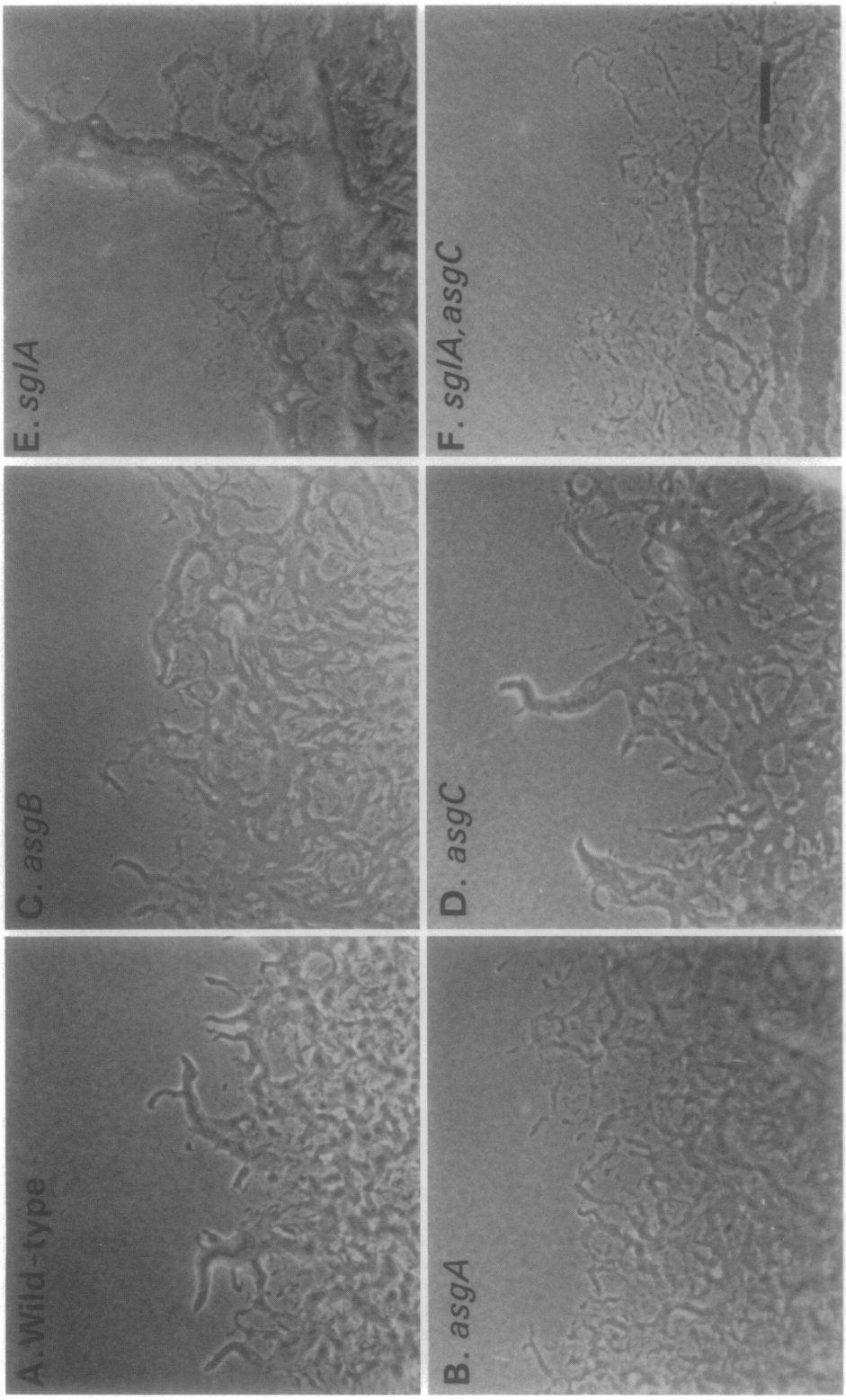

FIG. 3. Motility of *asg* mutants. Photographs of colony edges of strains grown for 24 h on CTT agar at 32°C are shown. Strains: A, *asg*$^+$-carrying strain DK5056; B, *asgA* mutant DK5057; C, *asgB* mutant DK4398; D, *asgC* mutant DK5061; E, *sglA1* mutant DK5058; F, *sglA asgC* double mutant DK5059. Bar, 100 μm.

