JOURNAL OF BACTERIOLOGY, Dec. 2000, p. 6900–6905 Vol. 182, No. 24
0021-9193/00/$04.00+0

Calcium and Malate Are Sporulation-Promoting Factors of *Physarum polycephalum*

STEFAN RENZEL,[1] SIGRID ESSELBORN,[1] HELMUT W. SAUER,[2]* AND ARMIN HILDEBRANDT[1]

Institut für Zellbiologie, Biochemie und Biotechnologie, Universität Bremen, 28334 Bremen, Germany,[1] and Department of Biology, Texas A&M University, College Station, Texas 77843[2]

Received 10 July 2000/Accepted 29 September 2000

Fruiting body formation (sporulation) is a distinctive, irreversible differentiation process in the life cycle of the slime mold *Physarum polycephalum*. The most important requirement for sporulation of *Physarum* is a period of starvation, and normally sporulation proceeds in the light. It is shown here that by omitting the liquid sporulation medium and elevating the temperature from 21 to 25°C, sporulation can occur routinely in the dark. It is further shown that this autocrine signaling in the dark requires calcium ions and malate. A putative sporulation control factor was detected in conditioned media derived from plasmodia starved in the dark, which was then identified as polymalate. As an additional role for this previously detected polyanion, specific for the plasmodial state of *Physarum*, it is suggested that the secreted compound serves as a source for both malate and calcium ions and thus promotes sporulation without light signaling.

Sporulation of *Physarum polycephalum* has long been considered a model system for the investigation of eukaryotic differentiation (7, 18). During an extended starvation period of several days, the plasmodium reaches a state of "competence" (6) which enables it to activate a sporulation pathway after an induction signal, usually light, is perceived. This is followed by a "point of no return" (commitment), a morphogenetic sequence of multiheaded sporangia formation, hence the name *P. polycephalum*, and cellularization of uninucleate spores (17). Little is known about the biochemical events that establish the state of competence and promote sporulation. Wilkins and Reynolds demonstrated the existence of sporulation control factors (SCFs) (26). They suggested that starving plasmodia release these substances into the medium. A threshold level of the unidentified SCFs in the medium was postulated for acquiring competence to sporulate after illumination of a starved plasmodium.

In this study, a new regimen to obtain sporulation in the light or in the dark has been established. It was used along with a solid matrix (agarose or agar) as a sensitive test for secretion of sporulation-promoting factors from the starved plasmodium and to assay active fractions obtained from conditioned medium. The same assay conditions were used to identify such activity among the components of the classical sporulation medium of Daniel and Rusch (6). We report here the involvement of three substances: calcium ions, malate, and polymalate. Calcium ions and malate are sufficient to promote sporulation in darkness; polymalate seems to be a sporulation control factor and may act as a source for calcium ions and malate.

MATERIALS AND METHODS

Organism and culture media. Two lines of the yellow, haploid CL strain of *Physarum polycephalum* were investigated which differed in their sporulation potential. Strain CL-A, which had the lower sporulation potential (on average, 2 to 4 out of 10 cultures sporulated), was used as the acceptor for putative sporulation-promoting factors in "dark sporulation assays" (i.e., sporulation without light induction). Conditioned medium of this strain was also analyzed as a reference sample by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy (or negative control, as it did not enhance sporulation). Strain CL-D, which sporulated very well (routinely, 9 to 10 of 10 cultures sporulated), was used as the donor of conditioned media and as an active (i.e., sporulation-promoting) sample for NMR spectroscopy. Strain CL-D was also studied in the constant light assays. Microplasmodia were grown as 50-ml shake cultures in 500-ml Erlenmeyer flasks in the citrate-hematin growth medium (5). The sporulation medium (SM) originally designed by Daniel and Rusch (6) consisted of the following (in mg/l): citric acid, 1,200; KH_2PO_4, 400; $CaCl_2 2H_2O$, 600; $MgSO_4 7H_2O$, 600; $FeCl_2 4H_2O$, 60; $MnCl_2 4H_2O$, 84; $ZnSO_4 7H_2O$, 34. The pH was adjusted to 5.0 with 2 M KOH (about 15 ml/liter).

Sporulation assays. Microplasmodia were harvested at the end of exponential growth by centrifugation at 250 × *g* for 20 s. Pellets (8- to 12-ml pellet, or 4 to 6 g freshweight after washing, depending on the age of the culture) were resuspended in 70 ml of 30 mM KCl washing solution and centrifuged again. Aliquots (0.5 ml) of the pellets were pipetted onto round filter papers (diameter, 2.6 cm; Schleicher & Schüll, no. 1574), which were placed on two layers of filter paper to blot off the remaining washing solution. After 15 min, the filter papers with the microplasmodia were transferred to agar or agarose dishes. Under standard conditions, 20 ml of 1% (wt/vol) agarose in double-distilled water was used in a 9-cm-diameter polystyrene Petri dish.

Constant light assays. Constant light assays were done at 25°C under normal room fluorescent light. Whereas the traditional method of starvation in the dark followed by light induction after 4 days (6, 18) is useful to obtain synchronously sporulating plasmodia, the constant light assay makes it possible to determine the sporulation time (the interval from placing the plasmodia on the gels to the visible onset of morphogenesis). This time interval is considered a more sensitive parameter of sporulation capacity than the standard two-step procedure of an extended starvation in the dark followed by illumination. Because of the consistant duration and exact temporal order of morphogenesis (from nodules over pillars to sporangia followed by melanization over a period of 8 h), it was sufficient to check plasmodia at intervals of 8 h.

