Autocide AMI Rescues Development in \textit{dsg} Mutants of \textit{Myxococcus xanthus}

AMY ROSENBLUH AND EUGENE ROSENBERG*

Department of Microbiology, Tel Aviv University, Ramat Aviv 69978, Israel

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Low concentrations of autocide AMI rescued aggregation and sporulation in the \textit{dsg} mutant class of \textit{Myxococcus xanthus} but were incapable of rescuing \textit{asg}, \textit{bsg}, or \textit{csg} mutants. AMI-induced spores of \textit{dsg} mutants were resistant to heat and sonication and germinated when plated on nutrient-rich agar. AMI accelerated aggregation and sporulation and increased the final spore number in submerged cultures of a wild-type strain of \textit{M. xanthus}. Development of \textit{M. xanthus} was accompanied by release of a fluorescent material (emission maximum, 438 nm) into the supernatant fluid. The release of this material began early and continued throughout development. All Spo$^{-}$ mutant strains tested released significantly reduced levels of this material. These levels were increased in the presence of AMI in all Spo$^{-}$ mutant classes, most dramatically in the \textit{dsg} mutants.

Myxobacteria are unique in the procaryotic world, displaying several atypical characteristics: (i) gliding motility, (ii) communal aspects during the entire life cycle, and (iii) a developmental program under starvation conditions culminating in the production of spore-bearing fruiting bodies (for a review, see reference 13).

In the 1970s, it was reported that an integral part of the developmental program is massive cell lysis (19, 20). This finding has been corroborated over the years by various investigators (e.g., see references 5, 7, and 15). It has been suggested that the lysed cells serve as signals or nutrients or both for the developing cells. Recently, however, the same laboratory which originally demonstrated this lysis has succeeded in interrupting lysis (by the use of a specific monoclonal antibody) without interfering with the developmental process (M. Dworkin, personal communication). Apparently, fruiting body formation, sporulation, and cell lysis interplay in complex ways which are only now beginning to be appreciated.

\textit{Myxococcus xanthus} produces at least five substances, termed autocides, which induce lysis in cultures of the producing and closely related strains (16). One of the two major autocides, AMI, has been shown to be a mixture of saturated and unsaturated free fatty acids (17). The other major autocide, AMV, is phosphatidylethanolamine (2). The autolytic activity of AMV depends on it being degraded to AMI by phospholipases. All strains of \textit{M. xanthus} tested were sensitive to 1 \(\mu\)g of AMI per ml. The observations that developing cells undergo autolysis and that \textit{M. xanthus} cells are peculiarly sensitive to lysis by the autocides led us to the hypothesis that these autocides play a role in developmental lysis.

One category of sporulation-deficient (Spo$^{-}$) mutants, those defective in cell-cell signaling, has been grouped into four classes (4, 11). This classification is based on the ability of two different mutants to complement each other extracellularly, thereby forming fruiting bodies and spores. Two mutants belonging to the same class are incapable of this phenotypic complementation, whereas two mutants capable of complementation will, by definition, be assigned to different classes. The four classes thus assigned are the \textit{asg}, \textit{bsg}, \textit{csg}, and \textit{dsg} mutant classes (11) (formerly SpoA, SpoB, SpoC, and SpoD, respectively). Results of the present study indicate that autocide AMI specifically induces development in \textit{dsg} mutants.

\section*{MATERIALS AND METHODS}

\textbf{Strains.} Wild-type strain \textit{M. xanthus} DK1622 was provided by D. Kaiser and has been maintained in our laboratory for several years. Sporulation-deficient mutants of DK1622 are listed in Table 1. The \textit{csg} mutant, DK2653, was obtained from L. Shimkets. All other mutant strains were provided by the laboratory of D. Kaiser. With the exception of the wild-type and \textit{bsg} strains, all strains used in this study are Tn5 or Tn5-derived transposon insertion mutant incapable of normal fruiting and sporulation. The \textit{bsg} strain is a developmental mutant with a tetracycline resistance gene inserted into the \textit{bsg} locus (3).

