Suppressors That Permit A-Signal-Independent Developmental Gene Expression in *Myxococcus xanthus*

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Progression through the early stages of *Myxococcus xanthus* fruiting body development requires the cell-to-cell transmission of soluble material called A signal. During these early stages, expression from the gene identified by Tn5 *lac* insertion Ω 4521 increases. A DNA probe of the Ω 4521 gene was constructed. Use of this probe showed that accumulation of mRNA corresponding to the Ω 4521 gene depends upon A signal. A-signal-deficient (*asg*) mutants fail to accumulate this RNA, and the external addition of A signal restores accumulation. To identify links between A signal and its responsive gene, Ω 4521, suppressors of an *asg* mutation were generated. All of the suppressor alleles restored *lacZ* expression from Ω 4521 in the absence of A signal, and they were demonstrated to be neither reversions of the *asgB* mutation nor mutations in the promoter of Ω 4521. Fifteen suppressor mutations map to two loci, *sasA* and *sasB* (for suppressor of *asg*). *sasA* and *sasB* mutants differ phenotypically during growth and development. Mid-logarithmic-phase *sasA asgB* double mutants, like *sas*⁺ *asg*⁺ strains, express low levels of *lacZ*, whereas *sasB asgB* double mutants express high levels. *sasA asg*⁺ mutants form abnormal colonies, are less cohesive than wild type, and are defective in fruiting body formation and sporulation. In contrast, *sasB asg*⁺ mutants form normal colonies, are as cohesive as wild type, and appear to develop normally. The characteristics of *sasA* suppressors implicate the *sasA*⁺ product as a negative regulator in the A-signal-dependent regulation of Ω 4521.

In response to nutrient limitation, a developmental program requiring a high cell density is initiated by *Myxococcus xanthus*. About 10⁵ rod-shaped cells aggregate to form a specialized mound called a fruiting body, in which cells differentiate into environmentally resistant, ovoid myxospores. Myxospores remain dormant until nutrients become available and they germinate (26). At least four cell-cell interactions are required to complete fruiting body development. Four classes of conditional mutants which form complete fruiting bodies only when the mutant cells are mixed with wild-type cells or cells from a different mutant class (9) have been isolated. Apparently each mutant class is defective in producing a different intercellular signal required for normal development but is able to respond to signals from wild-type cells. Each mutant class is arrested at a different stage of development. One class, the A-signal mutants (*asg*), is arrested early in development, prior to aggregation.

Developmentally regulated genes have been identified by transcriptional fusions to β -galactosidase generated by transposition of Tn5 *lac* (14, 15). In a wild-type (*asg*⁺) background, each of a selected set of 36 developmentally regulated fusions increases β -galactosidase activity at a characteristic time after development is initiated. In *asg* mutants, no β -galactosidase activity is observed from 18 of 21 of these *lac* fusions (defined as A-dependent fusions). β -Galactosidase activity is observed only in the strains containing fusions that are normally expressed during the first few hours of development (defined as A-independent fusions [18]). However, *asg* strains containing A-dependent fusions will express wild-type levels of *lacZ* if they develop

in the presence of material (termed A factor or A signal) that is released from developing wild-type cells (18).

The aim of this study was to uncover the steps between exogenous A signal and the expression of A-dependent genes by generating mutations that relieve dependence on A signal. The earliest known A-dependent gene monitored by a Tn5 *lac* insertion at site Ω 4521 was chosen as the reporter of suppression. β -Galactosidase specific activity in a wild-type strain containing the operon fusion Tn5 *lac* Ω 4521 increases more than 10-fold 1 to 2 h after development is initiated (15). This increase in β -galactosidase specific activity depends on A signal and is absent in *asgA* and *asgB* mutants (18). We report here the isolation and characterization of mutants containing suppressors of an *asg* mutation. Fifteen of the suppressor mutations map to two loci whose products are involved in the regulation of the Ω 4521 gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The strains and plasmids used in this study are listed in Table 1. DK1622 is used as the wild-type strain. DK101 is developmentally competent but is partially defective in social motility because of a *sglA* mutation. Myxophage Mx4 *ts18 ts27 hrm* has been described by Campos et al. (3) and Geisselsoder et al. (8). *P1cIrr100Cm::Tn5* and *P1cIrr100Cm::Tn5-132* were used for specialized transduction (1).

Growth and development. *Escherichia coli* JM107 was grown as described by Maniatis et al. (20) or on LB agar containing ampicillin (75 μ g/ml) or tetracycline (25 μ g/ml) when appropriate. *M. xanthus* was grown at 32°C in CTT liquid (1% Casitone [Difco Laboratories], 10 mM Tris hydrochloride (pH 8.0), 1 mM KH₂PO₄ (pH 7.7), 8 mM MgSO₄; final pH of the mixture, 7.6) with vigorous shaking or on CTT agar (CTT liquid with 1.5% Bacto-Agar [Difco]). Kanamycin sulfate (40 μ g/ml) or oxytetracycline (12.5 μ g/

