Phosphate Acquisition Components of the *Myxococcus xanthus* Pho Regulon Are Regulated by both Phosphate Availability and Development[▽]

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In many organisms, phosphatase expression and phosphate (P) uptake are coordinately regulated by the Pho regulon. In *Myxococcus xanthus* P limitation initiates multicellular development, a process associated with changes in phosphatase expression. We sought here to characterize the link between P acquisition and development in this bacterium, an organism capable of preying upon other microorganisms as a sole nutrient source. *M. xanthus* seems to possess no significant internal P stores, as reducing the P concentration to less than 10 μ M retarded growth within one doubling time. Pyrophosphate, polyphosphate, and glyceraldehyde-3-phosphate could support growth as sole P sources, although many other P-containing biomolecules could not (including nucleic acids and phospholipids). Several Pho regulon promoters were found to be highly active during vegetative growth, and P limitation specifically induced *pstSCAB*, *AcPA1*, and *pho3* promoter activity and repressed *pit* expression. Enhanced *pstSCAB* and *pho3* promoter activities in a *phoP4* mutant (in the presence of high and low concentrations of P) suggested that PhoP4 acts as a repressor of these genes. However, in a *phoP4* background, the activities of *pstSCAB* remained P regulated, suggesting that there is additional regulation by a P-sensitive factor. Initiation of multicellular development caused immediate down-regulation of Pho regulon genes and caused *pstSCAB* and *pho3* promoter activities to become P insensitive. Hence, P acquisition components of the *M. xanthus* Pho regulon are regulated by both P availability and development, with developmental down-regulation overriding up-regulation by P limitation. These observations suggest that when development is initiated, subsequent changes in P availability become irrelevant to the population, which presumably has sufficient intrinsic P to ensure completion of the developmental program.

The deltaproteobacterium *Myxococcus xanthus* exhibits a population-wide response to starvation, forming multicellular fruiting bodies within which sporulation occurs (4). This developmental program can be initiated by omitting phosphate (P) from the growth medium (13), and late events during development include expression of specific phosphatases. Phosphatase expression and P acquisition are coordinately regulated by the Pho regulon in many bacteria, including *Escherichia coli*, *Bacillus subtilis*, and *Streptomyces lividans* (8, 20, 22). The core of each Pho regulon comprises a two-component system (TCS) that responds to P limitation by induction of the *pstSCAB* operon (encoding a high-affinity P uptake system) and secreted phosphatases (which liberate P from extracytoplasmic P-containing compounds). TCSs typically comprise a sensor protein kinase (PhoR) and a partner response regulator protein (PhoB/P). Pho regulon response regulators serve as transcriptional activators when they are phosphorylated (8, 20, 22).

In *M. xanthus* seven TCS proteins (PhoRP1, PhoPR2, PhoPR3, and PhoP4) have been implicated in the regulation of phosphatase expression and late developmental events (3, 15,

17). Deleterious mutations in operons encoding the Pho1, Pho2, and Pho3 TCSs each cause a partial reduction in the activity of developmentally expressed phosphatases. The phenotypes of the *pho2* and *pho3* mutants are particularly similar and are also similar to the phenotype of the *pho2 pho3* double mutant, suggesting that the Pho2 and Pho3 systems act in the same pathway (15). A *phoP4* mutant exhibited almost complete abolition of phosphatase expression (17), implying that PhoP4 acts upstream of Pho1 and Pho2/Pho3 during late development. The *phoP4* gene is immediately upstream of the *M. xanthus* *pstSCAB* operon, and disruption of *phoP4* results in altered expression of *pstSCAB* (17). This suggests that PhoP4 has dual functions—regulating both P uptake and multicellular development. Several key questions thus arise. Is the Pho regulon sensitive to changes in P availability? What environmentally available P sources can be utilized for growth (*M. xanthus* can prey on other microorganisms as sole nutrient sources)? Is the Pho regulon regulated by developmental progression?

