The cyanobacterium *Synechococcus* sp. strain PCC 7942 contains a second alkaline phosphatase encoded by *phoV*

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A gene (*phoV*) encoding an alkaline phosphatase from *Synechococcus* sp. strain PCC 7942 was isolated by screening a plasmid gene bank for expression of alkaline phosphatase activity in *Escherichia coli* JM103. Two independent clones carrying the same alkaline-phosphatase-encoding gene were isolated. One of these clones (pKW1) was further analysed and the nucleotide sequence of a contiguous 3234 bp DNA fragment was determined. Two complete open reading frames (ORF1 and *phoV*) and an incomplete ORF3 were identified reading in the same direction. The deduced *phoV* gene product showed 34% identity to the alkaline phosphatase PhoA from *Zymomonas mobilis*, and the N-terminal part of the putative ORF3 protein exhibited 57% identity to a protein of unknown function from *Frankia* sp. Insertional inactivation of the *Synechococcus* PCC 7942 *phoV* gene failed, indicating an essential role for either the *phoV* or the ORF3 gene product. PhoV consists of 550 amino acid residues, resulting in a molecular mass of 61.3 kDa. To overexpress the *Synechococcus* PCC 7942 *phoV* gene in *E. coli*, plasmid pKW1 was transformed into a *phoA* mutant of *E. coli* (CC118). In *E. coli* strain CC118(pKW1) PhoV was expressed constitutively with high rates of activity, and was shown to be membrane associated in the periplasmic space. After partial purification of the recombinant PhoV, it was shown that, like other alkaline phosphatases, the *Synechococcus* PhoV had a broad pH optimum in the alkaline region and a broad substrate specificity for phosphomonoesters, required Zn²⁺ for activity, and was inhibited by phosphate. In contrast to several other alkaline phosphatases, PhoV was inhibited by Mn²⁺. Due to the lack of a *Synechococcus* PCC 7942 *phoV* mutant strain, the function of PhoV remains uncertain. However, the present results show that *Synechococcus* PCC 7942 has a second, probably phosphate-irrepressible, alkaline phosphatase (PhoV, 61.3 kDa) in addition to the phosphate-repressible enzyme (PhoA, 145 kDa) already described.

Keywords: alkaline phosphatase, *Synechococcus* PCC 7942, cyanobacteria, *phoV* gene

INTRODUCTION

Cyanobacteria and other micro-organisms constantly monitor environmental conditions and adjust their struc-

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Abbreviations: APase, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitroblue tetrazolium; 4-NP, 4-nitrophenol; 4-NPP, 4-nitrophenyl phosphate.

The GenBank accession number for the sequence reported in this paper is Z48801.

ture and physiology accordingly. Phosphate is a nutrient required at high level for cell growth and is frequently the limiting nutrient, since it often exists in forms not readily available. To cope with phosphate-limiting conditions, cells store polyphosphate reserves which allow them to withstand short periods of phosphate deprivation, and express extracellular phosphatases to obtain phosphate from organic substrates which are present in the surrounding medium, but which cannot penetrate the cell membrane (Carr & Mann, 1994; Grossman *et al.*, 1994). When growing in a phosphate-deficient environment, a number of micro-organisms induce the synthesis of

alkaline phosphatase (APase). Most of these APases are located in the periplasmic space as soluble enzymes (Cheng & Costerton, 1973; Bhatti *et al.*, 1976) but membrane-associated enzymes have also been found (Von Tigerstrom & Stelmashuk, 1986; Baoudene-Assali *et al.*, 1993). The most thoroughly investigated prokaryotic APase is that of *Escherichia coli* (Wanner, 1987; Wyckoff, 1987).

APase activity varies with phosphate composition in *Anabaena flos-aquae* (Bone, 1971; Healy, 1973), *Synechococcus* sp. strain PCC 7942 (Ihlenfeldt & Gibson, 1975; Block & Grossman, 1988), and *Plectonema boryanum* (Doonan & Jensen, 1977). Ultrastructural localization of APase in *P. boryanum* showed that the enzyme was present in layer 3 (periplasmic space) of the cell wall. Extensive studies have been carried out on a derepressible APase from *Synechococcus* PCC 7942 (Block & Grossman, 1988; Ray *et al.*, 1991). This atypical APase has a subunit molecular mass of 145 kDa and is located in the periplasmic space in association with the cell wall or cytoplasmic membrane. Insertional inactivation of the corresponding *phoA* gene resulted in loss of extracellular, phosphate-regulated phosphatase activity, but did not alter the capacity of the cells for phosphate uptake, nor their viability when maintained on phosphate-sufficient or -deficient medium. The mutant showed slightly greater overall external APase activity than the wild-type, but there was no further increase in APase activity after transfer of the mutant cells to phosphate-free medium. These results imply that *Synechococcus* PCC 7942 cells have at least one additional APase as well as the atypical 145 kDa PhoA (Ray *et al.*, 1991). This additional APase is not repressed by phosphate, in contrast to the phosphate-repressible PhoA. Here we report the identification and sequence analysis of a second gene (*phoV*) encoding an APase in *Synechococcus* PCC 7942, and the partial characterization of the *phoV* gene product.

METHODS

Bacterial strains, plasmids and growth conditions. *Synechococcus* PCC 7942 was obtained from the Pasteur Culture Collection of Cyanobacterial Strains, Paris, France. The *E. coli* JM103 host for the plasmids pUC19, pSVB28 and derivatives was the same as described by Messing *et al.* (1981), and the APase-free *E. coli* strain CC118 was the same as described by Manoil & Beckwith (1985). *Synechococcus* PCC 7942 was grown in BG11 medium (Rippka, 1988) as previously described (Engels *et al.*, 1992). *E. coli* strains were cultivated at 37 °C in LB medium (Miller, 1972) or on PA plates [1 l distilled H₂O contained 17.5 g antibiotic medium no. 3 (assay broth, Oxoid) and 16.0 g bacteriological agar (Oxoid)]. Antibiotics were added at the following concentrations: ampicillin, 150 mg l⁻¹ for *E. coli* and 0.5 mg l⁻¹ for *Synechococcus*; chloramphenicol, 50 mg l⁻¹ for *E. coli* and 7.5 mg l⁻¹ for *Synechococcus*. To achieve phosphate deprivation, cells were harvested by centrifugation at 5000 g for 15 min, washed with LB medium in which KH₂PO₄ was replaced by KCl (phosphate-free medium) and then resuspended in the same medium at about a threefold lower cell density.