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TABLE 1. Strain list

Strain or plasmid	Genotype	Derivation	Reference or source
<i>E. coli</i>			
JM107	$\Delta(lac-pro) endA1 gyrA96 thi-1 hsdR17 supE44 relA1$ (F' <i>traD36 proAB⁺ lacI^q-ZΔM15</i>)		30
pHBK102	Amp ^r	pUC18 (<i>Sma</i> I restriction site deleted)	This study
pHBK200	Amp ^r Tc ^r Tn5 <i>lac</i> Ω4521	18-kb fragment of DK6620 ligated into pHBH102	This study
<i>M. xanthus</i>			
DK101	<i>sglA1</i>	Spontaneous from FB	10
DK1622	Wild type	Transduction of DK1217 to [Agl ⁺]	12
DK4296	Tn5 <i>lac</i> Ω4445		15
DK4322	Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	DK4521(Mx8) × DK101	18
DK4324	<i>asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	DK4521(Mx8) × DK480	18
DK4366	Tn5 <i>lac</i> Ω4427		15
DK4521	Tn5 <i>lac</i> Ω4521		15
DK4746	Tn5 Ω4678 <i>asgB480</i>		A. Kuspa
DK5057	Tn5 Ω4560 <i>asgA476</i>		17
DK5061	Tn5 Ω4561 <i>asgC767</i>		A. Kuspa
DK5205	Tn5 <i>lac</i> Ω4442		15
DK6600	Tn5 <i>lac</i> Ω4521 (Tc ^r) <i>sglA1 asgB480</i>	DK6620(Mx4) × DK4324 Tc ^r [Km ^s] ^a	This study
DK6601	<i>sasA1 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6602	<i>sasA2 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6603	<i>sasA3 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6604	<i>sasA4 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6605	<i>sasB5 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6606	<i>sasA6 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6607	<i>sasB7 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6608	<i>sasA8 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6609	<i>sasA9 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6610	<i>sasA10 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6611	<i>sasA11 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6612	<i>sasA12 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6613	<i>sasA13 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6614	<i>sasB14 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6615	<i>sasB15 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6616	<i>sasB16 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6617	<i>sasB17 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6618	<i>sasA18 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6619	<i>sasA19 asgB480</i> Tn5 <i>lac</i> Ω4521 <i>sglA1</i>	UV of DK4324	This study
DK6620	Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1</i>	P1:Tn5-132 × DK4322 Tc ^{rb}	This study
DK6621	<i>sasA1</i> Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480</i>	DK6620(Mx4) × DK6601 Tc ^r [Km ^s]	This study
DK6622	<i>sasA4</i> Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480</i>	DK6620(Mx4) × DK6604 Tc ^r [Km ^s]	This study
DK6623	<i>sasB5</i> Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480</i>	DK6620(Mx4) × DK6605 Tc ^r [Km ^s]	This study
DK6624	<i>sasA6</i> Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480</i>	DK6620(Mx4) × DK6606 Tc ^r [Km ^s]	This study
DK6625	<i>sasB7</i> Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480</i>	DK6620(Mx4) × DK6607 Tc ^r [Km ^s]	This study
DK6627	<i>sasA10</i> Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480</i>	DK6620(Mx4) × DK6610 Tc ^r [Km ^s]	This study
DK6629	<i>sasA12</i> Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480</i>	DK6620(Mx4) × DK6612 Tc ^r [Km ^s]	This study
DK6630	<i>sasA13</i> Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480</i>	DK6620(Mx4) × DK6613 Tc ^r [Km ^s]	This study
DK6631	<i>sasB14</i> Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480</i>	DK6620(Mx4) × DK6614 Tc ^r [Km ^s]	This study
DK6632	<i>sasB15</i> Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480</i>	DK6620(Mx4) × DK6615 Tc ^r [Km ^s]	This study
DK6633	<i>sasB16</i> Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480</i>	DK6620(Mx4) × DK6616 Tc ^r [Km ^s]	This study
DK6634	<i>sasB17</i> Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480</i>	DK6620(Mx4) × DK6617 Tc ^r [Km ^s]	This study
DK6635	<i>sasA18</i> Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480</i>	DK6620(Mx4) × DK6618 Tc ^r [Km ^s]	This study
DK6636	Tn5 Ω6636 Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480</i>	Tn5 pool(Mx4) × DK6621 Km ^r [Sas ⁺] ^b	This study
DK6637	Tn5 Ω6636 Tn5 <i>lac</i> Ω4521(Tc ^r) <i>sglA1 asgB480 sasA1</i>	DK6636(Mx4) × DK6621 Km ^r [Sas ⁻]	This study
DK6638	Tn5 Ω6636 <i>sglA1</i>	DK6636(Mx4) × DK101 Km ^r [Sas ⁺]	This study
DK6639	Tn5 Ω6636 <i>sglA1 sasA1</i>	DK6637(Mx4) × DK101 Km ^r [Sas ⁻]	This study
DK6640	Tn5 Ω6636(Tc ^r) <i>sglA1</i>	P1:Tn5-132 × DK6638 Tc ^r [Sas ⁺]	This study
DK6641	Tn5 Ω6636(Tc ^r) <i>sglA1 sasA1</i>	P1:Tn5-132 × DK6639 Tc ^r [Sas ⁻]	This study
DK6642	Tn5 Ω6636	DK6636(Mx4) × DK1622 Km ^r [Sas ⁺]	This study
DK6643	Tn5 Ω6636 <i>sasA1</i>	DK6637(Mx4) × DK1622 Km ^r [Sas ⁻]	This study
DK6648	Tn5 Ω4560 <i>asgA476</i> Tn5 Ω6621(Tc ^r)	DK6640(Mx4) × DK5057 Tc ^r [Sas ⁺]	This study
DK6649	Tn5 Ω4560 <i>asgA476</i> Tn5 Ω6621(Tc ^r) <i>sasA1</i>	DK6641(Mx4) × DK5057 Tc ^r [Sas ⁻]	This study
DK6652	Tn5 Ω4678 <i>asgB480</i> Tn5 Ω6621(Tc ^r)	DK6640(Mx4) × DK4746 Tc ^r [Sas ⁺]	This study

Continued on following page

TABLE 1—Continued

Strain or plasmid	Genotype	Derivation	Reference or source
DK6653	Tn5 Ω 4678 <i>asgB480</i> Tn5 Ω 6621(Tc ^r) <i>sasA1</i>	DK6641(Mx4) $\times$ DK4746 Tc ^r [Sas ⁻]	This study
DK6656	Tn5 Ω 4561 <i>asgC767</i> Tn5 Ω 6621(Tc ^r)	DK6640(Mx4) $\times$ DK5061 Tc ^r [Sas ⁺]	This study
DK6657	Tn5 Ω 4561 <i>asgC767</i> Tn5 Ω 6621(Tc ^r) <i>sasA1</i>	DK6641(Mx4) $\times$ DK5061 Tc ^r [Sas ⁻]	This study
DK6658	Tn5 Ω 6658 Tn5 <i>lac</i> Ω 4521(Tc ^r) <i>sglA1 asgB480</i>	Tn5 pool(Mx4) $\times$ DK6625 Km ^r [Sas ⁺] ^b	This study
DK6659	Tn5 Ω 6658 Tn5 <i>lac</i> Ω 4521(Tc ^r) <i>sglA1 asgB480 sasB7</i>	DK6658(Mx4) $\times$ DK6625 Km ^r [Sas ⁻]	This study
DK6660	Tn5 Ω 6658 <i>sglA1</i>	DK6658(Mx4) $\times$ DK101 Km ^r [Sas ⁺]	This study
DK6664	Tn5 Ω 6658	DK6658(Mx4) $\times$ DK1622 Km ^r [Sas ⁺]	This study
DK6665	Tn5 Ω 6658 <i>sasB7</i>	DK6659(Mx4) $\times$ DK1622 Km ^r [Sas ⁻]	This study
DK6676	Tn5 <i>lac</i> Ω 4521 Tn5 Ω 6636(Tc ^r)	DK6641(Mx4) $\times$ DK4521 Km ^r Tc ^r [Sas ⁺]	This study
DK6677	Tn5 <i>lac</i> Ω 4521 Tn5 Ω 6636(Tc ^r) <i>sasA1</i>	DK6641(Mx4) $\times$ DK4521 Km ^r Tc ^r [Sas ⁻]	This study
DK6680	Tn5 <i>lac</i> Ω 4442 Tn5 Ω 6636(Tc ^r)	DK6641(Mx4) $\times$ DK5205 Km ^r Tc ^r [Sas ⁺]	This study
DK6681	Tn5 <i>lac</i> Ω 4442 Tn5 Ω 6636(Tc ^r) <i>sasA1</i>	DK6641(Mx4) $\times$ DK5205 Km ^r Tc ^r [Sas ⁻]	This study
DK6701	Tn5 <i>lac</i> Ω 4427 Tn5 Ω 6636(Tc ^r)	DK6641(Mx4) $\times$ DK4366 Km ^r Tc ^r [Sas ⁺]	This study
DK6702	Tn5 <i>lac</i> Ω 4427 Tn5 Ω 6636(Tc ^r) <i>sasA1</i>	DK6641(Mx4) $\times$ DK4366 Km ^r Tc ^r [Sas ⁻]	This study
DK6705	Tn5 <i>lac</i> Ω 4445 Tn5 Ω 6636(Tc ^r)	DK6641(Mx4) $\times$ DK4296 Km ^r Tc ^r [Sas ⁺]	This study
DK6706	Tn5 <i>lac</i> Ω 4445 Tn5 Ω 6636(Tc ^r) <i>sasA1</i>	DK6641(Mx4) $\times$ DK4296 Km ^r Tc ^r [Sas ⁻]	This study

