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Cloning, Nucleotide Sequence, and Mutagenesis of a Gene (*irpA*) Involved in Iron-Deficient Growth of the Cyanobacterium *Synechococcus* sp. Strain PCC7942

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We describe the cloning and sequencing of a gene from the cyanobacterium *Synechococcus* sp. strain PCC7942, designated *irpA* (iron-regulated protein A), that encodes for a protein involved in iron acquisition or storage. Polyclonal antibodies raised against proteins which accumulate during iron-deficient growth were used as probes to isolate immunopositive clones from a λgt11 genomic expression library. The clone, designated λgtAN26, carried a 1.7-kilobase (kb) chromosomal DNA insert and was detected by cross-reactivity with antibody against a 36-kilodalton protein. It was possible to map a 20-kb portion of the chromosome with various DNA probes from λgt11 and λEMBL-3 clones, and Southern blot analysis revealed that the *irpA* gene was present in a single copy and localized within a 1.7-kb *PstI* fragment. DNA sequencing revealed an open reading frame of 1,068 nucleotides capable of encoding 356 amino acids which yields a protein with a molecular weight of 38,584. The hydropathy profile of the polypeptide indicated a putative N-terminal signal sequence of 44 amino acid residues. IrpA is a cytoplasmic membrane protein as determined by biochemistry and electron microscopy immunocytochemistry. The upstream region of the *irpA* gene contained a consensus sequence similar to the aerobactin operator in *Escherichia coli*. This fact, plus a mutant with a mutation in *irpA* that is unable to grow under iron-deficient conditions, led us to suggest that *irpA* is regulated by iron and that the gene product is involved in iron acquisition or storage.

Iron is an essential element required for growth and development of living organisms. Although it is abundant in nature, the availability of this element is very limited owing to its poor solubility in aerobic environments (3, 25). In the presence of oxygen at physiological pH, the rapid oxidation of the ferrous form to the ferric form leads to precipitation of iron and its essential unavailability. Therefore, living organisms have developed various mechanisms to solubilize iron by secreting different forms of chelating agents called siderophores (25). Siderophores have been reported in higher plants, fungi, bacteria, and cyanobacteria (5, 25), including gram-positive bacteria such as *Bacillus* sp. and *Mycobacterium* sp. and the gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium* (25). *E. coli* has been extensively used in genetic studies of iron acquisition, and a significant portion of the *E. coli* genome is involved in cellular iron acquisition.

In *E. coli*, several genes involved in iron acquisition have been characterized (3); these studies demonstrate that aerobactin and enterobactin are the two high-affinity iron transport systems in *E. coli* (3, 6, 10). The well-characterized aerobactin operon consists of five genes which have now been cloned and sequenced (3). The enterobactin system utilized several genes for enterobactin biosynthesis, including genes that code for iron transport proteins (10). Some cyanobacteria are apparently capable of siderophore production; e.g., *Anabaena* spp. produce schizokinen, a high-affinity iron transport compound (37), and iron-deficient cultures of *Synechococcus* sp. strain PCC7002 synthesize a hydroxamate-type siderophore (2). However, no high-affinity iron transport system has yet been characterized for *Synechococcus* sp. strain PCC7942.

Iron is involved in a wide variety of biochemical processes in cyanobacteria including photosynthesis, chlorophyll biosynthesis, nitrate reduction, nitrogen fixation, and many other redox reactions (5, 17, 27, 33). Iron deficiency in *Synechococcus* sp. strain PCC7942 causes dramatic changes in cellular physiology and ultrastructure (15, 35). These alterations include a decrease in the number of thylakoid membranes and phycobilisomes per cell and a concomitant accumulation of glycogen granules (35). Spectral changes include a 4- to 5-nm blue shift in the in vivo chlorophyll absorption peak (14). In addition, chlorophyll fluorescence emission spectra at 77 K show a single major peak at 685 nm in iron-deficient cells instead of the normal three peaks at 685, 696, and 716 nm (14). These differences are mirrored by changes in the chlorophyll-protein complexes; e.g., chlorophyll protein complexes of photosystem I are decreased, whereas a new protein complex, called CPVI-4, is synthesized (29). This complex becomes the predominant chlorophyll species during low-iron growth, and readdition of iron leads to the disappearance of this complex (H. R. Riethman and L. A. Sherman, Plant Physiol., in press).

The induction of CPVI-4 under iron-deficient conditions represents the starting point for this study. The apoprotein was purified and used to prepare a polyclonal antibody. Western blots (immunoblots) utilizing this antibody preparation against iron-deficient membranes resulted in cross-reactivity to three polypeptides with apparent molecular masses of 34, 35, and 36 kilodaltons (kDa) (Reithman and Sherman, in press). We determined that these proteins are inducible under iron-deficient growth conditions and that they are distinctly different. The 34-kDa polypeptide is the chlorophyll-binding apoprotein of the complex CPVI-4,

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whereas the 35-kDa protein is a highly basic glycoprotein. The 34- and 36-kda polypeptides are intrinsic membrane proteins as determined by Triton X-114 fractionation (Reithman and Sherman, in press). This paper describes the cloning and sequencing of the gene (*irpA*) encoding the 36-kDa polypeptide. Additionally, we describe the construction of a site-directed mutant of *Synechococcus* sp. strain PCC7942 (generated by insertion of Tn*5* into the *irpA* gene) that is deficient in iron-limited growth. These results lead us to suggest that *irpA* is regulated by iron and that IrpA is involved in iron acquisition and storage.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The cyanobacterial strain *Synechococcus* sp. strain PCC7942 was grown in iron-sufficient BG-11 medium (1) under continuous light (0.5 mW/cm^2). Iron-deficient BG-11 medium was prepared as previously described (35). The *E. coli* strains, bacteriophages, and plasmids used in this study are listed in Table 1. *E. coli* Y1090 was used as a host strain in the construction and screening of the λgt11 library. *E. coli* NM539 was used as a host strain for λEMBL-3 recombinants, and strain JM83 was used to maintain pUC (45) and Bluescribe plasmids. M13mp18 and -19 (23) were grown on *E. coli* JM101. Bacteriophages λgt11 and λEMBL-3 were purchased from Promega Biotec (Madison, Wis.).

