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Intercellular Signaling Is Required for Developmental Gene Expression in *Myxococcus xanthus*

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Certain developmental mutants of *Myxococcus xanthus* can be complemented (extracellularly) by wild-type cells. Insertions of Tn*5 lac* (a transposon which couples β-galactosidase expression to exogenous promoters) into developmentally regulated genes were used to investigate extracellular complementation of the A group mutations. A⁻ mutations reduced developmental β-galactosidase expression from 18 of 21 Tn*5 lac* insertions tested and that expression was restored to A⁻ Tn*5 lac* cells by adding wild-type cells. The earliest A-dependent Tn*5 lac* normally expresses β-galactosidase at 1.5 hr of development indicating a developmental block at 1-2 hr in A⁻ mutants. A substance which can rescue the expression of this early Tn*5 lac* is released by wild-type (A⁺) but not by A⁻ cells. This substance appears in a cell-free wash of wild-type cells or in starvation buffer conditioned by wild-type cells 1-2 hr after development is initiated. The conditioned starvation buffer also restores normal morphological development to an A⁻ mutant.

INTRODUCTION

Myxococcus xanthus is a gram-negative soil bacterium which undergoes simple multicellular morphogenesis and cellular differentiation to form a fruiting body. Upon starvation, about 10^5 rod-shaped cells aggregate, forming a mound in which many of the cells lyse and the rest form ovoid myxospores (Wireman and Dworkin, 1977). Coordinating the construction of a fruiting body would seem to require communication between cells. Accordingly, intercellular communication has been implicated in the control of this developmental process by several experiments (Shimkets and Dworkin, 1981; Hagen *et al.*, 1978; LaRossa *et al.*, 1983; Janssen and Dworkin, 1985). Hagen *et al.* (1978) isolated four classes of conditional sporulation mutants (groups A, B, C, and D) which behave as if they are defective in intercellular signaling. The sporulation defect of each type of mutant can be extracellularly complemented if the mutant cells are allowed to develop, in mixture, with wild-type cells or cells of a different mutant group. Sporulation can also be rescued if a cell-impermeable filter is placed between mutant and wild-type cells, implying that diffusible factors provided by the wild-type cells are responsible for correcting the mutant defect (LaRossa *et al.*, 1983).

Each group of complementable mutants appears to arrest development at a different stage, as if each group were blocked at a different point on a developmental pathway (For review see Kaiser, 1984). Group A mutants appear to be blocked early in development (LaRossa *et al.*, 1983). In addition to their sporulation defect, they do not undergo developmental lysis or form tight aggregates, they fail to make detectable levels of myxobacterial hemagglutinin (protein H) which normally accumulates during aggregation (Cumsky and Zusman, 1979) and they delay production of the spore-coat protein S which is normally detectable by 5 hr after starvation (Inouye *et al.*, 1979). The phenotype of A⁻ mutants could be explained by a deficiency in an early, intercellular signaling substance that is necessary for the proper developmental expression of genes that are normally expressed later. To investigate this hypothesis, we have studied the expression of 21 different developmentally regulated genes in A⁻ genetic backgrounds.

The genes used in this study are part of a set of developmentally regulated genes identified by the transposable promoter-probe Tn*5 lac*. Tn*5 lac* generates transcriptional fusions to *lacZ* when it transposes into an operon in the proper orientation, and puts the expression of β-galactosidase under the control of chromosomal promoters (Kroos and Kaiser, 1984). Kroos *et al.* (1986) have used Tn*5 lac* to identify and study a set of genes in *Myxococcus* which are expressed at different and characteristic times of development. Of the 21 developmentally regulated genes examined, we found that 18 fail to express β-galactosidase in A⁻ mutants. Developmental β-galactosidase expression from these A-dependent Tn*5 lac* insertions in A⁻ cells can be rescued by the presence of wild-type cells or cell-free conditioned buffer from cells developing in suspension. These findings open a way to investigate the molecular basis of intercellular signaling in *Myxococcus* development.

MATERIALS AND METHODS

Myxobacteria and phage. Strain DK101 was the parental wild-type strain. It grows dispersed in liquid cul-

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ture, forms fruiting bodies on starvation agar, and carries the motility mutation *sglA1* (Hodgkin and Kaiser, 1979). The group A mutants DK476 and DK480 were derived from DK101 by UV mutagenesis (Hagen *et al.*, 1978). DK1622 is more cohesive than DK101 because it is *sglA*$^+$. It grows clumped in liquid culture and forms more compact fruiting bodies than DK101 on agar (Kaiser, 1979). The generalized transducing phage Mx8 has been described (Martin *et al.*, 1978).

Growth and development. Cells were grown in CTT liquid and maintained on CTT agar (Hodgkin and Kaiser, 1977) or CTT agar supplemented with 40 μg/ml kanamycin sulfate.

Cells were allowed to develop on agar as described (Kroos *et al.*, 1986). Development of two strains in a mixture (codevelopment) was carried out by overlaying previously dried spots of one strain with an equal quantity of the other strain. Developmental supernatants from cells developing on agar were prepared by scraping cells into MC7 buffer [10 m*M* 3-[*N*-morpholino] propanesulfonic acid (MOPS), pH 7.0, 1 m*M* $CaCl_2$], resuspending the cells by vortexing and then quickly centrifuging the cells out of the buffer as described below for development in liquid.

Submerged culture development was performed as previously described (Kuner and Kaiser, 1982) modified for multiwell plates and pH 7 by C. Zwizinski (personal communication). Cells in CTT broth at a density of 1.5 Klett units were allowed to grow for 24 hr at 32°C in a 24-well plate (0.4 ml per well, Falcon). This growth period results in the formation of a thin mat of cells which adheres to the well bottom. Development was initiated by aspirating the CTT and replacing it with 0.4 ml quartz-distilled water. After 15 min at room temperature, the water was removed and replaced with MC7 or supernatants from cells developing in MC7 (see below) and development was allowed to continue at 32°C. Both growth and development were performed in humid chambers to prevent losses by evaporation.