^a Strain constructions are described in an abbreviated form. For DK6600, a Mx4 phage stock was propagated on DK6620 and used to infect DK4324 and transductants were selected for Tc^r and screened for Km^r. Tc^r before the brackets is used to indicate the selection for oxytetracycline resistance was applied before screening.

^b See "Genetic methods" in Materials and Methods.

ml) was added when appropriate. Growing cells were assayed in the mid-exponential phase of growth (80 to 160 Klett units, $\sim 4 \times 10^8$ to 8×10^8 cells per ml). For development on TPM starvation agar, growing cells were harvested and prepared as described by Kroos et al. (15).

The number of heat- and sonication-resistant spores was determined as described by Kuspa and Kaiser (17), except developing cells were scraped into 0.5 ml of TPM starvation buffer (15) and heated for 2 h at 50°C.

Cloning of the DNA upstream of Tn5 *lac* Ω 4521. A 18-kb *Pst*I fragment from strain DK6620 which included about 6 kb of DNA upstream of Tn5 *lac* Ω 4521 and Tn5 *lac* DNA to the *Pst*I site in IS50_R was cloned into a pUC vector by using map data from Southern blot hybridization analysis by Kroos et al. (15), the restriction map of Tn5 (11), and the following procedure. Chromosomal DNA prepared from strain DK6620 was digested with restriction enzyme *Pst*I and ligated with *Pst*I-restricted pHBK102, a pUC18 derivative in which alpha complementation is eliminated. JM107 was transformed with a portion of the ligation reaction and plated onto LB agar containing ampicillin. Transformants were overlaid with the chromogenic substrate for β -galactosidase, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), at 0.5 μ g per plate. A dark colony, possibly containing the desired *Myxococcus* chromosomal DNA and adjacent Tn5 *lac* DNA, which contained a plasmid subsequently named pHBK200, was isolated. pHBK200 was found to confer tetracycline resistance and to contain the expected 18-kb insert. An approximately 0.2-kb *Xho*I-to-*Bam*HI fragment of pHBK200 called U4521 (for upstream of Ω 4521), which contains the *Myxococcus* chromosomal DNA directly upstream of Tn5 *lac* Ω 4521 and the 5' end of Tn5 *lac* (30 bp of IS50_L), was isolated for use as a probe in Northern (RNA blot) analysis (described below). All methods used are described by Maniatis et al. (20). Enzymes were purchased from Boehringer Mannheim or New England BioLabs.

Mutagenesis. To produce suppressor mutations, strain DK4324 was exposed to UV radiation. Exponentially growing cells in CTT broth were diluted to 2.5×10^8 cells per ml

and exposed to 14 ergs s⁻¹ mm⁻² for 3 min in a thin-layer suspension (1 to 2 mm thick). Survival was 2% of the input cells, as measured by the number of colonies which grew on CTT agar. A portion of the suspension of irradiated cells was plated on CTT agar containing kanamycin and incubated at 32°C for 6 days. Then each plate was overlaid with 1 mg of X-Gal in 2.5 ml of CTT soft agar. A total of 19 blue colonies were isolated from 55,000 colonies screened. The efficiency of mutagenesis was measured by the frequency of mutations in *carR*, a well-studied locus (23). Colonies of *carR* null mutants are red; 5 red colonies were observed among the 55,000 colonies.

Genetic methods. To isolate Tn5 elements genetically linked to the suppressor mutations, several preliminary steps were required. First, strain DK6620 was constructed by replacing the Tn5 *lac* Ω 4521 DNA encoding kanamycin resistance (Km^r) with the DNA encoding tetracycline resistance (Tc^r) from Tn10 (Table 1) by P1 transduction of Tn5-132 (Tc^r) by the procedure of Avery and Kaiser (1). Next, DK6620 was used as a donor in a generalized transduction with myxophage Mx4 to replace the Tn5 *lac* Ω 4521 (Km^r) of the parent strain DK4324 and 13 of the 19 suppressor mutants with Tn5 *lac* Ω 4521 (Tc^r). Transductants were selected on plates containing 12.5 μ g of oxytetracycline per ml. Sensitivity to kanamycin was verified. To verify each construction and the location of each transposon, chromosomal DNA was prepared, digested with appropriate restriction enzymes, transferred to nitrocellulose by the procedure of Southern (28), and hybridized by standard methods to a ³²P-labeled Tn5 probe.