MATERIALS AND METHODS

Culture conditions, media, and bacterial strains. Cultures of *M. xanthus* were incubated at 30°C. Double Casitone yeast (DCY) medium was used as a rich medium for maintenance and manipulation of strains (2% Casitone, 0.2% yeast extract, 10 mM Tris [pH 8.0], 8 mM MgSO₄). A1W defined medium is the same as A1 (2) except that it is supplemented with 1 mM potassium chloride. A1W lacking 1 mM potassium phosphate was designated A1W-P. The starvation media used were TM (10 mM Tris [pH 8.0], 8 mM MgSO₄) and TPM (TM with 1 mM potassium phosphate). For developmental assays, TM and TPM were

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TABLE 1. Plasmids used

Plasmid	Description or derivation ^a	Reference or source
pDAH274	Promoterless <i>lacZ</i> , Ap ^r Km ^r , P1 <i>incΔ</i> , p15A <i>ori</i>	19
pDEW400	NPA promoter (positions 3370645 to 3371672) cloned into pDAH274 (BamHI/EcoRI)	This study
pDEW401	AlPA1 promoter (positions 1632891 to 1633918) cloned into pDAH274 (BamHI/EcoRI)	This study
pDEW402	AlPA2 promoter (positions 5517533 to 5518544) cloned into pDAH274 (BamHI/EcoRI)	This study
pDEW403	AcPA1 promoter (positions 5511801 to 5512813) cloned into pDAH274 (BamHI/EcoRI)	This study
pDEW404	AcPA2 promoter (positions 8030693 to 8031721) cloned into pDAH274 (BamHI/EcoRI)	This study
pDEW405	<i>pho2</i> promoter (positions 3233720 to 3234749) cloned into pDAH274 (BamHI/EcoRI)	This study
pDEW407	<i>pho3</i> promoter (positions 7901235 to 7902248) cloned into pDAH274 (BamHI/EcoRI)	This study
pDEW409	<i>pstSCAB</i> promoter (positions 5986510 to 5987526) cloned into pDAH274 (BamHI/EcoRI)	This study
pDEW411	PAPK promoter (positions 6677564 to 6678608) cloned into pDAH274 (BamHI/EcoRI)	This study
pDEW412	<i>pit</i> promoter (positions 26494423 to 26495448) cloned into pDAH274 (BamHI/EcoRI)	This study
pDEW413	<i>pho1</i> promoter (positions 5975177 to 5976176) cloned into pDAH274 (BamHI/EcoRI)	This study

^a Ap^r, ampicillin resistance; Km^r, kanamycin resistance; P1 *incΔ*, incompatibility locus from coliphage P1 deletion variant; p15A *ori*, origin of replication from plasmid p15A. The positions are the positions in the promoter region in the *M. xanthus* genome sequence (GenBank accession number NC_008095).

solidified with 1.5% agarose. Phosphate levels in media were determined using the method of Itaya and Ui (9). The *M. xanthus* strains used in this study were wild-type strain DK1622 (10) and *phoP4* mutant VP963 (16). VP963 carries an unmarked in-frame deletion of *phoP4* engineered in a DK1622 background. The *Escherichia coli* strain used for plasmid construction and P1-mediated transduction was MC1061 [*hsdR mcrB araD139 Δ(araABC-leu)7679 galU galK rpsL thi ΔlacX74 (lacIPOZY)*].

Manipulation and physiological assays of *M. xanthus*. P1-mediated transduction of plasmids from *E. coli* into *M. xanthus* was performed by using the method described by Hodgson (6). Transductants were verified by their production of LacZ (as demonstrated by production of indigo upon incubation on medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal]) and by successful PCR amplification of the promoter-*lacZ* unit. LacZ expression assays were performed as described by Hodgson (6) in triplicate using triplicate biological samples. During analysis, only promoter activity changes that were two-fold or greater were considered significant. Multicellular development assays were performed as described by Higgs and Merlie (5) in triplicate. Assay reagents were obtained from Sigma.