Development in liquid ("shaken suspension") was initiated by washing vegetative cells (after centrifugation from CTT) in cold (4°C) MC7 buffer followed by centrifugation and resuspension in room temperature MC7 to a calculated cell density of 400–1000 Klett units (2–5 $\times 10^9$ cells/ml). Suspensions were shaken vigorously at 32°C. Supernatants were harvested from suspensions by centrifugation at 10,000*g* for 10 min at 4°C.

Genetic methods. The strains shown in Table 2 were constructed by generalized transduction with myxophage Mx8 *clp2* as previously described (Hodgkin and Kaiser, 1977) using the original Tn*5 lac* insertion isolates as donors (Kroos *et al.*, 1986). For all strain constructions, 20–50 kanamycin-resistant transductants were screened on CTT plates and TPM plates containing 40 and 20 μg/ml, respectively, of the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) as previously described (Kroos *et al.*, 1986). This assay provides an approximate measure of β-galactosidase expression as judged by colony color. Since spontaneous *lacZ*$^-$ mutants were found as approximately 0.2% of the transductants, only transductants which had the same colony color as the parental strains during growth (for A$^-$ mutant and wild-type insertion derivatives) and development (for wild-type insertion derivatives) were selected for use in this study. The physical distance between genetic loci was estimated from cotransduction frequencies as described previously (Sodergren *et al.*, 1983) assuming 12 and 56 kb, respectively, for the sizes of Tn*5 lac* and Mx8 DNA.

Determination of β-galactosidase activity. The specific activity of β-galactosidase was determined according to Kroos *et al.* (1986). In codevelopment experiments where only one-half of the cells contain Tn*5 lac*, samples were assayed as described but the total protein concentration was divided by 2 before calculating a specific activity.

The "expression time" for β-galactosidase was estimated as described by Kroos *et al.* (1986).

A-Factor assay. This assay is designed to detect the putative A-factor by measuring the increase in β-galactosidase expression of an A$^-$ mutant containing an A-dependent Tn*5 lac* insertion. DK4324 (DK480 containing Tn*5 lac* insertion Ω4521) cells were washed in room temperature MC7 and resuspended in MC7 to 1000 Klett units. This cell suspension (25 μl) was diluted into wells of a 24-well tissue culture plate (Falcon) containing cells (25–75 μl of a 1000-Klett unit suspension in MC7) to be tested for A-factor activity or supernatants from cells developed in shaken suspension (1000 Klett units). The final volume in all wells was adjusted to 0.4 ml with MC7. Plates were incubated in a humid chamber at 32°C for 20–24 hr. β-Galactosidase activity was determined by assaying 0.1 ml from each well in 0.4 ml Z buffer (Miller, 1972) containing 30 μl 0.1% sodium dodecyl sulfate ($NaDodSO_4$) at 37°C. One unit of A-factor is defined as the amount of factor required to stimulate the test cells, DK4324, to produce one unit of β-galactosidase activity (1 nmole of *o*-nitrophenol/min, for an entire test well) above background.

RESULTS

A-476 and A-480 Are Mutations in Different Genes of Extracellular Complementation Group A

Since the group A mutants are the largest class of extracellularly complementable mutants and since they were found several times more frequently than group C mutants, all of which have been found in one gene (Shimkets *et al.*, 1983), it is likely that mutations at sev-

eral loci can cause an A^- phenotype (Hagen *et al.*, 1978). To identify general characteristics of the A group, two different A^- mutants were studied. The A^- alleles employed, *A-476* and *A-480*, arose independently (Hagen *et al.*, 1978). Moreover, we found that the mutation *A-480* (isolated in DK480) was linked to the Tn*5 lac* at chromosomal site Ω4411, while *A-476* (isolated in DK476) was not (Table 1). Because Mx8, the general transducing phage employed, can package 56 kb of DNA (Martin *et al.*, 1978) and since *A-480* is 24% cotransduced with Tn*5 lac*-4411, the *A-476* and *A-480* sites are estimated to be more than 27 kb apart and therefore in different genes.

A-476 and A-480 Block β-Galactosidase Expression from Developmentally Regulated Tn5 lac Insertions

Twenty-one independent Tn*5 lac* insertions isolated in the *M. xanthus* chromosome which all show developmentally regulated expression of β-galactosidase were used in this study (Kroos *et al.*, 1986; Table 2). Developmentally-competent wild-type strains harboring each of these insertions display 4- to 190-fold increases in β-galactosidase activity during development and these increases occur at a time characteristic for each insertion (Kroos *et al.*, 1986; Table 3). The increases in β-galactosidase activity observed in these Tn*5 lac* insertion strains provide a series of reproducible markers that span the entire period of fruiting body development in *Myxococcus*.

To analyze the regulatory effect of *A-476* and *A-480* mutations, we introduced each of the 21 Tn*5 lac* insertions into the developmentally defective A^- strains DK476 and DK480, and into their developmentally competent A^+ parent (DK101) by generalized transduction with myxophage Mx8, yielding the strains listed in Table 2. Since this procedure utilizes genetic recombination within homologous *Myxococcus* DNA sequences outside the Tn*5 lac* element, it transfers the Tn*5 lac* insertion from the chromosome of the donor strain to the same site in the chromosome of the recipient strain. Mutant and wild-type strains containing each insertion were allowed to develop on starvation agar and the specific activity of β-galactosidase was determined at various times. The specific activity of β-galactosidase in each wild-type insertion strain increased at a characteristic time during development, the "expression time" (Fig. 1 and Table 3). Repetition of these experiments showed that the expression times were reproducible to within ±3 hr for strains which increase β-galactosidase activity after 6 h of development and ±30 min for those which express at 6 hr of development or before.