TABLE 5. Sporulation of *asg* mutants

Developmental condition and locus	% of viable spores[a]		
	asg mutant	Extracellular complementation (1:1 cell mixture)	
		asg mutant	Wild type
Starvation agar			
asgA	0.0004	29	1.5
asgB	43	650	27
asgC	100	1,100	19
Submerged culture			
asgA	0.0002	7.0	3.0
asgB	0.0003	59	14
asgC	2.0	45	18

[a] Heat- and sonication-resistant viable spores were assayed after 3 days of development by the ability to form colonies on CTT agar. Each mutant was compared with a congenic wild-type strain. The strains used were as follows: for *asgA*, DK5057 and DK5056 (*asgA*$^+$); for *asgB*, DK4398 and DK4399 (*asgB*$^+$); for *asgC*, DK5061 and DK5060 (*asgC*$^+$). Spore levels are given as percentages of the *asg*$^+$-carrying control, which ranged from 2×10^6 to 5.6×10^6 spores per 10^8 input vegetative cells. Thus, the number of spores produced by the wild type alone under each experimental condition was 100%.

number of spores produced by the wild type. As was observed for aggregation, the *asgB* and *asgC* mutants made fewer spores in submerged culture development than an agar (Table 5); no sporulation of the *asgB* mutant was detectable, and the *asgC* mutant produced only 2% of the wild-type level of spores.

Given the number of spores which *asgB* and *asgC* mutants produce on starvation agar (Table 5), it is not apparent why they would have been picked up in screening for sporulation-deficient mutants on starvation agar (9). Since the original screening had been performed on strain DK101, which carries the *sglA1* mutation, the effect of *sglA1* on the sporulation of *asg* mutants was examined. It was found that strains containing the *sglA1* and *asgB* or the *sglA1* and *asgC* mutations produced 10 to 20% the *asg*$^+$ *sglA1* (DK101) level of spores during development on starvation agar. Thus, the *sglA1* mutation enhanced the *asgB* and *asgC* sporulation defects even though there was no difference in spore number between *sglA1* and *sglA1*$^+$. This enhancing effect of *sglA1* on the phenotype of strains that carry *asg* may explain why the *asgB* and *asgC* mutations were isolated along with the stronger *asgA* alleles.

To test whether the striking difference between *asgB* mutant sporulation on agar (43%) and in submerged culture (0.0003%) might be due to the difference in the composition of the media used under the two conditions (Table 5), the *asgB* mutant was allowed to develop on the surface of a 1% agarose gel (agarose has a lower level of amino acids than commercial agar; 4) containing the submerged culture buffer, MC7 (Materials and Methods). With these changes in medium composition, the *asgB* mutant sporulated at 1% of the wild-type level. Therefore, the more severe sporulation defect of the *asgB* mutant in submerged culture than in starvation agar is in part related to the differences in chemical composition between the two media used, i.e., to buffer MC7 versus TPM and perhaps to substances, like amino acids, that are present in agar but absent from agarose (4).

Extracellular complementation for sporulation. The original *asg* mutants were isolated because their sporulation defect could be corrected by codevelopment with wild-type cells (9). To test whether this property is associated with the *asg* mutations themselves, the backcrossed *asg* mutants were assayed for extracellular complementation of their sporulation by wild-type cells. The *asg* mutants were each mixed 1:1 with wild-type cells and plated on starvation agar or allowed to develop in submerged culture. Under both conditions, the fruiting bodies produced by the mixtures morphologically resembled those of the wild type alone. The percentages of viable spores generated by the mixtures relative to wild-type controls are shown in the last two columns of Table 5. Each of the *asg* mutants produced at least 10 times more spores in mixtures with the wild type than by themselves (compare columns 1 and 2). The *asgA* mutant produced 7×10^4-fold more spores in the mixture than by itself during development on agar. Five other *asgA* mutants (*asgA418*, *asgA471*, *asgA473*, *asgA495*, and *asgA516*) were tested this way and showed comparable spore number increases (data not shown). The absolute level of sporulation of the mutants in the presence of wild-type cells ranged from 7 to 1,100% of that of the wild type alone in the same experiment. Since the rescue is at least 10-fold and sometimes 10^4-fold, it is likely that extracellular complementation is important for spore formation during normal wild-type development.

