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Alkaline, Acid, and Neutral Phosphatase Activities Are Induced during Development in *Myxococcus xanthus*

ROBIN A. WEINBERG AND DAVID R. ZUSMAN*

Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

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One of the signals that has been reported to be important in stimulating fruiting body formation of *Myxococcus xanthus* is starvation for phosphate. We therefore chose to study phosphatase activity during *M. xanthus* development. Many phosphatases can cleave the substrate *p*-nitrophenol phosphate. Using this substrate in buffers at various pHs, we obtained a profile of phosphatase activities during development and germination of *M. xanthus*. These experiments indicated that there are five patterns of phosphatase activity in *M. xanthus*: two vegetative and three developmental. The two uniquely vegetative activities have pH optima at 7.2 and 8.5. Both require magnesium and both are inhibited by the reducing agent dithiothreitol. The developmental (spores) patterns of activity have pH optima of 5.2, 7.2, and 8.5. All three activities are Mg independent. Only the alkaline phosphatase activity is inhibited by dithiothreitol. The acid phosphatase activity is induced very early in development, within the first 2 to 4 h. Both the neutral and alkaline phosphatase Mg-independent activities are induced much later, about the time that myxospores become evident (24 to 30 h). The three activities are greatly diminished upon germination; however, the kinetics of loss differ for all three. The acid phosphatase activity declines very rapidly, the neutral activity begins to decline only after spores begin to convert to rods, and the alkaline phosphatase activity remains high until the time the cells begin to divide. All three developmental activities were measured in the developmental signalling mutants carrying *asg*, *csg*, and *dsg*. The pattern of expression obtained in the mutants was consistent with that of other developmentally regulated genes which exhibit similar patterns of expression during development. The ease with which phosphatases can be assayed should make the activities described in this report useful biochemical markers of stages of both fruiting body formation and germination.

Myxococcus xanthus is a gram-negative soil bacterium that is of interest to study because its life cycle is unique for a procaryote (8, 32, 33, 40). Unlike most bacteria, the myxobacteria interact with each other in cooperative groups. When food is plentiful and the cells are growing vegetatively, the bacteria move as hunting groups, preying on other bacteria and organic material in their path by the excretion of degradative enzymes and antibiotics (36). When nutrients become limiting, cells (about 100,000) assemble to form mound-shaped fruiting bodies. Within the fruiting bodies, about 98% of the cells differentiate into dormant myxospores; the remaining cells which do not enter fruiting bodies (about 10% of the total population) remain as peripheral rods (27). Reintroduction of nutrients triggers the myxospores to germinate in numbers large enough to form new hunting groups.

Early experiments clearly indicated that starvation signals the developmental cycle (6). Studies have been done to determine whether starvation for specific nutrients is required to induce fruiting body formation (25). Limiting levels of amino acids which provide both carbon and nitrogen clearly induce fruiting body formation. However, it was also found that lack of phosphate will trigger the developmental cycle. This is not surprising since phosphate is required for growth (5). Not only does a lack of phosphate induce fruiting, it has also been demonstrated that phosphate can induce the initial stages of germination of glycerol spores (30). As a first step to understand how phosphate regulation is working in *M. xanthus*, we decided to study the expression of phosphatases during development and germination. Gonzalez et al. (11) have studied phosphatases in *Myxococcus coralloides* D. However, the only study of phosphatase expression during development of *M. xanthus* is one that compares the level of alkaline phosphatase activity in glycerol spores with that in vegetative cells (7, 9). Other microorganisms that sporulate, such as *Bacillus subtilis* (28) and *Dictyostelium discoideum* (23), express much higher levels of alkaline phosphatase in spores than in vegetative cells. Our results demonstrated that the same is true of *M. xanthus*. We identified five phosphatase patterns of activity in *M. xanthus*: two which are vegetative and three which are developmental. The different phosphatase activities should make useful biochemical markers of stages of fruiting body formation and germination.

MATERIALS AND METHODS

Media, growth conditions, and strains. Casitone-yeast extract (CYE) (1) was used for vegetative growth of *M. xanthus*. Cells were grown with vigorous aeration at 32°C. Development was studied on clone fruiting (CF) agar (12). Development was induced by growing cells vegetatively until the mid-log or late log phase, pelleting the cells at 12,000 × *g*, suspending them at 4×10^9 cells per ml in TM buffer (0.01 M Tris hydrochloride, 0.008 M $MgSO_4$, pH 7.6), and spreading 2.5×10^8 cells on a CF agar plate. The plates were then incubated at 34°C unless otherwise noted. Medium components were obtained from Difco Laboratories, Detroit, Mich.

The *M. xanthus* strains used in these studies are listed in Table 1. DZF1 was used as the wild type (26). The DK strains were kindly supplied by Dale Kaiser.

Phosphatase assays. Vegetative samples were collected by

* Corresponding author.

TABLE 1. Strain list

Strain	Genotype	Source
DZF1	*sglA1*	Morrison and Zusman (26)
DK1622	Wild type	Kaiser (16)
DK5057	Tn*5*Ω4560 *asgA476*	Kuspa et al. (22)
DK2657	Tn*5-132*Ω1519 *csgA731*	Shimkets and Asher (34)
DK3260	Tn*5*Ω1867 *dsg-429*	Cheng and Kaiser (3)

pelleting cells at 12,000 × *g* for 10 min and suspending the cells in TM buffer or 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4. Developmental cells were harvested from plates in TM buffer by scraping with a razor blade. Samples were then pelleted and resuspended in TM buffer or 20 mM HEPES. Samples were frozen at −20°C before breakage by agitation in a Mini Beadbeater (Biospec Products, Bartlesville, Okla.) with zirconium beads (0.010- to 0.015-mm diameter). Cells and spores were broken after five 30-s pulses. Breakage was monitored microscopically. Samples were centrifuged for 5 min in a microcentrifuge, and the supernatant fraction was used in enzyme assays. Sample preparation from fruiting bodies 6 days old or older differed slightly. Spores were first purified from peripheral rods as described in the germination section below. Spores were then disrupted in the Mini Beadbeater.