\textbf{Media and growth conditions.} Strains were grown routinely in 1C medium (1\% Casitone [Difco Laboratories, Detroit, Mich.] containing 0.2\% MgSO$_4$ 7H$_2$O). 1C solid medium (1C plus 1.8\% agar) was used for growth of cells on plates. All cultures were grown at 30\(^\circ\)C. Growth turbidity was determined on a Klett-Summerson photometer with a no. 54 filter. Development was carried out in submerged cultures (8) in 24-well plates (Cel-Cult Tissue Culture Products, Sterlin Ltd., Feltham, England). Cells were grown to the exponential phase in 1C medium and diluted to 3 to 6 Klett units. A 1-ml sample of this cell suspension was deposited into each well. After the cells were allowed to settle onto the polystyrene surface and multiply for ca. 20 h, the growth medium was removed and the wells were rinsed with MOPS (morpholinepropane-sulfonic acid) buffer (10 mM MOPS, 2 mM CaCl$_2$, 4 mM MgSO$_4$, pH 7.2). After the rinsing procedure was repeated several times to eliminate traces of the 1C growth medium (which interfered with the fluorometric assay), 1 ml of MOPS buffer was added to the adherent cells in each well. Under these conditions, the wild-type strain began to aggregate at approximately 18 h at 30\(^\circ\)C.

\textbf{Determination of spore number.} The entire contents of a submerged culture well were removed by scraping the adherent cells with a sterile broad-tipped toothpick, followed by pipetting the medium up and down several times with a
drawn-out Pasteur pipette. The well was then rinsed once with 1 ml of MOPS buffer, and the combined mixture was heated at 57°C for 15 min to destroy vegetative cells. The sample was cooled and then sonicated in a Labsonic 1510 sonicator at 100 W three times for 15 s each to disperse the spores. Appropriate dilutions were performed in 1CT medium; 10-μl drops were then plated on 1CT agar to determine the number of heat-resistant viable spores. The reported values are the average of two independent well samples.

**Measurement of extracellular fluorescence.** The extracellular concentration of an endogenous fluorescent material released by cultures of developing cells was determined in the overlying fluids of submerged cultures. After the fluid from the well was carefully removed with a Pasteur pipette, the sample was centrifuged at 10,000 × g for 5 min to remove remaining cells and cell debris. The assay consisted of diluting samples to a final volume of 3 ml of MOPS, exciting the sample at 360 nm, and reading fluorescence at 450 nm on a Kontron spectrofluorometer (SFM23).

**Fluorometric determination of DNA.** It is difficult to accurately determine cell numbers of *M. xanthus* during development because of the tendency of the cells to clump and to lyse during handling (12). In an attempt to circumvent these problems, we used a microfluorometric assay (1, 10, 18) to determine total DNA in the sample and then calculated cell number utilizing the DNA per cell value determined by Zusman et al. (22). To determine total cellular DNA per well at the beginning of development, we removed the entire contents of a well immediately after the growth medium was replaced with the MOPS buffer. The suspension was sonicated at 100 W for a total of 3 min, until no rods were visible by microscopic examination, and frozen for later assay.

The microfluorometric assay was performed as follows. Portions of the thawed, sonicated samples were mixed with MOPS buffer to a final volume of 2 ml. Dye solution (1 ml; 1/50th solution of Hoechst H33258, a thiazole blue [American Hoechst Corp., Chemical Plastic Division, Somerville, N.J.], stock solution prepared at 8 μg/ml in 0.154 M NaCl-0.015 M sodium citrate) was added to each sample. The sample was mixed by inverting it several times before measurement. Samples were excited at a wavelength of 360 nm, and fluorescence emission was measured at 450 nm. The data were corrected for endogenous fluorescence of the material (i.e., no dye). Standard curves were determined for calf thymus and *M. xanthus* DNA. The blank sample contained MOPS buffer and the dye solution without DNA. For an equivalent concentration of DNA, *M. xanthus* DNA gave a reading 20% that of calf thymus DNA. This observation is correlated, in part, with the different mole percent C+G content of the particular DNAs measured (18). All values reported in this study are based on the standard curve determined for *M. xanthus* DNA.