TABLE 6. A-factor release from *asg* mutants

Strain (genotype)	A-factor activity (U/ml)[a]	% Wild-type activity
DK5056 (*asgA*$^+$)	32	100
DK5057 (*asgA*)	1.6	5
DK4399 (*asgB*$^+$)	51	100
DK4398 (*asgB*)	0.70	2
DK5060 (*asgC*$^+$)	60	100
DK5061 (*asgC*)	2.7	4

[a] The A-factor activity released into a cell-free wash fraction was determined as described in Materials and Methods. The values shown are averages of three independent experiments.

Interestingly, all of the *asg* mutants depressed wild-type sporulation in the mixtures. The frequency of sporulation of wild-type cells in mixtures with *asg* mutants was always less than that of wild-type cells alone, which is set to 100% in Table 5. The *asgB* and *asgC* mutants reduced wild-type sporulation by 4- to 7-fold, and the *asgA* mutants reduced wild-type sporulation about 70-fold (Table 5; data not shown).

A-factor release and *asg* mutants. A-factor is a heat-labile substance which can rescue gene expression and sporulation of *asgB480* cells (19; unpublished data). A-factor is released by wild-type cells but not by DK480 or DK476 cells, beginning 1 to 2 h after initiation of their development (19). Strains containing mutations from each of the three *asg* loci were tested for the ability to release A-factor. Extracts were prepared from cells that were starving, and their A-factor activities were determined as described in Materials and Methods. The activity released from *asg* mutants was 5% or less of that released by wild-type cells (Table 6).

Colonies of *asg* mutants. As the backcrossed *asg* mutants were studied, it became evident that their colonies differed from those of *asg*$^+$-carrying organisms. Indeed, the tan color of *asg* mutant colonies had facilitated the isolation of linked insertions of Tn*5*. In addition to color, differences from *asg*$^+$-carrying organisms in the distribution of cells at the colony edge and the cohesiveness of *asg* mutants were found.

When the edges of *asg* mutant colonies were examined by phase-contrast microscopy, a consistent difference in cell distribution was observed compared with the wild type. The

edges of *asgA* mutant colonies had more individual cells and fewer groups of cells, and those groups had fewer cells in them than did those of the wild type (Fig. 3A). This alteration in cell distribution was less obvious in *asgB* mutants (Fig. 3C) and barely detectable in *asgC* mutants (Fig. 3D). However, the same type of alteration was clearly evident when the *asgC* mutation was combined with an *sglA1* mutation (compare Fig. 3F and E). An *sglA1* mutation was shown above to enhance the sporulation defect of *asg* mutants.

In the alteration just described, a decreased proportion of cells in groups relative to single cells, resembles the phenotype of a large class of motility mutants known as S^- (11). A genetic test was performed to see whether an *asg* mutant is a motility mutant of the S^- class. Mutation in any of some 10 different genes has been shown to yield S^- mutants (11), and the whole class has the property that addition of a second motility mutation belonging to the complementary class produces a completely nonmotile strain. Accordingly, *asgA*, *asgB*, and *asgC* mutations were each crossed into a strain that contained one of the complementary mutations, namely, DK1218. Transfer of an *asg* mutation was selected with the appropriate linked Tn*5*; however, all of the resulting transductants were motile. Specifically, the Km^r transductants that had received an *asg* mutation as judged by tan colony color were motile. (In these crosses, *asgA*, *asgB*, and *asgC* cotransduced with their respective Tn*5* markers at the expected frequencies.)

An independent test for an S^- motility mutation is the presence of pili at the ends of cells. All pilus-deficient mutants are S^-, and most S^- mutants are also deficient in pili (14). Accordingly, wild-type, *asgA*, *asgB*, and *asgC* strains were examined by negative staining and electron microscopy for the presence of pili. However, no differences between the wild type and the *asg* mutants were observed in the fraction of cell ends with pili (data not shown). The genetic test and the pilus counts agreed that although the distribution of cells at the edges of *asg* mutant colonies resembled that of S^- motility mutants, the *asg* mutants were not S^- and the *asg* loci do not belong to motility system S.

