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Isolation, Transcription, and Inactivation of the Gene for an Atypical Alkaline Phosphatase of *Synechococcus* sp. Strain PCC 7942†

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The alkaline phosphatase of *Synechococcus* sp. strain PCC 7942 is 145 kDa, which is larger than any alkaline phosphatase previously characterized and approximately three times the size of the analogous enzyme in *Escherichia coli*. The gene for the alkaline phosphatase, *phoA*, was cloned and sequenced, and the protein that it encodes was found to have little similarity to other phosphatases. Some sequence similarities were observed between the *Synechococcus* sp. strain PCC 7942 alkaline phosphatase, the α subunit of the ATPase from bacteria and chloroplasts, and the UshA sugar hydrolase of *E. coli*. Also, limited sequence similarity was observed between a region of the phosphatase and a motif implicated in nucleotide binding. Interestingly, although the alkaline phosphatase is transported across the inner cytoplasmic membrane and into the periplasmic space, it does not appear to have a cleavable signal sequence at its amino terminus. The half-life of the mRNA encoding the alkaline phosphatase, measured after inhibition of RNA synthesis, is approximately 5 min. Similar kinetics for the loss of alkaline phosphatase mRNA occur upon the addition of phosphate to phosphate-depleted cultures, suggesting that high levels of this nutrient inhibit transcription from *phoA* almost immediately. The *phoA* gene also appears to be the first gene of an operon; the largest detectable transcript that hybridizes to a *phoA* gene-specific probe is 11 kb, over twice the size needed to encode the mature protein. Other phosphate-regulated mRNAs are also transcribed upstream of the *phoA* gene. Insertional inactivation of *phoA* results in the loss of extracellular, phosphate-regulated phosphatase activity but does not alter the capacity of the cell for phosphate uptake.

Phosphate is one of the nutrients required at high levels for cell growth. It can be limiting in both freshwater and terrestrial environments (26), since it often exists in forms not readily accessible to most organisms. When the unicellular cyanobacterium *Synechococcus* sp. strain PCC 7942 is deprived of phosphate, it exhibits a variety of responses. Growth ceases after four to five divisions, the cells exhibit decreased levels of chlorophyll and light harvesting phycobilisomes (7), and the rate at which phosphate is transported into the cell is elevated between four- and fivefold (13). Several proteins accumulate during growth on medium depleted of phosphate, although the roles of most of these proteins in the acclimation process have not been established. One of the prominent proteins that accumulates during phosphate-limited growth is a high-molecular-weight alkaline phosphatase that has been isolated and localized to the periplasmic space (2, 16).

The production of an alkaline phosphatase in response to phosphate deprivation has been extensively studied in *Escherichia coli* and other prokaryotic and eukaryotic organisms (14, 34, 38, 41). Generally, the enzyme is a nonspecific phosphomonoesterase associated with zinc and in prokaryotes is located in the periplasmic space. Subunit molecular masses of most alkaline phosphatases range from 47 to 87 kDa (3, 5). The *E. coli* enzyme is a dimer with two active sites containing metal ion triplets. There is 30% homology between the sequences of the *E. coli* alkaline phosphatase and that from human placenta, and the two enzymes share many features of their three-dimensional structures (40).

In *E. coli* the alkaline phosphatase gene, *phoA*, is part of a phosphate-regulated system (*pho* regulon) that comprises over 20 genes encoding both structural elements required for elevated phosphate acquisition and regulatory elements involved in transcriptional control during the stress response (35). Some of the genes that compose the *pho* regulon encode components of a phosphate transport system (*pst* genes), components of a sugar phosphate transport system (*ugp* genes), and a protein responsible for creating a pore in the outer membrane that allows selective entry of phosphate into the periplasmic space (*phoE* gene). The regulatory components of the system are encoded by *phoB* and *phoR*, which are members of a two-component regulatory system involved in sensing the phosphate levels in the environment and initiating changes in gene regulation that aid in the acclimation process.

There are some conspicuous differences in features of the systems involved in the acclimation of *E. coli* and cyanobacteria to phosphate-limited growth. These differences are especially apparent in comparisons of the alkaline phosphatases. The *Synechococcus* sp. strain PCC 7942 alkaline phosphatase is located in the periplasmic space, but, unlike the *E. coli* enzyme, it appears to be associated with the cell wall or cytoplasmic membranes (2). In contrast to other alkaline phosphatases that have been characterized, it is irreversibly inhibited by zinc (2, 16) and has a subunit

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molecular mass of 145 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (2).

The regulation of phosphate transport in *Synechococcus* sp. strain PCC 7942 may also differ from that of the enteric bacteria. *E. coli* utilizes a low-affinity phosphate transport system when grown in phosphate-rich medium. A second high-affinity transport complex accumulates when growth becomes phosphate limited (35). The strains of *Synechococcus* that have been tested exhibit an elevated V_{max} for phosphate transport when grown in phosphate-limited medium; however, the $K_{1/2}$ remains the same (13, 24a), suggesting the presence of a single phosphate transport system in these cyanobacteria that accumulates to higher levels in cells limited for the nutrient. The $K_{1/2}$ of the cyanobacterial system is similar to that of the high-affinity *pst* transport system of *E. coli*.

Studies aimed at elucidating differences between the responses of *E. coli* and those of cyanobacteria to phosphate-limited growth were initiated with the characterization of the alkaline phosphatase of *Synechococcus* sp. strain PCC 7942 (2). Here, we describe the isolation and characterization of the gene encoding the periplasmic alkaline phosphatase of this organism and the modulation of *phoA* transcript levels during the acclimation of cells to changes in the phosphate status of the medium. We also discuss the consequences of in vivo inactivation of the *phoA* gene.

MATERIALS AND METHODS

Culture conditions. *Synechococcus* sp. strain PCC 7942 was obtained from the laboratory of Louis Sherman. Cells were grown at 30°C in liquid or on solid (1.5% agar) BG-11 medium (30) containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 8.0). Cultures were bubbled with air enriched to 3% CO_2 and illuminated with incandescent bulbs at a fluence of 50 μmol m^{-2} s^{-1}. Where appropriate, the growth medium was supplemented with spectinomycin (25 μg/ml) and/or ampicillin (1 μg/ml). For phosphate deprivation experiments, exponentially growing cells were harvested at 3,500 × *g* for 5 min at room temperature, washed in phosphate-free BG-11 (KH_2PO_4 was replaced by KCl), and resuspended in the same medium to a final concentration of 10^7 cells per ml (10-fold-lower cell density).

E. coli DH5α (Bethesda Research Laboratories), JM101, and NM539 were grown on LB, 2× TY, or NZC (19) medium supplemented with ampicillin (50 μg/ml) or spectinomycin (25 μg/ml) when appropriate.

Measurement of phosphatase activity. Cells (10^8 per ml) were harvested and resuspended in 0.2 M Tris-HCl (pH 8.5)–2 mM $MgCl_2$–3.6 mM *p*-nitrophenyl phosphate (Sigma Chemicals) in 1.5 ml (final volume) and incubated at 37°C for 20 min. The reaction was stopped by the addition of 150 μl of 4 M NaOH, and then the samples were centrifuged for 4 min in an Eppendorf microfuge (7,000 × *g*). The A_{400} of the supernatant was measured and compared with a standard absorbance curve for *p*-nitrophenol.

