The response regulator PhoP4 is required for late developmental events in *Myxococcus xanthus*

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Phosphate regulation is complex in the developmental prokaryote *Myxococcus xanthus*, and requires at least four two-component systems (TCSs). Here, the identification and characterization of a member of one TCS, designated PhoP4, is reported. *phoP4* insertion and in-frame deletion strains caused spore viability to be decreased by nearly two orders of magnitude, and reduced all three development-specific phosphatase activities by 80–90 % under phosphate-limiting conditions. Microarray and quantitative PCR analyses demonstrated that PhoP4 is also required for appropriate expression of the predicted *pstSCAB–phoU* operon of inorganic phosphate assimilation genes. Unlike the case for the other three *M. xanthus* Pho TCSs, the chromosomal region around *phoP4* does not contain a partner histidine kinase gene. Yeast two-hybrid analyses reveal that PhoP4 interacts reciprocally with PhoR2, the histidine kinase of the Pho2 TCS; however, the existence of certain phenotypic differences between *phoP4* and *phoR2* mutants suggests that PhoP4 interacts with another, as-yet unidentified, histidine kinase.

INTRODUCTION

The social bacterium *Myxococcus xanthus* undergoes a multicellular morphological differentiation process when starved of nutrients. This process requires precise spatial and temporal regulation for the cells to elaborate macroscopic structures, called fruiting bodies, within which vegetative cells differentiate into stress-resistant cysts called myxospores. For an *M. xanthus* swarm to determine the necessity for, and appropriateness of, activating this developmental programme, multiple nutritional and population-density signals must be monitored (for reviews, see Kaiser, 2000; Shimkets, 1999; Ward & Zusman, 2000).

One way in which bacteria sense and process such environmental signals is through signal transduction pathways known as two-component systems (TCSs). These typically consist of a sensor histidine kinase and a partner effector protein called the ‘response regulator’. For most TCSs, the pathway becomes activated upon detection of a signal by the histidine kinase, which then autophosphorylates a conserved histidine residue in its C-terminal H-box domain.

The signal is subsequently transduced by transfer of the phosphoryl group from the H-box histidine to a conserved aspartate residue in the receiver domain of the partner response regulator. In its phosphorylated form, the response regulator effects a variety of physiologic changes in the cell, which usually involve the activation or repression of genes to coordinate an appropriate response to the activating signal (for reviews, see Kenney, 2002; Parkinson & Kofoed, 1992; Stock *et al.*, 2000).

The recently completed genome of *M. xanthus* reveals the presence of 255 known and putative TCS components, 44 % of which are orphaned or unpartnered (D. E. Whitworth, unpublished data). One of these is a putative response regulator that we have named PhoP4, for phosphate regulation protein 4. It shows homology to PhoB in *Escherichia coli* and PhoP in *Bacillus subtilis*, which in those organisms regulate the Pho regulon of phosphate-starvation-inducible genes, including genes for phosphate transport and several phosphatases (Hulett *et al.*, 1994; Makino *et al.*, 1989).

The regulation of phosphate pools is important for development in *M. xanthus*, as evidenced by the fact that starvation for inorganic phosphate induces fruiting-body formation (Manoil & Kaiser, 1980). Furthermore, phosphatase activities with pH optima at 5.2, 7.2 and 8.5, known as the magnesium-independent (MI) acid, MI neutral and MI alkaline activities, respectively, have been reported that are specifically activated in development at discrete times

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Abbreviations: MI, magnesium-independent; QPCR, quantitative PCR; TCS, two-component system.

following fruiting-body formation (Weinberg & Zusman, 1990). The tight temporal regulation of these MI phosphatase activities suggests that they play an integral role in development; however, the number and identities of all the phosphatases contributing to these activity profiles remain to be elucidated.

Previously, the laboratory of Muñoz-Dorado identified and characterized three known Pho TCS operons named *phoP1-phoR1*, *phoR2-phoP2* and *phoR3-phoP3* (Carrero-Lerida *et al.*, 2005; Martinez-Canamero *et al.*, 2003; Moraleda-Muñoz *et al.*, 2003). Deletions in any of these systems affect specific developmental phosphatase activity patterns in *M. xanthus*, while leaving the vegetative phosphatase activities, which are magnesium dependent, unchanged. In addition, none of these mutations appears to affect sporulation.

We report here the identification and characterization of a member of a fourth Pho TCS, PhoP4. Our data show that PhoP4 is required for (I) expression of all three known development-specific phosphatase activities, (II) wild-type-level sporulation, and (III) expression of the predicted assimilatory inorganic phosphate *pstSCAB-phoU* operon. In addition, our yeast two-hybrid analyses show reciprocal interactions between PhoP4 and the histidine kinase of another *M. xanthus* Pho TCS, PhoR2. In sum, these data indicate that PhoP4 (and the TCS system it represents) is a very important component of the *M. xanthus* Pho regulatory circuit, specifically with regard to activities required for development.

METHODS

Strains, plasmids, cloning, and transformation. A complete list of strains and plasmids used in this study is shown in Table 1. Plasmids were propagated in *E. coli* JM109 (Yanisch-Perron *et al.*, 1985). An internal fragment of the *phoP4* ORF was amplified by PCR using forward primer PhoP4-F43 and reverse primer PhoP4-R575, which contain engineered *Pst*I and *Eco*RI restriction sites (underlined, see Table 2), respectively. The resultant amplicon was digested with the two restriction enzymes and inserted directionally into vector pBGS18 to generate plasmid pDV525. Wild-type *M. xanthus* strain DK1622, which does not maintain *E. coli* vectors, was transformed by electroporation with pDV525 using a GenePulser Xcell electroporator (Bio-Rad Laboratories), as described elsewhere (Kashefi & Hartzell, 1995). Kanamycin-resistant (Km^R) transformants were selected and then screened by Southern blot analysis for the insertion of pDV525 by homologous recombination to generate the *phoP4::pDV525* insertion strain VP965.