The plasmids used in this study were pUC19 (Yanisch-Perron *et al.*, 1985), pSVB28 (Arnold & Pühler, 1988) and pRB5, a pUC19 derivative carrying the *Synechococcus* PCC 6301 *psbO* gene inactivated by a Cm^r cassette (Bockholt *et al.*, 1991). Details of

the pUC19 and pSVB28 derivatives constructed in this paper are given in the legend to Fig. 1.

Cloning and hybridization procedures. Isolation of total DNA from *Synechococcus* PCC 7942 and of plasmid DNA from *E. coli*, cloning procedures and restriction analysis were performed by standard methods (Sambrook *et al.*, 1989). Hybridization experiments were carried out using the DIG labelling and detection kit (Boehringer Mannheim).

To isolate the APase-encoding gene (*phoV*) of *Synechococcus* PCC 7942, a size-fractionated genomic library containing 2–10 kb DNA fragments (from a partial *Sau3A*I digestion), cloned into the *Bam*HI site of vector plasmid pUC19, was transformed into *E. coli* JM103. Ninety-two percent of the clones were shown to contain an insert. Colonies were transferred onto nitrocellulose membranes (Millipore HAHYO8250, filter type HA, pore size 0.45 µm) and were incubated under an atmosphere of chloroform vapour for 15 min. Subsequently, the membranes were incubated in lysis buffer (100 mM Tris/HCl, pH 7.8, containing 150 mM NaCl, 5 mM MgCl₂, 1.5 % BSA, 1 µg DNase I ml⁻¹ and 40 µg lysozyme ml⁻¹) for 15 h at room temperature. Membranes were washed twice in TNT buffer (10 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, 0.05 % Tween 20) for 30 min at room temperature. Remaining cell debris was wiped from the membranes before washing in colony-assay buffer (100 mM Tris/HCl, pH 7.6, containing 1 mM ZnCl₂ and 0.1 % Triton X-100) for 10 min at room temperature. Each membrane preparation (about 50 cm²) was incubated in 10 ml fresh colony-assay buffer before 50 µl NBT (50 mg ml⁻¹ in 70 % dimethylformamide) and 25 µl BCIP (50 mg ml⁻¹ in 100 % dimethylformamide) were added. After 5–15 min incubation at room temperature, clones containing the APase-encoding gene (*phoV*) from *Synechococcus* PCC 7942 were identified by their red colour.

DNA sequencing. Appropriate restriction fragments were cloned into the pSVB28 sequencing vector (Arnold & Pühler, 1988), resulting in hybrid plasmids pKW13 and pKW14, and nested deletions were introduced with the exonuclease III deletion kit (Pharmacia). Sequencing of both strands was performed using the Automatic Laser Fluorescent DNA Sequencer (Pharmacia). DNA sequences were analysed using the Staden software package (Staden, 1986) and PC/GENE (IntelliGenetics). Homology searches were performed using the FASTA and TFASTA algorithms (Pearson & Lipman, 1988).

Biochemical methods. For APase measurements, either whole *E. coli* cell suspensions or cell extracts were used. Cells were harvested by centrifugation, washed once with APase buffer (0.1 M Tris/HCl, pH 7.6, containing 1 mM ZnCl₂ and 1 mM MgCl₂) and resuspended in the same buffer. Cell extracts were obtained by passing cell suspensions through a French press at 138 MPa. The osmotic shock procedure for *E. coli* cells was performed according to Neu & Heppel (1965). Protein determinations were performed according to Bradford (1976), and SDS-PAGE was done as previously described (Engels *et al.*, 1992).

APase activity was determined in a reaction mixture of 2.5 ml containing: 100 mM Tris/HCl, pH 7.6, 1 mM ZnCl₂, 1 mM MgCl₂, 2 % (v/v) Triton X-100, 10 mM 4-nitrophenyl phosphate (4-NPP), and the sample. After incubation for 5 min at 37 °C the reaction was stopped by adding 0.5 ml 1 M NaOH. After centrifugation at 5000 g for 15 min, the absorbance of the supernatant was measured at 405 nm and compared to a standard absorbance curve of 4-nitrophenol (Sigma).

Partial purification of PhoV. *E. coli* CC118(pKW1) cells were harvested by centrifugation and washed once with 10 mM

Tris/HCl, pH 7.6, containing 1 mM EDTA, and then resuspended in 10 mM Tris/HCl, pH 7.6, containing 1 mM MgCl₂ and 1 mM ZnCl₂ (buffer A). After breaking the cells by French press treatment at 138 MPa and centrifugation of the extract at 30 000 *g* for 35 min, the pellet was washed three times with buffer A. Subsequently, PhoV was extracted from the pellet by washing with 1 M Tris/HCl, pH 7.6, containing 10 mM MgCl₂, 10 mM ZnCl₂, and 2% Triton X-100 (buffer B). After centrifugation at 30 000 *g* for 35 min, the suspension was dialysed against buffer A. This resulted in precipitation of the major part of the PhoV. After centrifugation, the pellet fraction containing the PhoV was washed with buffer A containing 2% Triton X-100 to extract proteins which become solubilized by Triton X-100 without high Tris concentrations, and centrifuged again. The washed pellet containing the PhoV was suspended in buffer B, and the suspension was stirred overnight at room temperature. After centrifugation, the major part of the PhoV was present in the supernatant. This supernatant was subjected to ammonium sulphate fractionation. The PhoV precipitated in the 50–95% ammonium sulphate fraction, but was floating as a layer on top of the solution. This floating layer was redissolved in buffer B.