Alkaline phosphatase was assayed as described by Kreuzer et al. (21), using 1 M Na_2CO_3 to stop the reaction. The activity at pH 7.2 was assayed as above except in 1 M Tris hydrochloride (pH 7.2). The acid phosphatase was assayed in 0.1 M sodium acetate (pH 5.2). All activities were expressed as nanomoles of product formed per minute per milligram of protein. Protein concentration was measured with BCA protein assay reagent obtained from Pierce Chemical Co. (Rockford, Ill.) according to the instructions of the manufacturer. Bovine serum albumin was used as a standard. Chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.).

pH optima. Three different buffers were used in this experiment depending on the pH range being assayed. For the lower range, pH 3.8 to 5.6, 0.1 M sodium acetate was used. In the middle range, pH 6.0 to 6.8, 0.25 M PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)] was used. At the high end, pH 6.8 to 10.0, 0.5 M Tris hydrochloride was used.

K_m determination. The color development of the product of the phosphatase reaction with *p*-nitrophenol phosphate as the substrate can be observed spectrophotometrically at pH values above 7.0. The rate of the reaction at pH 7.2 and 8.5 was measured at four substrate concentrations, 5, 10, 25, and 50 mM. The K_m was determined by plotting the velocity versus substrate concentration on a Lineweaver-Burk plot.

Germination. Spores were harvested after 7 days on CF plates. The spores were harvested in TM buffer containing 1 mM KH_2PO_4 and 10% Ficoll into conical centrifuge tubes. Cells were sedimented for 10 min at 500 rpm in a Sorvall GLC-2B general laboratory centrifuge. The top layer and interface were drawn off with a Pasteur pipette. The pellet containing the spores from fruiting bodies was resuspended in TM buffer, transferred to a round-bottom centrifuge tube, and pelleted at 12,000 × *g* for 20 min. This protocol resulted in very clean preparations of spores. Very few rods were present in these samples. The spores were suspended in a small volume of CYE, and the sample was placed in a sonicating water bath for 5 min to disrupt clumps of spores. The spores were then diluted in CYE to a Klett reading of approximately 120 (with a red filter). The culture was subjected to heat shock at 48°C for 10 min, placed on ice for 5 min, and transferred to a 32°C shaking incubator. Heat shock was found to lead to more synchronous germination. Germination was monitored microscopically. Germination was approximately 80% synchronous.

RESULTS

pH optima studies. To determine at what pH the phosphatases in *M. xanthus* were most active, we measured phosphatase activity in a pH range from 3.8 to 10 using extracts from different stages of the life cycle: (i) vegetative cells, (ii) cells in the process of aggregation (12 to 14 h), and (iii) mature myxospores (7 days old). These three samples were chosen because they reflect distinct stages of the life cycle when different sets of genes should be expressed (4, 19, 20). The three extracts gave very different patterns (Fig. 1). The vegetative cells had two major peaks of phosphatase activity, one around pH 5 and another at pH 8.5 (Fig. 1A). The aggregating cells (Fig. 1B) showed an increased peak of activity around pH 5, but the alkaline peak was reduced. The spore extract (Fig. 1C) appeared to have two nearly equivalent peaks of activity at pH 5 and pH 7. Note that the scales of specific activity for the three samples are very different. For example, the highest specific activity measured in the vegetative samples for the acid phosphatase was almost 10-fold lower than that recorded for the spore sample. The difference in pattern of the three samples, as well as the differences in actual specific activities, suggested that the phosphatase activities we were measuring were subject to developmental regulation. As a result of this experiment, we chose pH 5.2, 7.2, and 8.5 to assay developmental induction of phosphatases.

Developmental expression. We observed the expression of the phosphatases at the three pH values listed above during development. Fruiting body formation was induced as described in Materials and Methods. Samples were harvested every 3 h and assayed for activity. All three phosphatase activities were greatly induced during development, to levels 10- to 20-fold over vegetative activities (Fig. 2). However, the timing of induction appeared to be different for acid phosphatase than for the neutral and alkaline phosphatases. The acid phosphatase appeared to increase within 3 h after the cells were plated on fruiting agar. The neutral and alkaline phosphatases were not induced until about 30 h into development. By this time in development, aggregation was complete and the spores were beginning to become refractile. These results suggest that the expression of the neutral and alkaline phosphatases is controlled in a similar manner, whereas the regulation of acid phosphatase appears to be distinct.

Defining isozymes. We wanted a way to determine whether the enzyme activity we were measuring over time at a given pH was the result of one isozyme. The method we used to approach this problem was to determine the cofactor requirements, if any, for the three phosphatase activities. We chose to test magnesium because alkaline phosphatase activity from both *D. discoideum* and *B. subtilis* is routinely assayed in its presence (10, 23). We also tested the sensitivity of the activities to the reducing agent dithiothreitol (DTT) since it has been documented that the alkaline phosphatase of *Escherichia coli* is sensitive to this compound (13). The results are presented in Table 2. The acid phosphatase activity measured in the vegetative and aggregation (12 h of