Finally, Tn5 insertions, Tn5 Ω 6636 and Tn5 Ω 6668 (Table 1), were isolated near suppressor mutations by the method of Kuner and Kaiser (16). Specifically, eight Mx4 transducing phage stocks, each propagated on a pool of 315 to 619 strains containing independent random Tn5 insertions in the chromosome of DK101, were used to infect two different suppressed strains DK6621 and DK6625, both containing Tn5 *lac* Ω 4521 (Tc^r). Km^r Tc^r transductants exhibiting low β -galactosidase activity on the original nutrient agar plates were

identified after being overlaid with X-Gal (1 mg per plate). These transductants, which carry wild-type alleles of the suppressor genes, arise when a transducing particle introduces Tn5 and adjacent DNA containing a wild-type copy of the suppressor allele.

To confirm linkage between Tn5 and a suppressor locus, transductions were performed between donor strains containing Tn5 and recipient strains containing the suppressor mutation. With DK6636 as the donor and DK6621 as the recipient, Tn5 Ω 6636 cotransduced with the wild-type allele at 71 to 80%. The range expresses variation in phage stocks. With DK6658 as the donor and DK6625 as the recipient, Tn5 Ω 6658 cotransduced with the wild-type allele at 90%. In the backcross, Mx4 phage stocks were prepared on mutant transductants (DK6637 or DK6659). These stocks were used to infect strain DK6600, a derivative of the parent strain (DK4324) containing Tn5 *lac* Ω 4521 (Tc^r). With DK6637 as the donor, Tn5 Ω 6636 cotransduced with the mutant allele at 79%, and with DK6659 as the donor, Tn5 Ω 6658 cotransduced with the mutant allele at 89%. In each transduction, the developmental β -galactosidase activity of several transductants was examined, and in all cases the activity was indistinguishable from the appropriate strain (either the parent, DK4324, or the original suppressor mutant).

To separate a suppressor mutation linked to Tn5 from the *lac* fusion, the Mx4 phage stocks propagated on the mutant transductants were used to infect strains DK101 and DK1622. Km^r transductants were selected and screened by Southern analysis and transduction. Once separated from the *lac* fusion, the Tn5 insertion strain could be infected with P1::Tn5-132 to isolate Tc^r versions of the markers linked to the mutations, facilitating the movement of suppressor mutations into strains already Km^r. Mx4 phage stocks propagated on DK6638, DK6640, and DK6660 were used to test for linkage among the suppressor mutations (Table 2).

Measurement of β -galactosidase activity. Quantitation of β -galactosidase specific activity was performed as described by Kroos et al. (15), except that β -mercaptoethanol was eliminated from the Z buffer of Miller (24). In addition, the samples were sonicated in a cup horn sonicator (Tekmar) with ice water cooling for 1.5 min at the maximum setting to disrupt fruiting bodies and cells; these samples were not sedimented before they were assayed. Protein concentrations were determined by the Bradford assay (2), using Bio-Rad reagent.

A-signal assay and cell cohesion assay. The A-signal assay was performed as described by Kuspa and Kaiser (17). The cell cohesion assay was performed as described by Shimkets (27).

RNA analysis. RNA preparation from cells in mid-exponential growth phase and Northern analysis were performed as described by Stephens et al. (29). RNA was prepared from developing cells in the same manner as described by Stephens et al. (29), except cells were spotted onto large TPM agar plates, and at the appropriate time the cells were scraped from plates with a razor blade and directly suspended in a guanidine thiocyanate solution. Total RNA was heated and electrophoresed through 1.2% agarose-formaldehyde gels. The separated RNA was then transferred to a Nytran membrane (Schleicher and Schuell) and hybridized to a ³²P-labeled, denatured, double-stranded U4521 DNA probe by standard methods. Size standards were ³²P-end-labeled *Hind*III restriction fragments of simian virus 40 DNA. The concentration of RNA was determined spectrophotometrically. In each case, a second identical filter was

hybridized to a *dsg* probe (6), which demonstrated that the appropriate amount of RNA was loaded in each lane.

The effect of A signal on accumulation of RNA homologous to U4521 was measured in DK4324, using a scaled-up A-signal assay. For each RNA preparation, cells were treated as in the A-signal assay except 1.25×10^{11} cells were incubated in a total volume of 200 ml in two plastic dishes (22.5 by 22.5 cm) and incubated at 32°C for 4 h. Partially purified A signal (3,400 U) was added (fraction II [16a]), which is equivalent to the activity present in the same number of wild-type cells. Cells were harvested by centrifugation ($6,000 \times g$, 10 min, 4°C). The pellet was resuspended in guanidine thiocyanate solution, and total RNA was prepared, separated, and hybridized as described above.

For slot blot hybridization of total RNA from *asg sasA* double mutants, RNA was prepared as described above from cells that were developing on TPM agar plates for 4 h. RNA (0.1 and 0.2 μ g) from each strain was transferred directly to a Nytran filter, using a slot blot apparatus. Filters were treated as described above and hybridized to a U4521 DNA or *dsg* probe.

RESULTS

The A signal is required for the accumulation of RNA homologous to U4521. Since β -galactosidase activity from the *lac* fusion Ω 4521 can be detected 1 to 2 h after starvation, the corresponding RNA transcript should also be present by that time. A probe (called U4521, for upstream of Ω 4521) designed to detect transcription of the *Myxococcus* gene interrupted by Ω 4521, was constructed from a DNA segment immediately upstream of the *lacZ* coding sequence (Fig. 1). The probe was hybridized to total RNA prepared from strain DK4322, which contains the fusion Tn5 *lac* Ω 4521, and to RNA from strain DK101, which does not contain Tn5 *lac* downstream of U4521. RNA was prepared from cells grown to mid-exponential phase and from cells allowed to develop for 2 and 4 h. Hybridization with probe was detected to RNA prepared from both DK4322 and DK101 developing cells but not from growing cells (0 h) of either strain (Fig. 1A). The appearance of RNA homologous to U4521 coincided with the appearance of enzyme activity (Fig. 2), suggesting that developmental regulation of Ω 4521 *lacZ* expression is at the level of RNA accumulation.

RNA which hybridized to the U4521 probe when isolated from DK101 was longer than that from DK4322 (Fig. 1), and both are shorter than 3 kb, the full length of *lacZ* (13). Apparently, fusion RNA is degraded to pieces smaller than wild-type RNA.

Since the addition of A signal restores Tn5 *lac* Ω 4521 β -galactosidase activity to an *asg* strain (18), it should be possible to test whether control by A signal is also at the level of RNA accumulation. RNA was prepared from the *asg* mutant DK4324 under starvation conditions in the presence or absence of added A signal. No RNA homologous to U4521 accumulated in DK4324 in the absence of A signal (Fig. 1B). The addition of A signal allowed RNA homologous to U4521 to accumulate (Fig. 1B). The size of this RNA was similar to RNA prepared from *asg*⁺ fusion-containing DK4322 cells without exogenous A signal (Fig. 1A). These data show that A signal is required for the accumulation of RNA homologous to U4521.