Dark sporulation assays. For dark sporulation assays, plasmodia were starved in darkness at 25°C and checked for sporulation every 24 h by inspection under green light (500 to 600 nm), which has no effect on sporulation competence (14).

Preparation and assay of conditioned media. Conditioned media were produced in glass petri dishes with an inner diameter of 18.5 cm. Petri dishes were lined with polyester tissue disks and autoclaved. Then 20 ml of 0.5% agarose solution (about 50°C) was pipetted into the petri dishes (theoretical gel membrane thickness, 0.75 mm). After the agarose solidified, the gel was floated by pipetting 100 ml of double-distilled water under the agarose membrane. The membranes were supported by stainless steel grids. To obtain conditioned media, large masses of microplasmodia were harvested as for sporulation assays. Then 10-ml portions of the pellets were pipetted onto each round filter (7-cm diameter), which were transferred after 15 min onto the top of the agarose membranes. After 4 days of starvation at 25°C either in constant light or in darkness, conditioned media were sucked off and filtered (0.2-μm pore size; Millipore) (recovery was 50 to 70 ml per dish). To determine the sporulation-promoting activity, 5-ml aliquots of the conditioned media were spotted on 1% (wt/vol) agarose gels (20-ml volume, 1 mM $CaCl_2$) and were allowed to dry on a clean bench. These "conditioned gels" were tested in dark sporulation assays.

Fractionation of conditioned media. Conditioned media were separated into three fractions by ultrafiltration with the Amicon thin-channel ultrafiltration

* Corresponding author. Mailing address: Department of Biology, Texas A&M University, College Station, Texas 77843-3258. Phone: (979) 845-7760. Fax: (979) 845-2891. E-mail: sauer@bio.tamu.edu.

system TCF 10 and Diaflo membranes PM 30 and YM 3 (filter nominal values, 30 and 3 kDa, respectively). Conditioned medium (typically 300 ml pooled from several cultures) was filtered to 95% through the PM 30 membrane, and retenate 1 was rediluted to the start volume and labeled UF>30kDa. Filtrate 1 was filtered to 95% through the YM 3 membrane, yielding retenate 2. Then retentate 2 and filtrate 2 were also rediluted to the start volume and labeled UF3-30kDa and UF<3kDa, respectively. UF3-30kDa samples used for ^{1}H-NMR spectral analysis were not diluted.

Analytical methods. ^{1}H-NMR spectra were recorded on a WH 360 NMR spectrometer (Bruker). To UF3-30kDa fractions (15- to 25-ml retenates of 300 to 500 ml of conditioned media), 1% (vol/vol) 10 mM Na_2EDTA solution (pH 5.0) was added to reduce interferences by Fe^{2+} and Mn^{2+} ions. They were then lyophylized and dissolved in 0.5 ml of D_2O. The high-pressure liquid chromatography (HPLC) purified poly(β-L-malate) (40 mg/ml in D_2O) was a gift of E. Holler (Regensburg). Anions were analyzed by Dionex ion chromatography with a conductivity detector.

RESULTS

Starved plasmodia of *Physarum* sporulate in light or in darkness. After numerous pilot experiments, the following standard procedure was established, which is modified from the regimen used previously (5, 9, 17, 18, 26). The minimal requirements did not include the permanent presence of the sporulation medium during the extended starvation period. Plasmodia were starved in polystyrene petri dishes in moist chambers in constant light or constant darkness at 21 and 25°C. The plasmodia kept in constant light at 25°C (12 of 12) sporulated after 2 days of starvation, and all those kept at 21°C (12 of 12) sporulated after 3 days. Plasmodia starved in constant darkness at 25°C had sporulated after 5 days (7 of 12 [58%]), but the plasmodia kept at 21°C failed to sporulate in the dark (0 of 12 [0%]; error probability, $P = 0.5\%$).

These results established a reliable sporulation assay. In contrast to most previous work, illumination seems not to be an absolute requirement for sporulation. However, in addition to the nutrient stress, light is very effective in reducing the sporulation time (2 versus 5 days at 25°C) as well as increasing the efficiency of sporulation (100 versus 0% at 21°C). One reason for successful sporulation in the dark could be the avoidance of dilution and diffusion of sporulation factors into the liquid sporulation medium used in the classical regimen. These observations, summarized in Fig. 1 and 2, are a sample of the results from many positive experiments done over a period of more than 5 years. The experiments shown in the figures were designed to demonstrate the general conclusions obtained under best controlled conditions (i.e., the tested macroplasmodia were set up from the same pool of microplasmodia to provide homogeneous samples and reliable results; see Materials and Methods).