Cell-cell cohesion of *asg* mutants. Growing cells of *M. xanthus* are cohesive (26). When they are taken from growth medium, suspended in buffer containing calcium and magnesium ions, and left undisturbed, wild-type cells agglutinate. They form a macroscopic network of cells which settles to the bottom of the tube, leaving the upper liquid clear. Figure 4 compares the turbidity of the top of a suspension of wild-type cells with those of representative *asg* mutants. All of the *asg* mutant strains tested were found to settle more slowly than wild-type cells. Samples of cell suspensions examined by phase-contrast microscopy at various times during these tests revealed that wild-type cells first formed small clumps of about 50 cells and then the clumps formed a filamentous network of cells at about the time of the steep fall in turbidity. The *asg* mutants did form small clumps of cells, but those clumps did not link into a network. Eventually, the clumps of *asg* mutant cells did settle out of suspension, usually within 18 h compared with less than 1 h for the wild type. Cells containing the *sglA1* mutation showed about the same level of cohesion by this assay as did the *asgA* mutant shown in Fig. 4 (data not shown). Interestingly, cells containing an *sglA1* mutation and the *asgB* mutation did not cohere within 4 h and did not settle out of suspension even by 18 h (data not shown). *sglA1* (*sglA* does belong to motility system S; 11) also appeared to enhance the mutant defect of *asg*.

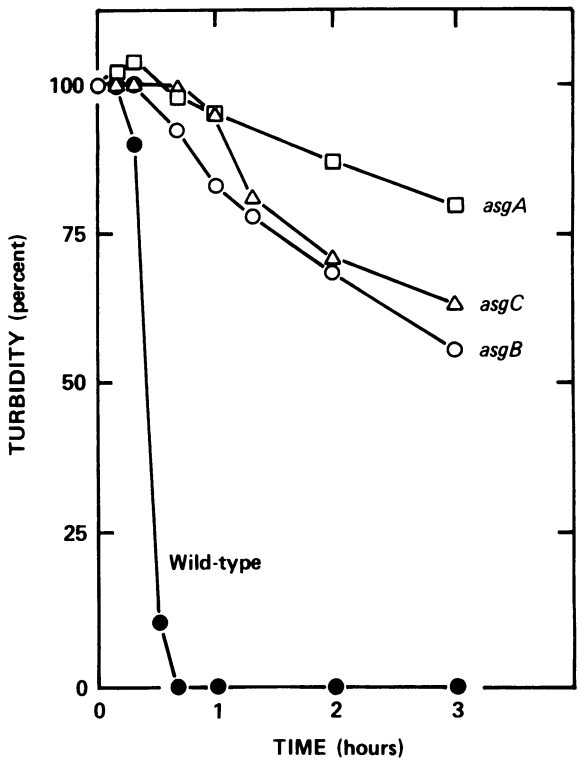

FIG. 4. Cell-cell cohesion of *asg* mutants. Cell cohesion was measured by monitoring the decrease in turbidity of a suspension of cells with a colorimeter. Cells were suspended in $CaCl_2$- and $MgCl_2$-containing buffer (see Materials and Methods) and incubated at room temperature without shaking. Strains: ●, asg^+-carrying DK1622; □, *asgA* mutant DK5057; ○, *asgB* mutant DK4398; △, *asgC* mutant DK5061. Each strain was tested in at least four independent experiments, and a representative experiment is shown.

Receptors for the diazo dye Congo red have been implicated in cell-cell cohesion in *M. xanthus* (2). Exposure of wild-type cells to Congo red blocks cohesion and inhibits fruiting body formation (1). In addition, *dsp* mutants, which are a class of S^- motility mutants, are noncohesive and lack Congo red receptors (1). Because the phenotype of *asg* mutants is similar to that of *dsp* mutants and wild-type cells treated with Congo red, binding of Congo red to the *asg* mutant was determined. Strains DK4399 (asg^+), DK5057 (*asgA*), DK4398 (*asgB*), and DK5061 (*asgC*) were tested for binding by the method of Arnold and Shimkets (1). The association constant for Congo red and all of these strains indicates that each of the *asg* mutants retains the high-affinity receptors previously described for *M. xanthus* (1). The association constants were measured to be 1.2×10^5 M^{-1}, 1.2×10^5 M^{-1}, 1.7×10^5 M^{-1}, and 0.62×10^5 M^{-1} for the wild type and the *asgA*, *asgB*, and *asgC* mutant strains, respectively. These are fairly close to the reported value for the wild type of 2.4×10^5 M^{-1} (1). The *dsp* mutants are reported to have an association constant of 8.5×10^3 M^{-1} (1). These results show that the *dsp* mutants and the *asg* mutants, though both are defective in cohesion, are nevertheless blocked in different ways.