An attempt to follow developmental cell lysis was made by assaying for release of DNA into the overlying supernatant fluid as a function of time of development. Overlying supernatant fluid was removed from wells at indicated time points, centrifuged to remove cells and debris, and frozen for later assay of DNA content as described above.

**Preparation and application of autocide AMI.** Autocide AMI was prepared as described previously (17). Different amounts of AMI in 5 μl of ethanol were added to 1 ml of developing submerged cultures. (E. coli demonstrated that 5 μl of ethanol without AMI had no effect on development in submerged cultures.) One unit of AMI is defined as that quantity which causes lysis of 1 ml of growing *M. xanthus* cells at 2 × 10^7 cells per ml and is equivalent to approximately 1 μg of the fatty acid mixture.

**Characterization of endogenous fluorescent material(s).** Wild-type strain DK1622 was allowed to develop in submerged cultures in 8.5-cm petri dishes or allowed to sporulate in MOPS buffer in shake flasks. Supernatant fluids from 6-day-old submerged cultures or from sporulated cell suspensions after 4 days of incubation in shake flasks were centrifuged to remove cells and debris. The clear supernatant fluid was lyophilized, and the resulting material was suspended in deionized water. This solution was applied to a Bio-Gel P-2 column (100/200 mesh; Bio-Rad Laboratories, Richmond, Calif.). The column was 61 cm in height, with a radius of 1.05 cm. The column was developed with 10 mM MOPS (pH 7.2) with a flow rate of 1.2 ml min^-1. Samples (4.5 ml) were collected and checked for fluorescence. Contents of peak tubes were pooled, lyophilized, dissolved in water, and applied to a Dowex AG1-X4 (Cl^- form) 200/400-mesh anion-exchange column (Bio-Rad). The column was developed with a formic acid gradient (0.5 to 2.0 N). Fractions containing the fluorescent material were pooled and dried under vacuum to remove water and formic acid.

**RESULTS**

**Effect of AMI on sporulation and fruiting body formation.** Autocide AMI, previously shown to be capable of lysing vegetative *M. xanthus* cells in liquid culture, was added to submerged cultures of the wild-type strain, DK1622 (Fig. 1). The addition of 6 U of AMI to 1 ml of MOPS buffer accelerated the appearance of heat- and sonication-resistant spores by 6 to 8 h and led to an average increase of 10% in the final number of spores. In addition, it was observed that aggregation, which normally took place at 18 h, took place at 12 h in the presence of AMI. Although there was some variation in the final level of sporulation from experiment to experiment, in all cases 6 U of AMI per ml caused a 50 to 100% increase in the number of heat-resistant spores and a 6- to 8-h acceleration in the onset of aggregation and spore formation. Lower levels of AMI (2.5 to 4.5 U ml^-1) increased final sporulation values by 20 to 30% (Fig. 2).

When increasing concentrations of AMI were added to submerged cultures of the *asg, bsg*, and *csg* mutant strains, slight variations in sporulation levels were measured, but all values remained between 0.1 and 1.0% of those of the wild type (Fig. 2). In addition to the *asg, bsg*, and *csg* strains shown in Fig. 2, the two other *asg* strains tested (DK5077 and DK5079) failed to aggregate or form spores in the presence of the autocide. However, the *dsg* mutant responded to the addition of AMI by a 10-fold increase in the
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FIG. 1. Effect of AMI on the formation of heat-resistant myxospores during development of DK1622 in submerged culture. Cells adhering to the polystyrene surface were exposed to MOPS buffer (○) or MOPS buffer containing 6 U of AMI per ml (●). At the indicated time points, the contents of a well were harvested, heated, sonicated, and plated for viable counts (see Materials and Methods).