Isolation of suppressor mutants of *asgB*. If A signal controls Ω 4521 expression through regulatory proteins, then mutations in the genes encoding these proteins might permit expression of an A-dependent fusion in the absence of A

FIG. 1. Accumulation of RNA that hybridizes to DNA directly upstream of *Tn5 lac Ω4521* (U4521). (A) Accumulation of RNA homologous to U4521 during growth and early development in strains with and without *Tn5 lac Ω4521*. Total RNA prepared from DK4322 (a strain containing *Tn5 lac Ω4521*) (5 μ g) and DK101 (a strain without a fusion) (2.5 μ g) cells was electrophoresed and probed as described in Materials and Methods. The probe used was 0.2 kb of DNA upstream of the fusion *Tn5 lac Ω4521* (U4521 [■]). The diagram shows the portion of the DK4322 chromosome into which the *Tn5 lac Ω4521* has inserted. The *Myxococcus* chromosome (—), *Tn5 lac Ω4521* (—), and the direction of transcription (—>) are shown. The positions of molecular size standards are indicated in kilobase pairs. (B) Exogenously added A signal restores the accumulation of RNA homologous to U4521 in an *asgB* strain. Total RNA (2.5 μ g) prepared from DK4324 (an *asgB* strain containing *Ω4521*) cells incubated for 4 h with MC7 buffer (17) (no added A signal) or with partially purified heat-labile A signal was electrophoresed and probed as described for panel A. The molecular size standards (right) are given in kilobase pairs.

signal. Strain DK4324 (*asgB Tn5 lac Ω4521*) was chosen for suppressor isolation because it expresses *lacZ* in very low amounts unless it is starved and A signal is added (18). DK4324 was mutagenized with UV light and plated on nutrient agar plates (see Materials and Methods). After incubation at 32°C for 6 days, the plates were overlaid with X-Gal to reveal colonies that expressed *lacZ*. From 55,000 colonies, 19 colonies expressing *lacZ* at greatly increased levels, as judged by their blue color, were found.

asgB mutants such as DK4324 are defective in A-signal production (17); a compensatory mutation at the *asgB* locus could in principle restore A signaling and increase *Tn5 lac Ω4521* expression. To test this possibility, assays of A-signal release were performed on buffer conditioned by starving suppressed strains. The newly isolated mutant strains DK6601, DK6607, and DK6615 were found to release low A-signal activity (0.43, 1.1, and 2.2 A-signal activity units per ml, respectively). These activities, though higher than the A-signal activity levels of the DK4324 parent (0.2 A-signal units per ml in the same assay), were at most 11% of the A-signal activity released by the *asg*⁺ strain, DK4322 (19.6 A-signal units per ml). The values for DK4322 and DK4324 are comparable with those reported previously (17). Considering that the suppressed strains exhibited much more β -galactosidase activity (see below) than an *asg*⁺ strain, their low level of A-signal activity argues that these strains are not *asgB* revertants and are not likely to have opened an alternative pathway for A-signal production.

FIG. 2. β -Galactosidase specific activity in representative suppressed strains during growth and early development. The specific activity of β -galactosidase in sonic extracts of *Tn5 lac Ω4521*-containing cells harvested at different times during development on starvation agar was determined as described in Materials and Methods. Profiles for representative suppressor strains, which also contain *asgB* mutations, mutant 6601 ($\blacktriangle$), 6607 ($\blacktriangledown$), and 6615 ($\blacklozenge$), are shown. Controls include the *asg*⁺ strain DK4322 ($\circ$), and *asg* mutant parent strain DK4324 ($\square$). Specific activity at 0 h reflects the level of β -galactosidase activity in growing cells. Each strain was tested in at least three independent experiments, and a representative experiment is shown. ONP, o-Nitrophenol.

Mutations to high *lacZ* expression might occur in the promoter of the gene identified by *Tn5 lac Ω4521* or might create new promoters. Promoter mutations would be expected to cotransduce with the kanamycin resistance encoded by *Tn5 lac*. However, no *Km*^r transductants that expressed high levels of *lacZ* were observed in any cross with suppressed strains as the donors and wild-type cells as the recipients. Because the mutations causing increased *lacZ* expression are not linked either to the *Ω4521* or to *asgB*, they can be considered extragenic suppressors.

Suppressed levels of β -galactosidase activity. All of the suppressed strains expressed more *Tn5 lac Ω4521* during development than their DK4324 parent (Fig. 2). Some suppressed strains also expressed high levels of *lacZ* during growth. Like the *asg*⁺ strain DK4322, which carries neither *asgB* nor a suppressor mutation, all of the suppressed strains exhibited an increase in their β -galactosidase specific activity 1 to 2 h after development was initiated. The suppressed strains can be divided into two classes depending on their β -galactosidase specific activity levels at time zero of development (Fig. 2). Thirteen mutant isolates exhibited indistinguishable β -galactosidase activity patterns represented in Fig. 2 by strain DK6601. The levels of β -galactosidase specific activity at the initiation of development were in the range of 5 to 15 U (Table 2), which is similar to those for the *asgB* parent and the *asg*⁺ strain (Fig. 2). Moreover, in all these mutants, which are called class I, the β -galactosidase specific activity closely paralleled the *asg*⁺ strain for the first 6 h of development. However, after 6 h the β -galactosidase

FIG. 3. Accumulation of RNA homologous to U4521 in representative suppressed strains during growth and early development. Total RNA was prepared from representative suppressed strains, containing *asgB* mutations and Tn5 *lac* Ω 4521 at 0, 2, and 4 h of development on starvation agar. Total RNA (2.5 μ g) was electrophoresed in each lane. The procedures were the same as described in the legend to Fig. 1 and as described in Materials and Methods. The molecular size standards (right) are given in kilobase pairs.

specific activity continued to increase in the class I mutants, whereas the specific activity plateaued at about 300 to 400 U in the *asg*⁺ strain. In six other suppressed strains (designated class II and represented in Fig. 2 by DK6607 and DK6615), the β -galactosidase specific activity ranged between 300 and 1,500 U at time zero (Table 2). By 12 h, β -galactosidase specific activity reached 1,000 to 2,000 U for both classes of suppressed strains, representing about a 50-fold increase over initial levels for class I strains and at best a 6-fold increase for class II strains. This high β -galactosidase specific activity may account for the ability to identify the suppressed strains on nutrient agar. Starving cells in old colonies may accumulate significant amounts of β -galactosidase.