To test whether substances released by wild-type cells might restore the ability of *asg* mutants to cohere, wash fluids from developing wild-type cells were added to mutant cells. Wash fluids prepared as described in Materials and

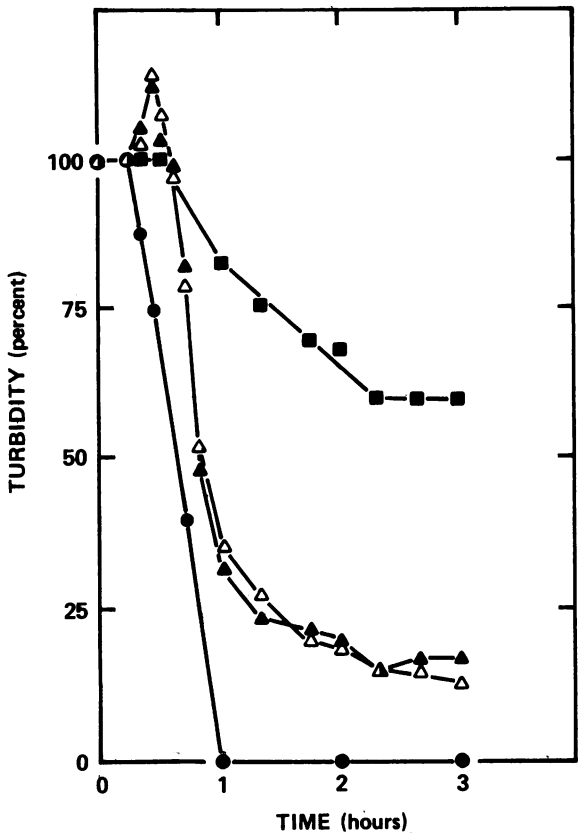

FIG. 5. Cohesion rescue of *asgB* mutant cells. Cell cohesion was measured for *asgB* mutant strain DK4398 as described in the legend to Fig. 4. Cells were suspended in buffer (■), a concentrated cell-free wash fraction from starving wild-type cells (△), or a wash fraction that had been boiled for 10 min (▲). Wild-type strain DK4399 (●), shown for comparison, was suspended in buffer.

Methods did restore, to some extent, the ability of *asgB* mutant cells to cohere (Fig. 5). The same wash fluid had little effect on the cohesion of wild-type cells. The wash fluid also restored the ability of an *sglA1* mutant to cohere (data not shown). The substance(s) responsible for the restoration is apparently heat stable, because similar results were obtained with wash fluids that were or were not heated for 10 min at 100°C. These results suggest that *asgB* mutants are defective in the production or release of a heat-stable substance which is involved in cell-cell cohesion. The heat stability of the substance distinguishes it from heat-labile A-factor.

DISCUSSION

asg mutants were obtained by mutagenesis and selection (9). Selection started with a round of fruiting body development and sporulation initiated at high cell density, which ensured that mutant cells would have asg^+ neighbors. Colonies formed by germination of individual spores from round 1 were then visually selected for loss of the ability to sporulate when separated from their neighbors. For sporulation, the resultant mutants were dependent on a function(s) that could be provided by adjacent wild-type cells. Mutants were also tested for the ability to rescue other mutants in mixtures. In this way, mutants that could be rescued by wild-type cells were divided into four extracellular complementation groups. Eighteen mutants were assigned to one of these groups, called *asg*.

The 15 *asg* mutations analyzed here proved to be genetically heterogeneous; they reside in three different loci: *asgA*, *asgB*, and *asgC*. Each locus is identified by one or several insertions of Tn*5* that are near enough to their *asg* mutations to cotransduce with them. For example Tn*5* Ω4560, Tn*5* Ω5090, and Tn*5* Ω5091 are all linked to *asgA*, and Tn*5* Ω4561 is linked to *asgC*. However, no linkage was detected among *asgA*, *asgB*, and/or *asgC* by cotransduction with myxophage Mx4 or Mx8. These phages could have shown cotransduction of sites closer than 50 kilobases (30). Most of the *asg* mutations (13) map to *asgA*, one maps to *asgB*, and one maps to *asgC*. The linked Tn*5* insertions facilitated backcrosses to bring all of the *asg* mutations into the same genetic background for comparison of their properties.