number of heat-resistant spores in the presence of 0.5 U of AMI per ml and by an approximately 100-fold increase in the presence of 2 to 11.5 U of AMI per ml. This 100-fold increase represents a sporulation level of the dsg mutant approaching that of the wild-type strain in the absence of exogenously added AMI. In addition to sporulation, complete aggregation of the dsg mutant was observed in those wells containing 0.5 U of AMI per ml or more. A typical experiment is displayed in Fig. 3, where 7.7 U of AMI per ml induced aggregation within 24 h in submerged cultures of the dsg strain DK3260; spore-filled fruiting bodies were observed by 48 h. The other dsg mutant tested (DK3261) aggregated and formed spores in the presence of AMI in a manner indistinguishable from that of DK3260.

Assay for release and degradation of DNA during development. An attempt was made to measure the release of DNA during development of the wild-type strain, DK1622, in submerged cultures. Samples were assayed by the microfluorometric technique for measuring DNA as described in Materials and Methods. Fluorescence was also measured in the absence of the dibenzimidazole dye to control for the release of endogenous fluorescent material throughout development. The sample readings in the presence of the dye were virtually identical to those in its absence (Fig. 4). These results demonstrated that (i) an endogenous fluorescent material accumulates in the extracellular supernatant fluid of developing wild-type (DK1622) submerged cultures and that (ii) there is no measurable extracellular DNA in the supernatant fluids of such cultures during the time course studied.

The fluorescent material is a water-soluble, heat-stable compound that eluted from a P-2 column with an apparent molecular weight of 360. The material bound to Dowex AG1-X4 and was eluted with 2 N formic acid, indicating that it contained a strong negative charge. The partially purified material had an excitation maximum at 368 nm and an emission maximum at 438 nm.

The failure to detect extracellular DNA during development could be due to the fact that there was no developmental lysis or to the fact that cells did lyse, but the released DNA was degraded. To examine the latter possibility, calf thymus or M. xanthus DNA was added to developing DK1622 submerged cultures at various times during development and the kinetics of its degradation were determined fluorometrically. Throughout development, the exogenous DNA was degraded rapidly (data not shown). The values for DNA degradation at different times of development were similar. A typical DNA degradation experiment is summarized in Fig. 5. M. xanthus and calf thymus DNA were degraded at linear rates, beginning immediately upon addition of the exogenous DNA and continuing to the last time point (i.e., 120 min). M. xanthus DNA added to the submerged culture decreased at approximately 135 ng ml⁻¹ h⁻¹.

In submerged culture experiments, in which 1-ml samples of cultures measuring 3 Klett units were allowed to multiply and adhere in 1CT for 24 h, a total of 8.5 × 10⁷ cells per ml at the initiation of development was calculated, based on a measurement of 1.7 μg of DNA per ml. No measurable DNA degradation was observed when DNA was added to separated cell-free overlying fluid obtained from developing submerged cultures, suggesting that the DNase activity was associated with the bound slime layer.

Fluorescence of submerged culture supernatants of Spo⁰ mutants. Sporulation-deficient mutants were assayed for the appearance of the fluorescent material in submerged cultures (Fig. 6). All strains tested released significant levels of the material, albeit lower than those of the wild-type strain. Concentrations of the fluorescent material began to increase at early times, attaining final values of 28, 41, 67, and 51% that of the wild-type strain for asg, bsg, csg, and dsg