DNA manipulation and plasmid construction. Table 1 shows the plasmids utilized in this study. Promoter regions were amplified by PCR using the oligonucleotide primers shown in Table 2. PCR products were introduced into the pCR2.1-TOPO vector (Invitrogen) by using the manufacturer's instructions. Promoter regions were then subcloned into pDAH274 (Table 1) using unique restriction sites (BamHI and EcoRI) engineered into the amplification oligonucleotide primers (Table 2). Screening for plasmids containing inserts was performed using the screening primers shown in Table 2 and a PCR protocol with annealing at 50°C and 30 cycles. All plasmids were verified by DNA nucleotide sequencing. Fine chemicals and enzymes were obtained from Sigma and Fermentas, respectively, and were used according to the suppliers' protocols.

RESULTS

***M. xanthus* can acquire P from a variety of P-containing compounds.** Initially, we determined if *M. xanthus* required inorganic P for growth or whether it could obtain P from more complex molecules. A modified form of the defined minimal medium A1 (2) was obtained by replacing potassium phosphate with potassium chloride (A1W-P), thus eliminating P from the medium (see Materials and Methods). By monitoring

the growth rate by determining changes in the optical density at 600 nm, it was observed that *M. xanthus* wild-type strain DK1622 exhibited no significant growth in A1W-P (Fig. 1) compared to growth in A1W (A1W-P which was supplemented with 1 mM potassium phosphate; doubling time, ~24 h). The kinetics of growth in A1W were virtually identical to those in A1 (2). Supplementing A1W-P with different concentrations of potassium phosphate showed that growth could be supported at a rate similar to that in A1W by phosphate concentrations as low as 10 μM. However, at a P concentration of 1 μM or lower, the growth rate was reduced to the growth rate in A1W-P.

We then sought to identify substances that could replace P in A1W and still support growth by adding them individually to A1W-P. We chose substances that might be available to *M. xanthus* either because of lysis of prey organisms or because of their presence in the soil environment. The substances tested included phosphatidylethanolamine, phosphatidylcholine, phytic acid, phosphonates (methylphosphoric acid, phosphonomethyl glycine, 3-*N*-phosphomethyl glycine, phosphonoacetic acid, 2-amino-3-phosphonopropionic acid, and 2-aminoethylphosphonic acid), RNA, DNA, deoxynucleoside triphosphate, nucleoside triphosphate, ATP, glucose-6-phosphate, glyceraldehyde-3-phosphate, glycerol-3-phosphate, sodium pyrophosphate, and sodium polyphosphate. Enough of each compound was added to ensure that phosphoryl groups were present at a molar equivalent of ≥1 mM. A substance was classified as a potential phosphate source for *M. xanthus* if its inclusion in A1W-P allowed the optical density at 600 nm (or number of CFU) of a culture to at least double after 7 days of incubation. When this criterion was used, only pyrophosphate, polyphosphate, glycerol-3-phosphate, and glyceraldehyde-3-phosphate were classified as potential P sources for *M. xanthus* (Fig. 1).

TABLE 2. Oligonucleotide primers

Gene or plasmid	Forward primer	Reverse primer
Primers for promoter regions ^a		
<i>pho1</i>	AGTCAGGGATCCCGAGCCCGTCGTCGTGGAGGC CGCC	AGTCACGAATTCGAAGCCCTCTCGAGTCCC TCCGCGC
<i>pho2</i>	TCTTCTGGATCCTCGCTGCGCCGCGAGGACCGG CCCG	TCTTCTGAATTCGTGGAGCCGCCGCTGACT ATCGCAC
<i>pho3</i>	TCTTCTGGATCCTCATCCGCTCCTTTGAATCAA TGGC	TCTTCTGAATTCGCCGGGCCACGCACGCCG TGACCAC
<i>pit</i>	GTGTGTGGATCCAGAAGTCGTGGCCGTGGGCC CGGC	GTGTGTGAATTCCTCCCTCCAAGTCGCC TGAGGGC
<i>pstSCAB</i>	GTGTGTGGATCCGACAGAGCTCCCTGGGGTTTA CCTC	GTGTGTGAATTCGGGGGACTTCTCCTGGCT GGAGGAT
<i>PAPK</i>	TCTTCTGGATCCGACGGGCATCTCCCCGCGCGC CATG	TCTTCTGAATTCGAGTACGCCGTGGAGCC GGTGGGC
<i>AcPA1</i>	GTGTGTGGATCCGCGCGCCGGTGATGGTGGCGT GGGC	GTGTGTGAATTCAGGCTGCCGTTTCTAGAA GAGCCCT
<i>AcPA2</i>	GTGTGTGGATCCACGAGAAGCACGCTCAAGAGC AGTG	GTGTGTGAATTCGTCCGGCTCCAGGCGCGC CCCCGCA
<i>NPA</i>	TCTTCTGGATCCACCCGGCTCAGCCGGAGTCCT ACGC	TCTTCTGAATTCGCCGAGCACTCCTTCCCGAG GCGAGG
<i>AlPA1</i>	GTGTGTGGATCCGCCGGAGTCGTCCGGTAAGAC GACG	GTGTGTGAATTCGCGATCGTGTCAACCACG CCCGGTG
<i>AlPA2</i>	GTGTGTGGATCCTCCACATAGACCTTCATCTTC TCCG	GTGTGTGAATTCCTCCGCCGGGATGGTGAG AAGCAAC
Primers for plasmids ^b		
pDAH274	CATCGCCACGCATAACCGA	GTTGTAAAACGACGGCCAGT
pCR2.1	GTAAAACGACGGCCAG	CAGGAAACAGCTATGAC