The volume effect suggests secreted sporulation-promoting activity. It was previously shown (26) that plasmodia can secrete materials with SCF activity that increase the competence to sporulate. If so, dilution of this secreted material should delay sporulation. Therefore, in preliminary experiments, sporulation in constant light of plasmodia strain Cl-D was tested on agar or agarose gels of different concentrations. The time interval from placing the plasmodia on the gels to the visible onset of morphogenesis was determined. This period, defined as the sporulation time, was dependent on the gel material, the concentration of the gel, the gel volume, and the composition of the aqueous phase. On 1% (wt/vol) and 2% agar gels and on 2% (wt/vol) agarose gels, sporulation time lasted 48 to 60 h if the gels were prepared with double-distilled water.

On 1% agarose (i.e., conditions of a higher diffusion rate), sporulation time lasted 84 h. These conditions define the standard procedure (see Materials and Methods). The results in Fig. 1A show that under these conditions, i.e., 20 ml of 1% agarose gel in water and constant light, sporulation initiated at 84 h and the last of the plasmodial set tested had begun sporulation by 90 h. All sporangia of the experiments summarized in Fig. 1 show normal morphology. Reducing the gel volume (gel thickness), which presumably increased the local concentration of secreted material, shortened the sporulation time. This observation was dubbed "volume effect" (greater volumes lead to longer sporulation times). This interval decreased as the gel volume was reduced (Fig. 1A).

The shortest sporulation time was 49 h, when the plasmodium was placed on an eightfold reduced volume. This was similar to the minimal period detected with plasmodia starved on moist filter paper alone, i.e., in the absence of any gel matrix or sporulation medium. This approach, focusing on the delay of sporulation by dilution and diffusion, cannot assay for positive-acting sporulation factors, which are assumed to be released from plasmodia during starvation.

$Ca2^+$ ions have sporulation-promoting activity. The surprising observation that both strains, Cl-A and Cl-D, could sporulate in the dark in the absence of liquid SM prompted an analysis of the liquid phase of the agarose gels. First the effect of SM was evaluated. Different dilutions of SM were compared with just water in a volume of 20 ml of 1% (wt/vol) agarose.

As in the volume effect experiments (Fig. 1A), the average sporulation time on water was about 84 h (Fig. 1B). Again, all cultures assayed sporulated. Undiluted SM did accelerate the sporulation time in constant light to 56 h. However, because the shortest sporulation time of 45 h was found with 1:4 diluted SM, not only positive but also negative effects of some components of the full-strength SM on sporulation time were concluded.

Therefore, the components of SM were examined one at a time, at different concentrations in 1% agarose gels. After pretests, cations were used as chlorides, anions were used as potassium salts, and the pH was adjusted to 5.0 if necessary. The results were as follows (for reference, note the concentrations for the various compounds in the SM [6] in parentheses): (i) K^+, Na^+, and Cl^- ions had no effect on sporulation time up to 50 mM; (ii) SO_4^{2-} ions (2.5 mM) or $HPO_4^{2-}/H_2PO_4^-$ ions (2.9 mM) accelerated sporulation only in at least fourfold higher SM concentrations; (iii) citrate (5.7 mM) permitted sporulation from 5 to 10 mM but plasmodia looked abnormal, and at 12 mM citrate was toxic; (iv) Fe^{2+} (0.3 mM) or Mn^{2+} (0.4 mM) or Zn^{2+} ions (0.1 mM) were already at SM concentrations that were slightly toxic; (v) Ca^{2+} (4.1 mM) and Mg^{2+} ions (2.4 mM), each tested separately, promoted sporulation in concentrations from 1 to 16 mM; and (vi) the vitamins of SM, niacin (0.8 mM) and niacinamid (0.8 mM) were also tested. In SM only niacinamid had sporulation-promoting activity. However, either of the two tested alone (i.e., in water) at concentrations from 0.8 to 8 mM had no effect. Niacin in concentrations higher than 8 mM was toxic.

To examine whether the dilution of secreted Ca^{2+} or Mg^{2+} ions could explain the volume effect, five SM with different concentrations of $CaCl_2$ and $MgCl_2$ were tested at a combined concentration of 7.2 mM. As shown in Fig. 1C, sporulation was much more strongly promoted at increasing Ca^{2+} concentrations (relative to the Mg^{2+} ion concentration). As the total molarity of the two ions was always the same, it seemed that the dilution of Ca^{2+} ions could not be compensated by Mg^{2+} ions. It is also apparent that at the two lowest calcium concentrations sporulation capacity is reduced, whereas at the highest concentration the fastest of all sporulation times, 39 h, and 100% sporulation were registered. Clearly, calcium ions are necessary for efficient sporulation but are not sufficient to