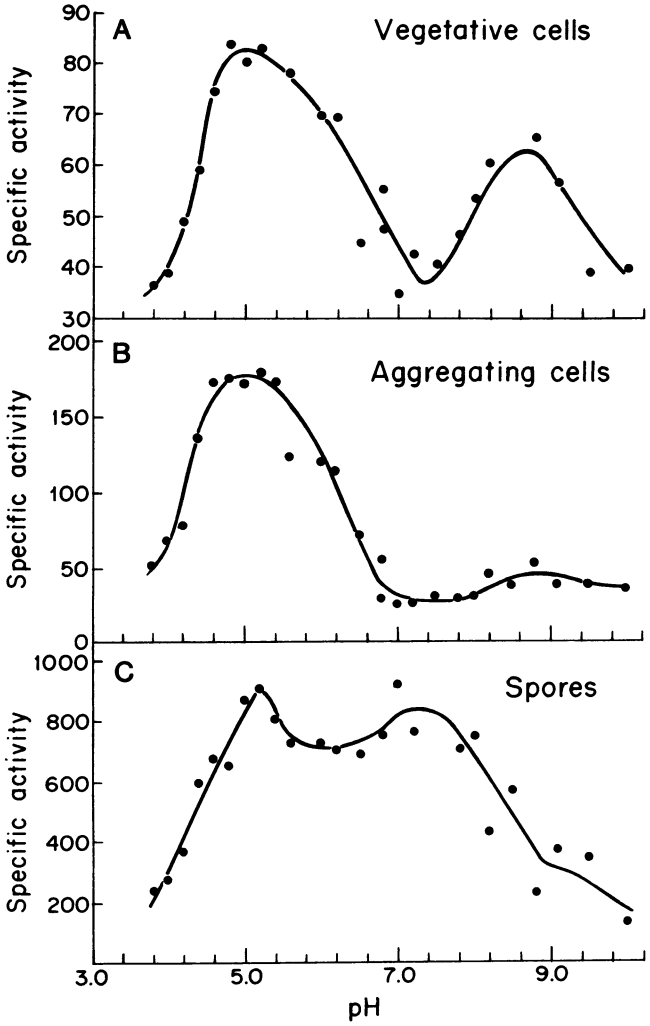

FIG. 1. pH optima of Mg-independent phosphatases from cells at three stages in the *M. xanthus* life cycle. Phosphatase activity was measured with *p*-nitrophenol phosphate as the substrate in a pH range from 3.8 to 10.0 in the absence of $MgCl_2$. Vegetative extracts were made from cells growing logarithmically in CYE broth (A). Extracts for developmental cells were prepared by scraping cells from CF plates that had been incubating at 34°C for 14 h (B), a time when cells are aggregating to form fruiting bodies, or 7 days (C), once fruiting body formation is complete and the spores are mature. Note the differences in scale used for specific activity for the three cell types.

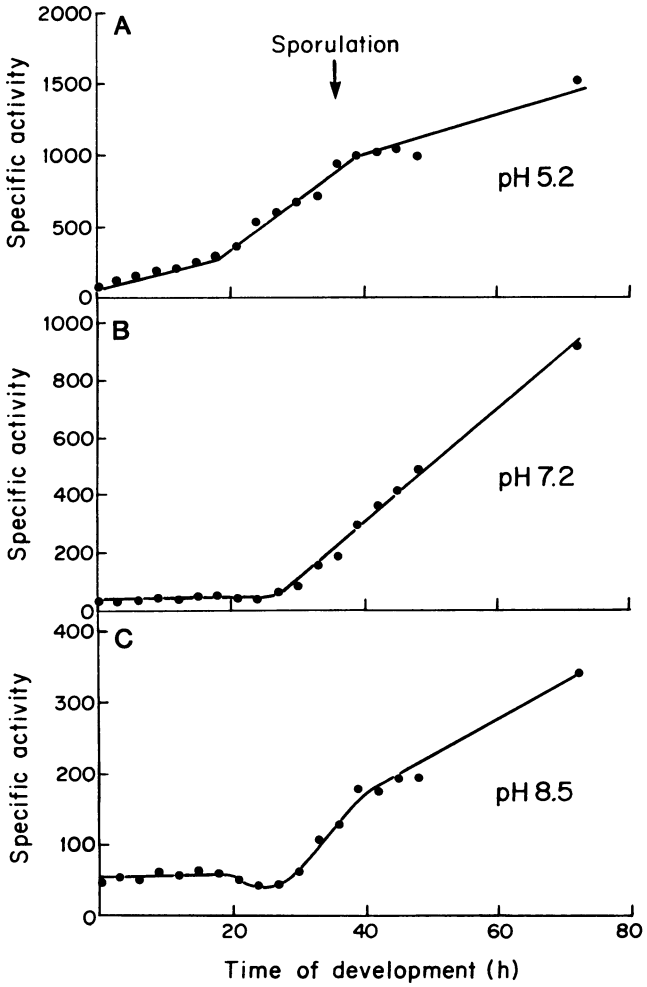

FIG. 2. Developmental induction of Mg-independent phosphatase activities. Vegetatively growing cells were plated on CF agar to induce fruiting body formation. Plates were incubated at 34°C. Cells were harvested every 3 h for the first 48 h and at 72 h. (A) Acid phosphatase. (B) Neutral phosphatase. (C) Alkaline phosphatase. The arrow indicates the time when 98% of the cells in aggregates have converted to spores.

development) stages and in spores (6 days old) was not affected by the addition of 10 mM $MgCl_2$ or 5 mM DTT. Thus, by these criteria, one enzyme might be responsible for all three activities.

At pH 7.2, the addition of 10 mM $MgCl_2$ stimulated activity in both vegetative cells and those 12 h into development. The activity in the spore sample was not affected by the addition of $MgCl_2$. The two activities also differed in their sensitivity to DTT. DTT was inhibitory to the Mg-dependent activities, as seen in the third column in Table 2. The Mg-independent spore activity, on the other hand, was unaffected by the addition of DTT (Table 2, second column). The Mg-independent activity in the other two samples was also more resistant to DTT than was the Mg-dependent activity (Table 2, compare second and third column). It therefore appeared that there were two neutral phosphatase activities, one that was present in vegetative cells and early in development that was Mg dependent and sensitive to DTT, and another that made up a bulk of the activity in spores which did not require $MgCl_2$ for full activity and was resistant to DTT.

When assayed at pH 8.5, the addition of $MgCl_2$ resulted in a greater increase in activity in the nonspore extracts than was observed at pH 7.2 (Table 2). The spore samples was again unaffected, suggesting that the enzyme present in the spore sample was distinct from that present in the other two samples. The addition of 10 mM EDTA reduced the activity of the alkaline phosphatase to one-third that of the control. It had little effect on any of the other activities.