The *phoP4* in-frame deletion strain was generated by allelic exchange using the GalK selection method (Ueki *et al.*, 1996). Briefly, sequences upstream and downstream of the *phoP4* gene were amplified by PCR using the primers listed in Table 2, then ligated into the multiple-cloning site of plasmid pBJ114 to form plasmid pCS66. Insertion of this plasmid at the *phoP4* locus was accomplished as described above. Km^R transformants were plated on 2% galactose CTTYE agar (CTT supplemented with 0.2% yeast extract) and Km^S Gal^R candidates (which had lost the plasmid by homologous recombination) were screened by Southern blot analysis for the presence of the deletion allele, yielding strain VP963, in which *phoP4* was deleted between bases 77 and 541.

Plasmids pDV888, pDV890 and pDV892 were generated by cloning the *Pst*SqF1/R1, *Pst*BqF1/R1 and *Pho*UqF1/R1 PCR amplicons, respectively, into the pCR 2.1-TOPO vector as directed by the TOPO TA Cloning Kit protocol (Invitrogen).

Culture media and conditions. *E. coli* strains were grown in Luria-Bertani broth (Sambrook *et al.*, 1989) in a roller drum at 37 °C and 60 r.p.m. Unless otherwise stated, *M. xanthus* strains were grown on CTTYE media at 33 °C, as previously described (Hodgkin & Kaiser, 1977). When needed, 40 µg kanamycin monosulfate (Sigma-Aldrich) was added per millilitre of medium.

Developmental media and conditions. Mid-exponential-phase *M. xanthus* cells were concentrated to a density of 5×10^9 cells ml⁻¹, and spotted onto TPM agar (10 mM Tris, pH 7.6, 1 mM potassium phosphate, pH 7.8, 8 mM magnesium sulfate; Kroos *et al.*, 1986) at 33 °C for developmental and sporulation assays. Fruiting-body formation was visually evaluated by light microscopy with a Nikon SMZ800 microscope. Myxospore preparations were performed as previously described (Pham *et al.*, 2005).

Yeast two-hybrid assays. The *Saccharomyces cerevisiae* strain used was EGY48 (Gyuris *et al.*, 1993). Cultivation and yeast two-hybrid methods were as described previously (Whitworth & Hodgson, 2001). Briefly, DNA encoding the receiver domains and H boxes of *pho* genes was amplified by PCR according to a protocol described by Whitworth & Hodgson (2001), using the primers listed in Table 2. PCR products were cloned into both pEG202 and pJG4-5, generating LexA and activation domain (AD) fusions, respectively (Table 1), using *Eco*RI and *Xho*I restriction sites. Testing the suitability of all LexA fusions for use in the two-hybrid system and subsequent screening for protein-protein interactions were also as described by Whitworth & Hodgson (2001). A positive control was provided by plasmids pDEW002 and pDEW020 (Table 1), encoding a pair of fusion proteins known to interact strongly and specifically (Browning *et al.*, 2003). Due to the semi-quantitative nature of the yeast two-hybrid assay, interactions were assessed visually on medium containing X-Gal, with the degree of indigo colouration scored in the range 1–3, as previously reported (Browning *et al.*, 2003; Higgs *et al.*, 2005; Whitworth & Hodgson, 2001). When tested with ONPG as the substrate, typical '1', '2', and '3' interactions were found to express β -galactosidase with specific activities of 2.9 ± 0.6 , 10.7 ± 1.0 and 2862 ± 1962 nmol (mg protein)⁻¹ min⁻¹, respectively. The lowest specific activities, at 0.7 ± 0.3 , corresponded to the absence of interaction, which is indicated with a – in Table 4.

RNA isolation. Total RNA samples used for all procedures described in this study were extracted by the hot phenol method (Sambrook *et al.*, 1989) from approximately 10^{10} quick-frozen *M. xanthus* cells. These cells were harvested either during vegetative growth at a cell density of 5×10^8 cells ml⁻¹, or 48 and 60 h after the initiation of starvation on TPM agar plates.

Quantitative PCR (QPCR). Analysis was performed as described previously (Diodati *et al.*, 2006). Expression of the *pstS*, *pstB* and *phoU* genes was calculated from DNA quantities obtained for concurrent standard curve reactions. Typically, a standard curve was generated by reacting a 10-fold dilution series of plasmid DNA (pDV888 for *pstS*, pDV890 for *pstB*, and pDV892 for *phoU*), ranging from 10^{10} to 10^1 copies per microlitre, with the appropriate primer set and the DyNAmo HS SYBR Green QPCR Master Mix (Finnzymes).

DNA microarrays. PCR-generated DNA microarrays containing probes to the 7235 *M. xanthus* ORFs of the M1 genome (Diodati *et al.*, 2006; Jakobsen *et al.*, 2004) were spotted onto poly-L-lysine coated glass slides by the Stanford Functional Genomics Facility (Stanford, CA). Samples from three independent biological replicates were collected as described above, and DNA microarray analyses were performed as previously described (Diodati *et al.*, 2006). Hierarchical

Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristics and derivation	Reference
Strains		
<i>E. coli</i>		
JM109	<i>endA1 gyrA96 hsdR17 supE44 Δ(lac-proAB) recA1 relA1 F'[traD36, proAB+, lacIq, lacZΔM15]</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>M. xanthus</i>		
DK1622*	Wild-type	Kaiser (1979)
VP963	<i>ΔphoP4</i>	This study
VP965	<i>phoP4::pDV525</i>	This study
<i>S. cerevisiae</i>		
EGY48	<i>MATα trp1 his3 ura3 leu2::LexAop-LEU2</i>	Gyuris <i>et al.</i> (1993)
Plasmids		
pBGS18	<i>nptII</i> (Km ^R)	Spratt <i>et al.</i> (1986)
pDV525	pBGS18 containing PhoP4-F43/R575 amplicon	This study
pBJ114	Km ^R , <i>galK</i>	B. Julien, Stanford University
pCS66	pBJ114 containing upstream PCR (PhoP4-A/B) and downstream (PhoP4-Y/Z) amplicons	This study
pCR 2.1-TOPO	Proprietary cloning vector, Km ^R , Ap ^R	Invitrogen
pDV888	pCR 2.1-TOPO containing PstSqF1/R1 amplicon	This study
pDV890	pCR 2.1-TOPO containing PstBqF1/R1 amplicon	This study
pDV892	pCR 2.1-TOPO containing PhoUqF1/R1 amplicon	This study
pDEW002	<i>HIS3</i> , 2 μm, Ap ^R , P ^{ADH} - <i>lexA</i> -QN	Browning <i>et al.</i> (2003)
pDEW020	<i>TRP1</i> , 2 μm, Ap ^R , P ^{GAL1} -B42-HA-RMN	Browning <i>et al.</i> (2003)
pDEW708	<i>HIS3</i> , 2 μm, Ap ^R , P ^{ADH} - <i>lexA</i> -PhoP1(R) pEG202 plus PCR (PhoP1F, PhoP1R) <i>EcoRI</i> - <i>XhoI</i>	This study
pDEW710	<i>HIS3</i> , 2 μm, Ap ^R , P ^{ADH} - <i>lexA</i> -PhoP2(R) pEG202 plus PCR (PhoP2F, PhoP2R) <i>EcoRI</i> - <i>XhoI</i>	This study
pDEW712	<i>HIS3</i> , 2 μm, Ap ^R , P ^{ADH} - <i>lexA</i> -PhoP3(R) pEG202 plus PCR (PhoP3F, PhoP3R) <i>EcoRI</i> - <i>XhoI</i>	This study
pDEW714	<i>HIS3</i> , 2 μm, Ap ^R , P ^{ADH} - <i>lexA</i> -PhoP4(R) pEG202 plus PCR (PhoP4F, PhoP4R) <i>EcoRI</i> - <i>XhoI</i>	This study
pDEW716	<i>HIS3</i> , 2 μm, Ap ^R , P ^{ADH} - <i>lexA</i> -PhoR1(H) pEG202 plus PCR (PhoR1F, PhoR1R) <i>EcoRI</i> - <i>XhoI</i>	This study
pDEW717	<i>HIS3</i> , 2 μm, Ap ^R , P ^{ADH} - <i>lexA</i> -PhoR2(H) pEG202 plus PCR (PhoR2F, PhoR2R) <i>EcoRI</i> - <i>XhoI</i>	This study
pDEW718	<i>HIS3</i> , 2 μm, Ap ^R , P ^{ADH} - <i>lexA</i> -PhoR3(H) pEG202 plus PCR (PhoR3F, PhoR3R) <i>EcoRI</i> - <i>XhoI</i>	This study
pDEW908	<i>TRP1</i> , 2 μm, Ap ^R , P ^{GAL1} -B42-HA-PhoP1(R) pJG4-5 plus PCR (PhoP1F, PhoP1R) <i>EcoRI</i> - <i>XhoI</i>	This study
pDEW910	<i>TRP1</i> , 2 μm, Ap ^R , P ^{GAL1} -B42-HA-PhoP2(R) pJG4-5 plus PCR (PhoP2F, PhoP2R) <i>EcoRI</i> - <i>XhoI</i>	This study
pDEW912	<i>TRP1</i> , 2 μm, Ap ^R , P ^{GAL1} -B42-HA-PhoP3(R) pJG4-5 plus PCR (PhoP3F, PhoP3R) <i>EcoRI</i> - <i>XhoI</i>	This study
pDEW914	<i>TRP1</i> , 2 μm, Ap ^R , P ^{GAL1} -B42-HA-PhoP4(R) pJG4-5 plus PCR (PhoP4F, PhoP4R) <i>EcoRI</i> - <i>XhoI</i>	This study
pDEW916	<i>TRP1</i> , 2 μm, Ap ^R , P ^{GAL1} -B42-HA-PhoR1(H) pJG4-5 plus PCR (PhoR1F, PhoR1R) <i>EcoRI</i> - <i>XhoI</i>	This study
pDEW917	<i>TRP1</i> , 2 μm, Ap ^R , P ^{GAL1} -B42-HA-PhoR2(H) pJG4-5 plus PCR (PhoR2F, PhoR2R) <i>EcoRI</i> - <i>XhoI</i>	This study
pDEW918	<i>TRP1</i> , 2 μm, Ap ^R , P ^{GAL1} -B42-HA-PhoR3(H) pJG4-5 plus PCR (PhoR3F, PhoR3R) <i>EcoRI</i> - <i>XhoI</i>	This study

*DK1622 was the parent strain for both *phoP4* mutants.

clustering and statistical analyses were performed using Cluster and Java TreeView software (Saldanha, 2004), and SAM (Tusher *et al.*, 2001). All DNA microarray data have been submitted to the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/projects/geo/>), and accession numbers are provided in the legend of Fig. 3.