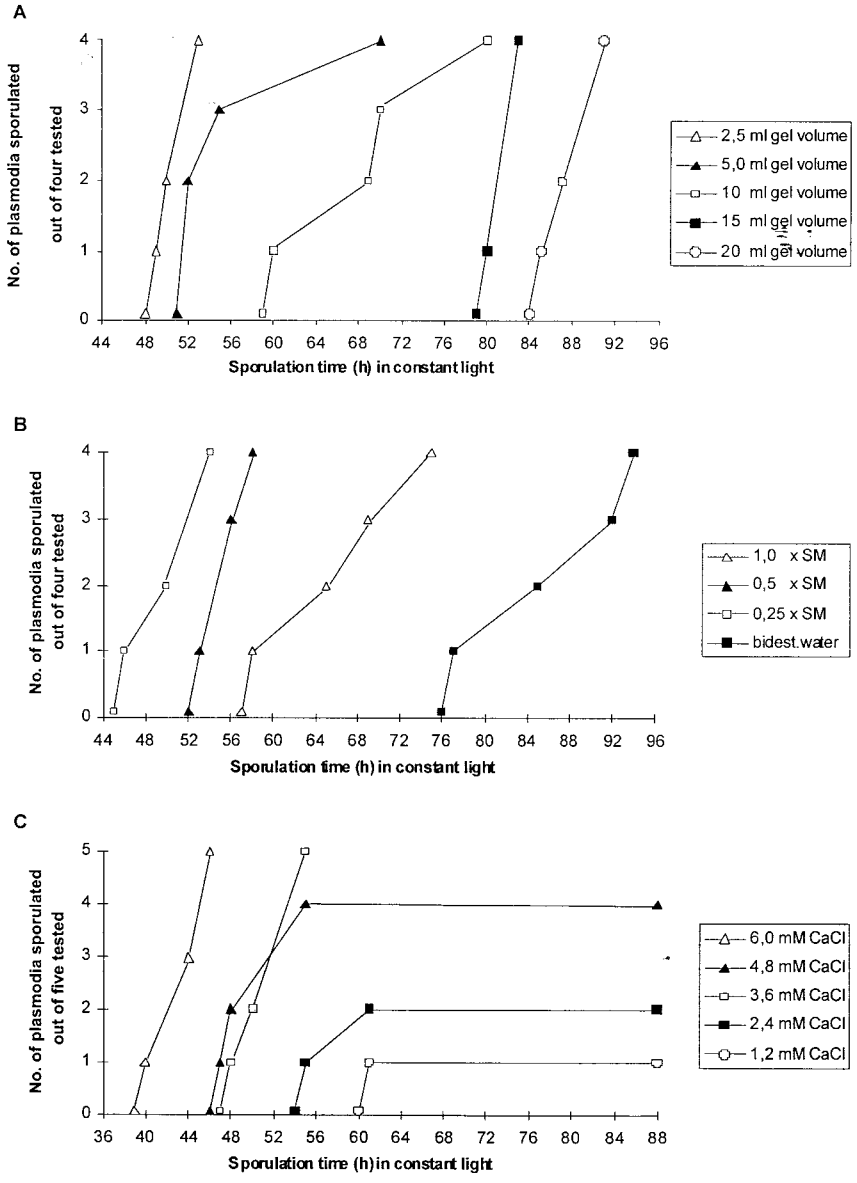

FIG. 1. Constant light sporulation assays. Measured were the sporulation times, i.e., the periods of time from placing the plasmodia on the gels to the visible onset of morphogenesis, at 25°C. Displayed are the number of plasmodia which had started visible morphogenesis at the time indicated or earlier. (A) Sporulation is dependent on the gel volume. Four plasmodia for each volume were tested on different volumes of agarose gels (1% [wt/vol]) prepared with double-distilled water. (B) Sporulation is dependent on the concentration of the SM. Four plasmodia for each concentration were tested on the agarose gels (20 ml, standard volume) prepared with different concentrations of SM or with double-distilled water. (C) Sporulation is dependent on the concentration of Ca^{2+} ions in the starvation medium. Five plasmodia for each medium were tested on starvation media derived from SM, with different concentrations of $CaCl_2$ and $MgCl_2$. The concentration of both together was always 7.2 mM.

make starved plasmodia competent to sporulate in the dark (see below).

Preparation of conditioned media and dark sporulation assays. For the isolation of further sporulation-promoting factors which allow sporulation in the absence of light, a new methodology was developed. Plasmodia were starved on a 0.75-mm thin agarose membrane above double-distilled water. The agarose membrane retains the bulk of the massive amounts of plasmodium stage-specific slime and prevents the accumulation of cell debris in the medium. As concluded from the volume effect described above (Fig. 1A), sporulation factors would diffuse through the agarose into the double-distilled water. The product is a liquid, conditioned medium without exogenous substances except for those derived from the starved plasmodia.

The sporulation-promoting activity of these media was tested in dark sporulation assays. To that end, aliquots of conditioned media were spotted on agarose gels (1% [wt/vol] agarose, 1 mM $CaCl_2$; see Materials and Methods). Plasmodia were starved on these conditioned gels in darkness (25°C), and sporulation was checked from day 4 to 9 every 24 h under green light.

With the dark sporulation assays, conditioned medium was effective. Conditioned media from plasmodia starved in the dark allowed three to five of six test plasmodia (50 to 83%) to sporulate, whereas conditioned media from plasmodia in the