Sixteen of the Tn*5 lac* insertions showed either no expression or greatly reduced developmental expression of β-galactosidase in both *A-476* and *A-480* strains (Figs. 1c–h and Table 3). In A^+ strains the expression time for these 16 Tn*5 lac* insertions would have ranged from 1.5 to 22 hr of development and the measurements in the A^- insertion strains were extended over 72 hr. The *A-476* and *A-480* mutations also abolished the develop-

TABLE 1
LINKAGE TESTS OF *A-476* AND *A-480* TO Tn*5 lac* INSERTION Ω4411

Recipient strain	No. agg^+/spo^+ Km^r transductants	No. tested	% Cotransduction with Ω4411
DK476 (*A-476*)	0	642	<0.2
DK480 (*A-480*)	64	266	24

Note. DK4411 containing Ω4411 is agg^+/spo^+ (aggregation and sporulation competent) and was used as the donor of Mx8 transductions into DK476 and DK480 in which kanamycin resistance (Km^r) was selected. The developmental phenotype of transductants was tested by toothpicking roughly 10^8 cells onto TPM agar and screening for aggregates with a dissecting microscope after 3 days of incubation at 32°C. The presence of spores was determined by phase-contrast microscopy of sonicated fruiting bodies for transductants which had aggregated.

TABLE 2
Tn*5 lac* INSERTION STRAINS

Tn*5 lac* insertion	Parental strains[a] DK101 (A^+)	DK476 (A^-)	DK480 (A^-)
Ω4273	DK4285	—	DK4281
Ω4401	DK4401[b]	DK4362	DK4363
Ω4403	DK4403[b]	DK4369	DK4370
Ω4406	DK4406[b]	DK4364	DK4365
Ω4408	DK4408[b]	DK4301	DK4302
Ω4411	DK4310	DK4311	DK4312[c]
Ω4414	DK4308	DK4306	DK4307
Ω4427	DK4366	DK4367	DK4371
Ω4435	DK4313	DK4314	DK4315
Ω4442	DK4442[b]	DK4374	DK4379
Ω4455	DK4455[b]	DK4372	DK4373
Ω4457	DK4457[b]	DK4375	DK4376
Ω4469	DK4377	DK4378	DK4379
Ω4473	DK4316	DK4317	DK4318
Ω4474	DK4380	DK4381	DK4382
Ω4480	DK4319	DK4320	DK4321
Ω4494	DK4386	DK4387	DK4389
Ω4506	DK4390	DK4391	DK4392
Ω4514	DK4325	DK4326	DK4327
Ω4521	DK4322	DK4323	DK4324
Ω4529	DK4393	DK4394	DK4395

[a] Parental strains are described under Materials and Methods.
[b] These strains were constructed by P1::Tn*5 lac* transduction as described in Kroos *et al.* (1985).
[c] DK4312 (*A-480*, Ω4411) was used to construct A^+ and A^- versions of DK1622 containing insertion Ω4411. DK4312 was used as the donor in an Mx8 transduction with DK1622 as the recipient. Kanamycin-resistant transductants were isolated and screened for A^- (DK4398) and A^+ (DK4399) phenotypes.

TABLE 3
SUMMARY OF DEVELOPMENTALLY REGULATED β-GALACTOSIDASE EXPRESSION FROM Tn*5 lac* INSERTIONS IN WILD-TYPE AND A⁻ STRAINS

Tn*5 lac* insertion	Time of expression in wild-type (hr)[a]	Wild-type veg. S.A.[b]	S.A. at wild-type peak[c]	Mutant S.A. at time of wild-type peak[d]	β-gal expression in A⁻ strains[e]
Ω4455	0	30	130	110, 100	+
Ω4457	1.5	3	55	9, 7	−
Ω4521	1.5	10	260	10, 16	−
Ω4494	2	26	140	40, 38	−
Ω4469	3	25	110	100, 85	+
Ω4442	3	70	285	40, 30	−[f]
Ω4408	5	7	55	65, 60	+[i]
Ω4273	6	3	50	—, 5	−
Ω4411	6	25	260	65, 30	−
Ω4403	8	2	60	8, 8	−
Ω4514	8	7	170	35, 25	−
Ω4473	9	7	270	70, 16	−
Ω4474	10	3	80	10, 8	−
Ω4506	16	12	180	14, 16	−
Ω4414	20	10	720	20, 20	−
Ω4529	21	2	86	6, 7	−
Ω4480	21	2	380	6, 10	−
Ω4427	22	8	63	9, 8	−
Ω4435	22	2	200	3, 5	−
Ω4401	—[g]	5	430[h]	16,[h] 8[h]	−
Ω4406	—[g]	5	350[h]	12,[h] 8[h]	−

[a] The time of β-galactosidase specific activity increase was determined as described under Materials and Methods.
[b] The units of β-galactosidase specific activity (S.A.) are nmole ONP/min · mg protein.
[c] Values are the highest specific activities measured for DK101 (wild-type, A⁺) insertion strains during a 72-hr developmental time course.
[d] Shown are the specific activities for DK476 and DK480 insertion derivatives, respectively, at the time of DK101 peak activity.
[e] Summarized are experiments of the type shown in Fig. 1. A plus sign indicates β-galactosidase expression occurred in both A⁻ backgrounds (DK476 and DK480) at close to the same time and extent as in DK101. Except where indicated, a minus sign indicates there was little or no increase in β-galactosidase activity in either A⁻ background.
[f] See Results.
[g] Expression times for these insertions were not determined because the level of β-galactosidase in rod-shaped cells does not increase more than threefold. There is an accumulation of β-galactosidase in spores, which is demonstrated by breaking spores by sonication with glass beads after 72 hr.
[h] Values shown are for sonication with glass beads at 72 hr of development.
[i] The expression time of Ω4408 in A⁻ strains is 0 hr.

mental expression of β-galactosidase in a strain containing an insertion of Tn*5 lac* in the *tps* gene (Fig. 1b), a well-characterized developmentally regulated *Myxococcus* gene which encodes the spore-coat protein S (Inouye *et al.*, 1983; Downard *et al.*, 1984). One other insertion, Ω4442, also showed reduced developmental expression in the A⁻ mutants, but in addition displayed a new pattern of β-galactosidase expression. Vegetative and peak developmental levels were reduced 10- and 4-fold, respectively, relative to the wild-type insertion strain and a 10-fold increase over the new vegetative level is observed during development (data not shown). The expression times for the A⁻ mutant and wild-type strains containing Ω4442 were the same. In sum, of 21 Tn*5 lac* insertions in developmentally regulated genes tested, 18 displayed greatly reduced or altered developmental expression in both the *A-476* and *A-480* mutants. We refer to these Tn*5 lac* insertions as A-dependent.