Phosphatase assays. Studies of other bacterial model organisms have shown that PhoB/PhoP regulation of phosphatases is most significant under phosphate-limiting conditions (Antelmann *et al.*, 2000; Hirani *et al.*, 2001; Wanner, 1996). Phosphatase assays were performed as previously described (Weinberg & Zusman, 1990), with the exception that cells used for the assay were cultured in CTT media without additional phosphate. We previously reported that CTT medium

without added phosphate contains ~0.75 mM phosphate (all of it presumably derived from casitone), while fully supplemented CTT medium contains 1.75 mM phosphate (Harris *et al.*, 1998). To perform the phosphatase assay, mid-exponential-phase cells grown on low-phosphate CTT media were concentrated to 5×10^9 cells ml⁻¹, spotted in triplicate sets onto non-phosphate-supplemented CF agar plates (Hagen *et al.*, 1978), and harvested at various time-points into TM buffer (TPM, buffer without the potassium phosphate) or 20 mM HEPES buffer. Cells were disrupted mechanically by sonication (Branson Sonifier 450, Branson Ultrasonics) with glass beads, and cell and spore breakage was confirmed by observation under light microscopy. Aliquots of cell lysates were added to appropriate buffers containing 0.01 M *p*-nitrophenol phosphate, incubated at 37 °C, and enzymic activity was assessed colorimetrically at A₄₁₀. Protein

Table 2. Sequences of oligonucleotide primers

Restriction enzyme sites engineered into each primer are underlined. Sites are: GAATTC for *Eco*RI, AAGCTT for *Hind*III, CTGCAG for *Pst*I, and CTCGAG for *Xho*I.

Primer	Sequence (5'–3')	Purpose
PhoP4-F43	GACCTGCAGGAGCTCATTGATTTCAACCTG	<i>phoP4</i> insertional disruption
PhoP4-R575	ATGGAATTCGTCTCCAGGAGGATGACAGG	
PhoP4-A	GGTAGGAAGCTTGCAGCAGGAGGATGAAGATGA	<i>phoP4</i> in-frame deletion (upstream amplicon)
PhoP4-B	GAGGCCCTGCAGCCGCGAGGTTGAAATCAATGAG	
PhoP4-Y	AAGAGGCTGCAGGCCTGTCACTCCTCCCTGGAGA	<i>phoP4</i> in-frame deletion (downstream amplicon)
PhoP4-Z	ATAGGGGAATTCCCGCCTCTTGTGACATTCTGTGT	
PhoP1F	GAGAGAGAATTCATGTGCGGCATCCTGATTATCG	PhoP1 yeast two-hybrid construct
PhoP1R	AAGGAACTCGAGCTAGCGCAGAAGGAGCTCGCGGACG	
PhoR1F	GTCTCGAATTCACCGAGCTGCGCCGGCTGG	PhoR1 yeast two-hybrid construct
PhoR1R	CTTCTTCTCGAGTCACAGGGTGACGCGGGCGAGCTT	
PhoP2F	CCACCCGAATTCACCGCCACATCCACCCCG	PhoP2 yeast two-hybrid construct
PhoP2R	CGTCGTCTCGAGCTAGCGGCGCACCAGGGCGCGTACC	
PhoR2F	CCTCCTGAATTCGTGGCCACCGTCTCCACG	PhoR2 yeast two-hybrid construct
PhoR2R	CCTTCCCTCGAGTCAGCGGCCCTTGTCGATGCGG	
PhoP3F	GGCGGCGAATTCATGACCTCATCCGTTCCGGCG	PhoP3 yeast two-hybrid construct
PhoP3R	TCCTCCCTCGAGTCAGGCCAGCAGCTCGCGCAGCCG	
PhoR3F	GGCGGCGAATTCCTGGAAGTGAAGAGCGAC	PhoR3 yeast two-hybrid construct
PhoR3R	CCTTCCCTCGAGTCAGCGCCCCTTGTCGATGCGG	
PhoP4F	CACCCAGAATTCGCCATGCCCCATGTCTCATCATC	PhoP4 yeast two-hybrid construct
PhoP4R	ACCACCTCGAGTCACAGCCGAGCACCAGCTCCCGC	
PstSqF1	CACCTACGTCTTCGTGAA	<i>pstS</i> QPCR standard curve template
PstSqR1	TGCCGCTCTTGATGTTGT	
PstBqF1	CAGAATCCCCGAGCGAAGATG	<i>pstB</i> QPCR standard curve template
PstBqR1	GCCCGAATGCGTGTTGC	
PhoUqF1	CGACCTGGCGGTGAACA	<i>phoU</i> QPCR standard curve template
PhoUqR1	CGCGAGTCCTCCATCATGTA	

concentration was determined by Bradford assay (Bio-Rad Laboratories). For specific activity calculations, extinction coefficients for each buffer were derived empirically.

Sequence analyses. Secondary structure predictions were performed using the GCG suite of sequence analysis tools, version 2 (Accelrys). Amino acid sequences were aligned using the CLUSTALW method (Thompson *et al.*, 1994).

RESULTS

PhoP4 is a putative PhoB-like response regulator

PhoP4 (TIGR annotation: MXAN4787) shows 30–40 % sequence identity to PhoB-like response regulators (Fig. 1), which in other bacteria regulate genes needed for phosphate scavenging. PhoP4 is predicted to be 229 amino acids in length, and contains a putative receiver domain between residues 5 and 119 (the putative conserved phosphoryl-acceptor aspartate is at position 52), and an OmpR-like DNA-binding/transactivation domain between residues 132 and 223. A putative winged helix–turn–helix structure (Okamura *et al.*, 2000), including the recognition helix

between residues 194 and 206, is predicted by the method of Chou & Fasman (1978).

Located 133 bp downstream of the *phoP4* gene is the predicted *pstSCAB–phoU* operon of Pi assimilatory genes (Fig. 2). Belonging to the ABC (ATP-binding cassette) superfamily of transporter genes, the *pstSCAB–phoU* operon encodes a periplasmic binding protein (PstS, MXAN4788), integral membrane-channel proteins (PstC, MXAN4789, and PstA, MXAN4790), an ABC family traffic ATPase (PstB, MXAN-4791), and a component hypothesized to participate in Pi incorporation into ATP (PhoU, MXAN4792). The gene order is identical to that of the homologous *E. coli* operon; however, in enteric bacteria, the *phoB* gene is not located near the *pstSCAB–phoU* operon (Blattner *et al.*, 1997; Karp *et al.*, 2002).