Extraction of total cellular proteins. Exponentially growing cells from 100 ml of culture were harvested by centrifugation at 4,000 × *g* for 5 min at 4°C. The cell pellet was drained, frozen in liquid nitrogen, and (when necessary) stored at −70°C. The frozen pellet was resuspended in 3 ml of ice-cold 2 mM HEPES (pH 8.0)–100 mM NaCl–1 mM benzamidine-HCl–1 mM *E*-aminocaproic acid–1 mM phenylmethylsulfonyl fluoride. The suspension was passed through a chilled French pressure cell at 100 mPa into a tube containing 20 μl of 0.1% DNase in 10 mM sodium acetate (pH 5.6)–1 mM $MgCl_2$ and incubated on ice for 15 min. Unbroken cells and cell debris were removed by centrifugation for 15 min at 7,500 × *g* at 4°C, and the supernatant was centrifuged for 1 h at 100,000 × *g* and 4°C in a 50 Ti fixed-angle rotor. Soluble proteins in the supernatant were precipitated by adding trichloroacetic acid to a final concentration of 10% and incubating the solution on ice for 30 min. The precipitated protein was pelleted by centrifugation at 12,000 × *g* for 15 min at 4°C and rinsed with 90% ice-cold acetone.

Electrophoresis of proteins. Both soluble protein and membrane pellets were resuspended by sonication in 0.1 M Na_2CO_3–0.1 M dithiothreitol to a concentration of between 1 and 5 mg of protein per ml. The proteins were solubilized by boiling in 0.5 volume of 5% SDS–30% sucrose–0.1% bromophenol blue. The polypeptides were resolved by electrophoresis on denaturing 7.5 to 15% polyacrylamide gradient gels with the Laemmli buffer system (18) and visualized by staining with Coomassie brilliant blue R-250.

Western blot analysis. Polypeptides separated by SDS-PAGE were electrophoretically transferred to nitrocellulose paper (36). Western immunoblot analysis was carried out as previously described (2).

Protein sequence analysis. The purified alkaline phosphatase (2) was resolved by preparative SDS-PAGE in Laemmli buffer and electroeluted from gel slices in a bicarbonate buffer system (15). The protein was subjected to automated Edman degradation on an Applied Biosystems model 470A gas-phase protein microsequencer.

DNA analysis. Genomic DNA was prepared from *Synechococcus* sp. strain PCC 7942 as described by Tandeau de Marsac et al. (32). DNA was digested with various restriction enzymes and blotted onto nitrocellulose paper (Schleicher and Schuell BA-85) as described by Maniatis et al. (21). The immobilized DNA was hybridized to a mixture of end-labeled oligonucleotides chemically synthesized on a Biosearch model 8600 oligonucleotide synthesizer. A mixed 17-mer, 5′ A(AG)(CT) TG(ACGT) AG(ACGT) GT(AG) AA(CT) TG 3′, was designed to hybridize to both the DNA and RNA encoding the amino acid sequence Gln-Phe-Thr-Leu-Gln-Leu (residues 2 through 7 of the amino terminus of the phosphatase, as determined by microsequencing of the protein). A *Pst*I fragment of approximately 4.2 kbp hybridized to this degenerate oligonucleotide. To isolate this specific DNA segment, fragments of this size were electroeluted from a 0.7% agarose gel and ligated into pUC8. Plasmid DNA containing inserts were isolated, digested with *Pst*I, resolved on a 0.8% agarose gel, transferred to nitrocellulose paper, and hybridized to the mixed oligonucleotide that was labeled at the 5′ end with T4 polynucleotide kinase (21). A clone containing the 4.2-kbp *Pst*I fragment that hybridized strongly to the oligonucleotide was used to screen a λEMBL3 library of *Synechococcus* sp. strain PCC 7942 DNA generated from a partial *Sau*3A digest of genomic DNA (21). Southern hybridizations were performed as described by Conley et al. (8), and the DNA fragment was labeled by using the random primer extension method of Feinberg and Vogelstein (9).

DNA sequence determination. DNA sequence analysis in M13mp18, M13mp19, and Bluescript (Stratagene) was by the dideoxy-chain termination method (28). The reactions were primed with either M13 universal or reverse primers or synthetic deoxyoligonucleotides, and extension was initiated with Sequenase II polymerase (U.S. Biochemical Corp.) (31). Subclones for sequencing were constructed by ligation

of specific restriction fragments into the appropriate sequencing vectors.

RNA isolation and Northern RNA hybridization. Total RNA from *Synechococcus* sp. strain PCC 7942 was isolated as described by Laudenbach et al. (19). RNA samples were resolved by electrophoresis in 1.5% agarose gels under denaturing conditions (8). The RNA was transferred to nitrocellulose without further treatment, and hybridizations were performed by the method of Thomas (33), except that the temperature during the hybridization was maintained at 65°C and washes were at room temperature. Commercially prepared RNA size markers (Bethesda Research Laboratories) were used to estimate sizes of the hybridization signals. Slot blot hybridizations (17) were used to quantitate RNA levels. The level of alkaline phosphatase mRNA was determined from a series of twofold dilutions of RNA, ensuring that measurements were within the linear response range of the film. Autoradiographs were scanned on a Hoefer gel scanning apparatus (GS 300).

Primer extension and RNase mapping. Total RNA from cells starved for phosphate for 24 h was coprecipitated with an end-labeled (27) oligonucleotide primer (GCAGAAGCTG AAGGGTAAATTGAGCCATCG), complementary to bases 170 through 199 of the coding region of the alkaline phosphatase, and resuspended in 30 μl of a solution containing 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.4), 1 mM EDTA, 0.4 M NaCl, and 80% formamide. The mixture was heated at 85°C for 10 min and then transferred to a 30°C water bath for 12 h to permit annealing of the labeled primer to the RNA. The RNA-primer hybrid was precipitated with ethanol, and the precipitate was dissolved in 20 μl of reverse transcriptase buffer containing 50 mM Tris-HCl (pH 7.6), 50 mM KCl, 10 mM $MgCl_2$, 1 mM each dATP, dCTP, dGTP, and dTTP, 1 mM dithiothreitol, 1 U of placental RNase inhibitor (RNAguard; Pharmacia) per μl, and 50 U of avian myeloblastosis virus reverse transcriptase. Extension of the primer toward the 5′ end of the RNA was allowed to proceed for 2 h at 37°C. The RNA-DNA complex was treated with 1 μl of 5-μg/ml of DNase-free pancreatic RNase for 30 min at 37°C, extracted with phenol-chloroform, and precipitated with ethanol. The pellet was resuspended in 6 μl of formamide loading dye and electrophoresed in a 6% polyacrylamide sequencing gel (27). To size the extension product, a sequencing ladder was generated by using the 30-bp oligonucleotide as a primer and a 0.6-kbp *Eco*RI-*Eco*RV fragment (leftmost fragment in Fig. 2) in Bluescript as a template.

Insertional inactivation with a drug resistance cassette. The plasmid pHP45Ω (24) was digested with *Sma*I to release a 2-kbp DNA fragment containing the spectinomycin resistance gene (*aadA*). This fragment was resolved on a 0.8% low-melting-point agarose gel and recovered from the gel by melting the agarose at 65°C, extracting the DNA with phenol, and precipitating it with ethanol. A 1.8-kbp *Eco*RI-*Sal*I fragment encoding the first half of the *phoA* gene was cloned into the *Eco*RI-*Sal*I site of pDPL13 (10); the new plasmid was designated pNPR16. To disrupt the *phoA* gene, a 1.4-kbp *Eco*RV fragment was excised from pNPR16 and replaced, by ligation, with the 2-kbp *aadA* gene, which confers spectinomycin resistance (Sp^r) to bacterial cells. Plasmids from colonies containing the recombinant DNA were transformed into DH5α and selected for growth on spectinomycin and ampicillin. Plasmids isolated from transformants were analyzed for the presence and orientation of the insert. The resulting plasmid, pNPR18, contained the coding region of *phoA* partially replaced by the *aadA* gene.

