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Expression in *Caulobacter crescentus* of the phosphate-starvation-inducible porin OprP of *Pseudomonas aeruginosa*

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1. SUMMARY

The gene for the phosphate-starvation-inducible outer membrane protein OprP, of *Pseudomonas aeruginosa* was introduced into *Caulobacter crescentus* CB2A on a plasmid vector. As is the case in *P. aeruginosa* and *Escherichia coli* the *oprP* gene was inducible under conditions of limiting phosphate in *C. crescentus*. However, the maximal medium concentration of phosphate which still permitted induction of OprP was lower in *C. crescentus* (50 μM) than in *P. aeruginosa* (200 μM). Induction of OprP was coincident with the process of stalk elongation, known to occur in *C. crescentus* under phosphate starvation conditions. When induced, OprP was localized to the cell envelope and became a major membrane protein, indicating that the *Pseudomonas* promoter was efficiently recognized in *C. crescentus* and that the gene product was targeted to the appropriate region of the cell. Our data provide support for the hypothesis that the mechanism for regulation of phosphate-starvation-inducible genes is highly conserved amongst the eubacteria.

2. INTRODUCTION

The mechanism for detecting and adapting to changes in environmental phosphate concentrations in *Escherichia coli* is based on a two component regulatory system consisting of a sensor and a regulator protein [1]. The genes involved in phosphate acquisition and assimilation have been collectively referred to as the pho regulon. One feature of pho regulon genes are a consensus DNA sequence in the promoter region of each gene, termed the 'pho box'. In *E. coli* this consensus region appears to be a binding site for a transcriptional "regulator" protein, the *phoB* gene product, and therefore has an essential role in the overall control of the regulon [2]. The PhoB protein is signaled by a change in the internal phosphate concentration which alters the interaction of the cytoplasmic "sensor" protein PhoR with PhoB.

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Phosphate limitation has been shown to result in the induction of outer membrane porins in a number of Gram-negative bacterial species, in addition to other polypeptides mediating phosphate acquisition, e.g., alkaline phosphatase and a peri-

3. MATERIALS AND METHODS

3.1. Bacterial strains and growth conditions

E. coli DH5α [13] was grown on LB medium [14] at 37°C and *C. crescentus* CB2A [15] was

0.300) grown in M-G (660 μM P_i) was washed twice with Hepes and resuspended into 25 ml of M-G containing no phosphate. Samples were taken at time 0, 5, 10, 15, 20, 30, 60, 120, 180 min and 16 h after the shift to low phosphate.

3.5. Comparison of phosphate concentrations required to induce OprP in C. crescentus and P. aeruginosa

To compare the maximum phosphate concentrations that will induce OprP, CB2A/pKT 230-XP and *P. aeruginosa* PAO H103 were separately grown overnight in M-G (660 μM P_i) to a final cell density of about 100 Klett units (Klett-Summerson colorimeter model 800-3, using filter 42). One ml of each culture was washed twice with 2 ml of Hepes and resuspended into 1 ml of Hepes. Fifty μl of washed cells were used to inoculate 5 ml of M-G supplemented with 660, 200, 100, 50, 25 or 0 μM phosphate. After 24 h growth at 30°C the cell density was measured and the samples were examined by Western blot analysis to detect induction of OprP.

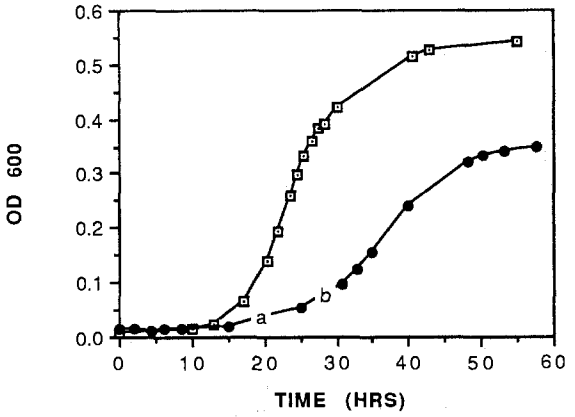

Fig. 2. Growth curve of *C. crescentus* CB2A/pKT230-XP in minimal media supplemented with 80 μM (sufficient) or 20 μM (limited) phosphate. a indicates time point where no free phosphate could be detected in the supernatant of the culture supplemented with 20 μM phosphate. b indicates the time point where stalk elongation was first noted by electron microscopy and OprP could first be detected by Western blotting. Cells grown in medium containing 80 μM phosphate did not express OprP or show stalk elongation at any time during the experiment.