Unlike the activities measured at pH 7.2 which showed differential effects to DTT, all the alkaline activities were strongly inhibited by the reducing agent. (The only exception was the Mg-independent vegetative activity, a very minor component of phosphatase activity.) This suggests that the Mg-independent phosphatase activities being measured at

TABLE 2. Effect of Mg and DTT on phosphatase activity as a percentage of control[a]

pH and age of culture	$MgCl_2$ (10 mM)	DTT (5 mM)	$MgCl_2$ and DTT[b]	K_m[c] (mmol)
pH 5.2				
Vegetative	104	104	103	ND[e]
12 h	100	89	—[d]	ND
6 days	116	99	—	ND
pH 7.2				
Vegetative	220	59	28	7.14
12 h	700	76	30	ND
6 days	111	97	—	7.14
pH 8.5				
Vegetative	557	76	9	4.17
12 h	875	36	16	10.00
6 days	84	0	0	10.00

[a] Samples were prepared in TM. The control of 100% was the activity measured in the absence of $MgCl_2$ for the first two columns.

[b] The control of 100% was the activity measured in the presence of 10 mM $MgCl_2$ and therefore is a measure of DTT sensitivity of the Mg-dependent activity.

[c] Cells were broken in 20 mM HEPES. The K_m was determined as described in Materials and Methods.

[d] —, The value was not determined because the activity present in the samples was almost completely Mg independent.

[e] ND, Not determined.

the two pHs, 7.2 and 8.5, are due to distinct enzymes, one resistant to DTT and the other sensitive.

Another way to determine whether or not activities are due to distinct enzymes is to compare the K_m of the reactions (Table 2). This was done for the three samples at pH 8.5 and the vegetative and spore samples at pH 7.2. Samples were prepared in HEPES and assayed in the absence of $MgCl_2$ since under these conditions Mg was only slightly stimulatory. At pH 8.5, the spore and 13-h-old sample had the same K_m, 10.0 mM. The K_m of the vegetative sample was 4.17 mM, again indicating that the vegetative and early developmental activity was distinct from that seen later in development. At pH 7.2, both the vegetative and spore samples had a K_m of 7.14 mM, suggesting that both activities arose from the same enzyme. This is contrary to the results shown in Table 2. It is possible that by preparing the cells in HEPES rather than TM buffer the solubility of enzymes may differ. It would appear from these results that the neutral Mg-independent activity was preferentially released as was the alkaline Mg-independent activity in the 13-h-old sample.

To examine the kinetics of induction during development of the Mg-dependent activities, we reassayed the samples used in the experiment shown in Fig. 2 in the presence of 10 mM $MgCl_2$. (These samples were prepared in TM buffer.) The results presented in Fig. 3 demonstrate that the neutral Mg-dependent phosphatase activity was present in vegetative cells at levels higher than the Mg-independent activity.

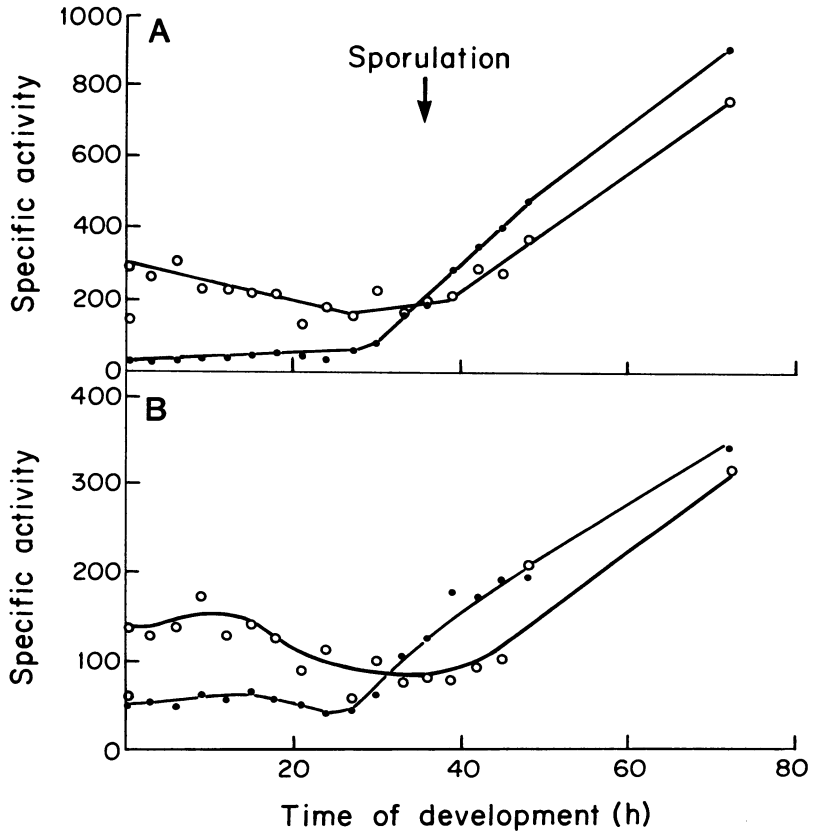

FIG. 3. Developmental induction of Mg-dependent phosphatase activities. Using the same cell extracts as were used in Fig. 2, phosphatase activity was measured in the presence of 10 mM $MgCl_2$ (○). This activity is a sum of both the Mg-dependent and -independent activity. The Mg-dependent activity can be calculated by subtracting the Mg-independent activity from that observed in the presence of $MgCl_2$. The Mg-independent activity was replotted for easier comparison (●). (A) Assays were done at pH 7.2. (B) Assays were done at pH 8.5. The arrow indicates the time when 98% of the cells in aggregates have converted to spores.

TABLE 3. Expression of Mg-independent phosphatases in sporulation-deficient mutants at 4 days of age as a percentage of parent strain DK1622[a]

pH	DK5057 *asg*	DK2657 *csg*	DK3260 *dsg*
5.2	25	84	120
7.2	35	14	31
8.5	0	8	21

[a] Cells were plated on CF agar and incubated at 28°C.