The *irpA*::Tn*5* derivative, K7, was diluted 1:10,000 from log-phase BG-11 cultures into iron-deficient BG-11 medium. After the cultures had become green (~2 × 10^6 cells per ml), the cultures were again diluted 1:1,000 in iron-deficient BG-11 medium. The secondary subcultures into iron-deficient BG-11 medium were monitored for growth at 750 nm. For the iron chelation experiments, log-phase cultures of wild-type and mutant cells growing in BG-11 medium were treated with 300 and 100 μM 2,2′-dipyridyl, respectively.

Preparation of cell envelopes. The procedure we used to isolate envelopes was similar to the method described by Resch and Gibson (32) for the preparation of cyanobacterial cell walls. *Synechococcus* sp. strain PCC7942 cells suspended in 50 mM (morpholino)ethanesulfonic acid (MES) (pH 6.5) were broken by two cycles of French pressure treatment at 40 MPa and centrifuged at 3,000 × *g* for 5 min to pellet unbroken cells. The supernatant was applied to the top of a 50 to 85% sucrose gradient made in MES buffer and centrifuged at 70,000 × *g* for 24 h. The resulting gradient yielded a chlorophyll-free carotenoid-containing band at 40% sucrose which contained both lipopolysaccharide and the carotenoid-binding polypeptides specific for the cytoplasmic membrane, as judged by immunoblotting (40) and periodic acid-Schiff staining of electrophoresed membrane samples (7, 48).

Immunocytochemistry. *Synechococcus* sp. strain PCC7942 cultures grown in normal and iron-deficient medium were fixed for immunocytochemistry precisely as described for yeast cells by van Tuinen and Riezman (42). Polyclonal antibodies to the IrpA protein were affinity purified (38) against the *lacZ-irpA* fusion protein expressed by pRB96 (36); this purified preparation was used directly to decorate thin sections of *Synechococcus* sp. strain PCC7942 embedded in Lowicryl HM20. Antibody-antigen complexes were visualized by treatment with gold-conjugated protein A prior to electron microscopy.

Spectroscopic methods. Absorbance spectra were obtained at 20°C with a Beckman DU-7HS spectrophotometer. Fluorescence emission spectra from dipyridyl-treated *Synechococcus* sp. strain PCC7942 cells were taken at 77 K with an SLM 8000 spectrofluorimeter (SLM Corp., Urbana, Ill.) with the excitation monochromator set at 435 nm. Prior to cooling to 77 K, the *Synechococcus* sp. strain PCC7942 cells were added to an equal volume of potassium glycerophosphate to prevent formation of ice crystals.

DNA isolation, construction, and screening of λgt11 expression library. *Synechococcus* sp. strain PCC7942 chromosomal DNA was prepared from 15 liters of stationary-phase cells as described previously (20); DNAs from λgt11 and λEMBL-3 recombinants were prepared by the DEAE-cellulose column procedure of Reddy et al. (31). The *Synechococcus* sp. strain PCC7942 λgt11 expression library was constructed essentially by the method of Young et al. (46) with the following modifications. Excess *Eco*RI linkers were separated by centrifugation on 10 to 40% linear sucrose gradients for 24 h at 25,000 rpm in a Beckman SW42 rotor. Gradient fractions containing 0.5- to 7-kilobase (kb) fragments were pooled and dialyzed against TE buffer (10 mM Tris hydrochloride, 1 mM EDTA, pH 7.6). DNA was extracted twice with phenol-chloroform and ethanol precipitated. The purified size-selected fragments were ligated to dephosphorylated λgt11 arms with T4 DNA ligase, and the ligated DNA was packaged with Promega Biotec in vitro packaging extracts.

Screening of the λgt11 expression library was performed by the method of Young et al. (46) except that bovine serum albumin was used for blocking instead of 20% fetal calf serum. Nitrocellulose filters were blocked in 5% bovine serum albumin–0.05% Tween 20 for 1 h and incubated in CPVI-4 primary antibody (diluted 1:10,000) for 1 h. Filters were washed six times for 10 min each in TBS (50 mM Tris hydrochloride, 150 mM NaCl, pH 8.0)–Tween after incubation in primary and secondary antibody.

Construction and screening of λEMBL-3 genomic library. *Synechococcus* sp. strain PCC7942 chromosomal DNA was subjected to partial digestion by the 4-base-recognizing enzyme *Sau*3A. The fragments were purified by ethanol precipation and treated with calf intestinal phosphatase. The dephosphorylated chromosomal fragments were ligated to λEMBL-3 arms (12) that contained *Bam*HI-compatible ends and packaged in vitro. The library was plated onto appropriate *E. coli* host strains on 86-mm plates to yield approximately 4,000 plaques per plate. Plaque screening was essentially similar to the method of Benton and Davis (4).

Cloning procedures and Southern hybridization. DNA fragments from λgt11 recombinants were subcloned into different plasmids and M13 vectors by standard procedures described by Maniatis et al. (22). DNA was transferred onto nitrocellulose filters as described by Southern (39). Prehybridization, hybridization, and washing conditions were those of Maniatis et al. (22). The 1.7-kb insert from λgtAN26 and the 1.3-kb insert from λgtAN103 were used to create DNA probes to map the *Synechococcus* sp. strain PCC7942 chromosome around *irpA*. Large-scale restriction mapping was done by using *Sal*I fragments from λEM129 and λEM130 as probes. DNA probes were labeled to high specific activity with [^{32}P]dCTP by the oligolabeling procedure (11).