4. RESULTS AND DISCUSSION

A protein of the same electrophoretic mobility as OprP was induced in CB2A/pKT230-XP under conditions of phosphate-limited growth but not in phosphate-sufficient medium (Fig. 1A). Western blotting using anti-OprP monomer-specific antibodies [3] confirmed that the induced protein was indeed OprP (Fig. 1B). Native CB2A cells did not produce a protein under phosphate-limited conditions that reacted with OprP specific antiserum. Western blots using anti-OprP trimer-specific antibodies indicated that OprP expressed in CB2A assembles into an oligomer (data not shown) in the same manner as it does in *P. aeruginosa* [3].

OprP induction and the appearance of long stalks provided evidence that the *oprP* gene was regulated by the *C. crescentus* phosphate-sensing system.

In the experiment where *C. crescentus* cells were shifted from growth in 660 to 0 μM phosphate, Western blots showed that OprP was undetectable until the 16 h time point (data not shown) indicating that there was a significant lag in the induction of OprP. This result suggested that induction of OprP may be a function of intracellular phosphate reserves. In preliminary experiments we learned that in medium with 0 μM phosphate, the time of stalk elongation and OprP induction, and the maximum optical density of the culture, varied greatly depending on the growth conditions of the

after which the cells were harvested and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [22]. Samples were heated in solubilization mix for 10 min at 100°C to observe OprP monomers and at room temperature to observe OprP trimers [23]. Outer membranes from *P. aeruginosa* grown in phosphate limited medium were isolated [23] and used to demonstrate authentic OprP.

3.4. Monitoring OprP induction in C. crescentus

To examine the effect of medium phosphate concentration on OprP induction, 1-ml samples of cells from logarithmic phase cultures of CB2A/pKT230-XP ($OD_{600} = 0.600$) grown in M-G (660 μM P_i) were washed twice with Hepes by centrifugation and resuspension, and used to inoculate 100 ml of M-G (20 or 80 μM P_i). In agreement with Poindexter [24], phosphate became growth rate limiting at 20 μM. Incubation at 30°C was initiated and at selected times, samples of cells were examined by electron microscopy to determine the length of the cell stalks and 1 ml samples were harvested by centrifugation. The cell pellets were used for Western blot analysis, as previously described [15], and the culture supernatants were analyzed for phosphate content using an adaptation of the stannous chloride method [25]. In a separate experiment, a 25 ml logarithmic phase culture of CB2A/pKT230-XP ($OD_{600} =$