^a Primers used to amplify promoter regions. Underlining indicates BamHI and EcoRI restriction sites in the forward and reverse primers, respectively.

^b Primers used to screen transformants for plasmids containing inserted promoter regions.

Identification of candidate Pho regulon phosphatases. Five phosphatase activities have been identified previously in *M. xanthus* (23). Two of them were vegetatively expressed magnesium-dependent activities (one with an acidic pH optimum

FIG. 1. Growth kinetics of *M. xanthus* on different P sources. Changes in cell number were monitored by recording the optical density at 600 nm at different times after inoculation (at zero time) into minimal medium (A1W-P) with different P sources. Optical density values were normalized to 0.1 at day 1, and the values are the averages of three independent experiments (the standard deviation was within 15% of the sample mean in all cases, and error bars are not shown). Pyrophosphate (PPi), polyphosphate (PolyP), and glyceraldehyde-3-phosphate (Gly-3-P) could support growth, while glucose-6-phosphate (Glu-6-P) could not.

and one with an alkaline pH optimum), and three were magnesium-independent activities that were induced during development (one acidic, one alkaline, and one neutral). In order to identify candidate genes that might encode previously observed phosphatase activities, we searched the predicted protein sequences derived from the *M. xanthus* genome using BLASTP (1) with the sequences of experimentally characterized phosphatases described previously as query sequences. The five most promising candidate genes were predicted to encode phosphatases with the correct pH optima, and these phosphatases were designated AcPA1, AcPA2, AlPA1, AlPA2, and NPA (acidic, acidic, alkaline, alkaline, and neutral, respectively). AlPA1 is a homologue of the PhoD alkaline phosphatase (COG3540), AlPA2 is predicted to be a member of the alkaline phosphate superfamily (COG1524), AcPA1 is homologous to the SurE acid phosphatase (COG0946), and AcPA2 is homologous to the NapD acid phosphatase, while NPA is homologous to the neutral phosphatase of *Staphylococcus aureus* (GenBank accession number P21222) and is predicted to be a member of the COG0637 group (phosphatases and phosphohexomutases).

Construction of Pho gene reporter strains. Based on previous experimental analysis and the bioinformatic analysis described above, 13 candidate Pho regulon promoter regions/operons were identified for further analysis; these regions/operons included the five phosphatase genes, four TCS operons, the *pstSCAB* operon, a *pit* operon, and the genes encoding PAPK and polyphosphate kinase (PPK) (Table 3). To characterize expression of promoters under different conditions, each promoter region was cloned upstream of a promoterless *lacZ* gene. This was