Most of the A⁻ strains containing A-dependent Tn*5 lac* insertions did eventually increase β-galactosidase activity 2- or 3-fold over vegetative levels by the time A⁺ strains had reached their peak expression (Table 3). These small increases may indicate that *A-476* and *A-480* mutant strains retain some residual capacity to develop.

For three of the Tn*5 lac* insertions tested, Ω4455, Ω4469, and Ω4408, β-galactosidase expression is unaffected by either the *A-476* or *A-480* mutation (Table 3). The developmental profiles of β-galactosidase activity for Ω4455 and Ω4469 in the A⁻ mutants are not significantly different from those in the wild type, which have expression times of 0 and 3 hr (Table 3). The third insertion, Ω4408, expresses β-galactosidase early in development whether it is in an A⁺ or A⁻ background with perhaps a slight difference in timing (Fig. 1a). The expression time for both A⁻ strains containing Ω4408 is 0 hr (Table 3). This

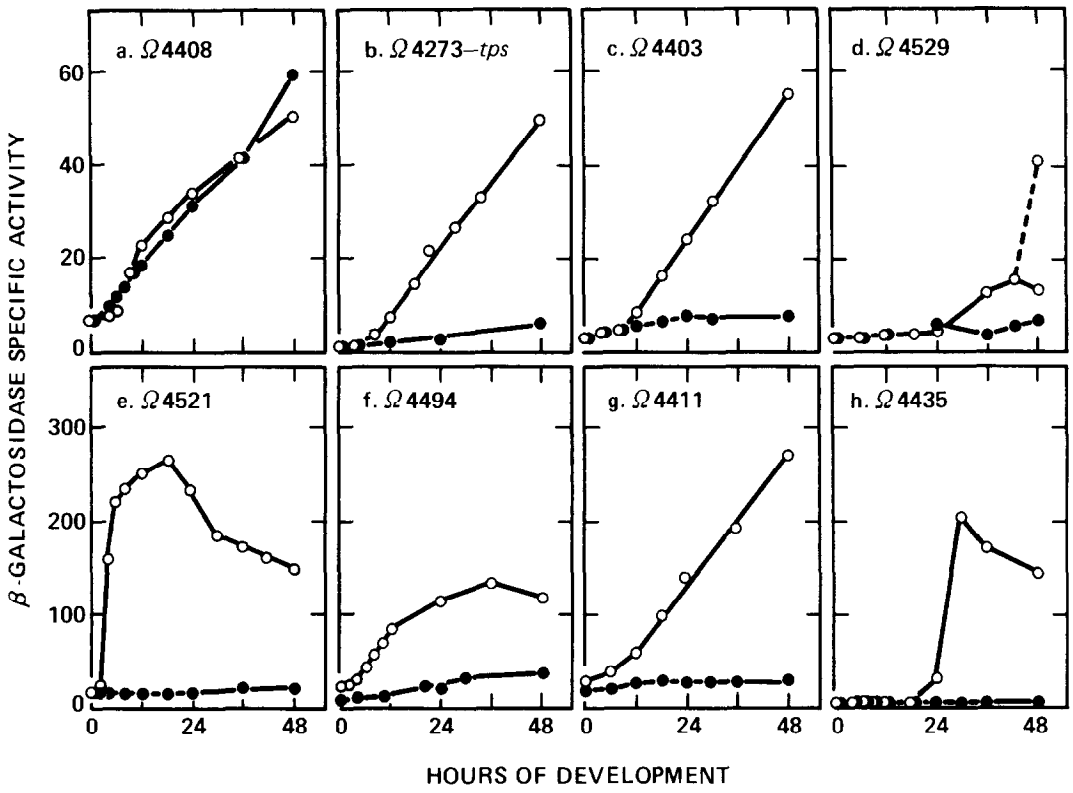

FIG. 1. β-Galactosidase expression in Tn*5* *lac*-containing strains. The developmental profiles of β-galactosidase expression are shown for wild-type (DK101, open circles) and group A mutant (DK480, closed circles) strains containing the Tn*5* *lac* insertions indicated in each panel. Cells were spotted on TPM agar, allowed to develop for the indicated times, scraped from the agar and assayed for enzyme activity (Materials and Methods). The dashed line in panel d shows the activity that was observed after spores were disrupted by sonication with glass beads.

insertion causes a developmental mutation (Kroos *et al.*, 1986).

Table 3 summarizes the β-galactosidase expression for all of the Tn*5* *lac* insertion strains tested. The three insertions that are unaffected by either A^- mutation begin accumulating β-galactosidase at or before 3 hr in the A^- mutants. Of the A-dependent Tn*5* *lac* insertions, four begin β-galactosidase expression at or before 3 hr of development and 14 begin after 3 hr. The earliest A-dependent effect is seen with insertions Ω4457, Ω4521, and Ω4494 which in A^+ strains express β-galactosidase at 1–2 hr. This shows that the function of the *A-476*$^+$ and the *A-480*$^+$ alleles may be required as early as 1–3 hr of development.

Extracellular Complementation for β-Galactosidase Expression

The mutants of extracellular complementation group A were originally isolated as conditional developmental mutants. Unable to form spores in fruiting bodies by themselves, they could sporulate when they were allowed to develop in the presence of wild-type cells. This rescue by wild-type cells raises questions about how and when the rescue by extracellular complementation occurs. Do wild-type cells restore normal development at the point at which it is blocked in group A mutants, or do they allow group A mutants to restart development at a later stage, leaving a gap of unexpressed functions between? To investigate these possibilities, we used some of the A-dependent Tn*5* *lac* insertions described above whose expression times in A^+ strains ranged from 1.5 to 22 hr of development. Corresponding A^- insertion strains were mixed with wild-type cells to determine if and when their β-galactosidase expression could be rescued during development.