Additionally, the region upstream of the first gene, *pstS*, contains at least two candidate PHO-box-like DNA-binding motifs (Fig. 2); these may serve as binding sites for PhoP4, in a similar manner to *E. coli* PhoB binding of PHO boxes in the promoter regions of its target genes, including the *pstSCAB–phoU* operon (Makino *et al.*, 1996; Wanner, 1996). However, it is important to emphasize that the consensus sequence of a PHO box varies from species to species (Fig. 2; Liu & Hulet, 1998; Makino *et al.*, 1996; Wanner, 1996), and

Fig. 1. Amino acid sequence alignment of PhoP4 with other *M. xanthus* Pho proteins and *E. coli* PhoB and *B. subtilis* PhoP. The sequence of the related *E. coli* response regulator OmpR is shown for comparative purposes. Residues are shaded according to degree of conservation, with black denoting identity, dark grey denoting strong similarity, and light grey denoting 'semiconservation', as defined by the European Bioinformatics Institute (EBI) (<http://www.ebi.ac.uk/clustalw/>). Conserved domains are demarcated as grey for the receiver domain, and black for the DNA binding/transactivation domain. The location of the recognition helix is identified by asterisks. Accession numbers are NP417864 for *E. coli* OmpR, P08402 for *E. coli* PhoB, P13792 for *B. subtilis* PhoP, AF133131 for *M. xanthus* PhoP1, AF157830 for *M. xanthus* PhoP2 and AF157829 for *M. xanthus* PhoP3. The PhoP4 sequence is tagged as MXAN4787 on the *M. xanthus* genome database maintained by The Institute for Genomic Research.

future direct DNA binding studies are needed to evaluate whether any of the candidate sites identified can actually be bound by purified PhoP4 protein.

***phoP4* mutants are defective for expression of the predicted *pstSCAB-phoU* operon**

DNA microarrays were used to compare the patterns of global gene expression between the $\Delta phoP4$ mutant and

wild-type cells at 0, 48 and 60 h post initiation of development (Fig. 3). During vegetative growth, i.e. at 0 h, no significant differences were observed between wild-type and the $\Delta phoP4$ mutant cells using DNA microarrays. However, at 48 and 60 h post initiation, the expression of several genes was affected in the $\Delta phoP4$ mutant. The data summarized in Fig. 3 show those genes whose expression either decreased (blue) or increased (yellow) by at least twofold in the $\Delta phoP4$ mutant as compared to wild-type. Many of

Fig. 2. Map of the *phoP4* chromosomal region. ORFs are represented as arrows pointing in the presumed direction of transcription, as defined by The Institute for Genomic Research. The nucleotide sequence of the region between *phoP4* and *pstS* is shown with candidate PHO boxes composed of dual binding sites (in bold type and underlined); the predicted stop codon for *phoP4* (TGA) and the predicted start codon for *pstS* (ATG) are indicated by boxes. For comparison, the consensus PHO box sequences in *B. subtilis* (Liu & Hulett, 1998) and *E. coli* (Makino *et al.*, 1996; Wanner, 1996) are also shown. The bases that comprise the binding sites are in bold type and underlined; for *E. coli* PHO boxes, some authors recognize only the internal heptamer (bold and underlined; Makino *et al.*, 1996), while others define them as encompassing the adjacent bases on each side as well (underlined only; Wanner, 1996). International Union of Biochemistry (IUB) codes for degenerate bases: W=A or T; Y=C or T; N=A, C, G or T.

the genes identified are 'hypothetical proteins'. However, several are genes whose expression is known or predicted to be developmentally regulated, e.g. myxobacterial haemagglutinin (Romeo *et al.*, 1986) and polyketide synthase. Most interesting was the identification of *pstS*, the putative *M. xanthus* homologue of the *E. coli* inorganic phosphate transporter gene. Further analysis revealed that transcription of other genes in the predicted *pstSCAB-phoU* operon was reduced by roughly 50 % (Fig. 4a). These data were then confirmed and quantified by QPCR using probes to the *pstB* gene (Fig. 4b): whereas expression of *pstB* in the wild-type was more than three times higher at 48 and 60 h into development than during vegetative growth (at 0 h), in the Δ *phoP4* mutant, the late development *pstB* transcript levels remained essentially unchanged compared to the 0 h time-point. This pattern was very similar to those observed for QPCR analyses of *pstS* and *phoU*, the two other genes in the predicted *pstSCAB-phoU* operon that were examined as part of this study (data not shown).

It should be noted, however, that the immediate downstream location of the predicted *pstSCAB-phoU* operon raises the possibility that unknown, *phoP4*-internal *cis* regulatory elements of the operon could have been removed, disrupted or displaced by the *phoP4* mutations (although the candidate PHO boxes identified in Fig. 2 were not affected in either the insertion or in-frame deletion strains). Hence, it is also possible that the expression defects observed by microarray and QPCR described above could have been

caused by the inadvertent mutation of unknown *cis* elements when the *phoP4* ORF was knocked out.