The Mg-dependent alkaline phosphatase activity was also more abundant in vegetative cells and early in development than was the Mg-independent counterpart. A small rise about 10 h into development was also evident. Both Mg-dependent activities appeared to decline before the onset of expression of the Mg-independent forms. It would appear from these studies that the Mg-dependent activities are vegetative, persisting early in development, while the independent forms are late developmental enzymes.

Expression of phosphatases in nonsporulating strains. Kaiser and co-workers (12) have isolated a series of mutants that are unable to form fruiting bodies by themselves but can be stimulated to develop by wild-type cells. These strains have proved useful in defining stages in the developmental pathway and have been grouped into four classes, *asg*, *bsg*, *csg*, and *dsg* (12). The four mutants are believed to be defective in producing signals that are necessary for transcription of developmentally regulated genes. Genes that do not require *asg* are expressed very early in development, within the first 2 h (22). Genes induced before 6 h of development do not require *csg* (19), and those expressed before 4 to 5 h do not require *dsg* (3). The differential timing of expression of the various phosphatases led us to investigate whether any of the signal functions were required for their expression. We assayed for developmental phosphatase activity (Mg independent) in an *asg* mutant (DK5057 [22]), a *csg* mutant (DK2657 [34]), and a *dsg* mutant (DK3260 [3]) as well as the parent strain DK1622 (16). The strains were plated on CF agar and incubated at 28°C. Samples were assayed after 4 days of incubation. Table 3 is a comparison of the phosphatase activities in the mutants and the parent strain, DK1622. It appears that in the *asg* strain, which is blocked earliest in development, none of the three phosphatase activities was induced to the wild-type level. In both the *csg* and the *dsg* mutants, the acid phosphatase activity was induced, but the neutral and alkaline activities were not. These results are consistent with the time of action that has been reported for the signal functions described above.

Effect of addition of phosphate to growth media on expression of phosphatases. It has been reported in many systems that the level of phosphatases, in particular, alkaline phosphatase, is repressed by the presence of phosphate in the media (2, 10, 38). We were interested to determine whether this was the case for the activities we were measuring in *M. xanthus* in the hope that this might give us an insight into the role of phosphate in inducing development. We tested the effect of exogenous phosphate during both vegetative growth and development. We found that the addition of phosphate affected cell growth. Adding 5 mM KH_2PO_4 to vegetative medium (CYE) almost doubled the generation time of DZF1. The addition of 10 mM had an even more profound effect. Whereas the control went through three divisions in 18 h, the culture to which 10 mM KH_2PO_4 had been added required 57 h to sustain an equivalent amount of growth after a 24-h lag.

TABLE 4. Effect of exogenous phosphate on expression of phosphatases

Growth condition[a]	Relative phosphatase expression with the following amt of phosphate:				
	Control[b]	0 mM[c]	1 mM	5 mM	10 mM
Vegetative					
pH 5.2	100		113	106	70
pH 7.2	100		120	81	102
pH 8.5	100		107	80	91
pH 7.2 + Mg[d]	100		101	64	73
pH 8.5 + Mg[d]	100		85	66	53
Developmental[e]					
pH 5.2	100[f]	117		168	137
pH 7.2	100[f]	56		154	120
pH 8.5	100[f]	92		173	107

[a] Cells (DZF 1) were grown in CYE broth at 32°C with vigorous aeration and harvested at approximately 100 Klett units.
[b] The value obtained without the addition of K_2HPO_4 was normalized as 100.
[c] No K_2HPO_4 was added to the media.
[d] $MgCl_2$ was added at 10 mM.
[e] Cells (DZF 1) were plated on CF agar, incubated at 34°C, and harvested after 66 h, once mature fruiting bodies had formed.
[f] CF agar contains 1 mM K_2HPO_4.

The effect of added phosphate on phosphatase activities was also measured (Table 4). All vegetative cultures were harvested at approximately the same cell density, 10^9 cells per ml. The extracts were assayed for five different activities, and assays were performed at three different pHs in the absence of Mg and at pH 7.2 and 8.5 in the presence of Mg (Table 4). Of all five activities tested, only the Mg-dependent alkaline phosphatase activity appeared to be repressed by the exogenous phosphate. The addition of 5 mM KH_2PO_4 reduced the level to two-thirds that of the control, and the addition of 10 mM KH_2PO_4 led to even greater repression, one-half that of the control (Table 4).

When the experiment was done on developing cultures, we found that the addition of 10 mM KH_2PO_4 to CF agar slowed development by approximately 24 h when the culture was incubated at 34°C. We tested only the Mg-independent activities in these samples since we believe they are spore specific. None of the three activities was repressed by the addition of phosphate (Table 4). However, normally CF agar contains 1 mM KH_2PO_4. When it was omitted, the activity at pH 7.2 was only one-half that of the control, suggesting that it requires phosphate for induction. The acid and alkaline activities were unaffected by this lack of phosphate.

Expression of phosphatases during germination. The high level of phosphatase activities in mature spores led us to consider whether or not they played a role during germination. As a first step in determining such a role, we measured the level of activity of the developmental (spore) phosphatases during germination. Spores that had been harvested after 7 days of development were germinated in CYE broth as described in Materials and Methods. The level of phosphatase activity was measured during this process and throughout growth. The three activities fell dramatically during germination but with different kinetics (Fig. 4). The acid phosphatase dropped almost 30% by 3 h, before any visual evidence of conversion of spores to rods. The neutral phosphatase began to drop when rods began to become evident, by about 5 h. The alkaline phosphatase did not drop until 8 to 10 h, when cells were beginning to divide as measured by an increase in turbidity. Thus, these three