Against a standard genetic background, the developmental phenotype of *asg* mutants could be seen to have several facets. One facet of the *asg* phenotype is reduced aggregation—a reduced capacity to assemble into mounds. None of the *asg* mutants formed aggregated by 14 h on agar, while asg^+-carrying cells aggregated into well-defined mounds by this time. Similarly, none of the *asg* mutants aggregated in submerged culture. However, *asgB* and *asgC* mutants did aggregate on agar after a delay. The aggregation defect of the *asg* mutants could be related to the observed reduced cohesivity, as has been suggested for *dsg* mutants (27).

asg mutations also led to reduced levels of sporulation. A reduction in sporulation was more evident in submerged culture than on agar medium and was more pronounced for *asgA* than for *asgB* or *asgC*. Residual sporulation in the *asgB* and *asgC* backcrossed strains closely paralleled their residual aggregation. By 3 days on agar, *asgB* formed some aggregates and *asgC* produced (with a delay) an almost normal number of aggregates. This suggested that the sporulation defect of *asg* mutants was secondary to an earlier defect that also reduced or slowed aggregation. In any case, the defect which reduced sporulation in *asg* mutants could be overcome by added wild-type cells. It is therefore clear that the ability to be extracellularly complemented or synergized is associated with an *asg* mutation and does not depend on other adventitious properties of the original mutants. Under conditions that would induce asg^+-carrying cells to develop, *asgA*, *asgB*, and *asgC* mutants released less than 5% as much A-factor, by bioassay (Table 6), as did asg^+-carrying cells. This common A-factor deficiency would explain the failure of *asgA*, *asgB*, and *asgC* mutants to complement each other in cell mixtures and, thus, their inclusion in the same extracellular complementation group.

Is there one primary defect that might account for the multiplicity of developmental changes? Previous work has revealed a dependent regulatory pathway that governs the expression of many genes during *Myxococcus* development (15, 19). This pathway includes the *tps* gene, whose product, protein S, coats mature spores and has been extensively characterized as a development-specific protein (12, 23). The dependent pathway leads to spores, because operon fusions of developmentally regulated promoters to the *lacZ* gene from *E. coli* leads to accumulation of β-galactosidase trapped within spores (16). Whether specific aggregation functions, as well as sporulation functions, are connected to this dependent pathway is not known. In any case, release or exposure on the cell surface of A-factor is an essential step on this dependent pathway. A-factor appears to be required at around 2 h after development starts, to permit the expression of pathway genes. Thus, failure to sporulate could be explained by the absence of A-factor.

If A-factor is one substance, why are there three *asg*

genes? One possibility is that A-factor or a precursor of it is already present in cells at the start of development and that the pathway for release or exposure of A-factor contains at least three steps specified by *asgA*, *asgB*, and *asgC*. Alternatively, one of the *asg* genes, most likely *asgA*, since *asgA* mutants have the strongest phenotype, may be the A-factor structural gene, and the other two gene products may be involved in modifying product of the first. A choice between these alternatives and others awaits purification and identification of A-factor and comparison with the protein predicted from the base sequence of the *asg* genes. A-factor purification is under way.

The *asg* genes are expressed not only during development but also during growth. Their growth phenotype includes colony color; colonies formed by most *asg* mutants are permanently tan colored; asg^+-carrying colonies can be tan, but they can also give rise, at high frequency, to bright yellow colonies (5). A second part of their growth phenotype is a subtle change in colony morphology; colonies of *asg* mutants have relatively fewer clusters of cells at their periphery than do asg^+-carrying colonies. A decrease in cell clustering could be a consequence of the decreased cohesivity of *asg* mutant cells. *asgA*, *asgB*, and *asgC* mutant cells do cohere less well, as measured by the ability to form macroscopic networks that spontaneously settle out of suspension. A common element that could underlie the growth phase characteristics of *asg* mutants is an altered cell envelope. The pigment of phase variation is believed to be either membrane associated or extracellular (22); cohesivity is likely to reflect the structure of the cell surface (2), and the absence of extracellular A-factor may reflect a reduced capacity for export. Perhaps mutations *asgA*, *asgB*, and *asgC* alter export in different ways, thus accounting for differences in the properties of the three kinds of mutants.

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