RESULTS AND DISCUSSION

Cloning of an alkaline phosphatase-encoding gene from *Synechococcus* PCC 7942

A gene coding for an APase was isolated from a *Synechococcus* PCC 7942 gene bank by screening for expression of APase activity in *E. coli* JM103, which expressed only a weak background of APase activity under the conditions used. About 17 000 clones were grown on ampicillin-containing PA plates until tiny colonies were visible; the colonies were subsequently transferred onto nitrocellulose membranes. Cells were lysed, and non-bound cell debris was removed by several washing steps. The membranes were incubated in colony-assay buffer containing BCIP and NBT. By this chromogenic assay two clones were identified which strongly expressed APase activity. Plasmid DNA of both clones was isolated and characterized by restriction analysis. The hybrid plasmids contained inserts of 5.8 kb (pKW1) and 11.2 kb (pKW2), respectively. The 5.8 kb DNA fragment of plasmid pKW1 (Fig. 1) was part of pKW2 (data not shown) indicating that the two original clones encoded the same APase. Using both plasmids as probes in hybridization experiments with total DNA from *Synechococcus* PCC 7942 confirmed that no rearrangements or deletions had occurred during the cloning procedure (data not shown).

For further physical mapping of the APase-encoding gene within the 5.8 kb DNA fragment, hybrid plasmid pKW1 was partially digested with *Sau*3AI and subfragments were ligated into the *Bam*HI site of pUC19. Two hybrid plasmids (pKW4 and pKW6) carrying subfragments that conferred APase activity are shown in Fig. 1. Plasmid pKW6 indicates that the maximum size of the APase-encoding gene was less than 2.5 kb. Plasmids pKW4 and pKW6 contained the APase-encoding gene in different orientations relative to the *lac* promoter; thus it seems likely that the APase-encoding gene was transcribed from a promoter located within the cloned *Synechococcus* PCC

Fig. 1. Physical and genetic map of the *phoV* gene region from *Synechococcus* PCC 7942. Plasmids pKW1, pKW4 and pKW6 contain different *Sau*3AI fragments (a 5.8 kb, 3.5 kb or 2.5 kb fragment, respectively) from *Synechococcus* PCC 7942 carrying the *phoV* gene cloned into the *Bam*HI site of pUC19. Plasmids pKW13 and pKW14 contain subfragments of pKW1 cloned into the sequencing vector pSVB28. pKW13 contains a 3.3 kb *Cla*I–*Hind*III fragment from pKW1, and pKW14 contains a 3.5 kb *Nsi*I–*Hind*III fragment from pKW1. Plasmids pKW14-A and pKW14-B contain the chloramphenicol resistance cassette (*Cm*^r) used for mutational analysis. These plasmids are derivatives of pKW14 containing the 1.8 kb *Pst*I fragment from pRB5. The physical map of all hybrid plasmids is given for the enzymes *Bam*HI (B), *Cla*I (C), *Hind*III (H), *Nsi*I (N) and *Pst*I (P). Restriction sites given in parentheses were destroyed during the cloning of DNA fragments with compatible ends which did not reconstitute the original sites. The hatched boxes represent the multiple cloning sites of pUC19 or pSVB28, and are not drawn to scale. The heavy line in pKW1 marks the DNA fragment which was sequenced.

7942 DNA fragment. However, this promoter recognized by *E. coli* is not necessarily the same promoter that drives expression in *Synechococcus* PCC 7942.

DNA sequence analysis of the *phoV* gene region from *Synechococcus* PCC 7942

The 3.3 kb *Cla*I–*Hind*III fragment and the 3.5 kb *Nsi*I–*Hind*III fragment from plasmid pKW1 were cloned into the sequencing vector pSVB28. The resulting hybrid plasmids, pKW13 and pKW14, respectively (Fig. 1), were subjected to exonuclease III/S1 treatment to generate