TABLE 3. Putative operons involved in the regulation of P uptake in *M. xanthus*

Operon	Proposed function	P-related gene(s)	CDS no.	Reference
<i>pho1</i>	Regulation	<i>phoP1, phoR1</i>	MXAN_4777-4778	3
<i>pho2</i>	Regulation	<i>phoR2, phoR2</i>	MXAN_2778-2779	15
<i>pho3</i>	Regulation	OmpA precursor, <i>phoR3, phoP3</i>	MXAN_6413-6415	15
<i>pho4</i>	Regulation	<i>phoP4</i>	MXAN_4787	17
<i>pstSCAB</i>	High-affinity P transport	<i>pstS, pstC, pstA, pstB, phoU</i>	MXAN_4788-4792	17
<i>pit</i>	Low-affinity P transport	Pit accessory protein, <i>pitA</i>	MXAN_2280-2281	
PAPK	Polyphosphate degradation	PAPK	MXAN_5363	
PPK	Polyphosphate synthesis	PPK	MXAN_0056	24
NPA	Phosphatase	NPA	MXAN_2886	
AlPA1	Phosphatase	AlPA1	MXAN_1389	
AlPA2	Phosphatase	AlPA2	MXAN_4457	
AcPA1	Phosphatase	AcPA1	MXAN_4450	
AcPA2	Phosphatase	AcPA2	MXAN_6509	

achieved by PCR amplification of a region of *M. xanthus* genomic DNA originating immediately upstream of the start codon of the gene/operon of interest and extending upstream for ~1 kb. The exception was the *pho3* operon, where an open reading frame was found 9 bp directly upstream of *phoR3* (MXAN_6415, an *ompA*-related protein precursor gene), which was in turn preceded by an 86-bp intergenic region. The *pho3* operon was therefore assumed to include MXAN_6415, *phoR3*, and *phoP3*, and the promoter probe was designed accordingly. PCR products were introduced into pCR2.1-TOPO (Invitrogen) and then directionally subcloned into pDAH274 using unique restriction sites (BamHI and EcoRI) engineered into the oligonucleotide primers used for amplification. Tables 1 and 2 show the plasmids and primers utilized. The resulting plasmids (promoter probes) were introduced into *M. xanthus* wild-type strain DK1622 by P1-mediated transduction (see Materials and Methods) with selection for kanamycin resistance. Kanamycin-resistant transductants contained promoter probe plasmids integrated into the chromosome by homologous recombination (i.e., merodiploids with tandem duplication of the cloned promoter regions). LacZ production by the resulting transductants was assumed to mirror the activity of the relevant promoter in its natural context. Unfortunately, efforts to produce promoter probes for the *phoP4* and *ppk* genes were unsuccessful. To ensure robust analysis, only changes in LacZ expression greater than twofold were considered physiologically relevant.

Vegetative expression of Pho regulon promoters. All 11 promoter probes were introduced into *M. xanthus* wild-type strain DK1622, and LacZ production during vegetative growth in shaken cultures (in DCY rich medium; P concentration, ~8 mM) was determined (Fig. 2). Several Pho regulon promoters were active during vegetative growth. The NPA, *pit*, *pstSCAB*, *pho1*, *pho3*, and PAPK gene promoters had high levels of activity (LacZ specific activity, >100 U), while the *pho2* and AcPA1 gene promoters had lower levels of activity (average specific activity, ~35 U). The remaining promoters were either inactive or initiated transcription at very low levels (average specific activity, <20 U).

For all promoters except the NPA gene promoter, LacZ expression did not change significantly upon the transition to stationary phase or upon introduction into starvation medium, whether phosphate was present or not (TPM and TM, respectively [see Materials and Methods]). However, the NPA gene promoter was activated approximately twofold by starvation and upon the transition to stationary phase (Fig. 2).

P-dependent changes in Pho regulon gene expression. To test the effect of P on expression of Pho regulon genes, wild-type *M. xanthus* strains containing the 11 promoter probes were subcultured into A1W and A1W-P, and LacZ production was then assayed after a suitable period of adaptation (2 days). For most promoters, there was a slight reduction in LacZ expression in A1W compared to rich medium, probably as a consequence of the reduced growth rate in A1W. However, the NPA gene promoter exhibited greater activity in A1W than in DCY medium, presumably reflecting the reduced levels of nutrients in A1W compared to DCY medium. Most promoter activities were not significantly different in A1W and A1W-P (Fig. 3). However the AcPA1, *pho3*, and *pstSCAB* gene promoters were induced around fourfold, twofold, and fourfold, respectively, by incubation in A1W-P, while the *pit* promoter activity was reduced twofold in A1W compared to A1W-P (Fig. 3).