PhoP4 is required for development-specific phosphatase activities under phosphate-limiting conditions

Mutations in the other *M. xanthus* Pho TCSs partially decrease the MI acid phosphatase activity (AcPA) and/or the MI neutral phosphatase activity (NPA), without impairing the MI alkaline phosphatase activity (ALPA) (Carrero-Lerida *et al.*, 2005; Moraleda-Muñoz *et al.*, 2003). In order to determine whether PhoP4 is similarly involved in their regulation, phosphatase activities over developmental time-courses were assayed for both the Δ *phoP4* mutant VP963 and the *phoP4*::pDV525 insertional disruption mutant VP965, along with the parent wild-type strain DK1622. These analyses revealed that both *phoP4* mutants were nearly identically defective for all three development-specific phosphatase activities: AcPA and ALPA were down about fivefold compared to wild-type, while NPA was decreased by 10-fold (Fig. 5). Close examination of the activity profiles also indicated that differences between the wild-type and either *phoP4* mutant began to become pronounced at around 44 h into development, which coincides with the formation of the first myxospores (Fig. 5). This is significant, because it has previously been demonstrated that most development-specific phosphatase activities originate within spores (Weinberg & Zusman, 1990). In contrast to their effects on the developmental phosphatase

Mx number	MXAN number	Time (h)	48 h			60 h			Annotation
			Av.	sd	Fold	Av.	sd	Fold	
Down-regulated			0	48	60				
Mx_4548	MXAN7061		0.25	0.12	4.07	0.23	0.21	4.39	Myxobacterial hemagglutinin
Mx_1238	MXAN4915		0.25	0.10	3.93	0.49	0.45	2.03	Cellulase
Mx_1237	MXAN4915		0.30	0.09	3.36	0.47	0.40	2.14	Laminin A
Mx_6464	MXAN5265		0.30	0.10	3.35	0.49	0.50	2.02	Putative copper-dependent oxidase
Mx_6412	MXAN2146		0.32	0.27	3.14	0.57	0.80	1.75	Putative calmodulin-binding protein
Mx_1785	MXAN1468		0.37	0.18	2.71	0.57	0.41	1.75	Predicted protein
Mx_4431	MXAN5074		0.37	0.02	2.68	0.43	0.30	2.32	SpoVG, septation in cell division
Mx_4932	MXAN0929		0.37	0.30	2.67	0.49	0.06	2.03	Hypothetical protein
Mx_3048	MXAN3592		0.39	0.04	2.59	0.39	0.24	2.56	Ribosomal protein L35
Mx_3284	MXAN2945		0.41	0.29	2.47	0.54	0.36	1.85	Hypothetical protein
Mx_6720	MXAN7433		0.42	0.16	2.40	0.44	0.26	2.28	Transposase
Mx_3811	MXAN4788		0.43	0.14	2.34	0.66	0.28	1.52	PstS
Mx_3042	MXAN2621		0.43	0.39	2.32	0.64	0.36	1.55	Putative histidine kinase
Mx_1227	MXAN7401		0.44	0.21	2.27	0.48	0.32	2.09	Vegetative protein
Mx_5865	MXAN6860		0.45	0.24	2.23	0.61	0.35	1.64	Similar to ExbD and TolR, adventurous gliding motility proteins
Mx_923	MXAN6867		0.45	0.25	2.23	0.66	0.09	1.51	Peptidase
hpkA	MXAN7512		0.46	0.10	2.19	0.54	0.08	1.85	HpkA, histidine kinase
Mx_553	MXAN0737		0.46	0.17	2.19	0.57	0.09	1.75	Polyketide synthase
Mx_3440	MXAN0964		0.46	0.18	2.18	0.36	0.40	2.74	Calcium-binding acidic-repeat protein precursor
Mx_2288	MXAN0578		0.46	0.22	2.18	0.51	0.28	1.95	Transposase
Mx_5051	MXAN4480		0.46	0.10	2.16	0.50	0.16	1.98	Putative transcriptional repressor
Mx_4270	MXAN1642		0.47	0.31	2.13	0.45	0.37	2.20	Hypothetical protein
Mx_4931	MXAN0928		0.48	0.10	2.09	0.60	0.35	1.67	Putative histidine kinase
Mx_1628	MXAN0962		0.49	0.31	2.05	0.49	0.34	2.04	Calcium-binding acidic-repeat protein precursor
Mx_2287	MXAN0578		0.49	0.43	2.04	0.42	0.10	2.38	Transposase
Upregulated			0	48	60				
Mx_6280	MXAN1724		6.31	10.00	6.31	203.25	350.86	203.25	Hypothetical protein
Mx_6787	MXAN0457		4.47	2.90	4.47	4.59	4.63	4.59	RedA, sigma-54 modulation domain containing protein
Mx_5790	MXAN1092		3.80	4.78	3.80	13.17	21.02	13.17	Heat shock protein
Mx_6923	MXAN5892		3.42	2.53	3.42	1.55	0.41	1.55	Hypothetical protein
Mx_3763	MXAN2230		3.40	4.20	3.40	21.79	35.34	21.79	LuxR-like, DNA binding regulator
Mx_598	MXAN3134		3.13	4.01	3.13	1.79	1.01	1.79	Hypothetical protein
Mx_2739	MXAN5485		2.97	4.15	2.97	11.80	18.68	11.80	Hypothetical protein
Mx_2041	MXAN6679		2.50	2.69	2.50	2.41	1.93	2.41	Rhs family protein, cell wall associated protein
Mx_5329	MXAN1578		2.46	3.10	2.46	9.07	14.31	9.07	Beta-lactamase-like
Mx_3028	MXAN6573		2.43	2.72	2.43	2.05	2.47	2.05	Hypothetical protein
Mx_100	MXAN4222		2.41	2.04	2.41	1.70	1.17	1.70	Hypothetical protein
Mx_3373.3	MXAN6082		2.41	2.31	2.41	5.59	7.65	5.59	Hypothetical protein
Mx_6923	MXAN5892		2.24	1.62	2.24	1.86	1.41	1.86	Hypothetical protein
Mx_3770	MXAN6225		2.14	1.58	2.14	2.45	1.61	2.45	Hypothetical protein
Mx_6858	MXAN4859		2.03	1.25	2.03	4.01	4.53	4.01	Fatty acid desaturase
Mx_2367	MXAN7299		2.02	2.01	2.02	2.65	3.45	2.65	Fatty acid desaturase