1 CATTGCAACTCAGAAAGCTAGGTTATAATTTAACTGACAAAGCAGTACTTGATGCCTTAAAAAGCGTAAAAAACTCAC
 81 TGGCTTACAAGGTAGATGGCAAACGCTTCATAAAAAACACCTTACCATTGTGTATACCGGACACAATAAAGCTGGCATC
 161 GCCGAAGTGGCACAAAACATCAAACAAACACCTTAATGAAAACCTACACATGGTGATTGGAATGGTCAAAGATAAAGATA
 I S A V L A L L P A N A N Y Y F C Q P N L E R A L P V
 241 TTAGTGCTGTACTGGCCTTGTGTCAGCCAACGCAAATTATTACTTTTGCCAGCCAAATTTAGAAAGGGCCCTACCTGTA
 E E L K Q Q A S Q Y Q L R G E A F T S G S D A L Q T A
 321 GAGGAACAGAAACAGCAAGCAGTCAATATCAACTCCGAGGCGAAGCGTTACATCGGGTAGTGACGCTCTGCAAACCTGC
 Q S A A N E K D L I F I G G S T F V V A E I I
 401 GCAAAGTGCTGCTAACGAAAAGGATCTCATATTCATTGGTGGTAGCACATTTGTAGTAGCAGAAATTATATAAAATCTTT
 M K I K L L
 481 ACAATGCGCACCAATTATCTTAACATCGTTTTAATACCTTTACAAAAACATCAATTTTATTAATGAAAATTAACTTTTG
 C I S L A V L F C S S A ↓ N A ↓ Q K K Q A K V Q P S V F P
 561 TGCATTTCTTTAGCTGTTTTGTTTTGTCAGCAGTGCAAACGCCAGAAAAACAAGCTAAAGTTCAACCTTCTGTATTTCC
 Q T V A R P K L V V G M V I D Q M R W D Y L Y R F Y
 641 ACAAAACGGTAGCTCGTCCCAATTAGTAGTTGGGATGGTGATAGATCAATGCGTTGGGATTATTTATATCGTTTTTATG
 A R Y G N G G F K R L I N E G F S A E N T L I P Y T P
 721 CCAGATATGGCAATGGTGGCTTCAAACGATTAATCAATGAAGGTTTTCTGCAGAAAATACTTTGATCCCTTATACACCA
 PstI
 T L T A C G H S S I Y T G S V P A I N G I I G N N . W . F
 801 ACCTTAACATGCATGCGGCCACTCTTCAATTTATAGTGGTCTGTACCGGTATTATGTTGGAATAACTGGTT
 D P Q L G R D V Y C V E D K S V K T V G S S S N E G
 881 TGACCCTCAGTTGGCGAGGATGTTTATTGTGTAGAAGATAAAGCGTTAAACCGTTGGAAGCTCAAGCAACGAAGGGC
 L M S P K N L L V T T V T D E L R M A T N F R S K V I
 961 TAATGTCACCAAAAAATTTGCTAGTTACTACGGTAACCGACGAGCTTAGGATGGCCACCACTTTAGAAGCAAGTAATT
 S V S I K D R G A I L P G G H T A N G A Y W Y D D M T
 1041 AGTGTCTTCTATTAAGATAGAGGTGCGATTCTTCTGCGGGCATAACGGCAATGGCGCTTACTGGTACGATGACATGAC
 G S F I S S T H Y M Q Q L P T W V N D F N A Q R L P
 1121 TGGCAGTTTTATTTCAGCAGCATTACATGCAGCAATTGCCTACTTGGGTAAATGATTTTAAATGCGCAAAGACTTCCCA
 N K Y F E Q D W N T L Y P I E T Y T E S T A D A K P Y
 1201 ATAAATACCTTTGAACAAGATTGGAATACCTTATATCTATTGAACTTATACGGAAGTACCGCCGATGCAAAACCATAC
 E R T F K G A K T S S F P H L F K Q Y A N K N Y S M M
 1281 GAGCGCACTTTTAAAGGTGCAAAAACCTCAAGTTTTCTCCTCACTTATTTAAACAATATGCTAATAAAACTATTTCGATGAT
 A S M P Q G N S F T L E F A K A A I P A E K L G Q T
 1361 GGCAAGCATGCCACAGGTAACAGCTTTACTTTAGAATTGCAAAAGCTGCGATACCTGCTGAAAACTGGCCAAACTG
 G N T D F L A V S L S S T D Y V G H Q F G P N S I E L
 1441 GAAATACAGATTTCTTAGCAGTAAGTTTATCTTCAACAGATTATGTAGGGCACCAATTGCGACCAATTCATCGAGCTG
 E D T Y L R L D K D L E D F F N Y L D K T I G K G N Y
 1521 GAAGATACGTATTTAAGGCTTGACAAAGATTGGAAGATTCTTCAACTATTTGGACAAAACCATCGGCAAAGGAACTA
 L L F L T A D H G A T H V P G F L R N K M P G G R L
 1601 TTTGTTATCTTAACATGCGGACCGGTGCAACTCATGTTCTGGTTTTTAAAGAAACAAATGCCTGGAGGTCGATTGC
 L L K V Q T D L D S L I F N E F K V R C N F T I I N N
 1681 TACTAAAAGTTCAAACCTGATTAGATAGTTTGATTTTAAATGAGTTTAAAGTGAGATGTAATTCACCATTTATTAACAAT
 Q V I F D T D A I K E A K A D Y A K I K Q S T I D Y L
 1761 CAAGTGATTTTGTACTGATGCAATTAAGGAAGCTAAAGCTGATTATGCTAAAATCAAACAATCCACGATAGATTATTT
 V K Q D G V L N A V D I K N M G A V T I P Q E I K N
 1841 GGTAAAACAAGACGGGTTTTAAATGCGGTAGACATTAAAAATATGGGTGAGTTACTATCCCTCAAGAAATTAAAAATA
 K I I N G Y N A R R S G D V Y I I L D A G W Y P T L T
 1921 AAATCATCAATGGGTATAATGCCCGTAGAAGTGGCGACGTATATATCATTCGATGCTGGTGGTATCTTAACT

ORF1

phoV

Fig. 2. For legend see facing page.

P G T G H A A W N P Y D S H I P A L F M G W G V K P G
 2001 CCAGGCACAGGGCATGCCGCATGGAATCCTTACGATAGTCACATTCCTGCACTTTTCATGGGTTGGGGCGTTAAACCTGG
 K T N K E Y Y M S D I A P T V S A L L H I Q Q P S G
 2081 TAAACAAATAAAGAATATTATATGTCCGATATTGCCCTACTGTTTCTGCCTTGTTGCATATTCAACAACCAAGCGGTA
 S I G K V I T D L L K
 2161 GTATAGGCAAAGTAATAACCGATTACTTAAATAGCTTTAAATTTTAATAATTCAACCCCTCATTTCTGAAATATGAATT
 2241 GAGGGTTTTTCATTATTAGGGCTATTTTAATACCAGATCTTCAAATTTACTACACATTTTACTCCTAAAATTTAGCGTT
 2321 TTCACCTAGCAAAAATCTTACTTTGGTACATATCTATTAGCCAAATATTAGCAAAAATTATATCAGGATTTATCGTATGG M
 D K I E I Y Q P K H K I R F V T V A A L F D G H D A T ORF3
 2401 ATAAAATTGAAATCTATCAGCCTAAACATAAAATAAGATTTGTAAGTGTGCTGCACTTTTGTATGGGCATGATGCTACC
 I N I M R R I L Q S S G A E V I H L G H N R S V E E V
 2481 ATTAATATCATGCGTAGAATTTGCAGTCCAGCGGTGCCGAGGTTATTCACTTAGGGCATAACCGTTCGGTGGAAGAAGT
 V N C A I Q E D V Q G I A M T S Y Q G G H I E Y F K
 2561 TGTAATTTGCGCAATACAAGAAGATGTTCAAGGTATTGCGATGACATCTTATCAGGGTGACACATTGAATACTTTAAAT
 Y M Y D L L Q E R G A N H I K I F G G G G G V I L P N
 2641 ATATGTATGATTTATTGCAGGAACGTGGTGCAAATCATATCAAATATTTGGTGGTGGAGGTGGTGAATTTTACCTAAT
 E I K E L E S Y G I A E I L F A D D G R R I G T Q G M
 2721 GAAATTAAAGACTAGAGAGCTACGGTATTGCGGAGATCTTATTCGCGGACGACGGTAGAAGAAATGGGACGCAAGGCAT
 I N D M L Q K N R F C I I E K Q F D L E K P L N R E
 2801 GATTAACGACATGCTTCAAAAAACAGATTTTGCATTATAGAAAAGCAATTCGATTTAGAAAAGCCATTAAATAGGGAGA
 T T E D R K R N L F G R N N A E A F E D S L A T L R K
 2881 CAACGGAAGATCGCAAACGCAATCTCTTGGCAGAAATAATGCCGAAGCCTTTGAAGACTCGTTAGCAACGCTGAGAAAA
 K Q P T N P P V L G I T G T G G A G K S S L V D E L V
 2961 AAACAGCCTACCAACCCACCTGTTTTAGGTATTACCGGAACAGGCGGCGCAGGAAAGTCTTCCTTAGTTGACGAATTGGT
 R R F L I E V D N K S I A I I S V D P S K R K T G G
 3041 TCGAAGATTTTAAATCGAAGTAGATAATAAATCTATTGCAATTATTTCTGTTGATCCTTCTAAAAGAAAACTGGCGGCG
 A L L G D R I R M N A I N N P R V Y M R S L A T R Q A
 3121 CCTTGTGGGAGATAGAATAAGAATGAATGCCATTAACAATCCAAGGGTTTATATGCGTTTCATTAGCTACTAGGCAAGCT
 N L A L S K K C T R K
 3201 AACCTCGCCCTATCTAAAAAATGTACAAGAAAGC