Suppression and RNA accumulation. Suppressed strains accumulated RNA homologous to U4521 (Fig. 3), whereas the parent DK4324 accumulated little, if any, RNA homologous to U4521 (Fig. 1B). RNA which hybridized to the U4521 DNA probe was found in developing cells of all three suppressor strains tested, DK6601, DK6607, and DK6615, at 2 and 4 h. Hybridization to RNA prepared from growing cells (0 h) revealed differences between strains. Hybridization was not detected to RNA prepared from growing cells of the class I strain (DK6601) which, like the *asg*⁺ strain DK4322, exhibited low levels of β -galactosidase specific activity during growth (Fig. 2). In contrast, hybridization was easily detected with RNA prepared from growing cells of the class II suppressed strain DK6615, which exhibited substantial β -galactosidase specific activity during growth. A longer exposure than that employed for Fig. 3 was required to see hybridization with RNA prepared from growing cells of the class II suppressor strain DK6607, which exhibited intermediate levels of β -galactosidase specific activity during growth. Hybridization to RNA prepared from the suppressed strains included molecules smaller than those hybridized to RNA prepared from DK4322, a *sas*⁺ strain, which also contained a Ω 4521 Tn5 *lac* fusion (Fig. 1). This might be associated with the longer exposure of the filter employed to detect the less abundant RNA homologous to

TABLE 2. Linkage tests of suppressor mutations

Recipient strain	β -Galactosidase specific activity during growth ^a	No. of <i>sas</i> ⁺ transductants/total no. of Km ^r or Tc ^r transductants tested for <i>sas</i> phenotype (% cotransduction) with the following donor:	
		DK6638 (Tn5 Ω 6636 Km ^r) or DK6640 (Tn5 Ω 6636 Tc ^r)	DK6660 (Tn5 Ω 6658 Km ^r)
DK6621	6	538/705 ^b (76)	0/398 ^b (<0.3)
DK6622	11	567/824 ^b (69)	0/396 ^b (<0.3)
DK6624	10	792/1,004 ^b (79)	0/491 ^b (<0.3)
DK6627	12	454/682 ^b (66)	0/497 ^b (<0.2)
DK6629	10	584/750 ^b (78)	0/423 ^b (<0.2)
DK6630	15	602/745 ^b (81)	0/440 ^b (<0.2)
DK6602	7	64/74 ^c (86)	0/49 ^c (<3)
DK6603	10	73/88 ^c (83)	0/50 ^c (<2)
DK6611	5	68/89 ^c (76)	0/50 ^c (<2)
DK6635	7	25/84 ^c (30)	0/50 ^c (<2)
DK6608	8	0/100 ^c (<1)	0/49 ^c (<3)
DK6625	300	0/398 ^b (<0.3)	192/210 ^b (91)
DK6623	1,021	0/431 ^b (<0.2)	106/174 ^b (61)
DK6631	577	0/503 ^b (<0.2)	131/194 ^b (67)
DK6632	442	0/397 ^b (<0.3)	110/166 ^b (66)
DK6633	1,468	0/530 ^b (<0.2)	121/195 ^b (62)
DK6634	588	0/387 ^b (<0.3)	92/149 ^b (62)

^a Data shown are the β -galactosidase specific activities measured during growth for *sas* mutants containing Tn5 *lac* Ω 4521(Km^r) or Tn5 *lac* Ω 4521(Tc^r).

^b Number of *sas*⁺ transductants/total number of Km^r transductants. *sas* colonies are blue on CTT X-Gal plates and *sas*⁺ colonies are tan on these plates as described in Materials and Methods.

^c Number of *sas*⁺ transductants/total number of Tc^r transductants.

U4521 from DK6607. Alternatively, RNA degradation may be more extensive in the suppressed strains.

Mapping two suppressor loci. Genetic linkage among suppressor mutations was detected with Tn5 Ω 6636 and Tn5 Ω 6658 (see Materials and Methods). These Tn5 markers were transduced from *sas*⁺ strains into 17 of the suppressed strains, and their cotransduction frequencies revealed two distinct clusters of suppressor mutations (Table 2). Of 11 class I suppressor alleles tested, 10 are linked to Tn5 Ω 6636. Wild-type alleles of the suppressors were cotransduced into nine class I recipients with Tn5 Ω 6636, replacing the suppressor alleles in 66 to 86% of the selected transductants (Table 2, upper part). In one class I mutant, DK6635, the wild-type allele cotransduced with Tn5 Ω 6636 in about 30% of the selected transductants. No cotransduction was observed with Ω 6636 in class I strain DK6608. Since Mx4 has a DNA capacity of about 50 kb, failure to cotransduce suggests a separation of the mutation and Tn5 of more than 50 kb or the presence of more than one contributing mutation in the recipient. None of the class I mutant alleles are linked to Tn5 Ω 6658. Thus, nine of the class I mutations define a genetic locus, which we call *sasA*, for suppressor of *asg*. Although the suppressor in DK6635 is clearly linked to *sasA*, its relation to the *sasA* locus is unclear at this time. Further tests will be required to see whether *sasA* is a single gene or a more complex locus. Interestingly, in all of the transductional crosses of class I strains, high β -galactosidase activity always cosegregated with a small colony phenotype and low β -galactosidase activity always cosegregated with a large colony phenotype. This suggests that small colony size is another characteristic associated with a suppressor mutation in *sasA*.

All class II suppressors are linked to Tn5 Ω 6658 (Table 2, lower part). Using strain DK6625 (from which Tn5 Ω 6658 was isolated) as a recipient, 91% cotransduction of the wild-type allele of the suppressor gene with Tn5 Ω 6658 was observed. DK6625 exhibited the lowest β -galactosidase specific activity (300 U) of class II during growth. The corresponding wild-type alleles were cotransduced with Tn5 Ω 6658 into the other five class II suppressor strains in 61 to 67% of the selected transductants. In no case did *sas*⁺ cotransduce with Tn5 Ω 6636 in a class II suppressor. Thus, six suppressor mutations define a second locus, *sasB*. Moreover, *sasB* and *sasA* do not appear to be linked to each other.

***sasA*⁺ is needed for normal development.** *sasA* and *sasB* suppressor alleles were transduced by means of their linkage to Tn5 Ω 6636 and Tn5 Ω 6658 into the wild-type strain DK1622, whose developmental characteristics are known in detail (5). The transductants are strains DK6643 and DK6665. These strains permit close comparisons of their phenotypes.