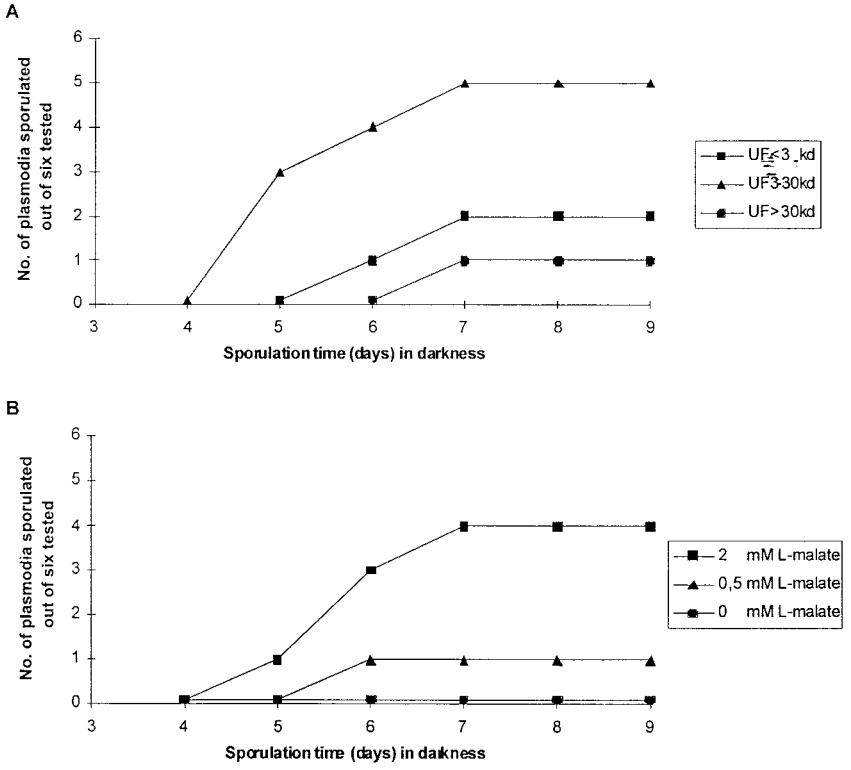

FIG. 2. Dark sporulation assays. Plasmodia were starved in the dark at 25°C. Sporulation was checked under green light every 24 h from day 4 to 9. Displayed are the number of plasmodia which had started visible morphogenesis at the time indicated or earlier. (A) Sporulation-promoting activity of ultrafiltration fractions of the conditioned medium of cultures kept in the dark. Five milliliters of the fractions called UF<3kDa, UF3-30kDa, and UF>30kDa (see Materials and Methods) were spotted onto agarose gels (1% [wt/vol]), 1 mM $CaCl_2$, 20-ml volume) and were dried on the clean bench. Six plasmodia were tested for each fraction. (B) Sporulation-promoting activity of L-malate. Six plasmodia for each concentration were tested on agarose gels (1% [wt/vol], 1 mM $CaCl_2$) containing different concentrations of L-malate.

light allowed only one to three of six test plasmodia (17 to 50%) to sporulate. It was concluded that conditioned media from plasmodia starved in darkness had more sporulation-promoting activity than media from plasmodia starved in constant light. For all test plasmodia, there were six control plasmodia (i.e., on unconditioned agarose, plain gel plus 5 ml of H_2O), none of which sporulated during the experiment. The effect of active conditioned medium was measurable above 1 ml but was more effective at 5 ml per conditioned plate. The dark starvation conditioned media did not lose their activity either by later exposure to light or by freezing and thawing.

The constant light assays could not be adapted to test for sporulation-promoting activity. With the demonstration of the calcium requirement in the light, calcium (1 mM) had to be included in the assay along with conditioned media. Under these conditions either all or none of the test plasmodia, or the six control plasmodia, sporulated, depending on the age of the stock cultures used to set up the test plasmodia. Therefore, conditions to tease out a dose-dependent effect of conditioned media, in the light plus calcium, could not be established.

Fractionation of conditioned media. The media were fractionated by ultrafiltration, because this technique enabled a fast physical separation without dilution. Conditioned media from separate sets of cultures kept either in the dark or in constant light were filtered with 30- and 3-kDa filters (filter nominal values), and retenates were diluted to the original volumes (see Materials and Methods). In dark sporulation assays (Fig. 2A), the fraction from 3 to 30 kDa (UF3-30kDa) had more sporulation-promoting activity with regard to sporulation time and frequency than the fraction lower than 3 kDa (UF<3kDa) and the fraction higher than 30 kDa (UF>30kDa).

Poly(beta-L-malate) (PMA) is a component of the active fraction of the conditioned medium. Ultrafiltration fractions from 3 to 30 kDa were prepared from conditioned media with strong or with weak sporulation-promoting activity (i.e., from plasmodia starved in darkness or in constant light; see above) and further analyzed. ^{1}H-NMR spectra of these fractions had characteristic differences. Spectra of the more active fraction had two additional signals at 3.0 and 5.2 ppm (Fig. 3B). These signals were not present, or were much reduced, in spectra of fractions with weak activity (Fig. 3A). These signals were also much smaller in the ^{1}H-NMR spectrum of a dark starvation medium of the *Physarum* strain with low sporulation potential, Cl-A (not shown).

The signals at 3.0 and 5.2 ppm were identified as PMA (Fig. 3C). This component had been previously detected in *Physarum* plasmodia and in the culture medium (8).