Fig. 2. Nucleotide sequence of the *Synechococcus* PCC 7942 *phoV* gene region. The DNA sequence is presented in the 5'→3' direction. The predicted amino acid sequences for two complete open reading frames (ORF1 and *PhoV*) and for one incomplete open reading frame (ORF3) are indicated by the single-letter code. Potential cleavage sites for a secretory leader sequence are indicated by vertical arrows. The *Pst*I site within the *phoV* coding region used for cassette mutagenesis is shown below the nucleotide sequence. A putative phosphorylation site of *PhoV* is underlined.

nested deletions. The deletion derivatives were used to determine the sequence of a contiguous 3234 bp DNA fragment in overlapping sequences for both strands (Fig. 2).

Three ORFs within the sequenced region could be defined in the same orientation. ORF1 might encode a small polypeptide of 87 amino acid residues, but computer searches did not reveal any homology to known genes. Since hybrid plasmid pKW6 (Fig. 1) did not contain the entire ORF1 but still confers APase activity, at least in *E. coli* ORF1 is not essential for APase activity.

ORF2 was located 72 bp downstream of ORF1 and codes for a protein of 550 amino acid residues with a calculated molecular mass of 61325 Da and an isoelectric point of

8.7. There are several lines of evidence that ORF2 corresponds to the APase-encoding gene from *Synechococcus* PCC 7942. (i) ORF2 was the only complete ORF encoded by the insert in pKW6, which confers APase activity. (ii) Insertion of an interposon carrying the chloramphenicol resistance gene in the *Pst*I site within ORF2 (Fig. 1) abolished APase activity in *E. coli* strains carrying the plasmid (see below). (iii) ORF2 showed 34% identity over its entire length with the *phoA* gene product from *Zymomonas mobilis*, strain CP4 (database accession number L36230; P. F. Gomez & L. O. Ingram, unpublished, 1994) (Fig. 3). Since another phosphatase-encoding gene from *Synechococcus* PCC 7942 has been described as *phoA* (Ray *et al.*, 1991), the ORF2 gene was called *phoV*.

(a)		
<i>SphoV</i>	MKIKLLCISLAVLCSSANAQKKQAKVQPSVFPQTVARP...K	40
	: * : * * : * : * : *	
<i>ZphoA</i>	MNSLLHHSFLKTVFSSLAIAIVTSSLSSVTIAATHPLDNHPKGEIAASSETAHNPWSGTR	60
<i>SphoV</i>	LVVGMVIDQMRWDYLYRFYARYGNGGFKRLINEGSAENTLIPYTPTLTACGHSSIYTG	100
	*: * : * * : * : * : * : * : * : *	
<i>ZphoA</i>	LIVAI SVDQFSSDLFSEYRGRF. RSGMKQL. QNGVVYPMAHSHAATETCPGHSVLLTGD	118
<i>SphoV</i>	VPAINGIIGNNWFD...PQLGRDVYCVEDKSVKTVGSSSNEGLMSPKNLLVTTVTDELRM	157
	* * * * * : * * * * * : * * * * *	
<i>ZphoA</i>	HPARTGIIANNWYDFSVKRAKDKVYCEDEPSLSADPQNYKPSV...HYLKVPPTLGDRMKK	175
<i>SphoV</i>	ATNFRSKVISVSIKDRGAILPGGHTANGA. YWYDDMTGSFISSTHYMQQLPTWVNDENAQ	216
	* : * * * * * : * * * : * : * : * : *	
<i>ZphoA</i>	ANP. HSRVISVAGKDRAAIMGGHMTDQIWFWSN...AYKTLADHKGEMPATVKTVNEQ	231
<i>SphoV</i>	RLPNKYFEQDWNLTLYPIETYTESTADAKPYERTFKGAKTSSFPHLFKQYANKNYSMMASM	276
	: * * : * : * : * : * : * : *	
<i>ZphoA</i>	. . VTRLMQQDEAPVMP SVCADHASALKIGNNRIIGLAPASRKTGDFKTFRVT.....	281
<i>SphoV</i>	PQGSFTLEFAKAAI PAEKLQGTGNTDFLAVSLSSDYVGHQFGPNSIELEDTYLRDKD	336
	*: : * : * * * * * * * * * * * : * : * : *	
<i>ZphoA</i>	PDYDRTTTIDIAIGLIDELKLGHNAPDILTVLSLSDAVGHAYGTEGAEMCSQMAGLDDN	341
<i>SphoV</i>	LEDFFNYLDKTIKGNLYLLFLTADHGATHVPGFLRNKMPGGRLLLVQTDLDSLIFNEFK	396
	* * * * * * * * * * * : * : * : *	
<i>ZphoA</i>	IARIIAALDSN. GVPY. VLVLTADHGGQDVPE.. RAKLRGVETAQRVDPALSPDQLS. LK	396
<i>SphoV</i>	VRCNFTIINNQVIF.....DTDAIKEAKADYAKIKQSTIDYLVKQDGVLNAVDI	445
	* * * : : : * * : * * : *	
<i>ZphoA</i>	LAERFQLSHNQPLFFANEPQGDWYINRNLFQTKAQLIQAAKSELSNHPQVAAVFTASEL	456
<i>SphoV</i>	KNMGAVTIPQEI...KNKIINGYNARRSGDVIILDAGWYPTLTP...GTGHAAWNPDY	498
	* * : : * * * : * * * * * * * : *	
<i>ZphoA</i>	THIPHPTRSPELWNLAEAEASFDPRLSGDLIVLLKPRVTPIAKPVSYVATHGSAWD. YD	515
<i>SphoV</i>	SHIPALFMGWGVKPGKTNKEYYMSDIAPTVSALLHIQQPSGSIGKVITDL.....LK	550
	: * * * * * * * * * * * : * * * * *	
<i>ZphoA</i>	RRVPIIFYTTPHAGFEQPMFVETVDIMPSLAALLQIPLRKGEVDGRCLDLDPTTEATTCPVK	576
(b)		
S-ORF3	MDKIEIYQPKHKIRFVTVAALFDGHDATINIMRRILQSSGAIEVIHLGH	48
	• • : * * * * * * * * * * * * * * *	
M55343	MSSDAMSSDNRHEPAATLHTPAHPVRIVTAASLFDGHDAAINIMRRLQAQGAIEVIHLGH	60
S-ORF3	NRSVEEVVNCAIQEDVQGIAMTSYQGGHIEYFKYMYDLLQERGANHIKIFGGGGGVILPN	108
	: * * * * * * * * * * * * * * * : * * * * *	
M55343	DRSVEEVVTAIQAQGIALLSSYQGGHVEYFSYLIERLRAGAGNVRVYGGGGGVILPA	120
S-ORF3	EIKELESYGIAEILFADDGRRIGTQGMINDMLQKNRFCIEKQFDLEKPLNRETTEDRKR	168
	* * * : * * * : * * * * * : * * : *	
M55343	EIDLLHARGVARI FSPQDGQRLGLAKMINMIEACDVLTEGPKLDAVAAGEHGALARA	180
S-ORF3	NLFGRNAEAFEDSLATLRKKQPTNPPVLGITGTGGAGKSSLVDELVRRFLIEVDNKSIA	228
M55343	ITVLE	185
S-ORF3	IISVDPSKRKTGGALLGDRI RMNAINNPRVYMRSLATRQANLALS KCKTRK	279