FIG. 2. Effect of AMI on the formation of myxospores in submerged cultures of the wild type and sporulation-deficient mutants. The ordinate is the number of heat-resistant spores of each strain after 144 h, relative to the number of spores of the wild-type strain (DK1622) in the absence of AMI. The wild type (○) and dsg mutant (DK3260) (●) were exposed to concentrations of AMI ranging between 0 and 11.5 U ml⁻¹; asg (DK5057) (■), bsg (M380), and csg (DK2653) (▲) mutants were exposed to 0, 2.8, 4.2, 6.0, and 7.7 U of AMI per ml. Values below 0.5% sporulation are not shown on this figure.
FIG. 3. Induction of aggregation by autocide AMI. The wild-type strain, DK1622 (panel 1), aggregates and forms fruiting bodies in submerged cultures in MOPS buffer. The dsg mutant, DK3260 (panel 2), is incapable of aggregating under these conditions unless AMI is added to the culture (panel 3). In this experiment, 7.7 U of AMI per ml were added (panel 3 only). The photographs were taken 48 h after the addition of MOPS buffer.

mutants, respectively, at 130 h. During the first 24 h under developmental conditions, there were smaller differences in release of the material(s) between the wild-type and sporulation-deficient strains.

Effect of AMI on appearance of fluorescent material. Addition of exogenous AMI to submerged cultures increased the concentration of released fluorescent material in all strains tested (Fig. 7). The wild-type strain displayed only a slight, if any, increase in this parameter as a function of concentration of AMI. The three mutant strains (asg, bsg, and csg), which did not aggregate or sporulate in response to exogenously added AMI, showed a moderate increase in release of this material during submerged culture (33 to 58%) when 7.7 U of AMI per ml were added. The dsg mutant (DK3260), which aggregated and sporulated efficiently in the presence of exogenously added AMI (Fig. 2 and 3), displayed the highest increase in autocide-induced release of fluorescent material (93%). At 2.8 U of AMI added per ml, the difference in AMI-induced fluorescence release between the dsg mutant and other sporulation-deficient mutants was even more dramatic—13, 15, and 28% for csg, asg, and bsg mutants, respectively, compared with 63% for the dsg strain.

DISCUSSION

As previously reported, autocide AMI, a mixture of saturated and unsaturated free fatty acids (17), lyases cells of the producing strain (16). The present study presents evidence for a role played by AMI in aggregation and sporulation. One

FIG. 4. Extracellular fluorescence in the presence and absence of Hoechst H33258 bibenzimidazole dye. DK1622 cells were developed in 24-well plates as described in Materials and Methods. At the indicated times during development, supernatant fluid was removed from the wells and fluorescence was measured in the absence (○) or presence (●) of the bibenzimidazole dye.

FIG. 5. Degradation of DNA in submerged cultures of DK1622. M. xanthus (●) or calf thymus (▲) DNA was added directly to submerged cultures of DK1622 after 1 day of development. Supernatant fluids were assayed for DNA at the indicated time points after the addition of DNA. The amount of DNA degraded per milliliter of supernatant fluid was calculated and plotted. M. xanthus (●) or calf thymus (▲) DNA was added to the cell-free system, and samples were assayed for DNA at the indicated time points. Results of a typical experiment are presented.
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FIG. 6. Kinetics of extracellular fluorescence production in submerged cultures of four sporulation-deficient mutants. Extracellular fluorescence levels were determined for four developmentally deficient mutants: asg (DK5057 [O]), bsg (M380 [Δ]), csg (DK2653 [■]), and dsg (DK3260 [ ]). The results of a typical experiment are presented. The ordinate is the ratio of extracellular fluorescence at each time point for each strain compared with extracellular fluorescence of the wild type at 132 h of development. The dotted line, indicating the wild-type strain DK1622 (from a similar experiment), is included for comparison.

class of Spo− mutants, the dsg mutants, could be induced to aggregate and sporulate in the presence of low concentrations of autocide AMI. In addition, the wild-type strain, DK1622, developmentally proficient, aggregated and sporulated earlier and more efficiently in the presence of microgram amounts of exogenously added AMI. AMI is most probably an enzymatic breakdown product of phosphatidylethanolamine, a major component of the cell membrane (2). It appears likely that endogenous AMI, a naturally occurring substance present during vegetative growth and, in higher concentrations, during stationary phase, exerts an effect on developing cells.