DNA sequencing strategy. Ordered *Bal* 31 deletions in the 1.3-kb insert in pBSP103 and pBSP103R were constructed by the method described by Poncz et al. (30). The DNA sequencing of M13 recombinants was done by the dideoxy chain termination method (34) with [^{35}S]dATP and the Pharmacia DNA-sequencing kit. *Bal* 31 deletion clones covered both strands of the 1.3-kb insert. Since the termi-

TABLE 1. Bacterial strains, phages, and plasmids

Strain, phage, or plasmid	Relevant characteristics	Source or reference
Escherichia coli		
Y1090[a]	Δ*lacU169 proA*$^+$ Δ*lon araD139 rpsL hsdR hsdM*$^+$ *strA supF trpC22*::Tn*10* pMC9 (Ap^r)	47
Y1089[a]	Δ*lacU169 proA*$^+$ Δ*lon araD139 strA hflA*[*chr*::Tn*10*] *hsdR hsdM*$^+$ pMC (Ap^r)	47
NM538	*supF hsdR*	12
NM539	*supF hsdR* (P2 *cox3*)	12
JM83	*ara* Δ(*lac proAB*) *rspL80 lacZ*ΔM15(r_K^+ m_K^+)	45
JM101	Δ(*lac proAB*) *supE thi* (r_K^+ m_K^+)/F′ *traD36 proAB lacI*^{q}ΔM15	45
BD1388	*his ara leu thr trpA sup*$^+$	44
Synechococcus sp. strain PCC7942	Wild type	This laboratory
Synechococcus sp. strain PCC7942 K7	R2 *irpA*::Tn*5*	This study
Bacteriophages		
λgt11	*lac5 cI857 nin5 S100*	47
λEMBL-3		12
λgtAN26	A derivative of λgt11 capable of synthesis of *lacZ-irpA* fusion protein; contains 1.7-kb fragment of *Synechococcus* sp. strain PCC7942 chromosome	This study
λgtAN103	Recombinant λgt11 with 1.3-kb insert	This study
λgtAN104	Recombinant λgt11 with 2.6-kb insert	This study
λgtAN105	Recombinant λgt11 with 3.1-kb insert	This study
λgtAN110	Recombinant λgt11 with 2.4-kb insert	This study
λgtAN104.17	Derivative of λgtAN104 bearing *irpA*::Tn*5*	This study
λEM120	Recombinant λEMBL-3 with 13.6-kb insert	This study
λEM127	Recombinant λEMBL-3 with 10.8-kb insert	This study
λEM129	Recombinant λEMBL-3 with 14.0-kb insert	This study
λEM130	Recombinant λEMBL-3 with 11.0-kb insert	This study
λ467	*rex*::Tn*5*	45
M13mp18		45
M13mp19		This study
M13mp18-1.7	1.7-kb *Eco*RI fragment from λgtAN26 in the *Eco*RI site	This study
M13mp18.103	1.3-kb *Eco*RI fragment from λgtAN103 in the *Eco*RI site	This study
M13mp18.103R	Same as M13mp18.103 but insert is in opposite orientation	
Plasmids		
pUC8	Ap^r	This study
pRB96	Derivative of pUC8 containing a 700-bp *Eco*RI-*Ava*I *irpA* internal fragment; expresses a 29-kDa fusion protein in *E. coli*	This study
pRB96.37	Derivative of pRB96 bearing Tn*5* inserted into the 700-bp *irpA* sequence	This study
pRB104.17	Derivative of pUC8 bearing the *Eco*RI *irpA*::Tn*5* insert from λgtAN104.17	45
pUC19	Ap^r	Stratagene[b]
Bluescribe plus	pUC19 derivative with T3, T7 promoters and intergenic region from M13	Stratagene[b]
Bluescribe minus	pUC19 derivative with T3, T7 promoters and intergenic region from M13	This study
pBSP103	1.3-kb *Eco*RI fragment from λgtAN103 in the *Eco*RI site of Bluescribe plus	This study
pBSP103R	Same as pBSP103 but insert is in opposite orientation	This study
pKJ110	2.4-kb *Eco*RI fragment from λgtAN110 in the *Eco*RI site of pUC19	This study
pKJ49	*lacZ-irpA* fusion at −231 bp in the *Sma*I site of Bluescribe minus	This study
pKJ34	*lacZ-irpA* fusion at −44 bp in the *Sma*I site of pUC19	This study
pKJ42	*lacZ-irpA* fusion at +64 bp in the *Sma*I site of Bluescribe minus	This study
pKJ33	*lacZ-irpA* fusion at +81 bp in the *Sma*I site of pUC19	This study
pKJ43	*lacZ-irpA* fusion at +422 bp in the *Sma*I site of Bluescribe minus	This study

[a] Restriction-minus derivatives of the original Young and Davis strain (47).
[b] Product from Stratagene, San Diego, Calif.

nation codon was not present in this insert, a 0.74-kb *Xho*I-*Eco*RI fragment from pKJ110 was cloned into the *Sal*I-*Eco*RI site of M13mp18. The DNA sequence at the fusion joint in the plasmid DNAs was obtained by sequencing double-stranded plasmid templates with the Promega Biotec K/RT system and reverse transcriptase. The plasmid DNA templates were prepared for sequencing by the rapid boiling method (18).

Construction of *irpA*::Tn*5* insertions. The clone λgtAN26 contains a 1.7-kb *Eco*RI linker-adapted insert expressing a *lacZ-irpA* fusion protein in *E. coli* Y1089. We subcloned a 700-base-pair (bp) *Eco*RI-*Ava*I fragment of λgtAN26 into the expression plasmid pUC8.2 (16); this construct, pRB96, expressed a 29-kDa immunopositive polypeptide in *E. coli* JM83 (45). Based on the DNA sequence of *irpA*, the *Eco*RI-*Ava*I fragment was an internal fragment of the gene and thus a suitable target for transposon mutagenesis with Tn*5*. This was performed essentially as described by deBruijn and Lupski (8) by transforming pRB96 into a *sup*$^+$ strain, BD1388, and infecting this strain with λ467 *rex*::Tn*5*. After