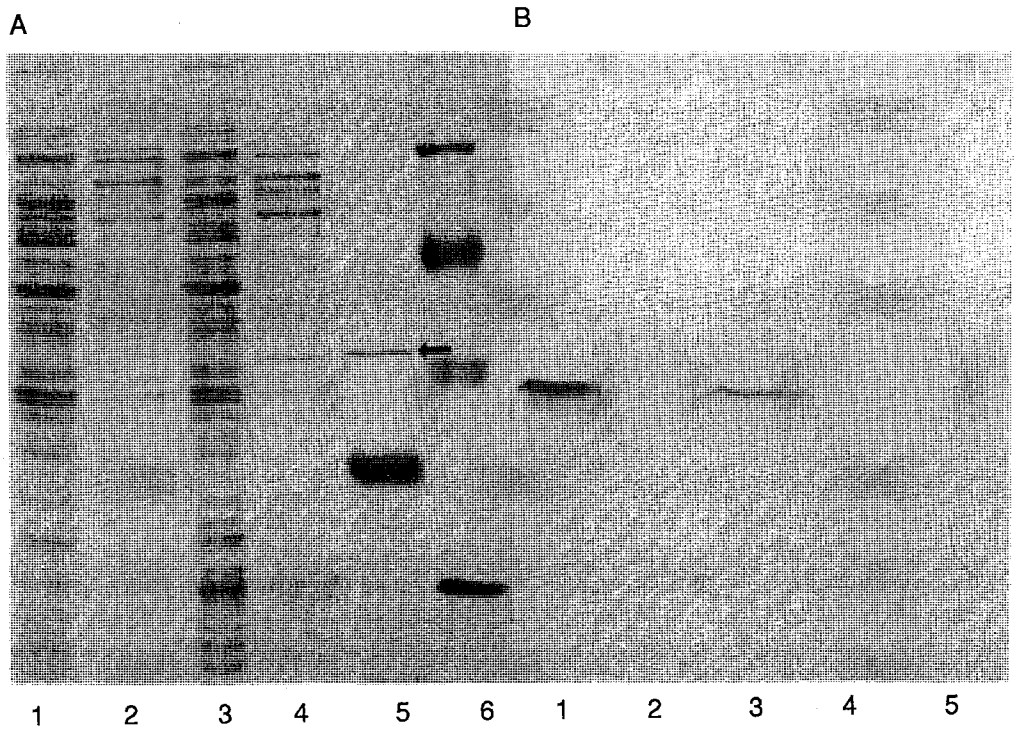

Fig. 1. Induction of OprP in *C. crescentus* CB2A/pKT230-XP under conditions of phosphate limitation. (A) 10–15% acrylamide gradient SDS-PAGE of whole cell extracts stained with Coomassie R-250. The cells were grown in high-phosphate (660 μM) or low-phosphate (10 μM) minimal salts medium as indicated. All samples were solubilized at 100°C and 30 μg of protein were loaded per lane: lane 1, CB2A (high); 2, CB2A (low); 3, CB2A/pKT230-XP (high); 4, CB2A/pKT230-XP (low); 5, purified *P. aeruginosa* outer membranes containing OprP; 6, protein standards. In order of decreasing molecular mass they are: rabbit muscle phosphorylase *b* (97400), bovine serum albumin (66200) and hen egg white ovalbumin (42699). The arrow indicates the running position of OprP. (B) Western blot of a 12.5% acrylamide SDS-PAGE of whole cell extracts reacted with rabbit antiserum directed against OprP monomers. The cells were grown in high phosphate (660 μM) or low phosphate (10 μM) minimal salts medium as indicated. All samples were solubilized at 100°C and 10 μg of protein were loaded per lane: lane 1, purified *P. aeruginosa* outer membranes containing OprP; lane 2, CB2A/pKT230-XP (high); lane 3, CB2A/pKT230-XP (low); lane 4, CB2A (high); lane 5, CB2A (low).

0.300) grown in M-G (660 μM P_i) was washed twice with Hepes and resuspended into 25 ml of M-G containing no phosphate. Samples were taken at time 0, 5, 10, 15, 20, 30, 60, 120, 180 min and 16 h after the shift to low phosphate.

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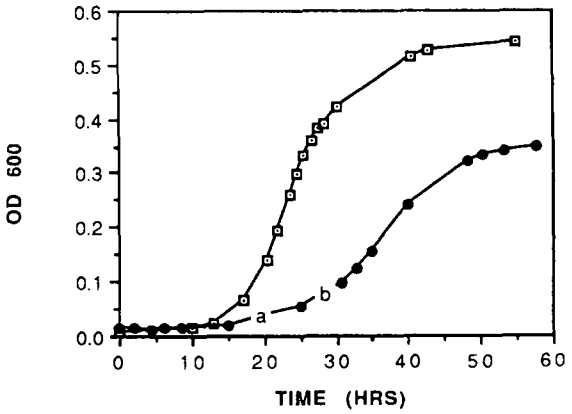

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OprP induction occurred coincident with low-phosphate-stress induced stalk elongation and neither stalk elongation nor OprP induction began until after phosphate in the culture medium was no longer detectable (Fig. 2). In past studies [18] stalk elongation was the most obvious consequence of phosphate limitation on the physiology of *C. crescentus*. The coincidence in the timing of OprP induction and the appearance of long stalks provided evidence that the *oprP* gene was regulated by the *C. crescentus* phosphate-sensing system.