Fig. 3. Alignment of predicted amino acid sequences of PhoV and ORF3 from *Synechococcus* PCC 7942 (S-PhoV and S-ORF3) and analogous proteins from *Zymomonas mobilis* (Z-PhoA) and *Frankia* sp. (F-ORF). The amino acid sequences are aligned for maximum matching and identical amino acid residues are marked by asterisks. Conservative substitutions are marked by colons. The putative phosphorylation site in S-PhoV and Z-PhoA is boxed. (a) Alignment of S-PhoV from *Synechococcus* PCC 7942 (this paper; database accession number Z48801) and Z-PhoA from *Z. mobilis* strain CP4 (database accession number L36230; P. F. Gomez & L. O. Ingram, unpublished 1994). (b) Alignment of S-ORF3 from *Synechococcus* PCC 7942 (this paper; database accession number Z48801) and F-ORF from *Frankia* sp. (database accession number M55343; Normand et al., 1992).

Analysis of the deduced amino acid sequence of the *Synechococcus* PCC 7942 *phoV* gene product using the PC/GENE programs revealed the presence of a prokaryotic leader peptide involved in secretion. Two potential cleavage sites were identified, either between positions 18 and 19 or between positions 20 and 21 (see Fig. 2). These predictions are in good agreement with the localization of the *phoV* gene product in the periplasmic space of *E. coli* strains carrying plasmid pKW1 (see below). In addition, a putative phosphorylation site that is conserved between PhoV of *Synechococcus* PCC 7942 and PhoA of *Z. mobilis* was identified in the central part of the APase (marked in Fig. 3). However, an involvement of this site in regulation of APase activity remains speculative.

The third open reading frame (ORF3; Figs 1 and 2) was separated by 204 bp from the *phoV* gene. ORF3 was incomplete in the subclones of pKW1 (Fig. 2), since the coding region exceeded the sequenced region of pKW13 at its 3' end. A high degree of identity (57%) was found for the N-terminal part of the deduced ORF3 gene product and a gene product from *Frankia* sp. (database accession number M55343; Normand *et al.*, 1992). The corresponding gene from *Frankia* sp. is associated with rRNA genes, but the exact function of the gene product is not known.

Mutational analysis of the *Synechococcus* PCC 7942 *phoV* gene

To analyse the function of the *phoV* gene product in *Synechococcus* PCC 7942 we attempted to construct a null mutant. For this purpose a 1.8 kb *Pst*I fragment carrying a chloramphenicol resistance gene (Cm^r) was isolated from hybrid plasmid pRB5 (Bockholt *et al.*, 1991) and ligated into the single *Pst*I site within the *phoV* coding region. The Cm^r cassette was inserted in both orientations into plasmid pKW14; the resulting hybrid plasmids pKW14-A and pKW14-B are shown in Fig. 1. In contrast to plasmid pKW14, hybrid plasmids pKW14-A and pKW14-B no longer expressed APase activity in *E. coli* JM103.