The expression of β-galactosidase in A^- strains containing various A-dependent Tn*5* *lac* insertions was compared in the presence and absence of wild-type cells. (The wild-type cells have no Tn*5* *lac* and no endogenous β-galactosidase so all enzyme activity in the mixture arises from the A^- Tn*5* *lac* cells.) As illustrated with four strains in Fig. 2, developmental β-galactosidase expression could indeed be restored when A^- mutants were mixed with the A^+ parental strain DK101. Similar results were obtained with all 8 A-dependent Tn*5* *lac* insertions tested in both *A-476* and *A-480* mutants (Table 4).

Restoration of β-galactosidase production in these experiments was not simply an effect of doubling the total

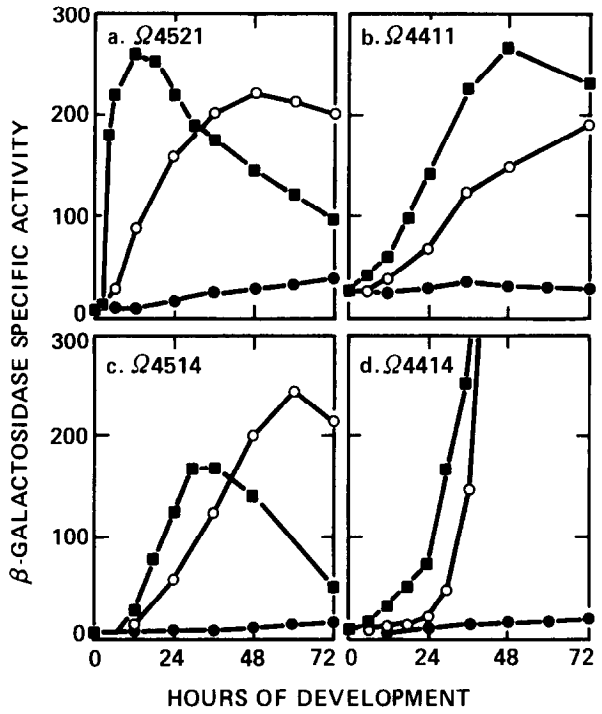

FIG. 2. Extracellular complementation for β-galactosidase expression. The developmental β-galactosidase expression for group A mutant strains (*A-480*) containing the Tn*5 lac* insertions indicated in each panel (see Table 2) was determined as described under Materials and Methods. Mutant strains were allowed to develop on TPM agar alone (closed circles) or mixed with wild-type cells (DK101, open circles). The developmental profiles of β-galactosidase expression for the wild-type (DK101) strains containing each insertion are shown (closed squares) for comparison with the mutant strains.

cell density since added A⁻ cells did not increase enzyme expression of the A⁻ Tn*5 lac* insertion strains and DK101 cells added to DK101 Tn*5 lac* insertion strains did not increase or change the timing of normal β-galactosidase expression (data not shown). The rescued β-galactosidase expression in the A⁻ mutants reached, and in some cases exceeded, the maximum level observed for the same insertion in A⁺ cells but the peak of rescued activity generally occurred later (Fig. 2 and Table 4). Nevertheless, the expression times estimated for the rescued β-galactosidase synthesis are, in most cases, close to the wild-type expression time (Fig. 2 and Table 4). In particular, Ω4521, the A-dependent insertion with the earliest expression time that was tested is rescued with an expression time very close to the wild-type value. This shows that the development of A⁻ mutants can be rescued at a very early point, one that apparently is near the time that they would have arrested development in the absence of wild-type cells.

Because the A⁻ mutants, DK476 and DK480, were isolated by UV mutagenesis of DK101 (see Materials and Methods) we wanted to test whether the A⁻ alleles were responsible for the properties observed in these mutants. To do this, we constructed DK4398 which carries *A-480* and the A-dependent insertion Ω4411 in the fully motile DK1622 background (see footnotes, Table 2). This strain displayed the same characteristics as DK4312 (the Ω4411 derivative of the original A⁻ strain, DK480). That is, DK4398 does not increase β-galactosidase expression during development unless mixed with wild-type cells (data not shown). DK101 and DK1622 genetic backgrounds differ in motility and in the timing of developmental morphogenesis (Hodgkin and Kaiser, 1979), and yet both A⁻ derivatives can be rescued. These data correlate the blockage of β-galactosidase expression from Ω4411 and its ability to be rescued with the *A-480* allele, which DK4312 and DK4398 have in common.

An Assay for A-Factor

Figure 3 shows that when A⁺ cells containing Tn*5 lac* insertion Ω4521 were starved in suspension, efficient induction of β-galactosidase was observed indicating that the gene associated with this insertion is developmentally regulated under these conditions. Identical incubation of A⁻ mutants containing Ω4521 failed to cause accumulation of β-galactosidase. As expected, starvation of wild-type cells (DK101) with A⁻ cells containing Ω4521 (DK4324) led to rescue of β-galactosidase expression. The time course of rescue in suspension was similar to that which occurred on agar plates. Again, only A⁺ cells, and not A⁻ cells, could rescue β-galactosidase expression.

Because it retained the proper specificity, rescue in suspension was used as an assay for the molecules responsible for the rescue of β-galactosidase expression from Ω4521 in A⁻ cells. Cells or extracts (see below) were mixed with a fixed number of A⁻ test cells (strain

TABLE 4
RESCUE OF β-GALACTOSIDASE EXPRESSION FROM A-DEPENDENT Tn*5 lac* INSERTIONS

Tn*5 lac* insertion	Expression time in wild type[a]	Expression time in A mutants mixed with A⁺ cells[b]	Wild-type S.A. at peak[a]	Mutant S.A. at rescued peak[b]
Ω4521	1.5	2, 2	260	250, 220
Ω4273	6	—, 7	50	—, 38
Ω4411	6	8, 8	260	240, 180
Ω4514	8	12, 8	170	460, 240
Ω4473	9	20, 16	270	590, 830
Ω4414	20	21, 20	720	710, 920
Ω4480	21	28, 28	380	270, 300
Ω4435	22	26, 28	200	250, 350

[a] From Table 3.