To determine whether P responsiveness was PhoP4 dependent, the *pho3*, *pit*, AcPA1, and *pstSCAB* gene promoter probes were introduced into *phoP4* mutant VP963, and LacZ expression was assayed in A1W. Expression from the *pstSCAB* promoter was threefold higher in the *phoP4* mutant than in the wild-type background (Fig. 3), suggesting that PhoP4 represses *pstSCAB* expression in the presence of high levels of P and that the induction of

FIG. 2. Promoter (LacZ) specific activities during vegetative growth (1 day after inoculation at an optical density at 600 nm of 0.1), during the stationary phase (5 days after inoculation), and after 2 days of incubation in starvation medium with phosphate (TPM) and starvation medium lacking phosphate (TM).

FIG. 3. Promoter (LacZ) specific activities with P limitation. Strains containing different promoter probes were incubated in A1W and A1W-P. The specific activities of P-responsive promoters (AcPA1 gene, *pho3*, *pstSCAB*, and *pit* promoters) were also determined for a *phoP4* mutant.

pstSCAB upon P depletion is a consequence of loss of PhoP4-mediated repression. However, expression from the *pstSCAB* promoter was still enhanced in A1W-P compared to A1W (Fig. 3), suggesting that another factor (factor X) activates *pstSCAB* expression under P-limiting conditions. The AcPA1 and *pho3* gene promoters also remained P responsive in a *phoP4* background, but the *pit* promoter was no longer down-regulated by P limitation (Fig. 3). This suggests that PhoP4 does not regulate the AcPA1 or *pho3* gene but rather represses expression of the *pit* operon under P-limiting conditions.

Developmental expression of Pho regulon promoters. Phosphatase activities are developmentally regulated in *M. xanthus*, and we therefore tested whether Pho regulon genes were also developmentally regulated. The *pstSCAB*, *pho3*, *pit*, and PAPK gene promoters were switched off during early development in

TM, as shown by an exponential decrease in the LacZ specific activity with time (Fig. 4). Other Pho regulon promoters were relatively unaffected by development. Changes in most promoter activities were not significantly affected by the presence of P (TM compared to TPM); the only exception was the NPA gene promoter, which was transiently up-regulated during early development, but only in the presence of P (Fig. 4). Thus, the P responsiveness of the Pho regulon *pho3*, AcPA1, *pit*, and *pstSCAB* components is lost during multicellular development.

DISCUSSION

The Pho regulon of *M. xanthus* appears to be far more complex than other prokaryotic examples, with seven TCS regulatory proteins rather than the usual two. Additionally, the

FIG. 4. Promoter (LacZ) specific activities at different times during development in the absence of phosphate (TM). The specific activities of the NPA gene, *pho3*, *pstSCAB*, and *pit* promoters during development in starvation medium with phosphate (TPM) were also determined. T, time (in days).

regulon regulates (and is regulated by) two discrete (although interdependent) physiological processes (P availability and uptake and multicellular development). While expression of P uptake genes is induced by P limitation, P starvation at high cell densities on a solid substrate can initiate progression through the developmental program, a process that shuts down expression of many P acquisition genes.

If a population of *M. xanthus* cells is transferred to medium lacking P, growth ceases within one doubling time (~24 h in A1W). This suggests that the amounts of phosphate stored in the cells (usually polyphosphate) are small. This agrees with the results of polyphosphate assays, which show that polyphosphate levels are low in exponentially growing cells, although polyphosphate transiently accumulates during early development (24). Cessation of growth required the concentration of P to be reduced below 10 μ M and was unaffected by previous incubation in different media, whether the medium was rich or minimal (DCY medium or A1W) (unpublished data). *M. xanthus* is able to grow with other microorganisms as sole nutrient sources (16). We therefore tested a range of P-containing biomolecules that might be expected to be released by lysis of prey to determine their abilities to act as sole P sources. Surprisingly, nucleic acids, phospholipids, and nucleotides could not act as sole P sources in our assays, nor could glucose-6-phosphate. We also tested phytic acid and phosphonates, as phytase and phosphonate transporter genes have been annotated in the *M. xanthus* genome (MXAN_5326 and MXAN_5978, respectively), although no homologues of phosphonate metabolism genes could be identified.