The Cm^r cassette had previously been demonstrated to be useful in the construction of a *Synechococcus* PCC 7942 *psbO* null mutant (Bockholt *et al.*, 1991). Therefore, plasmid pRB5 carrying the *psbO::[Cm^r]* mutant gene was used as a control during the attempts to construct a *phoV* null mutant. Transformation of *Synechococcus* PCC 7942 wild-type with pRB5 yielded about 52 Cm^r mutants per μg plasmid DNA. Since *Synechococcus* PCC 7942 has a very effective recombination system, more than 80% of the Cm^r mutants had lost the vector-encoded ampicillin resistance due to double recombination events (Bockholt *et al.*, 1991). However, transformation of *Synechococcus* PCC 7942 with either pKW14-A or pKW14-B did not lead to Cm^r mutants. Even merodiploid mutants carrying pKW14-A or pKW14-B integrated into the chromosome due to single recombination events were not viable. The most likely explanation for this result is that the 3.5 kb *Hind*III-*Nsi*I fragment from pKW14 belongs to one transcriptional unit and one gene of this putative operon

(either *phoV* or ORF3) is essential in *Synechococcus* PCC 7942. A single crossover would have regenerated a wild-type *phoV* copy and a truncated ORF3 plus the mutated *phoV* gene and an eventually untranscribed wild-type ORF3 which would indicate that ORF3 is required. However, this assumption does not explain why we did not find Cm^r Ap^r transformants which were not completely segregated and retained at least one wild-type copy (of the approximately 10 copies) of the genome to maintain viability. Since the *phoA* gene of *Z. mobilis* (ZM1) also could not be inactivated (Michel *et al.*, 1992), it seems likely that both *Synechococcus* PCC 7942 PhoV and *Z. mobilis* ZM1 PhoA might be essential for viability. However, from the information available in the literature, it is not clear whether the APase from *Z. mobilis* CP4 (database accession number L36230; P. F. Gomez & L. O. Ingram, unpublished, 1994), which shows 34% identity to the *Synechococcus* PCC 7942 PhoV, is identical with the APase isolated and characterized from *Z. mobilis* ZM1 (Michel & Baratti, 1989; Michel *et al.*, 1992; Baoudene-Assali *et al.*, 1993).

Expression of the *Synechococcus* PCC 7942 PhoV in *E. coli* CC118(pKW1)

For partial purification and characterization of the *Synechococcus* PCC 7942 PhoV, the APase-free *E. coli* strain CC118 (Manoil & Beckwith, 1985) was transformed with pKW1. In extracts of the transformed cells the detectable phosphatase activity corresponded to $1.7 \mu\text{mol}$ 4-NP formed $(\text{mg protein})^{-1} \text{min}^{-1}$ (as compared to zero activity when *E. coli* CC118 was transformed with pUC19). When the *E. coli* cells were transferred to phosphate-free medium, no further increase in phosphatase activity was obtained during a growth period of 24 h. This result implies that in *E. coli* CC118(pKW1) the expression of the *Synechococcus* PCC 7942 PhoV was not phosphate-regulated. For comparison, the expression of the phosphate-regulated *E. coli* PhoA in wild-type cells (*E. coli* JM103) was examined. Within 24 h under our incubation conditions in phosphate-free medium, the activity of the *E. coli* PhoA increased approximately 100-fold [from 0.02 to $2.50 \mu\text{mol}$ 4-NP formed $(\text{mg protein})^{-1} \text{min}^{-1}$]. It should be pointed out that the activity of the *Synechococcus* PCC 7942 PhoV in *E. coli* CC118(pKW1) was almost as high as the activity of the *E. coli* PhoA in *E. coli* JM103 under phosphate deficiency: 1.7 vs $2.5 \mu\text{mol}$ 4-NP formed $(\text{mg protein})^{-1} \text{min}^{-1}$, respectively.

Localization of the *Synechococcus* PCC 7942 PhoV in *E. coli* CC118(pKW1)

After applying the osmotic shock procedure commonly used to disrupt the outer cell membrane of *E. coli* (Neu & Heppel, 1965), APase activity was found in the spheroplast fraction. No, or only minor, activity was detectable in the soluble periplasmic protein fraction. Since 4-NPP cannot penetrate the cytoplasmic membrane of spheroplasts, it can be concluded that the recombinant *Synechococcus* PCC 7942 PhoV was extracellular and membrane-bound. It was most likely associated with the

Table 1. Partial purification of the *Synechococcus* PCC 7942 PhoV from *E. coli* CC118(pKW1)

Details of the purification procedure are given in Methods.

Purification step	Total protein (mg)	Phosphatase activity*	
		Total (units)	Specific activity [units (mg protein) ⁻¹]
French press extract:			
Total	4808	8525	1.8
Pellet	2640	6339	2.4
Supernatant	2117	2175	1.0
Extraction of pellet with buffer B†:			
APase activity in supernatant	432	2010	4.7
Dialysis of extract against buffer A†:			
APase activity in pellet	340	1822	5.4
Re-extraction of washed pellet with buffer B†:			
APase activity in supernatant	112	1049	9.4
Ammonium sulphate fractionation (50–95 %)	34	758	22.2

*1 unit of enzyme corresponds to 1 μ mol 4-NP formed min⁻¹ (at 37 °C).

† Buffer A: 10 mM Tris/HCl, pH 7.6, containing 1 mM MgCl₂ and 1 mM ZnCl₂. Buffer B: 1 M Tris/HCl, pH 7.6, containing 10 mM MgCl₂, 10 mM ZnCl₂ and 2% Triton X-100.

periplasmic side of the inner membrane in *E. coli* CC118(pKW1) (results not shown). A periplasmic location of the PhoV is in agreement with the presence of a prokaryotic leader peptide involved in secretion (Fig. 2).

Extraction and partial purification of the *Synechococcus* PCC 7942 PhoV from *E. coli* CC118(pKW1)

The membrane-associated PhoV enzyme was solubilized from *E. coli* CC118(pKW1) with Triton X-100 in concentrated Tris buffer (1 M Tris/HCl, pH 7.6, containing 10 mM MgCl₂, 10 mM ZnCl₂ and 2% Triton X-100). For optimal extraction a high Tris concentration, in addition to the presence of Triton X-100, was necessary. We were not successful in isolating PhoV in a homogeneous form. However, a partial purification was achieved by a differential extraction procedure as described in Methods. The results of such a partial purification are given in Table 1 and show that an approximately 10-fold increase in specific activity was obtained. This partially purified PhoV fraction contained rather low quantities of proteins in the 40–100 kDa region except the PhoV (61 kDa), but was greatly enriched in low molecular mass proteins of 10–30 kDa (not shown). The fact that the PhoV was recovered as a floating layer after ammonium sulphate fractionation implies that it may be a lipoprotein. Column chromatography for further purification was unsuccessful because of aggregation, low solubility and inactivation problems when the Tris concentration was reduced to allow chromatography on ion-exchange columns. Chromatography on hydrophobic column materials was also unsuccessful. A successful purification will most likely require empirical testing of several different ionic

detergents to find one which will keep the PhoV in solution in the absence of high Tris concentrations; such an approach was recently successful in purifying the PhoA from *Z. mobilis* (Baoudene-Assali *et al.*, 1993).