Compared with the *sas*⁺ strain DK6642, the *sasA* mutant DK6643 delayed aggregation until about 18 h of development (Fig. 4A). These late-forming aggregates failed to condense or to darken (Fig. 4A). Sporulation efficiency was reduced in *sasA* mutants. In one representative experiment, the number of viable spores produced after 72 h of development by strain DK6643 (1.5×10^5 spores per ml) was 14% that of the *sas*⁺ strain DK6642 (1.1×10^6 spores per ml). A prolonged developmental period was found to further decrease the viability of *sasA* mutant spores. When the *sasA* mutant strain, DK6643, was allowed to develop for 6 days, the number of viable spores was determined to be about 1% of that of the *sas*⁺ strain. In contrast, DK6665, the *sasB* mutant, sporulated at wild-type levels, producing 1.1×10^6 spores per ml after 72 h of development. Morphological development of *sasB* mutants was not distinguishable from that of the *sas*⁺ strain (Fig. 4A). These results indicate that the *sasA*⁺ gene product, but perhaps not the *sasB*⁺ gene product, is required for normal development.

None of the suppressed strains which contain an *asgB* mutation along with either a *sasA* or *sasB* suppressor formed fruiting bodies under development-inducing conditions. Figure 4B shows the failed development of two representative strains, DK6601 and DK6607. Because *sasA* suppressor alleles prevent normal development, it was to be expected that the suppressed *asgB* strains would not complete development as observed. Accordingly, it is possible that *sasA* suppressor alleles will not restore expression of all the A-dependent genes in the way they do for Ω 4521 (Fig. 2).

***sasA* suppressor alleles differentially regulate expression of A-dependent Tn5 *lac* fusions.** To examine the effect of a *sasA* suppressor allele on expression of different A-dependent genes, *sasA1* was transduced into 18 *asg*⁺ strains containing different developmentally regulated Tn5 *lac* fusions. The developmental time course of β -galactosidase specific activity of each fusion strain was compared within *sasA* and *sasA*⁺ pairs (Fig. 5). A *sasA1* allele increased the developmental expression of Tn5 *lac* Ω 4521 in an *asg*⁺ background (Fig. 5A), similar to the effect of *sasA* on Tn5 *lac* Ω 4521 expression in an *asgB* background (Fig. 2). The A-dependent Tn5 *lac* fusion Ω 4445 also showed an increase in expression during development over wild-type expression when *sasA1* was present (Fig. 5B). At 10 h of development, the β -galactosidase level was more than 100-fold that of *sas*⁺. Although the presence of *sasA1* increased Ω 4521 and Ω 4445 developmental expression, it decreased expression from the A-de-

pendent fusions Ω 4442 and Ω 4427 (Fig. 5C and D). Even the zero time level of β -galactosidase in *sasA1* Ω 4442 was 10-fold lower than that of *sasA*⁺. Expression of 14 other fusions, which include *asg*-, *bsg*-, and *csg*-dependent fusions, was not significantly affected by *sasA1* (data not shown). The fusions tested were Ω 4273, Ω 4401, Ω 4403, Ω 4408, Ω 4411, Ω 4414, Ω 4435, Ω 4455, Ω 4457, Ω 4469, Ω 4491, Ω 4494, Ω 4514, and Ω 4530.

***sasA* mutants have defects during vegetative growth phase.** Colony morphology and cell cohesiveness were both altered by *sasA* suppressor alleles. *sasA* colonies were smaller (about 1/2 the diameter of the wild-type after incubation for 72 h at 32°C on CTT agar containing kanamycin) and more glossy compared with the rough-surfaced colonies of the wild type. When embedded in soft agar, *sasA* mutants grew as extremely small lens-shaped colonies. The small colony size of *sasA* mutants might indicate that *sasA* belongs to either the A or S motility system of *M. xanthus* (10). Since a strain carrying mutations in both motility systems is nonmotile, transductions of the *sasA1* mutation from DK6601 into strains already carrying either an A⁻ or an S⁻ mutation were performed. These crosses failed to give any nonmotile recombinants. The A⁻ *sasA1* strain exhibited reduced S motility but was not nonmotile. The *sasA1* allele had no apparent effect on A motility. Because cell cohesion often correlates with S motility (12, 27), the cohesive properties of a *sasA* mutant were examined. *sasA1* cells were much less cohesive than their *sasA*⁺ siblings (Fig. 6). In contrast, the *sasB7* allele had no obvious effect on vegetative colony morphology, cell cohesion (Fig. 6), or motility (data not shown).

***sasA* restores the accumulation of RNA homologous to U4521 in *asgA*, -B, and -C backgrounds.** If the *sasA*⁺ gene product functions between the A signal and the gene identified by Tn5 *lac* Ω 4521 in a regulatory pathway, then a *sasA* suppressor allele would be expected to restore Tn5 *lac* Ω 4521 expression, not only in a strain containing the *asgB* mutation with which it was isolated but also in other mutants that block A-signal production. The *sasA1* allele was transduced into three strains each containing a mutation in one of the three *asg* loci. Each *asg sasA* double mutant and corresponding *sasA*⁺ strain was allowed to develop for 4 h. RNA was prepared and analyzed by hybridization to a U4521 probe. The *sasA* mutation restored the accumulation of RNA homologous to U4521 in the *asgA*, -B, and -C backgrounds (Fig. 7).

DISCUSSION

Extracellular A signal is essential for early development and for the expression of most developmentally regulated genes. We demonstrate here that extracellular A signal is absolutely required for the accumulation of the developmentally regulated mRNA homologous to U4521. RNA homologous to U4521 was observed during development when A signal was either produced by the cells or added exogenously to an *asg* mutant (Fig. 1). The experimental results do not distinguish between regulation of RNA accumulation at the level of message synthesis or stability.

A search for the regulatory molecules that compose the pathway between extracellular A signal and the accumulation of RNA homologous to U4521 led to identification of two genetic loci. Strains containing suppressor mutations were isolated on the basis of their ability to allow A-signal-independent expression of Ω 4521. Fifteen of the suppressor mutations map to two loci, *sasA* and *sasB* (for suppressor of

FIG. 4. Developmental morphology of *sas* mutants. (A) Developmental morphology of *sas* mutants in otherwise wild-type backgrounds spotted on starvation agar and incubated at 32°C is shown at various times during development. The wild-type strain is DK6642 (*sasA*⁺ *sasB*⁺). The mutant strains are DK6643 (*sasA*) and DK6665 (*sasB*). The bar represents 0.25 cm. (B) Morphology of suppressed strains at 72 h of development. The following Tn5 *lac* Ω 4521-containing strains were treated as described for panel A: parent strain, DK4324 (*asgB sas*⁺), DK6601 (*asgB sasA*), and DK6607 (*asgB sasB*).