L-malate has sporulation-promoting activity. Three signals in the ^{1}H-NMR spectra (2.6, 2.8, and 4.4 ppm) of the fractions from 3 to 30 kDa pointed to the presence of monomeric L-malate (Fig. 3A and B). This was confirmed by HPLC anion chromatography (not shown). Therefore, L-malate was tested in dark sporulation assays. Groups of six plasmodia were assayed on 1% agarose gels in the absence or presence of increasing concentrations of malate. As shown in Fig. 2B, under these conditions no sporulation was detected without malate (none of six plasmodia), while one of six sporulated on 0.5 mM

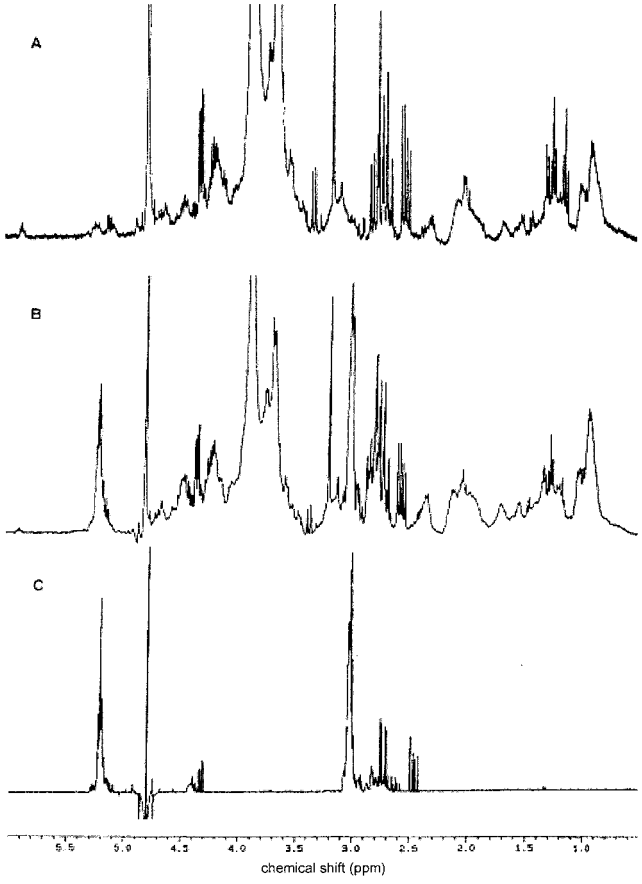

FIG. 3. ^{1}H-NMR spectra of ultrafiltrates from 3 to 30 kDa (filter nominal values) of conditioned media harvested from the following. (A) Cultures starved 4 days in light. (B) Cultures starved 4 days in darkness. (C) ^{1}H-NMR spectrum of poly(β-L-malate). (A and B) The two additional signals in panel B at 3.0 and 5.2 ppm correspond to the main signals of poly(β-L-malate). The strong signals at 3.6 and 3.9 ppm in panels A and B are generated by the added EDTA; all spectra are in D_20 (see Materials and Methods).

malate and five of six test plasmodia sporulated in the presence of 2 mM malate. In pilot experiments, the effect of malate became apparent. Additional experiments confirmed the sporulation-promoting effect of 2 to 10 mM malate. However, in no case did all plasmodia sporulate, perhaps indicating that malate is ineffective during a window in the cell cycle. These observations suggest that malate may be an active component of the sporulation pathway in the dark.

A possible direct effect of PMA—rather than monomeric malate—has not been studied because of its degradation by a specific hydrolase, polymalatase (12), which is also secreted by the plasmodium. Also, the higher amount of malate shown in Fig. 3A does not suggest higher sporulation activity of the conditioned medium fraction from the illuminated cultures because a larger sample size of the retinates of the medium from illuminated cultures was used to generate the spectrum depicted in Fig. 3A, to demonstrate the low level of PMA in conditioned medium derived from illuminated cultures.

An effect of malate on sporulation efficiency could not be shown in constant light. However, this does not exclude the effect of malate derived from PMA secreted from the illuminated test plasmodium to above threshold levels prior to the addition of exogenous malate to the test plate in the light sporulation assay.

DISCUSSION

Sporulation is an irreversible differentiation process in the life cycle of *Physarum* (18), and its analogies to embryonic induction have been discussed (17). The mechanism by which the plasmodium becomes competent to react to inducers of sporulation are poorly understood. However, starvation is a prerequisite for plasmodia to become competent to sporulate (4, 7, 17, 18, 22, 26). Progress has been made in unraveling the perception of light by a starved plasmodium. Three parallel pathways, triggered by either blue light, far-red light, or just heat shock, converge on a common photomorphogenesis pathway (4, 13, 14, 18, 23, 24).

To date most sporulation studies of *Physarum* have used the classical regimen (6, 18, 26) where an extended starvation period, usually 4 days, precedes illumination of different duration (pulses to 4 h). All happens to plasmodia on top of an ample supply of a liquid SM. This procedure, which is practical and reproducible, may not mimic either the conditions or the sequence of events in the natural habitat.

In preliminary experiments, several parameters were modified with the unanticipated result that a plasmodium, just on its moist filter paper support at 25°C, will sporulate in darkness. It seems that no exogenous signal is required, although illumination speeds up the sporulation process. One possible mechanism for self-promoting sporulation would be an autocrine stimulation by secreted factors. This possibility had been previously explored (26) by testing conditioned media. Evidence for SCF activity was obtained but no factors could be identified.