Preliminary characterization of the *Synechococcus* PCC 7942 PhoV

A preliminary enzymic characterization was performed with the enriched PhoV fraction. APase activity on 4-NPP was observed between pH 6 and pH 11, with optimal activity in the broad pH range of 7–10. These results are in agreement with the pH range reported for other APases in bacteria. APase activity increased with incubation temperature between 15 and 35 °C and decreased upon further heating; no activity was observed at 65 °C. Preincubation of the PhoV for 10 min at 50 or 60 °C caused 50% or 100% inactivation, respectively. Renaturation of the heat-inactivated PhoV was not possible. Thus, in contrast to several of the well-characterized APases which have been shown to be fairly heat resistant (Von Tigerstrom & Stelmashuk, 1986; Garen & Levinthal, 1960), PhoV is fairly heat-labile. Heat-labile APases have seldom been described in the literature. The APase of Antarctic bacteria was inactivated by incubation for 10 min at 55 °C (Kobori *et al.*, 1984). The PhoA of *Z. mobilis* ZM1 remained stable for 1 h at 60 °C, but further increase in temperature caused inactivation (Michel & Baratti, 1989).

Optimal activity of the *Synechococcus* PCC 7942 PhoV was dependent on the presence of Triton X-100 in the reaction mixture. Triton X-100 at a concentration of 0.05% increased the activity about fivefold relative to that in its

Table 2. Substrate activity

APase activity on various phosphomonoesters was determined as described in Methods. The specific activity obtained with 4-NPP was set as 100%.

Phosphomonoester	Relative specific activity (%)	K_m (mM)
4-NPP	100	0.1
ATP	41	1.0
ADP	33	0.5
AMP	29	1.5
D-Glucose 6-phosphate	48	1.9
Fructose 1,6-phosphate	30	1.4
Fructose 6-phosphate	19	2.3
Fructose 1-phosphate	62	3.8
3-Phosphoglycerate	50	1.9
3-Phosphocreatin	136	2.4

absence. Maximum activity was obtained with Triton X-100 in a concentration range of 0.01–2%. The enzyme was even active in Triton X-100 concentrations up to 80%, indicating a particular resistance to Triton X-100.

PhoV has a broad substrate specificity (Table 2) and thus can be classified as a nonspecific phosphomonoesterase. The lowest K_m values were obtained for ADP, ATP and 4-NPP, while the highest relative specific activities were observed with phosphocreatine and 4-NPP. As for other APases, phosphate was a competitive inhibitor of the reaction, with a K_i value of 0.57 mM. The enzyme was sensitive to chelators, such as EDTA (50% inhibition at 4 mM EDTA) or citrate (50% inhibition at 48 mM sodium citrate), a common feature of most APases (McComb *et al.*, 1979).

After dialysing the PhoV preparation against EDTA, $ZnCl_2$ addition was required to obtain maximal activity, implying that Zn^{2+} was partially lost by the EDTA treatment (Fig. 4). $ZnCl_2$ increased PhoV activity up to a concentration of about 1 mM. Further increase in $ZnCl_2$ concentration caused a drastic reduction of activity, an observation made for several phosphatases (Cathala & Brunel, 1975). At suboptimal Zn^{2+} concentrations $MgCl_2$ was stimulatory, but in contrast to $ZnCl_2$, $MgCl_2$ concentrations up to 0.2 M were not inhibitory. $MgCl_2$ caused no further increase in activity when the $ZnCl_2$ concentration was optimal (1 mM $ZnCl_2$). Other divalent cations, such as Ca^{2+} or Co^{2+} , had essentially no effect on the PhoV activity. Mn^{2+} was strongly inhibitory, giving 50% and 100% inhibition at 0.1 and 1 mM $MnCl_2$, respectively. Thus, like several other APases, PhoV requires Zn^{2+} for activity, and Mg^{2+} is stimulatory. Inhibition by high Zn^{2+} concentrations might be caused by Zn^{2+} binding to the Mg^{2+} site (see, for example, Cathala & Brunel, 1975). For several APases, such as bovine kidney APase (Cathala & Brunel, 1975), Mn^{2+} was shown to be stimulatory, replacing Mg^{2+} . However, Mn^{2+} was strongly inhibitory

Fig. 4. Influence of various cations on the *Synechococcus* PCC 7942 PhoV activity. The partially purified PhoV preparation was dialysed against 10 mM Tris/HCl, pH 7.6, containing 10 mM EDTA. APase activity was determined as described in Methods with the modification that cations (added as $ZnCl_2$, $MgCl_2$ or $MnCl_2$) were present in the reaction mixture as indicated in the figure.

to the *Synechococcus* PCC 7942 PhoV, most likely by replacing the essential Zn^{2+} from its specific binding site.

Concluding remarks

Based on the results here presented it can be concluded that *Synechococcus* PCC 7942 has at least two APases. One APase is phosphate-repressible (PhoA, 145 kDa) (Block & Grossman, 1988; Ray *et al.*, 1991), while the one here described is most likely not repressed by phosphate (PhoV, 61.3 kDa). The presence of a second APase in *Synechococcus* PCC 7942 could be important in providing the cells with sufficient phosphate under stress conditions, since Ray *et al.* (1991) speculated that the atypical 145 kDa PhoA might have functions in addition to hydrolysing phosphomonoesters. Similar results with respect to the presence of two APases have been obtained with *Bacillus subtilis*, which contains a phosphate-repressible and a phosphate-nonrepressible APase (Hulett *et al.*, 1990). Elucidation of the exact function of the PhoV in *Synechococcus* PCC 7942, and of the regulation of its expression and activity (especially since a regulation by phosphorylation/dephosphorylation seems possible) requires further work.

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