Plasmid DNA from pNPR18 was used to transform *Synechococcus* sp. strain PCC 7942 to Sp^r by the method of Laudenbach et al. (19). Colonies were screened for ampicillin sensitivity (Ap^s), which would be the consequence of a double-crossover event in which the genomic copy of *phoA* was replaced by the deleted, *aadA*-containing sequence. The gene replacement was confirmed by Southern hybridizations.

Phosphate uptake assays. Phosphate uptake assays were performed by a modification of the methods of Green and Grossman (12) and Grillo and Gibson (13). Briefly, aliquots of cells were harvested and washed with phosphate-deficient BG-11 medium and resuspended in the same medium to a concentration of between 2.5×10^7 and 5×10^7 cells per ml. One milliliter of the suspension of cells was placed in a water-jacketed glass chamber maintained at 30°C and illuminated at an intensity of 150 μmol m^{-2} s^{-1} from a slide projector. The cells were preequilibrated in the chamber for 2 min before the assay was initiated by the addition of $NaPO_4$ (pH 8) to a final concentration of 0.025 to 10 mM and containing 10 μCi of carrier-free $^{32}P_i$ (New England Nuclear Corp.). Samples (100 μl) were withdrawn at timed intervals (20, 40, 60, 80, and 100 s), and the cells were collected on 0.45-mm-pore-size GA-6 cellulose acetate filters (Gelman Sciences, Inc.) that were prewet by boiling in wash solution (0.5 M LiCl, 1 mM PO_4 [pH 9]). The filters were then washed with 2 ml of wash solution, transferred to glass vials, and resuspended in 5 ml of Budget Solve Complete Counting Solution (Research Products International) for scintillation counting. Total phosphate uptake was calculated after determining, by counting the cells on a hemacytometer grid, the total number of cells in the reaction chamber and the specific activity of the $^{32}P_i$ stock solution. The initial rate of uptake was calculated from the slope of a regression line through the linear portion of the curve depicting total phosphate uptake as a function of time.

RESULTS

Cloning the alkaline phosphatase gene. A clone containing a 4.2-kbp *Pst*I fragment was isolated based on hybridization to the degenerate oligonucleotide encoding the amino terminus of the *Synechococcus* sp. strain PCC 7942 alkaline phosphatase. This *Pst*I fragment was used as a hybridization probe to isolate a λEMBL3 clone, λPA1, containing a 12-kbp insert of *Synechococcus* sp. strain PCC 7942 DNA. A map showing restriction fragments A through E, generated by cleavage of the 12-kbp cloned DNA with *Eco*RI and *Sal*I, is presented in Fig. 1 (the region of identity between the 4.2- and 12-kbp fragments is indicated by a dotted line). The degenerate oligonucleotide encoding the amino terminus of the alkaline phosphatase hybridized to fragment C. The precise region of homology between the oligonucleotide and fragment C, indicated by a dark bar above the restriction endonuclease map, was established after the DNA fragment was sequenced (see below).

Northern blot hybridization. Total RNA from phosphate-sufficient and phosphate-deprived cells was resolved on denaturing agarose gels and hybridized to specific fragments, A to E (Fig. 1). Fragments C and B hybridized to an identical set of transcripts of 11, 8, and 5 kb that accumulated in phosphate-depleted cells. A hybridization signal is also seen at approximately 2 kb, which is probably an artifact resulting from radioactive accumulation under the rRNA band. It is also possible that it is a degradation product of the other RNA species. Transcripts of 2.5 and 5 kb, elevated in cells grown in medium lacking phosphate, hybridized to restric-

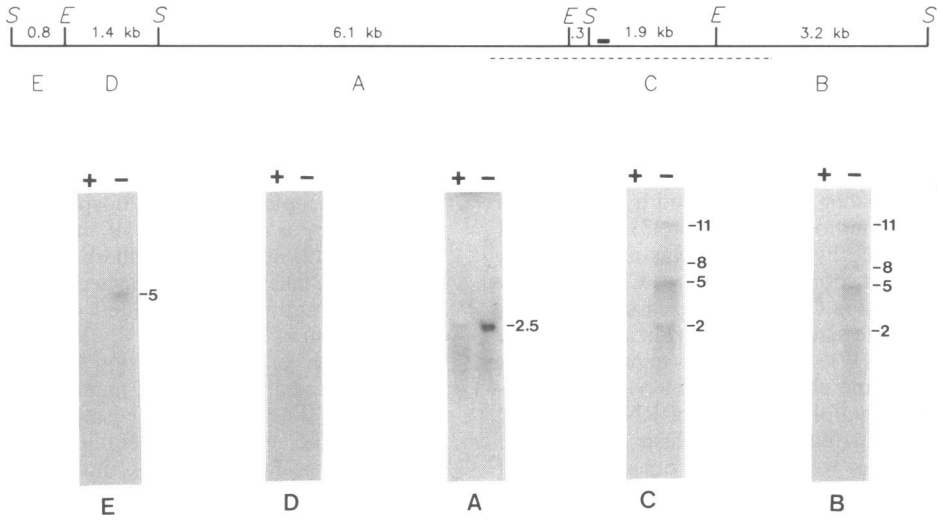

FIG. 1. Restriction endonuclease map and transcript analysis of λPA1. Homology to the 4.2-kbp *Pst*I clone isolated with the oligonucleotide probe is shown with a dotted line. The black bar overlying fragment C depicts where the gene-specific oligonucleotide, encoding the amino terminus of the alkaline phosphatase, hybridizes. The enzymes used to map this region were *Eco*RI (E) and *Sal*I (S). Fragments labeled A through E (below the map) were used to probe *Synechococcus* sp. strain PCC 7942 RNA (panels A through E). RNA was isolated from exponentially growing cultures 12 h after transfer of cells to phosphate-free (−) or phosphate-sufficient (+) medium.

tion fragments A and E, respectively. There was no detectable hybridization of fragment D to an mRNA species. Furthermore, we could not detect hybridization of the 0.3-kbp fragment, located between fragments A and C, to mRNA (data not shown), probably because this fragment only contains 50 bases of the *phoA* transcript.

DNA sequence analysis. Segments of a 5.1-kbp *Sal*I fragment, composed of fragments B and C from λPA1, were subcloned into M13mp18 and M13mp19 after digestion with various restriction enzymes. A restriction map of the 5.1-kbp *Sal*I fragment and the strategy for sequencing this fragment are presented in Fig. 2. The nucleotide sequence of the fragment and the amino acid sequence of an open reading frame encoded by the fragment are shown in Fig. 3. This open reading frame extends from the AUG to the TAG positioned under the restriction map in Fig. 2. The protein encoded by this open reading frame contains 1,344 amino acids and has a molecular mass of 145 kDa, which is in good agreement with the apparent molecular mass of the alkaline phosphatase protein as determined by SDS-PAGE (2). Furthermore, the amino termini deduced from the gene sequence and from microsequencing the isolated, periplasmic alkaline phosphatase were identical, establishing the identity of the gene as *phoA*.