FIG. 5. Developmental β -galactosidase specific activity in representative strains containing different A-dependent *Tn5 lac* fusions that are affected by the presence of a *sasA* mutation. β -Galactosidase specific activity was measured as described in Materials and Methods. In each panel, a representative experiment is shown in which a *sasA*⁺-carrying strain (○) and a *sasA* mutant-carrying strain (●) are shown. ONP, o-Nitrophenol.

A signal). Each locus may include more than one gene if differences in cotransduction frequencies prove to be significant (Table 2). All of the suppressor mutations examined restore the accumulation of RNA homologous to U4521 (Fig. 3). However, fruiting body development is not restored in any of the suppressed strains which contain the three genetic elements, *asgB*, *Tn5 lac* Ω 4521, and *sasA* or *sasB*, showing that the suppressor alleles do not substitute for A signal for all A-dependent genes.

The *sasA* and *sasB* suppressor alleles are distinguished by their effects on the expression of *Tn5 lac* Ω 4521. *Tn5 lac* Ω 4521 expression in *sasA* suppressed strains was low in growing cells, whereas *Tn5 lac* Ω 4521 expression in *sasB* suppressed strains was high in growing cells (Fig. 2 and 3). Nevertheless, in all of the suppressed *asgB* strains an increase in β -galactosidase specific activity and mRNA accumulation from *Tn5 lac* Ω 4521 was observed 1 to 2 h after development was initiated. Furthermore, in *asg*⁺ strains an increase in *Tn5 lac* Ω 4521 expression at 1 to 2 h was observed, whether the suppressor alleles were present or absent (Fig. 1, 2, and 5A). Hence, the mechanism responsible for this timed increase in expression following exposure

FIG. 6. Cell-cell cohesion of *sas* 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 DK6642 (*sas*⁺), DK6643 (*sasA*), and DK6665 (*sasB*) were used. Each strain was tested in at least three independent experiments, and a representative experiment is shown.

to starvation conditions is not dependent on the A signal or the wild-type alleles of either *sasA* or *sasB*.

The *sasA* and *sasB* mutants arose at about the same frequency as null mutations in a known gene *carR* (23), suggesting that the suppressor alleles are hypomorphic (causing a loss or decrease of function). It is unlikely that

FIG. 7. Accumulation of developmental RNA homologous to U4521 from *asg sasA* double mutants. Total RNA prepared from DK6649 (*asgA sasA*), DK6653 (*asgB sasA*), and DK6657 (*asgC sasA*) strains and the corresponding congenic strains DK6648 (*asgA sas*⁺), DK6652 (*asgB sas*⁺), and DK6656 (*asgC sas*⁺) at 4 h after development was initiated. Total RNA (0.1 and 0.2 μg) was transferred to Nytran filters, using a slot blot apparatus as described in Materials and Methods. One filter (left two columns) was hybridized to a U4521 DNA probe (Fig. 1). A second filter (right two columns) was hybridized to a *dsb* probe (6), which demonstrated that the appropriate amount of RNA was loaded in each slot.

many hypermorphic mutant alleles (causing a gain or increase of function) would arise at this level of mutagenesis with such indistinguishable phenotypes, as the *sasA* mutants; usually the generation of hypermorphic alleles is rare. Furthermore, the *sasA* mutation appears to be recessive to the wild-type allele when present in a heterozygotic tandem duplication (13a). Assuming they are hypomorphic, the phenotypes of *sasA* and *sasB* mutants and their effects on the expression of Tn5 *lac* Ω 4521 suggest several properties of the *sasA* and *sasB* gene products and how they would regulate Ω 4521 expression. First, both genes are likely to encode negative regulators, because the (presumed hypomorphic) *sasA* and *sasB* mutations restore Tn5 *lac* Ω 4521 expression to *asgB* mutants and hence are epistatic to *asgB*. Second, the *sasA*⁺ gene product may be a component of a putative A-signal transduction pathway which is dependent on starvation and cell density. This is based on the finding that the *sasA1* mutation restored expression of Ω 4521 in the absence of A signal during growth and early development to a level similar to that of the wild type. In addition, *sasA asg*⁺ strains are defective in fruiting body formation and sporulation, indicating that a suppressing *sasA1* allele is detrimental to the developmental program. Third, the *sasB*⁺ gene product likely has regulatory roles independent of A signal, because *sasB* mutations cause expression of Tn5 *lac* Ω 4521 during growth at higher levels than that of the wild type, yet *sasB* strains had no apparent loss of starvation-induced fruiting body formation or sporulation. Fourth, another factor independent of A signal, *sasA*, and *sasB* is responsible for the increased expression of Tn5 *lac* Ω 4521 at 1 to 2 h after starvation because Tn5 *lac* Ω 4521 expression increased at this time in *asgB sasA* and *asgB sasB* strains.

A multiplicity of controls on genes expressed early in development is suggested by the identification of at least two different suppressor loci, by the apparent 1- to 2-h timing mechanism, and because either nitrogen, carbon, or phosphate starvation induces fruiting body development (21, 22). Positive and negative controls also regulate *spoVG*, a *Bacillus subtilis* sporulation gene whose transcription is induced at the onset of sporulation (19). The transcription of *spoVG* appears positively regulated by Spo0H, a RNA polymerase sigma factor called σ^H (4, 7) and negatively regulated by Spo0A (31). The *spo0A* gene product represses the synthesis of the AbrB regulator during the early stages of sporulation, which is believed to allow transcription of *spoVG* (25). Our work suggests that *M. xanthus*, like *B. subtilis*, monitors several aspects of its environment before initiating development. Both *M. xanthus* and *B. subtilis* may need to predict whether the lack of nutrient is likely to be a short- or long-term condition in order to respond either with slow growth or with sporulation, preceded in the case of *M. xanthus* by aggregation to form a multicellular structure. Knowledge of the circuit that links external signals to responsive genes is essential for understanding how *M. xanthus* senses its nutritional status and cell density and translates that information into the initiation of the complex behavioral response of fruiting body formation.

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