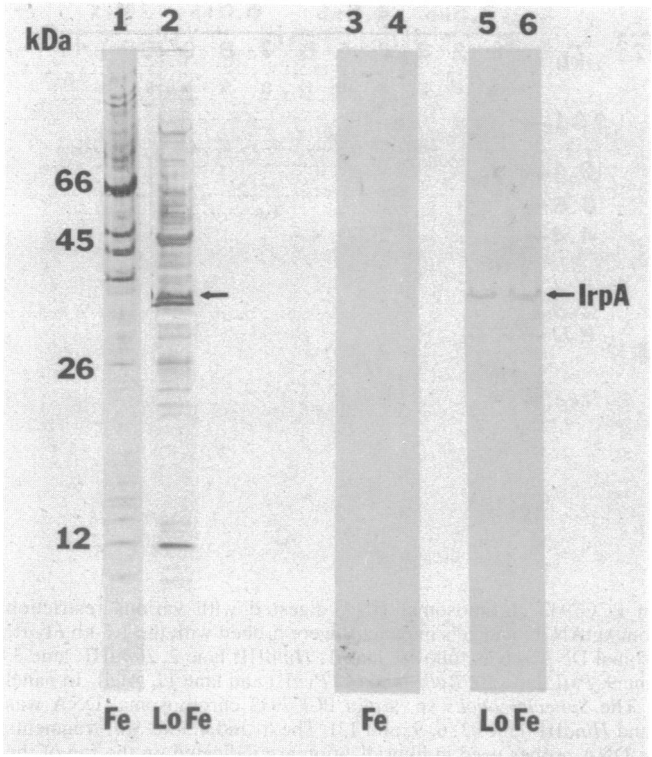

FIG. 1. Identification of the *irpA* gene product in cell envelopes. Lane 1, Polypeptide composition of envelope membranes from normally grown cells; lane 2, polypeptides of iron-deficient (LoFe) cell envelopes; lanes 3 and 4, immunoblot of envelopes from normally grown cells probed with antibody to the IrpA protein; lanes 5 and 6, immunoblot of iron-deficient envelope membranes probed with the IrpA antibody. The arrow in lane 2 identifies the immunoreactive polypeptide.

the cells were plated on LB medium that contained 20 μg of kanamycin per ml, Km^r survivors were pooled. Total plasmid DNA from the Km^r population was isolated and transformed into *E. coli* JM83. Eighty-nine Km^r Ap^r colonies were retained for DNA blot analysis to determine the site of Tn*5* in each plasmid clone. From this population, one plasmid, pRB96.37, was chosen for further study. The site of the Tn*5* insertion was 100 bp upstream from the *Ava*I site, which in turn is 220 bp from the *irpA* termination codon (36).

The short regions of homology shared by pRB96.37 and the *Synechococcus* sp. strain PCC7942 chromosome may have been insufficient to promote site-directed gene replacement following transformation. To generate a construct suitable for transformation into R2, we required an *irpA*::Tn*5* insertion bearing large (~1 kb) flanking DNA sequences to promote recombination. Our strategy to retrieve such flanking sequences involved recombination between pRB96.37 and a λgt11-derived clone, λgtAN104, a phage bearing a 2.4-kb *Eco*RI-adapted fragment of *Synechococcus* sp. strain PCC7942 DNA covering the *irpA* region. To accomplish this, we transformed pRB96.37 into *E. coli* Y1090, the host for λgt11 growth (46). A culture of Y1090(pRB96.37) was infected with λgtAN104 at a multiplicity of infection of 2, and the resulting transducing lysate was then tested for the ability to form Km^r lysogens on *E. coli* Y1089. A total of 24 Y1089 lysogens were picked and screened by DNA blot analysis, and 19 of these yielded the same restriction pattern as the original *irpA*::Tn*5* insertion into pRB96 (36). One of these lysogens was induced, yielding phage λgtAN104.17. Since Tn*5* lacks an *Eco*RI site, the 8.0-kb *Eco*RI insert containing the *irpA*::Tn*5* insertion was subcloned into the *Eco*RI site of pUC8 (43). This plasmid, pRB104.17, was used to transform *Synechococcus* sp. strain PCC7942 to Km^r (13). Km^r R2 colonies arose at a frequency of 2.5×10^{-7} per recipient, and these isolates were checked for altered growth in iron-deficient BG-11 medium. One clone, *Synechococcus* sp. strain PCC7942 K7, was retained for further characterization.

RESULTS

Identification of *irpA* gene. Immunoscreening of the λgt11 expression library with CPVI-4 antibody resulted in the identification of an immunopositive clone designated λgtAN26. When λgtAN26 DNA was digested with *Eco*RI, a 1.7-kb insert was released. The orientation of the insert within the *lacZ* gene was deduced by digesting λgtAN26 DNA with *Kpn*I and *Pst*I alone and also by double digestion

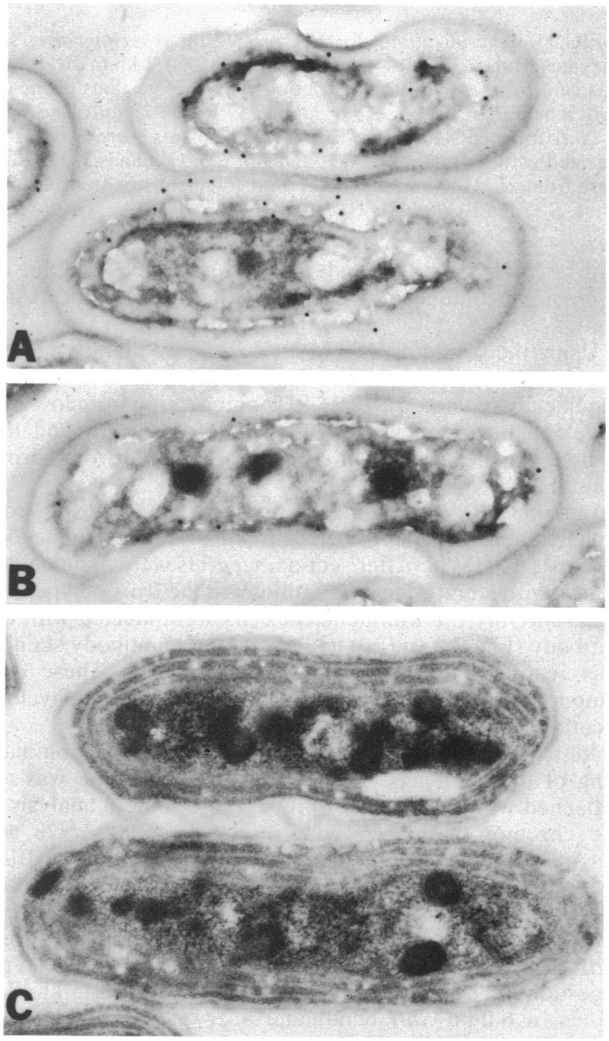

FIG. 2. Immunocytochemical localization of the IrpA protein. Cells were fixed and embedded as described previously (42). (A and B) Electron micrographs of iron-deficient cells after immunodecoration with anti-IrpA. (C) Electron micrograph of normally grown *Synechococcus* sp. strain PCC7942 cells after immunodecoration with the IrpA antibody.