The alkaline phosphatase contains no cysteines and only three histidines. Approximately 15% of the amino acids are Asp or Glu, which contributes to the protein's estimated pI of 3.85. The deduced amino acid sequence does not show

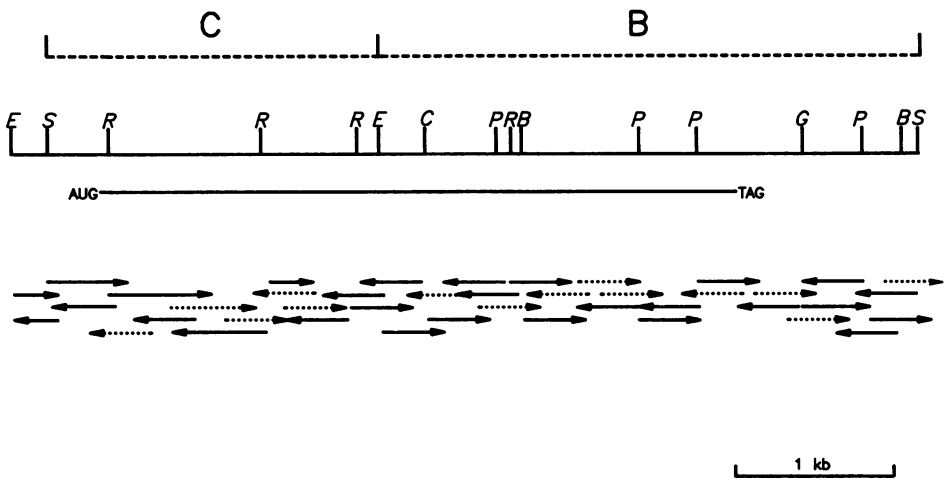

FIG. 2. Restriction endonuclease map of *phoA*. The restriction enzymes used were *Eco*RI (E), *Sal*I (S), *Eco*RV (R), *Cla*I (C), *Pst*I (P), *Bam*HI (B), and *Bgl*II (G). The locations of subclones C and B are indicated by the dashed line above the restriction endonuclease map. The length of the coding region of *phoA* is indicated by the solid line punctuated by the start (AUG) and stop (TAG) codons. The horizontal arrows show the subsets of sequences obtained to assemble the complete gene sequence. Sequencing was either from specific restriction sites (—►) or from internal sequences of the single-stranded M13 templates with synthetically synthesized oligonucleotides as primers (··►).

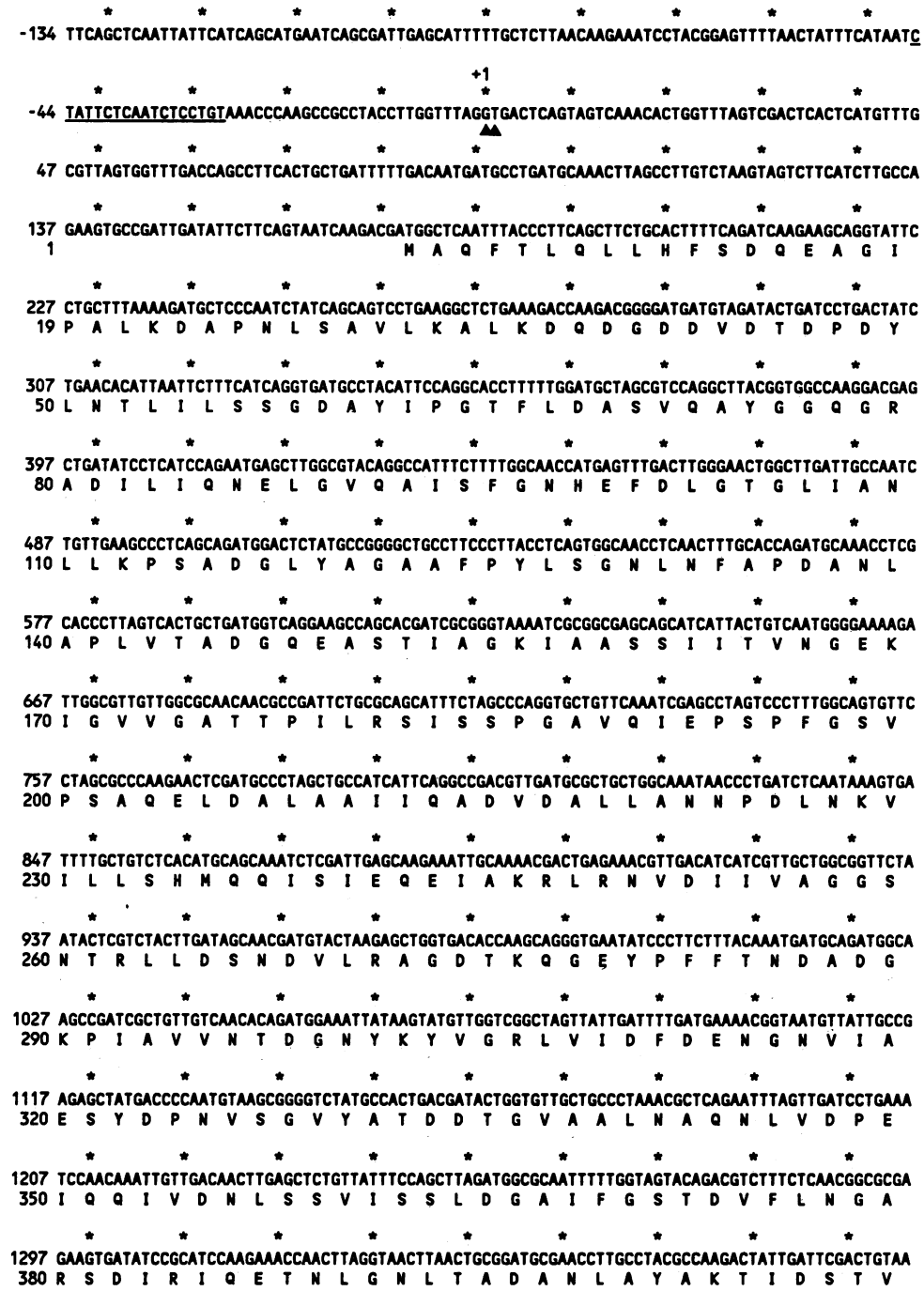

FIG. 3. Sequence of the *phoA* gene. The predicted amino acid sequence is shown below the nucleotide sequence. Arrowheads at nucleotides 1 and 2 indicate the position of transcription initiation. A possible *pho* box at positions −44 to −28 is delimited with solid line below the sequence. A sequence between nucleotides 4473 and 4509 that has the potential to form a hairpin loop in the RNA is indicated by a bold underline.

significant similarity with previously sequenced alkaline phosphatases. There is, however, weak identity between the first 500 amino acids of the cyanobacterial alkaline phosphatase and the α subunits of the F_1 and CF_1 ATPases of bacteria (22) and chloroplasts (25) (Fig. 4A). There is also a similarity of 37% conserved and 20% identical amino acids between residues 426 through 608 of the alkaline phosphatase and residues 369 through 560 of the *ushA* gene product (4), a UDP-sugar hydrolase present in enteric bacteria. The homology is most striking between residues 531 through 568 of the alkaline phosphatase and residues 492 through 527 of UshA. A similarity is also observed between a motif thought to be involved in nucleotide binding in the ComGI protein (1) and amino acids 759 through 771 of the