We argue here that the liquid sporulation medium in the previous studies dilutes secreted factors. Specifically, the volume effect of gels (Fig. 1A) can be best explained as a delay in sporulation by diffusion of sporulation-promoting factors. The sensitive parameter "sporulation time" and conditions of constant light or darkness now revealed that sporulation can occur at as early as 40 h in light and in 5 days in the dark. Sporulation on filter disks also rules out the possibility that enhancing factors are derived from the agarose gels. We further conclude that the assays used previously, like 4-day starvation in the dark, are insensitive to detect the factors shown in this study (because the starved plasmodia used to test for secreted factors presumably already supplied them at the above-threshold concentration). A further distinction of our assay is that it exposed fresh plasmodia (at the end of exponential growth phase but without any prior starvation on SM) to the test plates or conditioned gels (i.e., plasmodia not already partially autoinduced to sporulate).

Using the constant light sporulation assay and agarose plates, it was found that a fourfold dilution of the SM actually enhanced the sporulation process (i.e., shortened sporulation time by more than 10 h). This finding suggested inhibitory components in the SM. Screening individual components of SM, citrate, iron, manganese, and zinc ions were found to inhibit sporulation.

Under limiting conditions (i.e., the volume effect due to 20 ml of agarose in modified sporulation medium) calcium, at above 1 mM, was identified as a crucial requirement for sporulation. However, it was also demonstrated that sporulation happens without any exogenous calcium on agarose plates prepared in water and kept in the light. It is probable that under these conditions of plasmodial stress, calcium ions to above-threshold levels are released. It was previously shown that calcium was released from plasmodia after illumination (3), and this may explain why 1 mM calcium always had to be included in the dark sporulation assay (Fig. 2).

The most astonishing result of this work was the demonstration that the addition of malate was sufficient for sporulation in the dark. A possible physiological role for malate in sporulation can be deduced from the presence of PMA in that fraction (3 to 30 kDa) of conditioned medium (Fig. 3B and data not shown), which has sporulation-promoting activity when derived from plasmodia with a high sporulating capacity (Fig. 2A). PMA was discovered in *Physarum* and first identified as an inhibitor of the homologous DNA polymerase alpha (8) and its primase (11). Further investigations showed that PMA was synthesized only by plasmodia (1, 12, 19).

We propose that PMA is a competence factor of sporulation and suggest that the plasmodium secretes factors that induce its sporulation. These factors are calcium ions and malate. The latter could be derived from the action of the secreted extracellular PMAse on PMA (9, 12). It can be further argued that secreted PMA (a polyanion) captures calcium ions from the environment or transports calcium out of the plasmodium and makes free calcium available for sporulation after hydrolysis by PMAse. If so, the unexplored activation of PMAse may control the availability of both sporulation-enhancing factors described here: calcium ions and malate. Polymalate has been detected along with other high-molecular-weight microbial storage material, such as sPHA (poly-R-3-hydroxyalkanoates) and calcium polyphosphate (20). Interestingly, degradation of nuclear polyphosphate was detected during sporulation of *Physarum* (15).

Calcium seems to be required for the sporulation program of *Physarum*. A new role for calcium has recently been demonstrated for *Dictyostelium*, a cellular slime mold that is molecularly closely related to *Physarum*, a plasmodial slime mold (2). It was concluded (16) that a high calcium level at S phase was not required for cell cycle progression but was required for cell type choice at the onset of starvation.

This study has confirmed that growth conditions inhibit sporulation. However, starvation, while necessary, is not sufficient to permit sporulation. Coincident starvation and illumination significantly shorten the sporulation time. However, light is not necessary for successful sporulation, and possible roles for calcium and malate have been proposed. Until a specific signaling pathway for malate has been worked out, it is possible that a general state of stress triggers the sporulation program.

This is a classical problem with inducing and reacting developmental systems. It has been contemplated by H. Spemann (21) whether the amphibian organizer instructs the reaction system to develop nerve cells or permits it to select one of at least two available pathways (epidermis or nerve cells) already present in the reaction system.

Since PMA is secreted already under conditions of growth (19), it can be argued that sporulation may be the ground state of the developing plasmodium (just as nerve cell differentiation is in Xenopus [25]), which is repressed by a sporulation inhibitor in the growth medium (perhaps glucose). In Xenopus it has been shown that the inducer is an inhibitor of the neurulation inhibitor BMP4, a growth factor (25). The recently discovered mutant Cos (continuous sporulation), together with an elegant somatic complementation assay (24), may allow the genetic analysis of a putative inhibitor of the sporulation inhibitor and other aspects of the sporulation program of *Physarum*.

ACKNOWLEDGMENTS

We appreciate constructive criticism of drafts of the manuscript by Debby Siegele and M. Belyavskyi, College Station, Texas, and valuable comments by two anonymous reviewers.

This work was supported by a grant from the State of Bremen, Germany, to A.H.