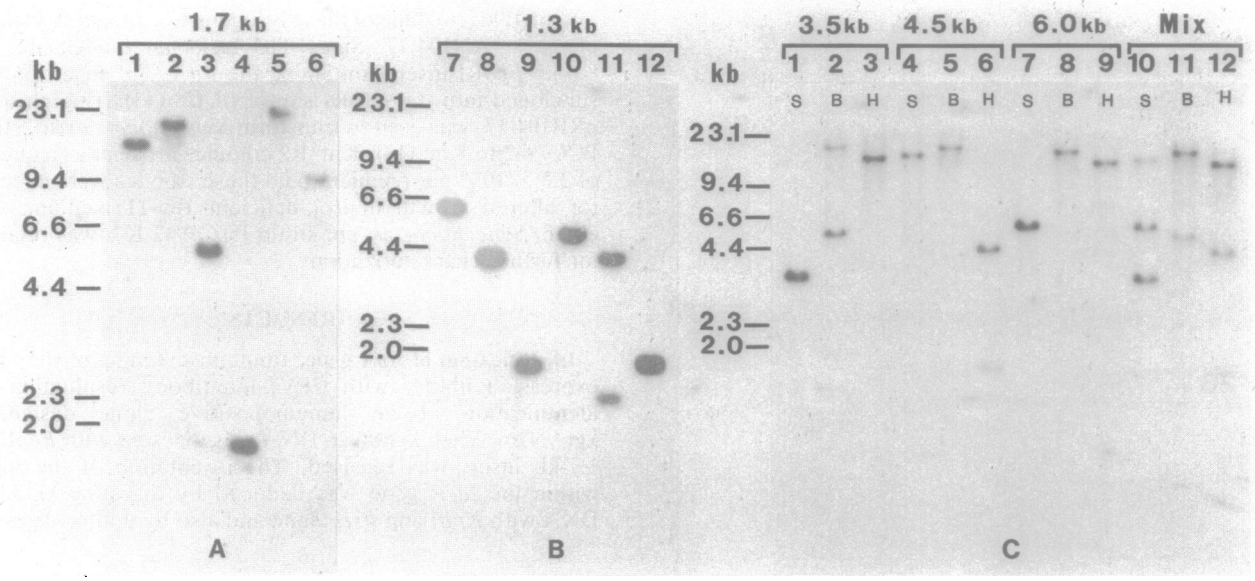

FIG. 3. Hybridization of DNA probes to *Synechococcus* sp. strain PCC7942 chromosomal DNA digested with various restriction enzymes. Gels in panel A were probed with the 1.7-kb *Eco*RI fragment from λgtAN26, and gels in panel B were probed with the 1.3-kb *Eco*RI fragment from λgtAN103. Restriction enzyme digestions of the chromosomal DNA are as follows: lane 1, *Hin*dIII; lane 2, *Bam*HI; lane 3, *Sal*I; lane 4, *Pst*I; lane 5, *Kpn*I; lane 6, *Sst*I; lane 7, *Sal*I; lane 8, *Acc*I; lane 9, *Pst*I; lane 10, *Bgl*I; lane 11, *Pvu*II; and lane 12, *Ava*I. In panel C, *Sal*I fragments from λEMBL-3 recombinants were used as probes. The *Synechococcus* sp. strain PCC7942 chromosomal DNA was digested with *Sal*I (lanes 1, 4, 7, and 10), *Bam*HI (lanes 2, 5, 8, and 11), and *Hin*dIII (lanes 3, 6, 9, and 12). The 6- and 4.5-kb *Sal*I fragments were from λEM130 and the 3.5-kb *Sal*I fragment was from λEM127. The DNA probes used in hybridization are indicated on the top of the lanes. S, *Sal*I; B, *Bam*HI; H, *Hin*dIII.

with *Eco*RI and *Pst*I and *Eco*RI and *Kpn*I. The insert contained a unique *Pst*I site 1.4 kb away from the fusion joint.

Using the fusion protein of *lacZ-irpA*, we affinity purified the CPVI-4 antibody (38). This affinity-purified antibody specifically reacted with a 36-kDa polypeptide from low-iron envelope membranes on Western blots (Fig. 1), and this protein was not present in envelopes prepared from normal cells (Fig. 1).

Immunocytochemical localization of IrpA protein. Iron-deficient and normally grown cells were fixed and embedded in Lowicryl HM20. After sectioning, grids were immunodecorated with affinity-purified antibody to the IrpA polypeptide (Fig. 2). Only the iron-deficient cells were labeled with the antibody (Fig. 2A and B); additionally, the antibody staining was restricted to the periphery of the cell. These data support the biochemical studies suggesting an envelope location of the IrpA protein.

Restriction mapping of *irpA* gene region. Restriction mapping of the *irpA* gene region in the chromosome was approached with two types of probes: (i) Southern analysis of the chromosomal DNA with inserts from λgtAN26 and λgtAN103, and (ii) larger *Sal*I fragments from the λEMBL-3 recombinants (λEM127 and λEM130) (Fig. 3). Southern blots of the chromosomal DNA with different DNA probes are shown in Fig. 3. The 1.7-kb *Eco*RI fragment from λgtAN26 hybridized to a unique band in many enzyme digestions. The probe identified two 1.7- and 1.5-kb *Pst*I bands, a 6.0-kb *Sal*I band, and a 12.5-kb *Sst*I fragment. When the 1.3-kb *Eco*RI fragment from λgtAN103 was used as a probe, the Southern blot revealed a single band in *Pst*I, *Acc*I, and *Sal*I digestions. Enzymes such as *Bgl*I and *Pvu*II showed hybridization to two bands (Fig. 3).