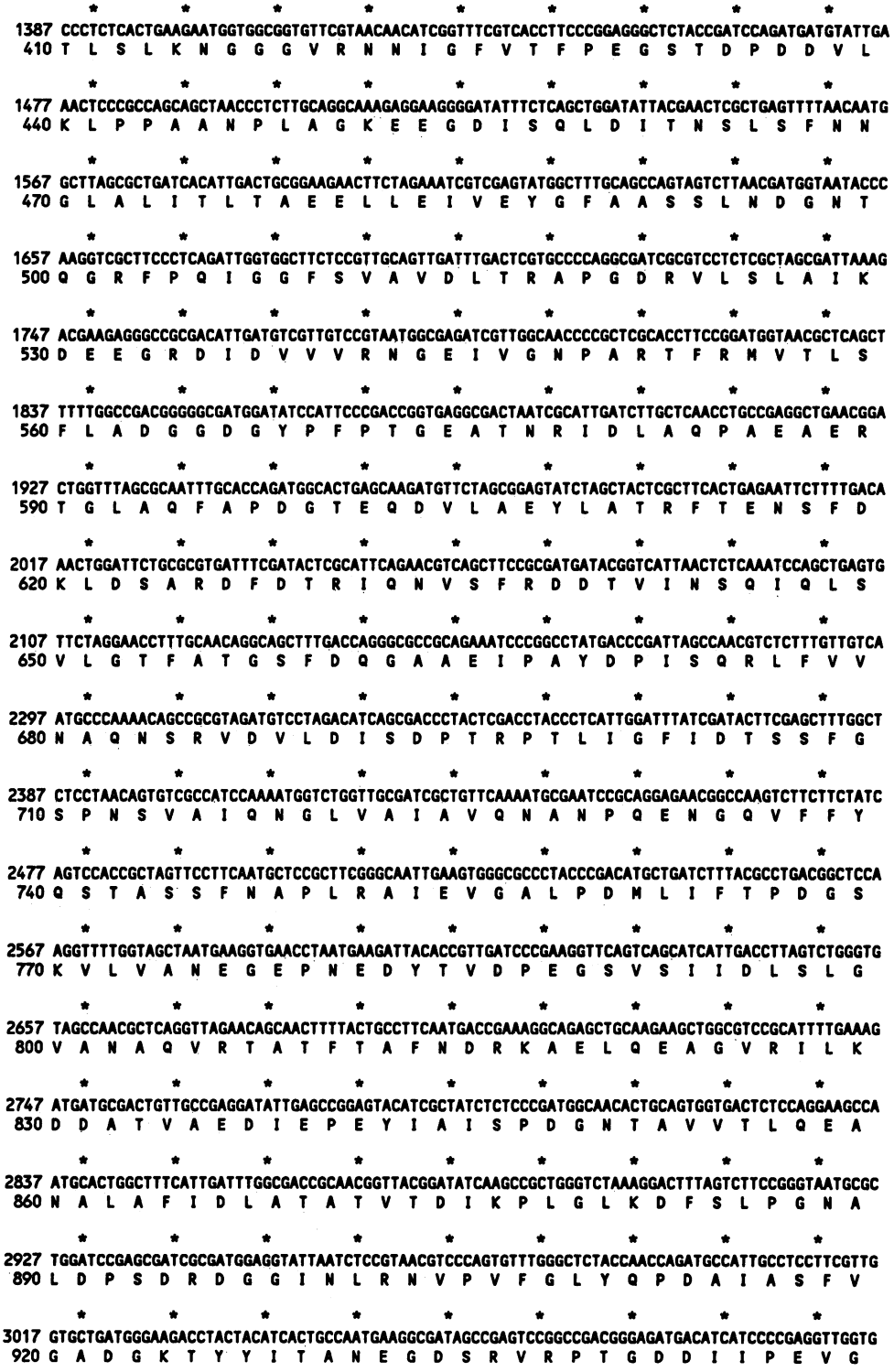

FIG. 3—*Continued.*

alkaline phosphatase (Fig. 4B). The sequence has a Ser Lys Val found in the P-loop region of some of the phosphoglycerate kinases (29). This is preceded by a hydrophobic region of five amino acids that is identical to the analogous sequence in the P loop of ComGI (1). Another possible P loop-like region is between amino acids 1311 through 1322, although this is less convincing.

Interestingly, the periplasmic alkaline phosphatase appears to have no cleavable signal sequence at the amino terminus. The methionine at the amino terminus of the

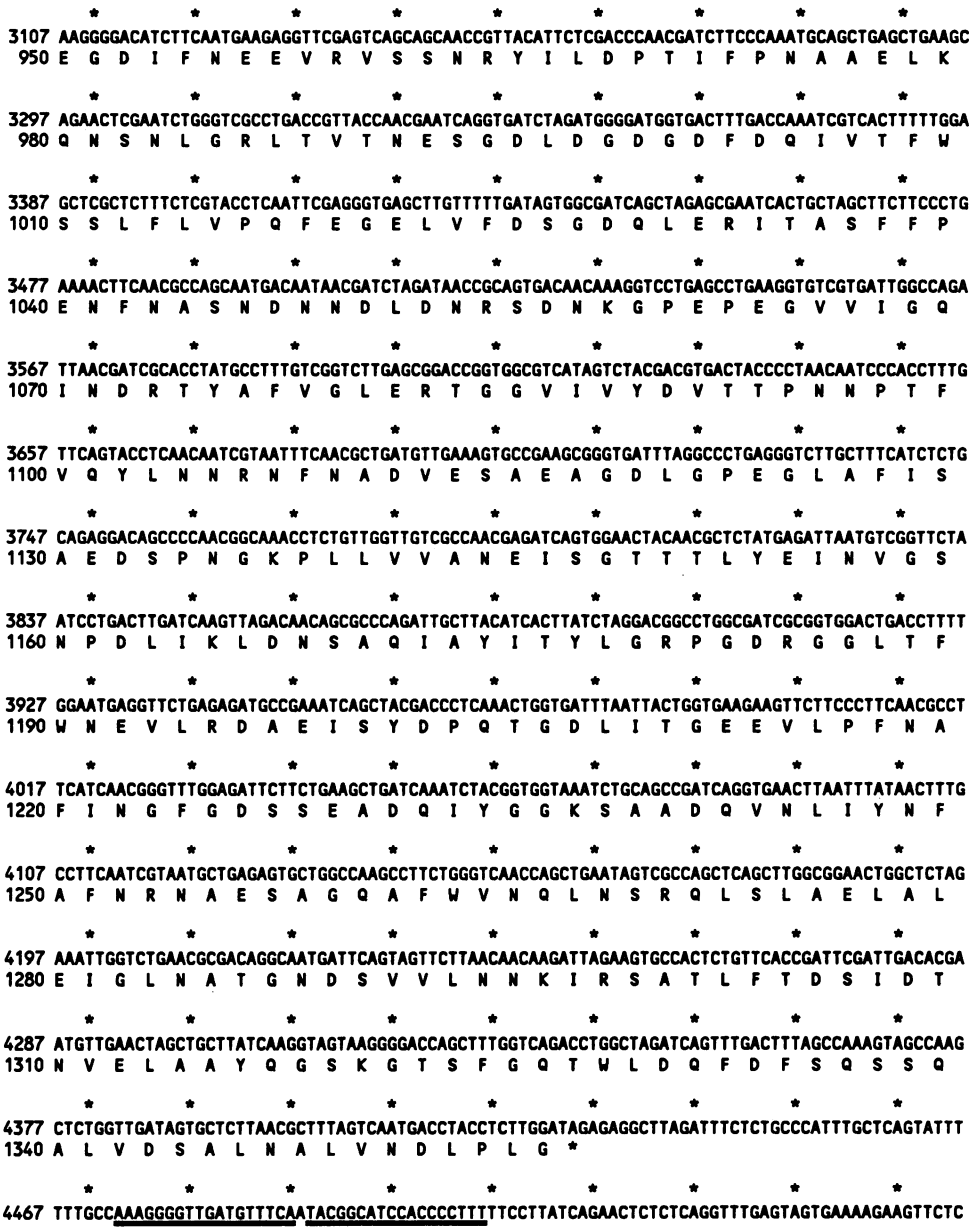

FIG. 3—*Continued.*

mature protein, as determined by microsequencing of the polypeptide, is the only likely site at which translation could initiate. The reading frame preceding the N-terminal methionine terminates seven potential codons upstream of the initiator AUG codon. No typical ribosome binding site for the initiation of translation has been identified. The first 30 amino acids of the phosphatase, as depicted in hydropathy profiles (data not shown), have hydrophobic character, raising the possibility that this region can function as a noncleaveable signal sequence. Other hydrophobic regions are also present in the polypeptide, both internally and at the carboxy terminus.