Our assays did show that pyrophosphate, polyphosphate, glycerol-3-phosphate, and glyceraldehyde-3-phosphate could be utilized by *M. xanthus* as sole P sources. With polyphosphate, there was a lag phase prior to exponential growth longer than that observed with P itself (Fig. 1). This suggests that there is a delay in utilization, which may be a consequence of the requirement for accumulation of secreted polyphosphatases prior to uptake of liberated P or due to a requirement for de novo expression of polyphosphatase genes. Genes encoding four pyrophosphatases are predicted to be present in the *M. xanthus* genome (MXAN_0862, MXAN_1671, MXAN_2473, and MXAN_5577), and one of them (MXAN_1671) is predicted to encode a V-type H⁺-translocating pyrophosphatase. There is no homologue of the gene encoding the UhpT transporter of hexose phosphates in the *M. xanthus* genome sequence. The *glpT* gene of *M. xanthus* has been characterized (14) and shown to be developmentally regulated, and it is induced early in development (0 to 24 h). Glycerol-3-phosphate was found to inhibit swarming and aggregation, and disruption of *glpT* caused cells to aggregate even on rich medium (14). The ability to utilize glycerol-3-phosphate and glyceraldehyde-3-phosphate as sole P sources suggests that there is uptake of glycerol-3-phosphate by cells, which can be readily produced from glyceraldehyde-3-phosphate, presumably liberated by extracellular lipolysis of prey organisms. As the doubling time of our cultures in A1W was ~24 h and *M. xanthus* doubles every ~3 h in rich medium, care must be taken when our conclusions are extrapolated to rich medium. However, it is still not clear to what degree utilization of different P sources occurs during predation. It is likely that multiple P sources are utilized simultaneously, and it would be interesting to quantify the relative contributions of different prey biomolecules to *M. xanthus* P uptake.

During development three phosphatase activities are induced, while two further activities are expressed during vegetative growth (23). Developmental phosphatase expression is significantly reduced in *pho2* and *pho3* mutants and *pho2 pho3* double mutants (15). However, the developmental defects due to *pho2* and *pho3* mutations and *pho2 pho3* double mutations are not apparent when exogenous P is supplied (15). This suggests that developmental phosphatases are important in P scavenging late in development. We utilized bioinformatics to identify the five genes most likely to encode these activities, expecting to see changes in promoter activity during development. To our surprise, four of the promoters were almost completely inactive and not affected by development, while the fifth appeared to be induced by nutrient limitation and transiently activated early in development, but only in the presence of P. This suggests that either we were unsuccessful in identifying the genes encoding the developmental and vegetative phosphatase activities described here (this could have been the case if, for instance, the phosphatase activities belong to phosphoprotein phosphatases) or the phosphatase activities are regulated posttranslationally. One putative phosphatase promoter (the AcPA1 promoter) was activated by P limitation, so AcPA1 may be a Pho regulon phosphatase. However, AcPA1 is regulated only by P availability and not by development.

Several other promoters were found to be highly active during vegetative growth, including the PAPK gene, *pho1*, *pho3*, *pit*, and *pstSCAB* promoters. For the *pho1* promoter, we observed a typical expression activity of ~180 U of LacZ, which agreed well with previous studies (3). The previously reported down-regulation of *pho1* promoter activity during development (3) was also observed (Fig. 4), with lower activities in media containing P (data not shown). We expected to see significant promoter activity for the *pit* promoter during growth with high P levels; however, the levels of activity were rather low. In contrast, the activities of the *pstSCAB* and PAPK gene promoters were remarkably high. It is particularly intriguing that the PAPK gene promoter activity was so high, given that the substrate for the gene product (polyphosphate) is essentially absent from cells during vegetative growth. Perhaps the PAPK gene is expressed at high levels to keep polyphosphate levels low during vegetative growth. This might be the case if polyphosphate acts as an alarmone signal for activation of the Lon protease rather than as a phosphate storage molecule in *M. xanthus*, as suggested by Zhang et al. (24). In *B. subtilis* *pstSCAB* promoter activity is induced ~5,000-fold upon P limitation, from ~0 to ~4,500 U of LacZ (18). In contrast, P limitation in *M. xanthus* increases the *pstSCAB* promoter activity from ~150 to ~650 U of LacZ (Fig. 3). Thus, in *M. xanthus* *pstSCAB* expression is unusually high in the presence of P. This may suggest that *M. xanthus* has adapted to a lifestyle or environment where free P is typically rare and where high constitutive expression of the *pstSCAB* operon would confer a selective advantage. It would be interesting to know what concentrations of P are typically found surrounding cells engaged in predation in the natural environment.