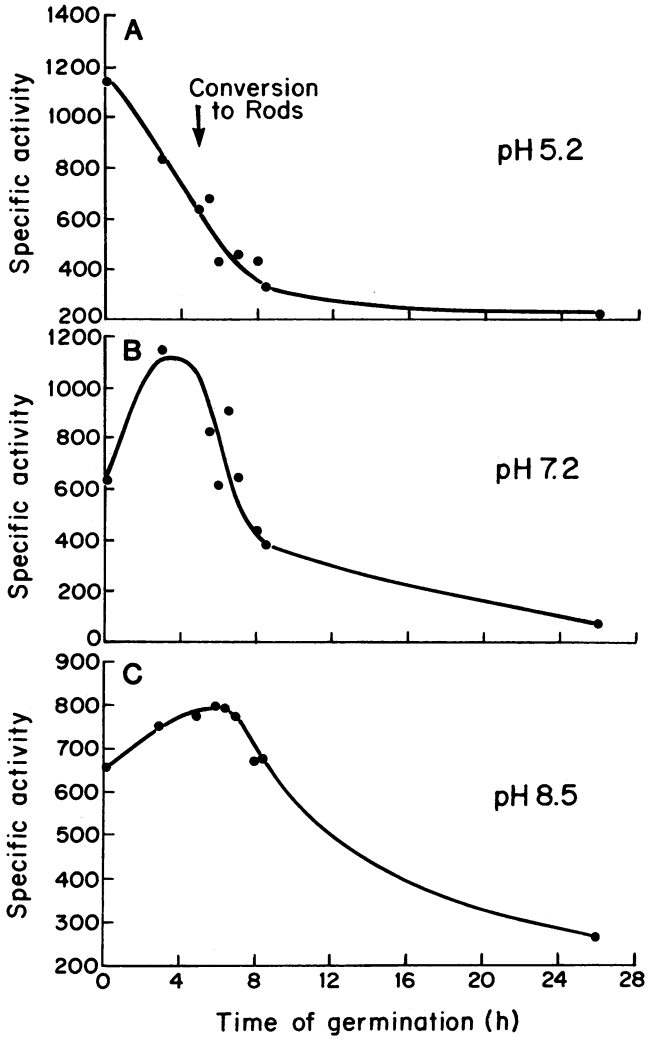

FIG. 4. Loss of Mg-independent phosphatase activities during germination. Spores were harvested from CF plates that had been incubated at 34°C for 7 days. Germination was induced as described in Materials and Methods and monitored microscopically. By this method the turbidity began to drop after 4 to 4.5 h. Rods were apparent after 5 to 6 h and began to divide by 10 h as evidenced by a rise in turbidity. Phosphatase assays were done in the absence of $MgCl_2$. (A) Acid phosphatase. (B) Neutral phosphatase. (C). Alkaline phosphatase. The arrow indicates the time when spores are beginning to convert to rods.

activities may be used to define three stages of the germination process and outgrowth.

Solubilization of activity from spores. Throughout these studies, we noticed that the amount of phosphatase activity in the spore samples varied depending on the manner in which the samples were prepared. It seemed possible that the differences in activity observed were due to our inability to completely solubilize the phosphatase activities. The protocol was varied to maximize the amount of activity present in the supernatant fraction. We found that the maximum amount of phosphatase activity was solubilized from myxospores when the sample was adjusted to 0.1% Triton X-100 before it was disrupted with the Mini Beadbeater. This was especially true when samples were prepared in HEPES as opposed to TM buffer. If Triton X-100 was omitted, the activity could be solubilized by adding

TABLE 5. Solubilization of alkaline phosphatase from myxospores[a]

Procedure	% Soluble activity[b]
Break in TM[c]	60
Break in HEPES	3
Break in HEPES + 0.1% Triton X-100	75
Break in HEPES, treat with 0.1% Triton X-100[d]	9
Break in HEPES, treat with Triton X-100 and sonicate[e]	15
Break in HEPES, treat with Triton X-100 and 10% $(NH_4)_2SO_4$[f]	54
Break in HEPES, treat with Triton X-100, sonicate, 10% $(NH_4)_2SO_4$[g]	115

[a] Spores were harvested from CF plates after 7 days.
[b] Soluble activity is that remaining in the supernatant fraction after the samples were spun in the microcentrifuge (14,000 × *g*) for 10 min. The activity present in extracts before centrifugation was used as 100%.
[c] Spores were disrupted in the Mini Beadbeater as described in Materials and Methods.
[d] Triton X-100 was added at 0.1% after breakage in the Mini Beadbeater, and the samples were incubated for 30 min at 37°C.
[e] The sample was treated as in footnote *d* except it was sonicated for 1 min before incubation at 37°C.
[f] The sample was treated as in footnote *d* except 0.10 volume of saturated $(NH_4)_2SO_4$ was added before incubation.
[g] The sample was treated as in footnote *f* except it was sonicated immediately before the addition of $(NH_4)_2SO_4$.

0.1% Triton X-100 to the disrupted sample, sonicating briefly, and immediately adding 0.10 volume of saturated $(NH_4)_2SO_4$ followed by an incubation at 37°C for 30 min. It was necessary to perform each of the steps described in the protocol above to fully solubilize the activity (Table 5). These results suggest that the activity was associated with the spore coat or membrane. Alternatively, the enzyme may be difficult to rehydrate from the interior of the spore.

The possible association of the activities with spore coat or membrane and the nonphysiological pH at which two of the phosphatases are most active led to the hypothesis that the phosphatases were on the outer surface of the spore. To address this question, we assayed intact spores for phosphatase activities. We were able to detect 50% of the total acid phosphatase activity, 29% of the neutral activity, and 14% of the alkaline phosphatase activity. The total activity was measured when spores were disrupted in the Beadbeater. These results suggested that the acid phosphatase was the most accessible. Experiments were then done to try to solubilize the phosphatase activities from intact spores. The spores were treated as described above with Triton X-100 and $(NH_4)_2SO_4$. At best, 15% of the acid phosphatase activity was solubilized. A similar result was obtained when 10 mM EDTA with or without 1 M NaCl was tried. These results suggest that the phosphatases are probably not on the outermost surface of the spores. The ability to measure phosphatase activity with intact spores may result from the permeability of the cells, especially immature spores, to the substrate, *p*-nitrophenol phosphate, which is a small molecule.