Transcript accumulation and stability. Slot blot hybridizations were used to quantitate the levels of the alkaline phosphatase transcript under various conditions. A restriction fragment containing a portion of the coding region of *phoA* (fragment C, Fig. 2) was hybridized to total RNA isolated from cells harvested every 2 h after they had been transferred to phosphate-free medium. Transcripts encoding the alkaline phosphatase accumulated to high levels 8 to 12 h after the culture had been depleted of phosphate (Fig. 5). High levels were maintained for at least 24 h after cells were transferred to phosphate-depleted medium.

The stability of the alkaline phosphatase mRNA was determined by treating cells that had been phosphate starved for 24 h with 100 μM rifampin, a concentration sufficient to prevent transcription in *Synechococcus* sp. strain PCC 7942 (unpublished data), and quantitating transcript levels at various times after the addition of the transcription inhibitor (Fig. 6). Alkaline phosphatase mRNA was also quantitated

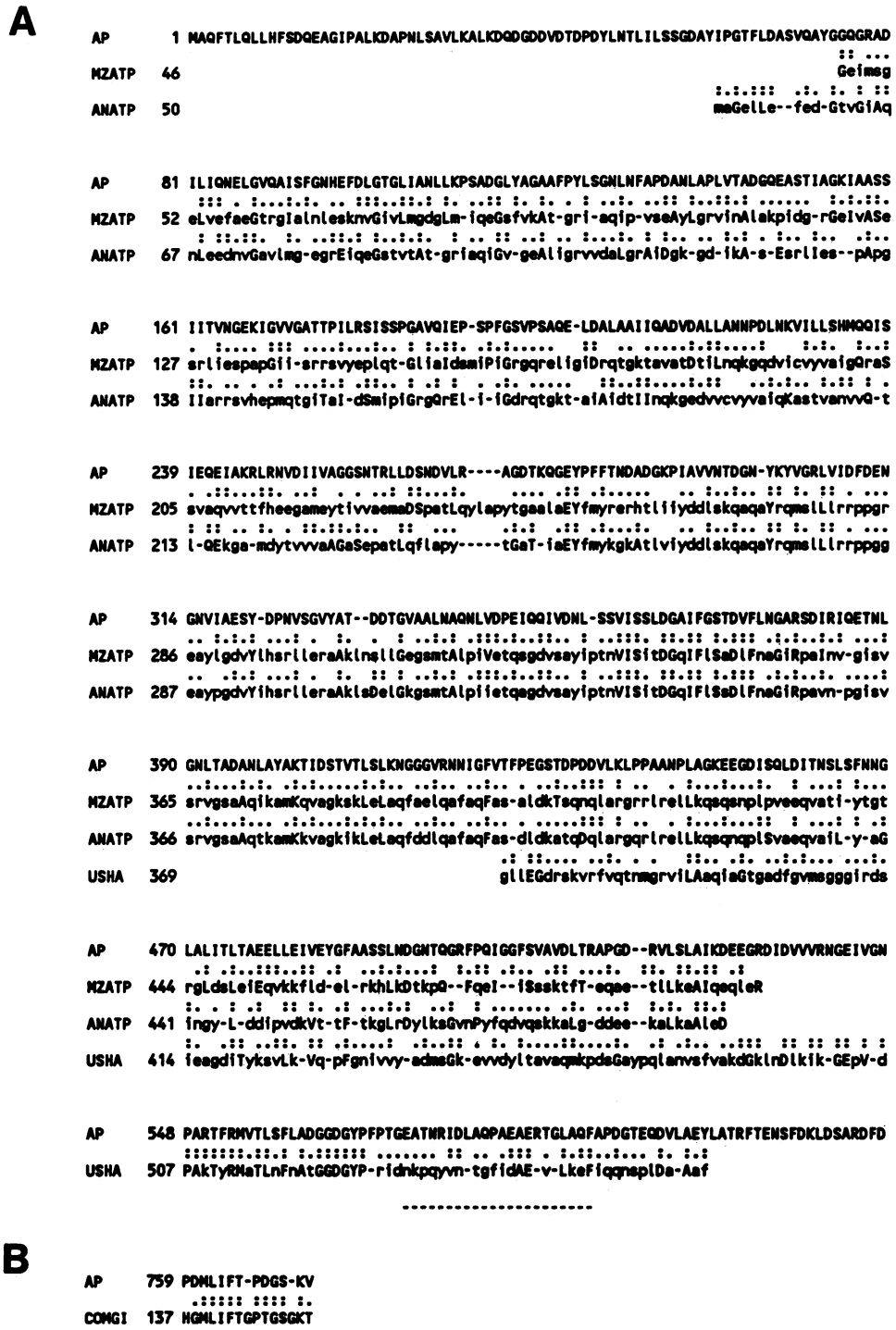

FIG. 4. (A) Comparison of the amino acid sequence of alkaline phosphatase to the α subunits of the *Zea mays* chloroplast coupling factor (23), the *Anabaena* coupling factor (20), and the UDP-sugar hydrolase of *S. typhimurium* (4). (B) Comparison of the nucleoside triphosphate binding region of ComGI with a region of the alkaline phosphatase. Highly conserved and identical residues are indicated by capital letters and colons. Neutral substitutions are represented by lower case letters and periods. Nonconserved substitutions are indicated by lowercase letters and blank spaces. Gaps (–) were introduced into the sequence to attain optimal alignments. AP, *Synechococcus* sp. strain PCC 7942 alkaline phosphatase; MZATP, chloroplast α subunit of the coupling factor of *Z. mays* (23); ANATP, subunit of the *Anabaena* sp. strain PCC 7120 coupling factor (20); USHA, UDP-sugar hydrolase (4); COMGI *Bacillus subtilis* ComGI (1).

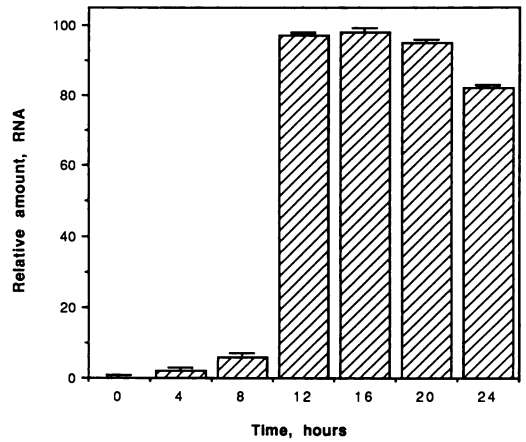

FIG. 5. Accumulation of the *phoA* transcript. Exponentially growing *Synechococcus* sp. strain PCC 7942 was transferred to phosphate-free medium, and RNA was isolated from cells harvested at 4-h intervals. The RNA was immobilized on nitrocellulose and quantitated via slot blot analysis with fragment C of the *phoA* gene for hybridizations (Fig. 1). Quantitation of RNA from phosphate-starved and unstarved cells was standardized to total RNA. The data were averaged from three experiments, the variation between the experiments was less than 10%.

after the addition of P_i (0.23 mM) to phosphate-starved cultures. Within 10 min after the addition of rifampin to phosphate-starved cells, the level of mRNA dropped to approximately 10% of that of untreated cells, and the half life of the transcript was estimated to be approximately 5 min. All three of the transcripts encoding the alkaline phosphatase decreased at similar rates, based on Northern blots, after the addition of rifampin (data not shown). A similar decay in mRNA levels was observed after the addition of either phosphate (Fig. 6) or phosphate and rifampin (data not shown) to starved cultures.