To retrieve large fragments of the *Synechococcus* sp. strain PCC7942 chromosome containing the *irpA* gene, we screened the λEMBL-3 library by probing with the 3.1-kb DNA fragment from λgtAN105. DNAs from four positive λEMBL-3 clones were digested with *Sal*I to release chromosomal fragments; all four clones retained chromosomal DNA ranging in size from 10 to 14 kb. Three DNA probes from two λEMBL-3 recombinants were used to map the entire *irpA* region. The 6.0- and 4.5-kb *Sal*I fragments from λEM130 and the 3.5-kb *Sal*I fragment from λEM127 were used individually and in combination to probe chromosomal DNA digested with *Sal*I, *Bam*HI, and *Hin*dIII (Fig. 3C). These Southern blots allowed us to map a 20-kb region on the *Synechococcus* sp. strain PCC7942 chromosome. Figure 4A shows the location of the *irpA* gene region including various portions represented in the λgt11 and λEMBL-3 recombinants.

DNA sequence analysis of *irpA* gene and flanking regions. The lysogen of λgtAN26 was tested for the production of immunopositive proteins in *E. coli*. The antibody reacted to a single polypeptide with a molecular mass of about 145 kDa which indicated that the 1.7-kb *Eco*RI fragment expressed a portion of the 36-kDa protein-coding sequence as a fusion protein. To obtain the 5′ terminus of the missing sequence, we rescreened the λgt11 library using the 1.7-kb fragment from λgtAN26 as a probe. Twelve positive clones were picked, and DNA was extracted from four of them. Figure 4A shows the DNA overlaps relative to λgtAN26 and the additional 5′ and 3′ sequences. One of the clones, λgtAN105, had an insert with 600 bp at the 5′ end which was not represented in λgtAN26. Three of the clones (λgtAN104, λgtAN105, and λgtAN110) also retained the 1.7-kb internal *Pst*I fragment.

The 1.7-kb *Eco*RI fragment of λgtAN26 was subcloned into the *Eco*RI site of M13mp18 and designated as M13mp18-1.7. λgtAN103 contained the additional 600 bp of the 5′ end of the gene, and the 1.3-kb insert from λgtAN103 was

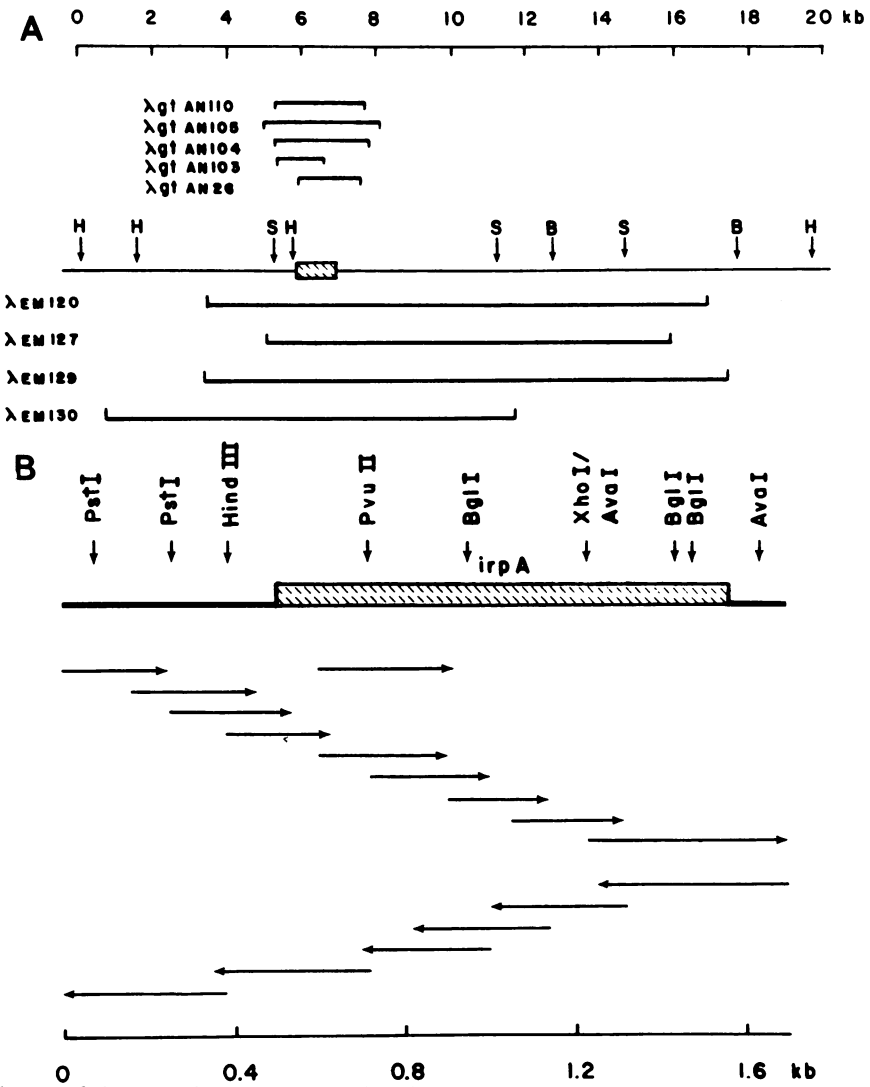

FIG. 4. (A) Physical map of the *Synechococcus* sp. strain PCC7942 chromosome in the vicinity of the *irpA* gene. The portions of chromosomal DNA represented in various λgt11 and λEMBL-3 recombinant phages are indicated (Table 1). Cleavage sites of three restriction enzymes (*Hin*dIII [H], *Sal*I [S], and *Bam*HI [B]) are shown. (B) Nucleotide sequencing strategy used to sequence the *irpA* gene and its flanking regions. Clones used for sequencing were obtained from the *Bal* 31 ordered deletions procedure, and the sequenced region of each clone is shown below the map. The positions of restriction sites are indicated.

cloned into the *Eco*RI site of M13mp18 in both orientations. These recombinants were designated M13mp18.103 and M13-mp18.103R. The same fragment was also cloned into the Bluescribe plasmid vector and designated pBSP103 and pBSP103R. The 2.4-kb insert from λgtAN110 was also cloned into the *Eco*RI site of pUC19 and designated as pKJ110. Plasmids pBSP103 and pKJ110 were subsequently used to create ordered *Bal* 31 deletions.