[b] Estimated times of expression and peak specific activities are from the type of experiment shown in Fig. 2 for DK476 and DK480 derivatives, respectively.

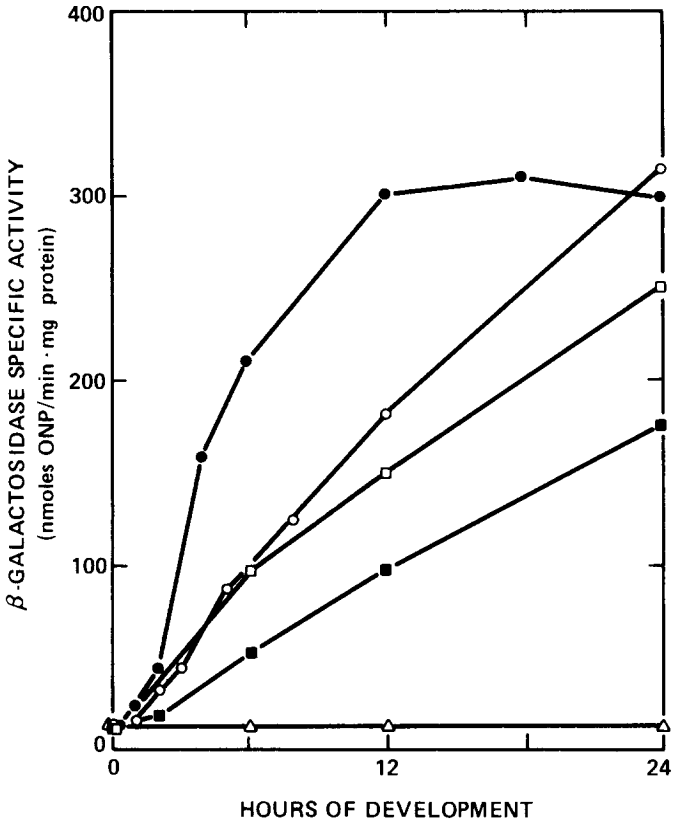

FIG. 3. Extracellular complementation of β-galactosidase expression from Tn*5* *lac*-4521. The expression of β-galactosidase from Tn*5* *lac* insertion Ω4521 in wild-type (DK4322) and A⁻ (DK4324) backgrounds is shown for various conditions of development. β-galactosidase expression in the wild-type strain DK4322 was determined for development on TPM agar (●) and, in suspension (○) under the conditions of the A-factor assay (75 μl of cell suspension per well, see Materials and Methods). β-galactosidase expression in the A mutant DK4324 is shown for development when mixed with an equal number of wild-type cells (DK101) on agar (■), with wild-type cells in suspension (□), and with A⁻ cells (DK480) in suspension (△). Suspension development was carried out under conditions of the A-factor assay (see Materials and Methods) with 25 μl of DK4324 cell suspension and 75 μl of either DK101 or DK480.

DK4324 containing *A-480* and Tn*5* *lac* insertion Ω4521) and incubated in multi-well tissue culture plates for 20 to 24 hr. A-factor activity was indicated by an increase in β-galactosidase activity over the levels produced by the unsupplemented test cells. One activity unit of A-factor was taken to be the amount necessary to induce the expression of one unit of β-galactosidase in the test cells of a single well (Materials and Methods).

Release of A-Factor from Developing Cells

When whole cells (DK101) were used as the source of A-factor, in the assay just described, a dose-dependent rescue of β-galactosidase expression from Ω4521 to near wild-type levels was observed (data not shown). If prior to their addition to the assay the source cells were allowed to undergo development on agar, their ability to provide A-factor decreased by 29% and 70% after 4 and 9 hr, respectively (average of two experiments). This suggested that A-factor is consumed during development and/or is released from the cells in a diffusible form.

To test the possibility that A-factor was released extracellularly, A⁺ cells were removed from developmental agar plates at various times, washed with buffer and this cell-free wash was assayed for A-factor activity. Figure 4 shows that A-factor was detectable in such washes. The amount of factor in wash fractions peaked at 2 hr of development and then declined to near background levels by nine hours. Identically treated washes of A⁻ cells contained less than 10% of the wild-type activity (Fig. 4).

Release of A-factor in suspension was also explored. When wild-type cells were shaken in nutrient-free (starvation) buffer, then removed by centrifugation, A-

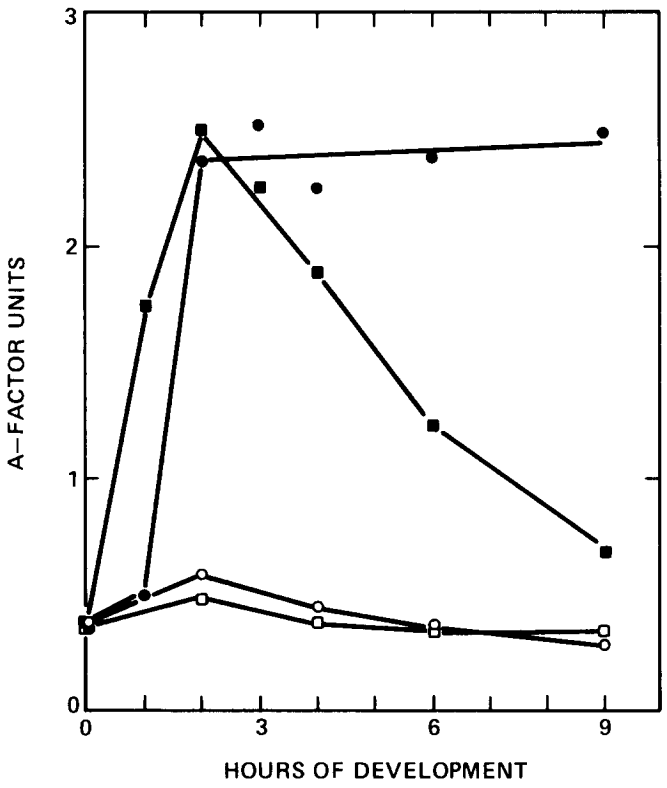

FIG. 4. Developmental release of A-factor. The release of extracellular A-factor activity from DK101 (closed symbols) and DK480 (open symbols) was monitored by the A-factor assay (see Materials and Methods). Cells were allowed to develop in shaken suspension (circles) or on agar (squares) and cell-free supernatants were harvested as described under Materials and Methods. Each point represents the average of two and five experiments for development on agar and in shaken suspension, respectively. The activity at 0 hr is equal to the amount of β-galactosidase activity in vegetative cells and is converted to A-factor "units" to indicate the background of the assay.