For several of the Spo− mutants, the time during development when the gene product of the interrupted gene affects gene expression at other loci has been determined. The bsg gene product affects expression within the first 0.5 h of development (3, 7), asg affects expression at approximately 1.5 h (9), and csg affects expression at approximately 6 h (7). The dsg mutant has been shown to be blocked early in development (i.e., within the first 0.5 h) (Y. Cheng and D. Kaiser, manuscript in preparation). It is interesting that AMI rescued only the dsg mutants. One possibility would be that dsg mutants are blocked before AMI normally exerts its influence. Addition of AMI either corrects or circumvents the block, thus allowing development to proceed. The asg- and csg-dependent expression occurs at later times, and therefore, adding more of this early-acting substance cannot correct or circumvent the deficiency. The action of AMI can thus be helpful in determining the order of events in early development. It is not clear whether bsg-dependent expression occurs before or after that of dsg. If bsg is blocked after dsg, then the explanation for asg and csg may also apply to bsg. Alternatively, if bsg is blocked at an earlier time than dsg, AMI may be unable to correct it because the bsg lesion is unrelated to the AMI-induced event.

Experiments are in progress to determine the production levels of AMI during submerged culture development of the wild-type and mutant strains. One possible explanation for AMI-induced rescue of development of dsg is that these mutants do not produce sufficient quantities of AMI to support normal development. Since AMI is apparently cleaved from phosphatidylethanolamine, dsg mutants may lack the necessary phospholipase activity to effect this cleavage. Alternatively, the dsg cell membrane may be altered so that AMI cannot be easily cleaved from its phosphatidylethanolamine. It has not been possible to isolate dsg mutants with the Tn5 transposon situated in the middle of the dsg locus (Cheng and Kaiser, in preparation). This observation has led to the hypothesis that such a mutation would be lethal. Only mutants with Tn5 situated at the 3′ end have been isolated. Total loss of the protein encoded by the dsg locus might be lethal if such a protein were an essential phospholipase or played a role in forming an integral component of the membrane.

In attempting to link the autolytic activity of AMI with its role in aggregation and sporulation, we experimented with a sensitive fluorometric assay for released DNA during development as a measure of lysis. However, we found that such a measurement is not feasible because of the presence of potent DNase activity in submerged cultures.

The DNase activity was tightly associated with the cell or slime layer and was not recoverable in the supernatant fluid. The rate of DNA degradation, 135 ng ml−1 h−1, is sufficient to rapidly degrade any DNA which might be released by lysing cells (which contain a total of 1.7 μg of DNA per ml).

An unexpected outcome of the fluorometric DNA assay
was the discovery of released fluorescent material by wild-type cells in submerged culture. This material was released at a steady rate throughout development. At early times of development, a similar rate of release was observed in all mutant strains studied. After approximately 24 h, however, these rates began to fall off and the resultant levels of released material were significantly, and reproducibly, lower than those of the wild type in all cases. Only when the development of one class of mutant (dsg) was rescued by AMI was the release of fluorescent material also rescued to wild-type levels. Since the level of released material was severalfold higher than the total amount present at the onset of development (measured after sonication), the fluorescent material was actively synthesized by cells under the sporulation conditions.

Although appearance of the extracellular fluorescent material may reflect lysis, it is also possible that the material is released by intact cells. In either case, extracellular fluorescence might be a useful developmental marker.

In summary, autocide AMI is capable of inducing (i) lysis of *M. xanthus*, (ii) accelerated aggregation and sporulation in the wild-type strain, (iii) increased levels of sporulation in the wild-type strain, (iv) aggregation and sporulation rescue in the dsg mutant class, specifically, and (v) increased levels of fluorescent material released during development in all *Spo*^-^ mutants studied, most dramatically in the dsg mutant class. Further studies are in progress to determine the specific component(s) of the AMI mixture that is responsible for each of the various activities listed above.

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LITERATURE CITED