Fig. 3. Genes showing at least twofold changes in expression by DNA microarray. Comparative expression in $\Delta phoP4$ strain VP963 with reference to the wild-type strain DK1622 is represented graphically as heat maps (blue is down-regulated in VP963, and yellow is upregulated in VP963) and as non-log-transformed expression ratios of VP963 to DK1622. Samples from three independent biological replicates were analysed, and the data within each experiment were normalized to the wild-type/mutant expression ratio at 0 h, which is set at a ratio of 1, since PhoP4 does not appear to be active during vegetative growth (=0 h). All microarrays have been submitted to GEO (platform, GPL2848; series, GSE3795; samples, GSM87287–GSM87295). Abbreviations: Av., average; SD, standard deviation. Fold changes are shown in italic type. Notes: Mx number designations are microarray spot assignments based on those of the M1 genome (Jakobsen *et al.*, 2004; R. D. Welch, personal communication), while MXAN number designations and annotations are based on the *M. xanthus* sequence completed by The Institute for Genomic Research and the Monsanto Company.

activities, the *phoP4* mutants, like other *M. xanthus pho* mutants, caused no defects in the vegetative phosphatase activities (data not shown).

PhoP4 mutants are defective for sporulation, but not for fruiting-body formation

Because *phoP4* mutations impaired the activities of phosphatases that are active only during development, the *phoP4* mutant strains were assessed for developmental phenotypes. We first tested whether the *phoP4* mutants were defective for organic nutrient sensing, because earlier work has shown that $\Delta phoR2$ –*phoP2* cells, unlike the wild-type, fruit and sporulate when grown on media containing

concentrations of 0.2% casitone or less (Moraleta-Muñoz *et al.*, 2003). In the case of the *phoP4* mutants, this defect was not observed when either *phoP4*::pDV525 or $\Delta phoP4$ cells were spotted onto media containing 0.1, 0.2, 0.5 or 1% casitone (data not shown). However, when sporulation was assessed on TPM starvation media, the data revealed that *phoP4* mutants produced viable spore levels nearly two orders of magnitude lower than those of wild-type cells (Table 3).

PhoP4 physically interacts with PhoR2

As part of a large-scale screen of interactions among *M. xanthus* TCSs, a strong interaction was observed between the

Fig. 4. PhoP4 regulation of the predicted downstream *pstSCAB–phoU* operon. All data were compiled from three independent biological replicates sampled as described in Methods. (a) Microarray analysis of *pstS*, *pstC*, *pstA* and *pstB* expression at 0, 48 and 60 h into development. Microarray analyses were performed and submitted to GEO as described for Fig. 3. (b) QPCR analysis of *pstB* expression in wild-type (white bars) and $\Delta phoP4$ (black bars) cells at 0, 48 and 60 h into development. Data were normalized to the wild-type level of expression at 0 h. Parallel experiments for other genes in the predicted *pstSCAB–phoU* operon, namely *pstS* and *phoU*, showed very similar expression profiles (data not shown).

orphan response regulator PhoP4 and the histidine kinase PhoR2. Three Pho-type systems have been previously described in *M. xanthus* (Pho1, Pho2 and Pho3), each composed of a histidine kinase and a response regulator. The response regulators are very similar to each other, and to PhoP4 (Fig. 1). Cross-communication among different Pho regulon TCSs is known in other systems (Amemura *et al.*, 1990), and therefore interactions among the *M. xanthus* Pho TCS proteins were systematically tested using the yeast two-hybrid system.

The results showed strong and specific interactions among some of the Pho protein fusions (Table 4). Strong reciprocal interactions were observed between PhoP1 and PhoR1, as would be expected of true TCS partners, while PhoR1 interacted with itself, suggesting homodimerization. An expected interaction between the protein fusions of the putative TCS partners PhoP2 and PhoR2 was also observed, though this interaction was not reciprocal. The PhoP2 receiver domain was apparently able to interact with itself, which is known to be the case for some response regulators (Lewis *et al.*, 2002; Muller-Dieckmann *et al.*, 1999). No interactions were seen for the Pho3 proteins, except that PhoR3 interacted strongly with PhoP2, which is not

surprising given the high similarity of the Pho2 and Pho3 systems (Moralada-Muñoz *et al.*, 2003). Some other predicted interactions for these two systems were also not observed (or were not reciprocal), suggesting a significant rate of false negatives in the assay results.

Most interestingly, the PhoP4 fusion proteins, regardless of whether assayed as bait or prey, interacted strongly with both PhoR2 fusions. Such reciprocal interactions provide solid evidence of a PhoP4–PhoR2 interactional relationship. While PhoP4 also interacted with both the PhoP1 and PhoP2 fusions, these interactions were not reciprocal, and their significance must therefore be considered more warily; this is also true for the non-reciprocal PhoP2–PhoR3 and PhoP2–PhoR2 interactions.

DISCUSSION

Studying signalling pathways is crucial for understanding how cells sense and respond to environmental stimuli. The soil bacterium *M. xanthus* possesses 255 components predicted to participate in pathways involved in signal reception and transduction (D. E. Whitworth, unpublished data). At least four of these pathways regulate Pho regulon

Fig. 5. Effect of *phoP4* mutations on regulation of development-specific phosphatase activities. (a) Acid, (b) neutral, and (c) alkaline phosphatase activities. Wild-type (◇) and *phoP4* (■) cells were induced to undergo development on CF starvation agar. Threefold replicate samples were harvested at various time-points between 0 and 72 h into development, and phosphatase activities at pH 5.2, 7.2 and 8.5 were separately monitored by the production of *p*-nitrophenol (pNP). The phosphatase activity curves for VP963 (Δ *phoP4*) and VP965 (*phoP4*::pDV525) cells were nearly identical, and only the VP965 results are shown to maintain figure clarity.

components in *M. xanthus*, possibly because having multiple regulatory systems allows *M. xanthus* to fine-tune more precisely the relevant responses associated with the maintenance of internal phosphate pools.