REFERENCES

1. **Angerer, B., and E. Holler.** 1995. Large complexes of beta-poly (L-malate) with DNA polymerase alpha, histones, and other proteins in the nuclei of growing plasmodia of *Physarum polycephalum*. Biochemistry **34:**14741–14751.
2. **Baldauf, S. L., and W. F. Doolittle.** 1997. Origin and evolution of the slime molds. Proc. Natl. Acad. Sci. USA **94:**12007–12012.
3. **Belyavski, M., S. Beylina, B. Berenfeld, E. Rashevskaya, and L. Fateeva.** 1986. Light-stimulated changes in calcium exchange and their relation to the cell cycle control in *Physarum polycephalum* plasmodium. Stud. Biophys. **116:**205–215.
4. **Chapman, A., and J. G. Coote.** 1982. Sporulation competence in *Physarum polycephalum* CL and the requirement for DNA replication and mitosis. J. Gen. Microbiol. **128:**1489–1501.
5. **Daniel, J. W., and H. P. Rusch.** 1961. The pure culture of *Physarum polycephalum* on a partially defined medium. J. Gen. Microbiol. **25:**47–59.
6. **Daniel, J. W., and H. P. Rusch.** 1962. Method for inducing sporulation of pure cultures of the myxomycete *Physarum polycephalum*. J. Bacteriol. **83:** 234–240.
7. **Dove, W. F., and H. P. Rusch (ed.).** 1980. Growth and differentiation in physarum. Princeton University Press, Princeton, N.J.
8. **Fischer, H., S. Erdmann, and E. Holler.** 1989. An unusual polyanion from *Physarum polycephalum* that inhibits homologous DNA polymerase alpha in vitro. Biochemistry **28:**5219–5226.
9. **Gasslmaier, B., and E. Holler.** 1997. Specificity and direction of depolymerization of beta-poly(L-malate) catalyzed by polymalatase from *Physarum polycephalum*—fluorescence labeling at the carboxy-terminus of beta-poly(L-malate). Eur. J. Biochem. **250:**308–314.
10. **Hildebrandt, A.** 1986. A morphogen for the sporulation of *Physarum polycephalum* detected by cell fusion experiments. Exp. Cell. Res. **167:**453–457.
11. **Holler, E., G. Achhammer, B. Angerer, B. Gantz, C. Hambach, H. Reisner, B. Seidel, C. Weber, C. Windisch, C. Braud, et al.** 1992. Specific inhibition of *Physarum polycephalum* DNA-polymerase-alpha-primase by poly(L-malate) and related polyanions. Eur. J. Biochem. **206:**1–6.
12. **Karl, M., and E. Holler.** 1998. Multiple polypeptides immunologically related to beta-poly(L-malate) hydrolase (polymalatase) in the plasmodium of the slime mold *Physarum polycephalum*. Eur. J. Biochem. **251:**405–412.
13. **Kroneder, R., A. R. Cashmore, and W. Marwan.** 1999. Phytochrome-induced expression of lig1, a homologue of the fission yeast cell-cycle checkpoint gene hus1, is associated with the developmental switch in *Physarum polycephalum* plasmodia. Curr. Genet. **36:**86–93.
14. **Nakagaki, T., S. Umemura, Y. Kakiuchi, and T. Ueda.** 1996. Action spectrum for sporulation and photoavoidance in the plasmodium of *Physarum polycephalum*, as modified differentially by temperature and starvation. Photochem. Photobiol. **64:**859–862.
15. **Pilatus, U., A. Mayer, and A. Hildebrandt.** 1989. Nuclear polyphosphate as a possible source of energy during the sporulation of *Physarum polycephalum*. Arch. Biochem. Biophys. **275:**215–223.
16. **Saran, S.** 1999. Calcium levels during cell cycle correlate with cell fate of Dictyostelium discoideum. Cell Biol. Int. **23:**399–405.
17. **Sauer, H. W.** 1982. Developmental biology of physarum. Cambridge University Press, Cambridge, England.
18. **Sauer, H. W., K. L. Babcock, and H. P. Rusch.** 1969. Sporulation in *Physarum polycephalum*: a model system for studies on differentiation. Exp. Cell Res. **57:**319–327.
19. **Schmidt, A., C. Windisch, and E. Holler.** 1996. Nuclear accumulation and homeostasis of the unusual polymer beta-poly(L-malate) in plasmodia of *Physarum polycephalum*. Eur. J. Biol. **70:**373–380.
20. **Seebach, D., and M. G. Fritz.** 1999. Detection, synthesis, structure, and function of oligo(3-hydroxyalkanoates): contributions by synthetic organic chemists. Int. J. Biol. Macromol. **25:**217–236.
21. **Spemann, H.** 1988. Embryonic development and induction. Garland Publishing, Inc., New York, N.Y.
22. **Starostzik, C., and W. Marwan.** 1994. Time-resolved detection of three intracellular signals controlling photomorphogenesis in *Physarum polycephalum*. J. Bacteriol. **176:**5541–5543.
23. **Starostzik, C., and W. Marwan.** 1995. Functional mapping of the branched signal transduction pathway that controls sporulation in *Physarum polycephalum*. Photochem. Photobiol. **65:**930–933.
24. **Starostzik, C., and W. Marwan.** 1998. Kinetic analysis of a signal-transduction pathway by time-resolved somatic complementation of mutants. J. Exp. Biol. **201:**1991–1999.
25. **Streit, A., and C. D. Stern.** 1999. Neural induction. A bird's eye view. Trends Genet. **15:**20–24.
26. **Wilkins, A. S., and G. Reynolds.** 1979. The development of sporulation competence in *Physarum polycephalum*. Dev. Biol. **72:**175–181.