factor activity was detected in the extracellular medium (Fig. 4). As with development on agar, extracellular A-factor was first released after 1 to 2 hr of starvation. The extracellular levels of A-factor then remained constant from 2 to 9 hr of incubation. Control experiments confirmed that the number of residual cells present in these developmental supernatants was insufficient to account for the amount of A-factor observed.

A-factor activity from whole cells and activity released into starvation buffer from cells in suspension is compared for wild-type and mutant strains in Table 5. In both whole cell and conditioned starvation buffer fractions, A^- mutants have less than 10% of the A-factor found in A^+ cells. Mixtures of samples of starvation buffer conditioned by A^+ and A^- cells contained approximately the sum of the individual activities, showing that the reduced activity in A^- supernatants is not due to the presence of an inhibitor.

Developmental Supernatants Rescue Fruiting in A^- Mutants

Starvation buffer conditioned by wild-type cells was tested for its ability to rescue the entire developmental cycle of A^- mutants. When DK4398, which carries the *A-480* mutation, is starved in submerged culture (see Materials and Methods) it fails to form fruiting bodies or spores (Fig. 5A). When DK4398 is allowed to develop in submerged culture with starvation buffer conditioned for 2 hr by wild-type cells (DK4399, which is an A^+ sibling of DK4398; see footnotes, Table 2), it constructs spore-filled fruiting bodies (Fig. 5B). The fruiting bodies and spores produced by the rescued cells appeared normal but there was a delay of approximately 24 hr in the aggregation of cells into fruiting bodies. The number of aggregates and spores increased with increasing amounts of conditioned buffer added (data not shown).

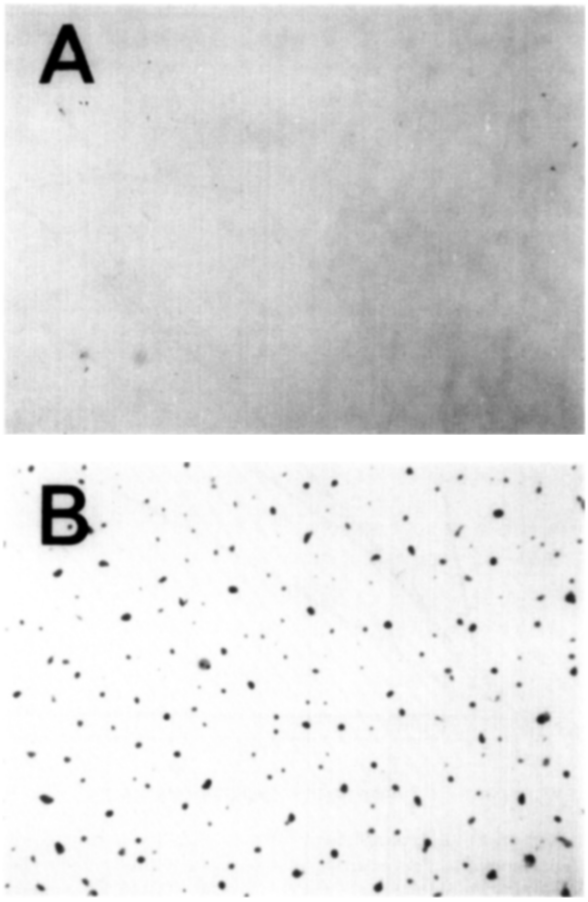

FIG. 5. Developmental rescue of a group A mutant. DK4398 (*A-480*) was allowed to develop in submerged culture in the presence of MC7 buffer (A) or a supernatant from 16 cell-equivalents of DK4399 (A^+) which had been developing in shaken suspension for 2 hr (B). Photographs (9× magnification) were taken 72 hr after development was initiated by starvation and spore-filled fruiting bodies appear as darkened spots.

TABLE 5
SPECIFICITY AND YIELD OF A-FACTOR ACTIVITY

Strain tested	A-Factor activity (U/ml)[a]	
	Whole cells[b]	Conditioned starvation buffer[c]
DK101 (A^+)	290 ± 36 (11)	12 ± 1.4 (11)
DK480 (A^-)	5.3 ± 2.6 (6)	0.80 ± 0.54 (5)
DK476 (A^-)	3.6 ± 0.43 (3)	0.61 ± 0.40 (3)
DK4399 (A^+)	—	15 ± 0.90 (7)
DK4398 (A^-)	—	1.2 ± 0.37 (7)

[a] Values are shown as units per milliliter of cells (or per milliliter of supernatants recovered from shaken suspensions) at a density of 1000 Klett units prepared as described under Materials and Methods for liquid development. The mean and standard error is given for the number of experiments shown in parentheses.

[b] Activities were determined with 75 μl of cells (1000 Klett units) in the standard A-factor assay, an amount which is within the linear response of the assay.

[c] Developmental supernatants from cells starving in liquid suspension were harvested as described under Materials and Methods at 2–6 hr (DK101, DK476, and DK480) or 2–3 hr (DK4398 and DK4399). Activities were determined from the linear portion of a curve of 20 to 350 μl of the supernatants added to the standard assay.