Mapping the transcription initiation site. To identify the transcription start site for *phoA*, primer extension experiments were performed with a synthetic oligonucleotide

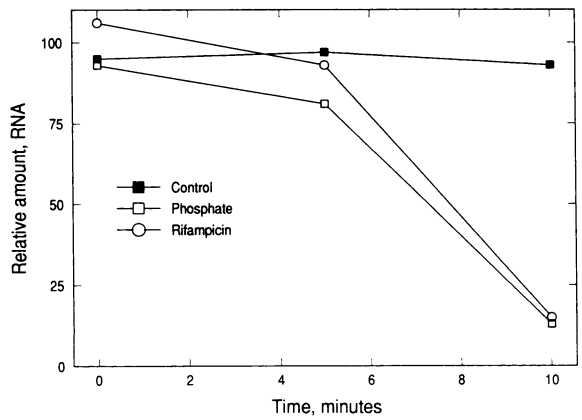

FIG. 6. *phoA* mRNA stability. Cultures were transferred to phosphate-free medium and allowed to grow for 16 h. Rifampin or phosphate was added to the cultures, and RNA was isolated 5 and 10 min after the additions. The RNA was immobilized on nitrocellulose and quantitated by serial dilution on a slot blot apparatus with fragment C of *phoA* as a probe. RNA was also extracted from control cultures that were not exposed to rifampin or phosphate.

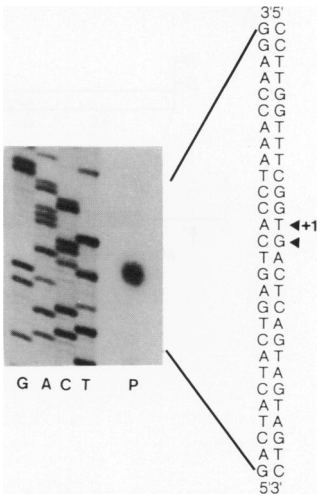

FIG. 7. Primer extension analysis of *phoA*. Total RNA was isolated from cells grown in phosphate-free medium for 24 h. RNA (50 μg) was annealed to a 30-base oligonucleotide (complimentary to bases 280 through 309 of Fig. 3). The primer was extended with 50 U of avian myeloblastosis virus reverse transcriptase in the presence of radiolabeled dATP. The primer extension product (P) was resolved on a polyacrylamide gel beside a sequencing ladder generated with the same primer and a 0.6-kb *Eco*RI-*Eco*RV fragment (Fig. 2) as a template. The positions at which initiation of transcription occur are indicated by arrows to the right of the nucleotide sequence.

primer and total RNA from phosphate-sufficient and -starved cells. With RNA from starved cells a band was observed that represented an extension of the primer to a position 171 nucleotides upstream of the initiation codon (Fig. 3 and 7, arrowheads). This extension product was not observed when total RNA from phosphate-sufficient cells was used in the reaction. RNase mapping was used to confirm the position of the transcription initiation site (data not shown).

We do not find an *E. coli*-like −35 region, but there is the sequence TAcctT located 6 nucleotides upstream of the transcription start site that resembles a −10 sequence. A sequence (underlined in Fig. 3) at positions −45 to −28 has weak homology to the conserved *pho* box that is involved in controlling transcription from genes of the *pho* regulon of *E. coli* (20). This sequence matches the *pho* box consensus sequence at 10 of 18 positions.

Insertional inactivation. To define the function(s) of the alkaline phosphatase and to determine phenotypic and physiological changes that occur when the protein is absent from the cell, we constructed a strain in which *phoA* was inactive (Fig. 8). A region of this gene in the plasmid pNPR12 was deleted and replaced with the gene that confers Sp^r to the host organism. This new plasmid, pNPR18, was used to transform wild-type cells of *Synechococcus* sp. strain PCC 7942 to Sp^r. Since pNPR18 has no origin of replication that will function in *Synechococcus* sp. strain PCC 7942, a Sp^r phenotype would be the consequence of integration of the plasmid into the genome. Sp^r transformants were screened for Ap^s, a phenotype indicative of a double homologous recombination event that would result in the replacement of the wild-type copy of *phoA* with the interrupted, deleted sequence (11).

Synechococcus sp. strain PCC 7942 colonies that exhibited a Sp^r Ap^s phenotype were purified to homogeneity.

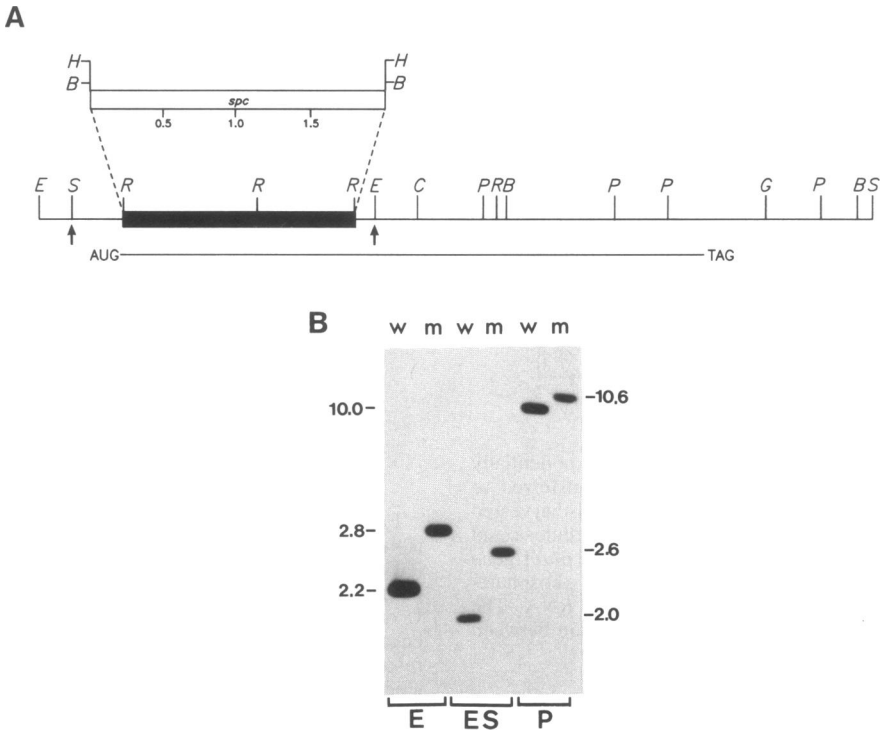

FIG. 8. Inactivation of the *phoA* gene. (A) Construction of the interrupted gene. A 1,425-bp *Eco*RV (*R*) fragment (indicated by the solid rectangle on the restriction map) was removed from *phoA*, and a 2,000-bp *spc* gene was inserted in its place (indicated above the restriction map). The resulting plasmid was transformed into *Synechococcus* sp. strain PCC 7942 and recombined into the chromosome, replacing the wild-type copy of the *phoA* gene (see Materials and Methods). (B) Southern hybridization of genomic DNA from wild-type and *phoA* mutant strains. Chromosomal DNA from wild-type and Sp^r Ap^s transformants was digested with *Eco*RI (E), *Eco*RI-*Sal*I (ES), and *Pst*I (P) and hybridized to the 1.8-kb *Sal*I-*Eco*RI fragment that includes the region of the *phoA* gene deleted in the transformant (indicated with arrows). The lanes contain DNA from the wild-type (w) and mutant (m) strains.

Genomic DNA from both wild-type and transformed strains was isolated, digested with various restriction enzymes, transferred to nitrocellulose, and hybridized to an *Eco*RI fragment from pNPR12 that spans the region of deletion-insertion. The results of these experiments (Fig. 8) confirm the occurrence of a double recombination event. The sizes of the fragments that hybridize in the mutant strain are exactly those predicted upon deletion of the 1.4-kbp *Eco*RV fragment and insertion of the 2.0-kbp *aadA* cassette. We could not detect unaltered copies of *phoA* in the mutant strain.