The plasmid pBSP103 was linearized by *Sph*I digestion within the multiple cloning region that is outside the insert and treated with *Bal* 31. The R2 insert with *Bal* 31 deletions was released by *Eco*RI and force cloned into the *Sma*I-*Eco*RI site of M13mp18. A similar strategy was used to create deletions from the opposite side in pBSP103R for obtaining the sequence of the noncoding strand (Fig. 4B).

M13 clones carrying deletions on either side of the insert were sequenced with a universal primer. The DNA sequencing strategy of various M13 clones is shown in Fig. 4B, and both strands of the gene were completely sequenced. The DNA sequence (Fig. 5) shows a 1,068-bp open reading frame with a 300-bp 5′ and 200-bp 3′ sequence. This open reading frame is capable of coding for a protein of 356 amino acids with a predicted molecular mass of 38,584 Da. The stop codon TAG was immediately followed by two more stop codons in another reading frame. The 5′ terminus of the *irpA* gene contains a 9-bp inverted repeat about 200 bp upstream from the predicted start codon, and the inverted repeats are separated by a 9-bp A+T-rich segment (Fig. 6).

Analysis of derived amino acid sequence. The derived amino acid sequence of the *irpA* gene was analyzed with the aid of the Dnastar protein analysis programs. The protein contained 142 hydrophobic and 107 polar amino acids, yielding an approximate derived isoelectric point of 4.87. Several repeats of three or four amino acids were found within the sequence; Val-Ala-Ala-Gly and Gln-Gly-Leu-Val were each repeated twice. Similarly, Pro-Thr-Leu was also repeated twice. The hydropathic index of the protein revealed some interesting features, as the amino terminus

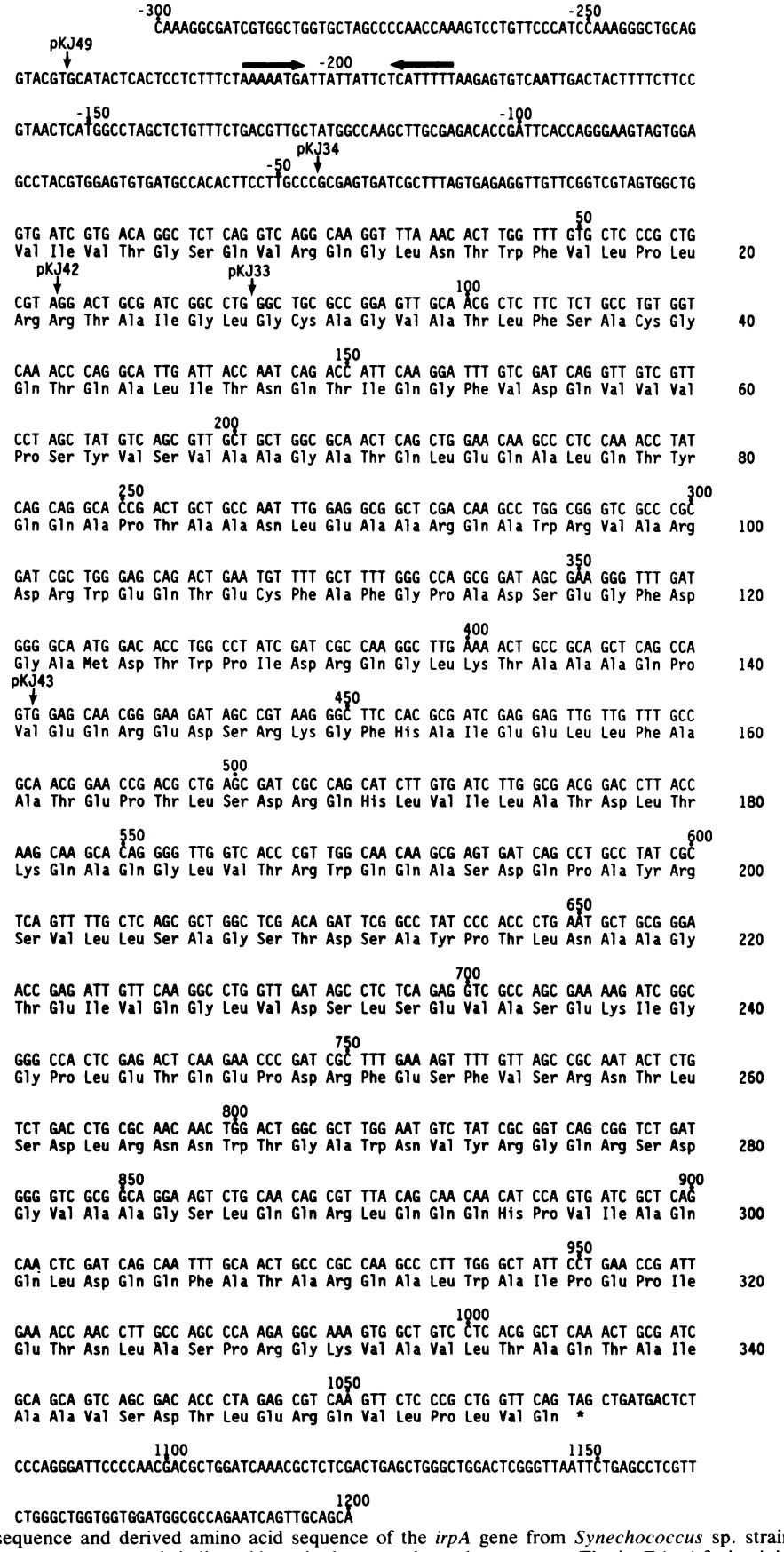

FIG. 5. Nucleotide sequence and derived amino acid sequence of the *irpA* gene from *Synechococcus* sp. strain PCC7942. The dyad symmetry found in the upstream sequence is indicated by paired arrows above the sequence. The *lacZ-irpA* fusion joints of various plasmids are indicated.