DISCUSSION

Of the four classes of extracellularly complementable developmental mutants of *Myxococcus* isolated by Hagen *et al.* (1978), the A^- mutants appear to arrest earliest in development because they fail to sporulate, fail to lyse, fail to synthesize protein H (which is normally synthesized at about 12 hr of development), and they delay synthesis of protein S which is normally synthesized by

5 hr (LaRossa *et al.*, 1983). Members of the other three classes of complementable mutants express one or more of these developmental markers at their normal time. (LaRossa *et al.*, 1983). If A^- mutations do cause a defect in a necessary and early developmental function, most of the Tn*5 lac* insertions used in this study would be expected to be A-dependent. Indeed, the expression of β-galactosidase from 18/21 insertions of Tn*5 lac* in developmentally regulated genes was found to be either drastically reduced or altered in A^- mutants. The three A-independent Tn*5 lac* insertions express β-galactosidase at 0–3 hr in A^- mutants. The 18 A-dependent Tn*5 lac* insertions would, in A^+ strains, express β-galactosidase at 1.5 to 22 hr of development. That both the *A-476* and *A-480* mutations block expression of genes which would normally be expressed at 1.5 hr of development and later implies that the A^- defect is manifest before 1.5 hr.

The *tps* gene is one that is A-dependent (Fig. 1b). This gene encodes spore-coat protein S (Inouye *et al.*, 1983) and measurements of protein S antigen indicated that some antigen is produced at late times in A^- mutants (LaRossa *et al.*, 1983). This delayed appearance of protein S antigen might be explained by the residual capacity of A^- mutants DK476 and DK480 to produce A-factor activity at low levels (Table 5). These low levels of A-factor may be insufficient to allow progress to the later stages of development such as synthesis of protein H and lysis.

The A^- mutants were initially identified because their developmental defect, the failure to form spores in fruiting bodies, could be corrected by the addition of wild-type (A^+) cells (Hagen *et al.*, 1978). A strong prediction of the hypothesis that the A^- mutants are defective in the production of some extracellular developmental signal but are still able to respond to this signal, is that developmental β-galactosidase synthesis by A^- strains containing A-dependent Tn*5 lac* insertions should be restored by added wild-type cells. Rescue of β-galactosidase expression from A-dependent insertions in A^- mutants was observed for all eight strains tested. In most cases, the maximum level of β-galactosidase expression observed was equal to that seen in the wild-type background. However, there was always a delay in the time the maximum rescued expression was reached. These delays suggest that development might normally be limited by the amount of A-factor available. In a rescue mixture the amount of A-factor available may be 50% of normal since the mutant cells provide little factor but can still utilize it. In one case, Ω4473, more β-galactosidase is produced after rescue than from the A^+ strain. Since A^- mutants fail to lyse during development (Janssen and Dworkin, 1985), it is possible that less efficient lysis in a mixture of A^- Tn*5 lac* and A^+ cells would prolong β-galactosidase synthesis from Tn*5 lac* insertions that would normally be expressed around the time of developmental lysis. This could lead to higher levels of enzyme in the rescued A^- mutant cells. Be that as it may, the results are consistent with the idea that developmentally regulated genes are not expressed properly in A^- mutants because these mutants are deficient in the production of an extracellular signal that can be provided by wild-type cells.

β-galactosidase expression from the earliest A-dependent Tn*5 lac* insertion tested, Ω4521 (with an expression time of 1.5 hr in wild-type cells), can be rescued in A^- mutant cells by added wild-type cells. When rescued, the A^- mutant cells containing this insertion begin to express β-galactosidase at 2 hr. Thus both the time of the block in the A^- mutants and the time they are rescued by A^+ cells must fall within the first 2 hr of development. Earlier studies (Hagen *et al.*, 1978; LaRossa *et al.*, 1983; Janssen and Dworkin, 1985) did not distinguish between rescue by replacement of a missing signal and a bypass mechanism in which late functions, perhaps only those essential for aggregation and sporulation, are activated but other intermediate processes are not. The facts that all Tn*5 lac* insertions tested were rescued by A^+ cells and that the earliest time of rescue is the same (within the time resolution of the experiments; ±0.5 hr) as the time of the developmental block strongly argue that A^- mutants can be rescued by replacing the missing A signal. Rescue by large-scale developmental lysis of the complementing cells, as suggested by Dworkin (1984), seems an unlikely explanation for the developmental rescue of the group A mutants since several A-dependent genes which are expressed before the time of developmental lysis can nevertheless be rescued with the proper timing.

An assay for A-factor has been developed, based on the rescue of β-galactosidase expression in an A^- strain containing an A-dependent Tn*5 lac* insertion. This assay utilizes A^- cells, containing the Tn*5 lac* insertion Ω4521, developing in suspension culture at moderate cell density. The complete process of fruiting body development has been shown to require a high cell density (Shimkets and Dworkin, 1981) and is ordinarily carried out on a solid surface. Nevertheless, some of the early steps in development have also been shown to take place at low cell density and in liquid suspension: expression of *tps* which normally occurs at 5 hr (Downard *et al.*, 1985), for example. Another example is the Tn*5 lac* insertion, Ω4521, which is used to assay A-factor. The timing of β-galactosidase expression from Ω4521 under standard A-factor assay conditions corresponds to that observed on agar at high cell density. Therefore, by the tests available at this time, the A-factor assay conditions do permit normal early development.

The A-factor assay detects an extracellular activity which is produced by developing cells. This activity meets several criteria for an intercellular signal essential to development: (1) the activity rescues the expression of the earliest A-dependent gene, (2) the activity is released by A^+ cells and in much lower amounts by A^- cells, and (3) activity is realeased from A^+ cells at 1–2 hr of development, the time at which A^- mutants arrest their development. Furthermore, the same extracts which contain the activity that rescues the β-galactosidase expression from an A-dependent Tn*5 lac* insertion contain an activity which rescues aggregation, sporulation and fruiting body development of a group A mutant. Whether the same molecule is responsible for both the rescue of β-galactosidase expression and the rescue of fruiting body development in A^- is not yet known.

The results presented here support the existence of an extracellular signal, A-factor, which is required early for proper developmental gene expression and for the completion of fruiting body development. We suggest that the primary defect in group A mutants is the failure to synthesize or release active A-factor or adequate amounts of A-factor. We are currently pursuing the purification of A-factor in order to test these ideas.

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