DISCUSSION

It had previously been demonstrated that the level of phosphate in the medium was important in determining whether *M. xanthus* would enter the developmental phase of its life cycle. Manoil and Kaiser (25) have shown that in addition to nitrogen and carbon limitation, phosphate starvation can also trigger fruiting body formation. At the other

end of the developmental cycle, Ramsey and Dworkin (30) have shown that phosphate can induce the initial stages of germination of glycerol spores. Our results support the hypothesis that regulation of phosphate pools is important since we measured a large increase in phosphatase activity during development. It is interesting that one of the earliest observations about alkaline phosphatase expression in *E. coli* was that it was synthesized when cells had stopped growing and had entered the stationary phase (35). The phosphate-regulated *phoA* gene in *E. coli* can be induced not only by phosphate starvation but by nitrogen and carbon starvation as well. This scenario is similar to the triggers of fruiting body formation in *M. xanthus*. Killeen and Nelson (17) have recently shown that heat shock will also induce alkaline phosphatase in *M. xanthus*.

The data presented in these studies suggest that vegetative cells of *M. xanthus* have a phosphatase profile similar to that described for *E. coli*. The pH optima in Fig. 1A for vegetative cells resemble those published for *E. coli* (29), with a majority of the activity falling in the acid range (pH 4 to 5) but with a second, smaller peak in the alkali range (pH 8 to 9). Furthermore, the repression of the *M. xanthus* alkaline phosphatase by 10 mM phosphate is akin to the type of regulation described for alkaline phosphatases studied in *E. coli*, *Pseudomonas aeruginosa* (2, 38), and vegetative cells of *B. subtilis* (10). The increase of Mg-dependent alkaline phosphatase early in *M. xanthus* development may be similar to the induction of alkaline phosphatase in *E. coli* when cells enter the stationary phase.

The inhibition of the vegetative form of *M. xanthus* alkaline phosphatase by the reducing agent DTT is also similar to the *E. coli* enzyme (13). The cellular location of both the acid and alkaline phosphatases in vegetative cells of *M. xanthus* has been determined cytologically by Voelz and Ortigoza (37), and as in many other bacteria, the enzymes reside in the periplasm. The DTT sensitivity of the Mg-dependent neutral phosphatase suggests that it too resides in the periplasm, since the cytoplasm is a reducing environment.

Both our work and that of Dworkin (5) suggest that *M. xanthus* can tolerate only a narrow range of phosphate concentrations in vegetative medium. Growth in CYE was severely inhibited by 10 mM phosphate, and even 1 mM phosphate was slightly inhibitory. A minimal medium used for vegetative growth, A1, contains 1 mM phosphate (32). Dworkin (5) found that when cells were grown in 2% Casitone, the addition of 10 mM phosphate was optimal for growth and that 3 and 30 mM were both inhibitory. The reason for the discrepancy is not clear, but it is likely that yeast extract (which is present in CYE broth) contains a fair amount of phosphate. The reason for this range is unclear at this time but may reflect an inability of the cell to maintain a phosphate balance under these extreme conditions.

Perhaps the most interesting aspect of these studies was the dramatic increase in phosphatase activities observed during *M. xanthus* development. It is clear that at least three activities are induced during fruiting body formation: the acid phosphatase, the Mg-independent, DTT-resistant neutral phosphatase, and the Mg-independent alkaline phosphatase. It also appears that the two vegetative phosphatases, the Mg-dependent neutral and alkaline phosphatases, are repressed late in development since the level of Mg-dependent activity decreased to a point at which all the activity appeared to be Mg independent.

Synthesis of the acid phosphatase was induced very early in development. Experiments presented involving expression in the *asg*, *csg*, and *dsg* mutants demonstrated that the *asg* function was necessary for developmental induction of the acid phosphatase. This result suggests that the rise in activity observed was most likely due to de novo synthesis and not modification of a preexisting enzyme. (Although it is formally possible that transcription of a modifying enzyme is regulated by *asg*.) The results also suggest that acid phosphatase is transcribed between 2 and 5 h of development since it was not induced in the *asg* mutant but was induced in both *csg* and *dsg* mutants (3, 19, 22).

It is clear that the neutral and alkaline phosphatases that are induced during spore formation are distinct from their vegetative counterparts (Table 2). The results obtained from the *asg*, *csg*, and *dsg* mutants suggest that induction of these activities is later than 6 h of development.

It appears that in at least three sporeforming microorganisms, *B. subtilis* (14, 28), *D. discoideum* (23), and *M. xanthus*, the induction of alkaline phosphatase requires commitment to spore formation. We have observed in other mutants of *M. xanthus* which do not aggregate normally but still form spores, e.g., the *frz* mutant (39), that alkaline phosphatase is induced to wild-type levels, suggesting that induction is associated with spore formation rather than aggregation. Furthermore, the overwhelming majority of alkaline phosphatase in fruiting bodies is associated with spores and not peripheral rods (K. A. O'Connor and D. R. Zusman, manuscript in preparation). Dworkin (7) has shown that glycerol spore formation also involves the induction of alkaline phosphatase. The increase in activity in *B. subtilis* is associated with stage II. A free protoplast must be formed to allow synthesis of alkaline phosphatase (28). Studies in *Bacillus licheniformis* (14) have shown that there are two structural genes for alkaline phosphatase and that they appear to be transcribed with different sigma factors. One uses the vegetative sigma factor, σ^{55}, and the other uses σ^{37}. Expression of this second activity is not affected by exogenous phosphate (28), similar to the result we obtained in *M. xanthus*. Last, studies in *D. discoideum* revealed that in this organism alkaline phosphatase is most strongly induced when the pseudoplasmodia culminate to form fruiting bodies (23).