In the experiment where *C. crescentus* cells were shifted from growth in 660 to 0 μM phosphate, Western blots showed that OprP was undetectable until the 16 h time point (data not shown) indicating that there was a significant lag in the induction of OprP. This result suggested that induction of OprP may be a function of intracellular phosphate reserves. In preliminary experiments we learned that in medium with 0 μM phosphate, the time of stalk elongation and OprP induction, and the maximum optical density of the culture, varied greatly depending on the growth conditions of the inoculum culture. In general, pregrowth in media rich in phosphate led to a delay, after shift to phosphate-free medium, in OprP induction and stalk elongation and also led to higher maximum cell densities (data not shown). Apparently the *Caulobacter* cells accumulated phosphate reserves (possibly as polyphosphate) which had to be exhausted before the OprP induction occurred.

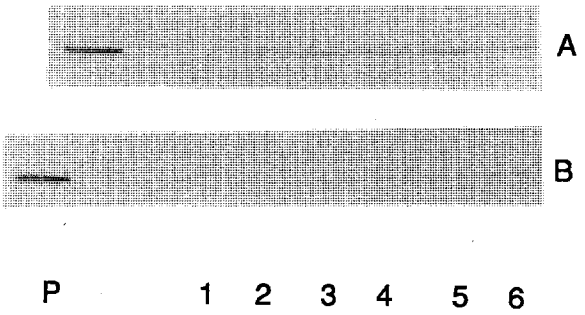

Fig. 3. Comparison of the phosphate concentrations required to induce OprP in *C. crescentus* and *P. aeruginosa*. Western blot of a 12.5% acrylamide SDS-PAGE of whole cell extracts of (A) *P. aeruginosa* and (B) *C. crescentus* reacted with rabbit antiserum directed against OprP monomers. Samples were solubilized at 100°C and 10 μg of protein was loaded per lane. The cells were grown in minimal media containing: lane 1, 660 μM; 2, 200 μM; 3, 100 μM; 4, 50 μM; 5, 25 μM; 6, no added phosphate; P, porin P control.

The maximal phosphate concentration permitting induction of OprP in *C. crescentus* was significantly lower than that needed for *P. aeruginosa* (Fig. 3). In *P. aeruginosa* OprP was induced by growth on 200 μM phosphate [23] whereas *C. crescentus* did not express the porin until phosphate levels were reduced in the initial culture medium to approximately 50 μM (Fig. 3).

The soluble and membrane fractions obtained after cell disruption in a French pressure cell were examined by Western blot analysis, using anti-OprP monomer antibodies, which indicated that OprP was completely segregated to the membrane fraction (data not shown). We were unable to further delineate the ultimate position of the OprP molecule in the *Caulobacter* cell. We have not been able to reliably separate the proteins of the inner and outer membranes of *Caulobacter* by standard methods involving sucrose density gradient centrifugation or differential detergent solubility of membranes (Hancock and Nikaido, unpublished data; Walker and Smit, unpublished data). Despite reports by others of successful segregation of *Caulobacter* membranes [26,27], in our experience the methods yield membrane fractions with nearly identical protein profiles. Surface labeling with porin P antibody gave negative results, yet was not definitive, since the antibodies used in this study are unable to label *P. aeruginosa* cells grown under phosphate limitation with rough or smooth LPS.

Our data imply that, although sensitive to different phosphate concentrations, the *C. crescentus* phosphate-starvation-inducible (pho) regulon shared sufficient similarity to the pho regulon of *P. aeruginosa* that it recognized the *P. aeruginosa* phosphate regulon control signals contained within the *oprP* gene, i.e. the consensus pho box region. It may also be inferred that *C. crescentus* produced an activator molecule which was functionally related to the PhoB-like protein of *P. aeruginosa* [28] in that it was capable of interacting with the regulatory signals present in the oprP gene. At present, native *C. crescentus* phosphate regulated genes have not been characterized but we predict, based on these results, that they contain DNA sequences homologous to the pho box found in OprP.

Inter-species control of pho regulons has been shown to occur between *E. coli* and *P. aeruginosa* [5], *V. parahaemolyticus* [6], *K. pneumonia* and *E. cloacae* [7]. By the criterion of ribosomal RNA sequence comparisons these species are phylogenetically related; all are members of the γ-purple bacteria subgroup 3 [11]. In contrast, *C. crescentus* is a member of the distantly related α-purple bacteria category [10,12]. Thus the data presented here imply extensive conservation throughout the Gram-negative eubacteria of control elements of the pho regulon.

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