The data in this study showed that the function of one Pho TCS, represented here by PhoP4, is tied closely to processes linked to phosphate regulation. Not only did *phoP4* mutations decrease spore viability by nearly two orders of magnitude (Table 3), perhaps by disrupting the developmental response to overall phosphate starvation, but they also impaired the magnesium-independent phosphatase activities that are associated with myxospores (Fig. 5). Furthermore, mutating *phoP4* also decreased expression of the predicted downstream *pstSCAB-phoU* operon (Fig. 4), which is homologous to systems responsible for inorganic phosphate assimilation (Wanner, 1996). Interestingly, the PhoP4-dependent Pst system appeared to be active very late in development (at 60 h), when most *M. xanthus* cells either have lysed or are developing into myxospores (Julien *et al.*, 2000). Possibly, the scavenging of inorganic phosphate by the Pst system at this stage, combined with the co-occurring high phosphatase activities, may be important for spore formation; this possibility is further strengthened by the observed defect in spore viability for the *phoP4* mutants. Moraleda-Muñoz *et al.* (2003) have reported that a Δ *phoR2-phoP2 Δ *phoR3-phoP3* double mutant, while having no defect in overall spore viability, nevertheless does produce a fraction of myxospores (5–10%) that do not reshape completely: they are ellipsoid rather than being roughly round like wild-type myxospores. Similar high-resolution microscopic imaging of the few *phoP4* myxospores produced may reveal whether structural defects are caused by the *phoP4* mutations. Any defects found will likely be more severe than the simple misshaping of a fraction of the *phoP4* myxospores formed (given the observed deficit in spore viability), and may include deformities in the spore coat, intermediate coat or cortex. Indeed, visual inspection of *phoP4* myxospores by phase-contrast microscopy revealed no discernible differences compared to wild-type myxospores in terms of phase brightness, shape or size.*

Beyond examining the various phenotypes caused by *phoP4* mutants, which are quantitatively the strongest among the *M. xanthus pho* mutants so far reported, the question

Table 3. Spore viability assay

Spores were harvested at 72 h after development was initiated by spotting 5×10^8 cells onto TPM starvation agar, as described in Methods. Values shown in the 'No. of viable spores' column represent the means of three independent experiments.

Strain	No. of viable spores	As a percentage of wild-type (mean $\pm$ range)
Wild-type	8.90×10^6	100.0 ± 6.7
<i>phoP4</i> ::pDV525	3.36×10^5	3.8 ± 1.1
Δ <i>phoP4</i>	1.34×10^5	1.5 ± 0.3

Table 4. Yeast two-hybrid interactions

Pairwise yeast two-hybrid interactions between bait and prey protein fusions were conducted as indicated in Methods. The strength of interaction, based on the intensity of the reporter indigo colour, was scored on a scale of 1–3, with a 3 assigned to the greatest colour intensity and hence the strongest interaction. No interaction is signified by –. See Methods for corresponding β -galactosidase specific activity values. Interactions involving PhoP4 or PhoR2 are highlighted in bold type. The interaction between RNM and QN was used as a positive control against which experimental interactions were standardized. AD, activation domain.

LexA–bait fusion	AD–prey fusion							
	RNM	PhoP1	PhoR1	PhoP2	PhoR2	PhoP3	PhoR3	PhoP4
QN	3	–	–	–	–	–	–	–
PhoP1	–	–	3	–	–	–	–	2
PhoR1	–	2	3	–	–	–	–	–
PhoP2	–	–	–	3	–	–	–	2
PhoR2	–	–	–	3	–	–	–	3
PhoP3	–	–	–	–	–	–	–	–
PhoR3	–	–	–	3	–	–	–	–
PhoP4	–	–	–	–	2	–	–	–

regarding the identity of the histidine kinase partner(s) to PhoP4 has yet to be fully answered. We noted that, whereas the mutants of each *M. xanthus* Pho TCS displayed different complements of phenotypes (Carrero-Lerida *et al.*, 2005; Martinez-Canamero *et al.*, 2003; Moraleda-Muñoz *et al.*, 2003), they all nevertheless affected certain subsets of the as-yet unidentified, developmentally active phosphatases (Weinberg & Zusman, 1990). This suggested to us that the four Pho TCSs may be functionally interlinked, rather than acting entirely separately from one another. Intriguingly, as reported above, a yeast two-hybrid analysis for interactions among the seven known Pho TCS components showed strong, reciprocal interactions between the receiver and H-box domains of PhoP4 and PhoR2, respectively (Table 4). It is possible that inputs from signals that activate the Pho2 and Pho4 systems, if received simultaneously, may lead to cross-communication between them, and amplify the expression of genes under their common control, e.g. those connected with the acid phosphatase activities.

However, besides interacting with PhoR2, we hypothesize that PhoP4 must be partnered with at least one other histidine kinase to regulate sporulation, and with the developmental alkaline and neutral phosphatase activities, since these processes are not affected in the *phoR2* mutant. The recently completed genome of *M. xanthus* may allow for a systematic approach towards identifying this histidine kinase partner of PhoP4. Since PhoP4 is a homologue of *E. coli* PhoB, it is predicted that its histidine kinase partner would resemble *E. coli* PhoR, the PhoB partner. The *M. xanthus* genome has been parsed for TCS components, and they have been classified based on domain architecture, gene order and deduced sequence phylogenies (D. E. Whitworth & D. A. Hodgson, unpublished results). These analyses have revealed a grouping of 13 histidine kinases, termed class II histidine kinases, which share these features with *E. coli* PhoR. In the next step, yeast two-hybrid analyses similar to the ones described in this study will be used to assess

whether any of these components interact with PhoP4. Coupled with expression studies to evaluate the physiological relevance of any interactions, and phenotypic analyses comparing *phoP4* mutants to mutants in genes of candidate partners, these future experiments will all help decipher the complexity of the *M. xanthus* phosphate regulatory circuitry.

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