Since the activity of phosphatase was so high in spores, it seemed reasonable to postulate that the activities might be important during germination. Contrary to the findings on germination of glycerol spores (5, 7), we did not see a large increase in any phosphatase activity during germination. In fact, the acid phosphatase activity dropped very abruptly. Our findings are also contrary to what has been reported for *M. coralloides* D (11), in which an increase in both acid and alkaline phosphatases was observed early in development followed by a decrease as spores began to form. We postulate that the difference seen in our results compared with previous studies occurred because we were able to measure spore-associated activities more efficiently than other investigators. We found that unless the spores were broken in the Mini Beadbeater, we were unable to detect all the spore activity. The increase in phosphatase activities reported during germination by other investigators may be caused by the relative ease in disrupting spores as they start to germinate.

It is interesting that the kinetics of loss of activities during germination for the three activities are different. The time at which the activity is lost seems to correlate well with our ability to assay for the activity using intact spores. The acid phosphatase, which decreased almost immediately upon putting spores in vegetative medium, was the most accessi-

ble. The neutral phosphatase activity, which dropped once spores converted to rods, was less accessible, and the alkaline phosphatase activity, which did not drop off until the cells began to divide, was the least accessible. It is unclear what role these phosphatases play during germination, but they may be involved in scavenging phosphate. The acid phosphatase may scavenge phosphate from the medium, while the alkaline phosphatase may be used to depolymerize polyphosphate that may have been stored in the spores. It has been reported that *E. coli* requires alkaline phosphatase to grow on long-chain polyphosphate as the sole phosphate source (31). It is also common for cells to store phosphate as polyphosphate. It seems reasonable that upon germination phosphorus would be required for the synthesis of a large number of essential cell components, and it would be worth investigating to see whether myxospores contain polyphosphates.

Besides serving as a preliminary study of phosphatases during development of *M. xanthus*, we think that these studies have some practical applications. When studying any developing system, it is useful to have biochemical markers that can serve as landmarks for particular stages of development. With this in mind, Kroos et al. (20) isolated a collection of developmentally regulated Tn*5-lac* transcriptional fusions (18). Although these strains have proved useful, they do not substitute for endogenous markers for many kinds of experiments. Endogenous markers have the advantage that they can be assayed in any strain and do not require further strain construction. Furthermore, it does not require the introduction of another mutation, namely, the Tn*5-lac*. Last, one is assaying for expression of a gene of known function. Relatively few developmental markers have been described for *M. xanthus*. The most commonly used markers in our laboratory are myxobacterial hemagglutinin, which is expressed during aggregation (4), and protein S, which is a spore-specific protein (15). While myxobacterial hemagglutinin is readily assayed by using an erythrocyte hemagglutination assay, quantitation of protein S requires immunological assays and therefore requires antibodies. The phosphatases, on the other hand, are very easy and inexpensive to assay. We therefore think that the acid phosphatase is an excellent marker of early development. The neutral and alkaline Mg-independent activities serve well as markers of maturing spores. We presented data which demonstrated that these markers behave as would be predicted in the developmental mutants *asg*, *csg*, and *dsg*, whose mutations define stages of development.

Our experiments suggest that the phosphatases can also be used to define stages in the germination process. In this instance, one would look for loss of activity rather than gain of activity. The first stage would be loss of acid phosphatase; the second stage, which morphologically corresponds to the conversion of round spores to rods, would be marked by the loss of the neutral activity; and the start of cell division could be identified with loss of alkaline phosphatase activity.

A word of caution should be made about using the activities as developmental markers. When these experiments were repeated with samples that had been disrupted in 20 mM HEPES instead of TM buffer, the addition of $MgCl_2$ had only a minor stimulatory effect, the largest being approximately 2.5-fold. Furthermore, DTT sensitivity was only observed when the samples were assayed at pH 8.5. We are unsure why we saw a difference in stimulation by Mg when cells were broken in HEPES as opposed to TM buffer. It had been observed that in both *P. aeruginosa* (2) and *M. xanthus* (37) when cells for cytological studies were washed in the absence of Mg, the alkaline phosphatase tended to stick to the cell wall. It is possible that by breaking the cells in HEPES in the absence of Mg only the Mg-independent activity was solubilized. Alternatively, in HEPES buffer the Mg may remain more tightly bound to the enzyme and therefore there was less stimulation by the addition of Mg.

Last, with the advent of the use of the transposon Tn*5-phoA* to probe for protein export signals (24), it is important to be able to distinguish the alkaline phosphatase activity resulting from the transposon from that of the parent cell. We think that the Mg-dependence of the *M. xanthus* vegetative enzyme may be useful to distinguish the two activities when studying secretion during vegetative growth. However, we advise caution in using this construction to measure activity during development. Studies must employ appropriate controls.

In the studies presented here, we focused on the developmental regulation of phosphatase activities and referred to them as activities rather than specific enzymes. We are uncertain whether the activity we were measuring under any given set of conditions was the result of only one enzyme or due to a combination of enzymes which can all cleave the substrate *p*-nitrophenol phosphate. It has been demonstrated in *E. coli* that a number of enzymes contribute to the overall acid phosphatase activity that is measured in crude extracts (29). It is also possible that during development a single phosphatase present in vegetative cells is modified in some way to alter its activity. The increase in activity we observed during development may be due to modification of a preexisting enzyme rather than de novo synthesis of an isozyme. Our results on Mg dependence and DTT sensitivity argue against a single-enzyme hypothesis but certainly do not rule it out.

In conclusion, we identified five patterns of phosphatase activity in *M. xanthus*. There are two vegetative activities, both of which require Mg and are inhibited by DTT; however, one has a pH optimum of 7, while the other is an alkaline phosphatase. The three developmental activities are an acid phosphatase (pH 5.2) that is Mg independent and DTT resistant, a neutral phosphatase (pH 7.2) that is Mg independent and DTT resistant, and an alkaline phosphatase (pH 8.5) that is Mg independent and DTT sensitive.

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