It was reported previously that a *phoP4* mutant exhibited levels of *pstSCAB* transcripts different than those in the wild-type strain (17). The vegetative expression of the *pstSCAB* promoter probe in a *phoP4* mutant was approximately threefold greater than that in the wild type, which suggested that

PhoP4 represses expression of *pstSCAB* in the presence of high P levels and that P limitation relieves repression by PhoP4. However, *pstSCAB* promoter activity remained P responsive in the *phoP4* mutant (Fig. 3), and there was a further fivefold induction of promoter activity upon P limitation. Thus, the *pstSCAB* promoter is regulated by PhoP4 (negatively) and also by a separate P-responsive “factor X.” The only regulatory operon of the Pho regulon to be transcriptionally regulated by P availability is *pho3* (Fig. 3), and the Pho3 system might therefore be factor X. Alternatively, factor X could be PhoU, which is encoded at the end of the *pstSCAB* operon.

The *pho3* and AcPA1 promoters were also induced by P limitation (twofold and fourfold, respectively), while the *pit* promoter was twofold less active under P-limiting conditions (Fig. 3). Induction of the AcPA1 gene and *pho3* was not *phoP4* dependent. However, the down-regulation of the *pit* genes in the absence of P was *phoP4* dependent (Fig. 3). Thus, it seems that PhoP4 acts as a repressor of *pstSCAB* expression independent of P, but under P-limiting conditions PhoP4 also represses expression of the *pit* operon. The AcPA1 gene and *pho3* promoters are P responsive but are not affected by a *phoP4* deletion, suggesting that another factor (factor X?) regulates induction of these promoter activities upon P limitation.

It appears that the promoters of P acquisition genes are turned off early during development, independent of P availability. In addition, P-responsive promoters become P unresponsive during starvation and indeed during development. This suggests that starvation induces a response that makes subsequent availability of P irrelevant. Presumably at such a point sufficient P is available to the population to allow completion of the developmental program, and the population may commit to completing this program at an early stage. This has parallels with the process of sporulation in *B. subtilis*, during which accumulation of phosphorylated Spo0A leads to down-regulation of the Pho regulon ~2 h after starvation (7). We also observed that the profile of promoter activities during development was not affected by mutation of *phoP4*, suggesting that the primary role of PhoP4 is in the regulation of P acquisition rather than in developmental events. However, this is difficult to reconcile with the dominance of the *phoP4* developmental phenotype over the phenotypes of *pho1*, *pho2*, or *pho3* mutants, unless PhoP4 modifies the activity of Pho1, Pho2, and Pho3 posttranscriptionally.

Finally, since four PhoP homologues are involved in the Pho regulon of *M. xanthus*, we attempted to find Pho boxes (PhoP binding sites), as defined by similarity to the consensus Pho box sequences of *E. coli*, *B. subtilis*, and cyanobacteria (11, 12, 21), in Pho gene promoter regions. No sequences that significantly resembled known Pho boxes could be identified in these regions. This perhaps should not have been surprising, as there would presumably be four sets of Pho box sequences (one per PhoP homologue) and an uncertain degree of sequence similarity.

In summary, it seems that the Pho regulon of *M. xanthus* has several novel features: the regulon is bifunctional, regulating both P acquisition and multicellular development (responding to P availability and developmental progression); it involves four transcriptional regulators, one of which acts as a repressor; and during starvation-induced fruiting body formation the regulon becomes insensitive to P levels.

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