Phenotype of the alkaline phosphatase-deficient strain. The strain with a deletion in the putative *phoA* gene was analyzed for the presence of externally accessible, phosphate-regulated alkaline phosphatase activity. The wild-type strain hydrolyzed 3.8 ± 0.3 and 42 ± 1 nmol of *p*-nitrophenyl phosphate per 10^7 cells in 20 min after growth in phosphate-replete and phosphate-deficient (24 h) medium, respectively. The mutant strain, designated *phoA18*, exhibited no increase in alkaline phosphatase activity after transfer of cells to phosphate-free medium (12 ± 3 compared with 13 ± 2 nmol of *p*-nitrophenyl phosphate hydrolyzed per 10^7 cells in 20 min for cells grown in phosphate-replete and -deficient medium, respectively). The slight increase in overall external phosphatase activity in *phoA18* relative to that in the wild-type strain when cells were maintained in phosphate-containing medium was reproducible. Furthermore, there was no accumulation of the 145-kDa alkaline phosphatase polypeptide in the mutant strain maintained in phosphate-deficient medium for 16 h (Fig. 9).

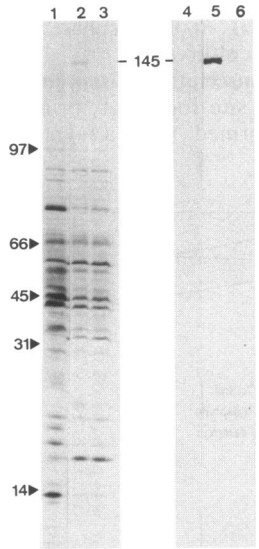

FIG. 9. Accumulation of the alkaline phosphatase during phosphate deprivation. Membrane proteins were extracted from cultures harvested every 4 h after the transfer of cells to phosphate-free medium and resolved on a 7.5 to 15% polyacrylamide gradient gel. The proteins were either stained with Coomassie brilliant blue R-250 (lanes 1 through 3) or transferred to nitrocellulose and screened with polyclonal antibodies that were raised against the purified alkaline phosphatase (lanes 4 through 6). Lanes: 1 and 4, wild type with phosphate; 2 and 5, wild type (16 h) without phosphate; 3 and 6, *phoA18* (the strain mutated for *phoA*) (16 h) without phosphate.

Phosphate uptake. The initial rates of phosphate uptake were measured in the wild-type and mutant strains to determine whether the alkaline phosphatase that accumulates during phosphate stress has a role in the transport of phosphate into the cell. There was no significant difference between the $K_{1/2}$s for phosphate uptake by the two strains when the cells were grown either in the presence or absence of phosphate (data not shown).

DISCUSSION

Synechococcus sp. strain PCC 7942 synthesizes a number of soluble and membrane-associated proteins in response to phosphate stress. Attempts were made to isolate phosphate-regulated genes from *Synechococcus* sp. strain PCC 7942 by using genes of the *pho* regulon of *E. coli* as heterologous probes. However, most of the genes did not hybridize to cyanobacterial DNA (data not shown). We succeeded in cloning the *phoA* gene of the cyanobacterium with the aid of chemically synthesized oligonucleotides designed to encode the amino terminus of the alkaline phosphatase that has been previously purified (2).

The *phoA* gene encodes a protein of 1,344 amino acids with a molecular mass of 145 kDa, which is almost three times that of the *E. coli* monomer. There is little sequence similarity between the cyanobacterial alkaline phosphatase and other alkaline phosphatases in the GenBank data set. A short but highly conserved stretch of identity does exist between the cyanobacterial enzyme and UshA, a UDP-sugar hydrolase found in *E. coli* and *Salmonella typhimurium* (4). No function has been assigned to this region in UshA. Another significant similarity was found between the alkaline phosphatase and the α subunit of the F_1 ATPase from bacteria (22) and the CF_1 ATPase of plant chloroplasts (25). The α subunit of the ATPase is involved in assembly of coupling factor subunits (40) and does appear to have a nucleoside triphosphate binding site (P loop) (37). Since few of the residues that are identical in the cyanobacterial alkaline phosphatase and the α subunit of CF_1 have defined functions, the significance of the homology noted is difficult to evaluate. However, these sequence similarities may ultimately help us localize important structural features shared by these proteins.

A short sequence similarity was found between a region in the second half of the alkaline phosphatase and a P loop that is thought to bind nucleoside triphosphate in ComGI (Fig. 4B) (1). Another sequence in the alkaline phosphatase with some similarity to the P-loop sequence was also noted. The conserved Lys residue of the P loop probably binds the γ phosphate of nucleoside triphosphate (29). Although the significance of the similarity is uncertain, we could speculate that P loop-like sequences might be important in binding the phosphate moiety of phosphate ester molecules that serve as substrates for the alkaline phosphatase. Two additional sequences that have regions of similarity, specifically to the ComGI P-loop motif, have been observed in phosphate-regulated open reading frames in another region of the cyanobacterial genome (24a).

Analysis of the *phoA* gene sequence raises intriguing questions concerning the biosynthesis of alkaline phosphatase and the way that it is targeted to the periplasmic space. Examination of the deduced amino acid sequence of the alkaline phosphatase with the amino-terminal sequence as determined by protein microsequencing suggests that the nascent polypeptide does not have an amino-terminal, cleavable signal sequence. However, the first 30 amino acids form a hydrophobic domain that, although not cleaved from the protein, may function in transport across the inner, cytoplasmic membrane. A number of bacterial proteins have noncleavable targeting sequences located at the amino terminus of the protein that enable them to cross the inner cytoplasmic membrane in an energy-dependent manner (23). It is also possible that internal or carboxy-terminal hydrophobic regions are important in the passage of the protein across the membrane. This is the first discription of a periplasmic protein in cyanobacteria that does not have a cleavable signal sequence at the amino terminus.

Several transcripts that accumulate in cells deprived of phosphate hybridize to the *phoA* gene. The largest transcript that contains the sequence encoding the alkaline phosphatase is 11 kb. The sequence of the alkaline phosphatase, encoded by approximately 5 kb of mRNA, has been localized to the 5′ end of this transcript, which suggests that there is an additional 6 kb of mRNA that encodes other proteins specifically synthesized during phosphate stress. The 5-kb transcript seen in Northern blots probably encodes only the alkaline phosphatase, whereas the 8- and 11-kb transcripts probably encode the alkaline phosphatase plus other phosphate-regulated proteins. We do not yet know whether these three transcripts arise by RNA processing or inefficient termination at various places in the gene cluster. Certain gene clusters in *E. coli* controlled by phosphate availability also exhibit a number of open reading frames in an operon structure (6, 39).

The *phoA* transcripts are degraded rapidly and have a half-life of approximately 5 min. The transcript declines with similar kinetics when phosphate is added back to cells maintained in phosphate-free medium. Therefore, phosphate triggers an immediate decline in mRNA levels and probably acts at the level of transcription.

The nucleotide sequence of *phoA* in the region −35 relative to transcription initiation has no resemblance to *E. coli* counterparts. There is a resemblance to a −10 *E. coli* sequence six nucleotides upstream of the transcription initiation site. A sequence with some similarity to that of the *pho* box has also been identified upstream of the transcription initiation site, but the significance of this sequence is questionable since the homology to the sequence of the *E. coli* *pho* box is weak.

Insertional inactivation of *phoA* results in a loss of the extracellular, phosphate-regulated alkaline phosphatase activity and no accumulation of the 145-kDa protein as determined by SDS-PAGE and immunological analyses. These results confirm the identity of the gene as *phoA*. Loss of the enzyme alters neither the kinetics of phosphate uptake by the cells nor their viability when maintained on phosphate sufficient or deficient medium (24a). These results suggest that the phosphatase is not important to phosphate transport and is of little benefit to the cells under conditions in which we culture them. However, it may be important in scavenging phosphate from organic compounds during the growth of *Synechococcus* sp. strain PCC 7942 in its natural environment.

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