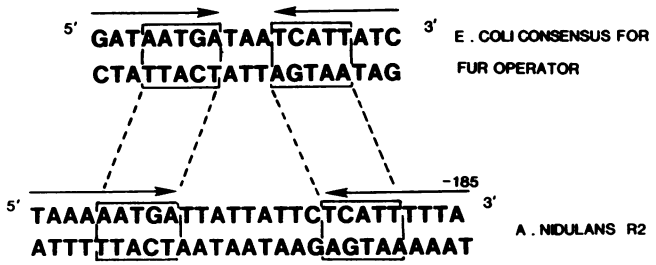

FIG. 6. Sequence comparison between the iron-regulated *fur* operator of *E. coli* and the 5′ upstream region of the *Synechococcus* sp. strain PCC7942 *irpA* gene. The *Synechococcus* sp. strain PCC7942 sequence is 185 nucleotides away from the presumptive valine start codon.

through residue 44 had characteristics typical of a signal sequence. In *Synechococcus* sp. strain PCC7942, one of the thylakoid lumenal proteins involved in water oxidation has been shown to possess a signal sequence of 28 amino acids (20). When the presumptive signal sequence was excluded from the IrpA protein, the remaining sequence showed bilateral symmetry in the hydropathy plot. The two ends of the protein were hydrophobic, and amino acid sequences from 75 to 145 and 240 to 310 were hydrophilic in nature (Fig. 7).

Characterization of Tn*5* insertion into K7. DNA was prepared from *Synechococcus* sp. strain PCC7942 K7, digested with restriction endonucleases, and transferred to nitrocellulose. Probing these blots with a 1.3-kb fragment containing the *irpA* gene revealed that Tn*5* was inserted into the correct site in the *Synechococcus* sp. strain PCC7942 genome (Fig. 8). Previous studies have shown that *irpA* resides on a 24-kb *Eco*RI fragment and that Tn*5* lacks *Eco*RI sites (19); the Southern blot of K7 DNA revealed that *irpA* was carried on a larger *Eco*RI fragment than in the wild-type strain (Fig. 8). Furthermore, knowing both the position of *Ava*I sites 500 bp from the ends of Tn*5* and the restriction map of the *irpA* region (19), *Pst*I-*Ava*I double digests confirm the position of Tn*5* 100 bp upstream of the *Ava*I site in *irpA*. Insertion of Tn*5* yields two fragments of 1,300 and 600 bp which are

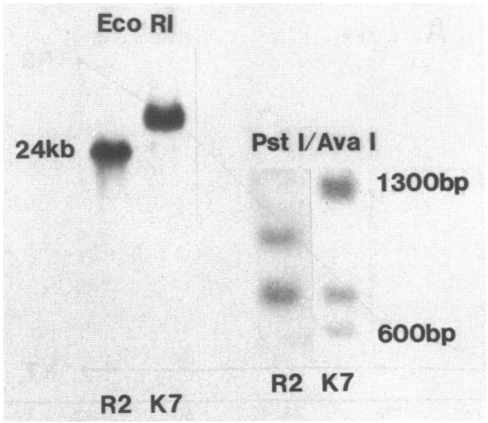

FIG. 8. Analysis of the Tn*5* insertion in *Synechococcus* sp. strain PCC7942. Left panel, Southern blots of *Eco*RI digests of *Synechococcus* sp. strain PCC7942 (R2) and K7 DNA were probed with the *Eco*RI fragment of λgtAN103; right panel, λgtAN103 was used to probe *Pst*I-*Ava*I double digests of wild-type (R2) and K7 DNA.

absent in the wild-type strain (Fig. 8). From these studies, it is evident that the *irpA*::Tn*5* insertion constructed in *E. coli* has replaced the *irpA* gene by homologous recombination following transformation into *Synechococcus* sp. strain PCC7942.

Growth characteristics of *Synechococcus* sp. strain PCC7942 K7 mutant. K7 was chosen from the Km^r population of *Synechococcus* sp. strain PCC7942 transformants because K7 grew very poorly in iron-depleted BG-11 medium. Whereas the wild type (R2) could be continuously subcultured in iron-deficient BG-11, K7 could not grow after two subcultures in this medium (Fig. 9A). Furthermore, BG-11-grown K7 exhibited increased sensitivity to the iron-chelating agent 2,2′-dipyridyl (Fig. 9B). Such compounds can be used to induce iron stress in media containing iron. Addition of dipyridyl to 300 μM rapidly killed log-phase K7 cultures, but the wild-type strain survived this treatment. Dipyridyl, however, inhibited growth of the wild-type strain; presumably this is due to secondary toxic effects of the agent (Fig. 9B).

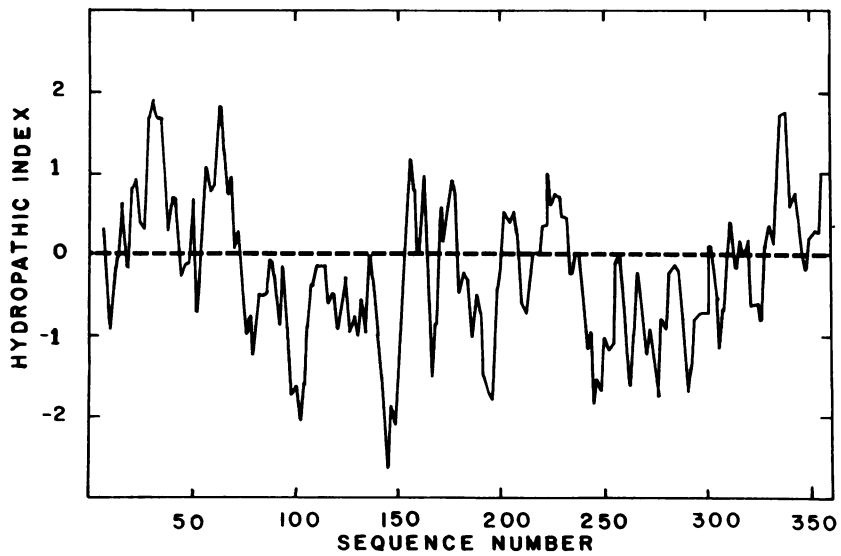

FIG. 7. Hydropathy profile of the IrpA protein determined by the method of Kyte and Doolittle (21), using a window of 11 amino acid residues.

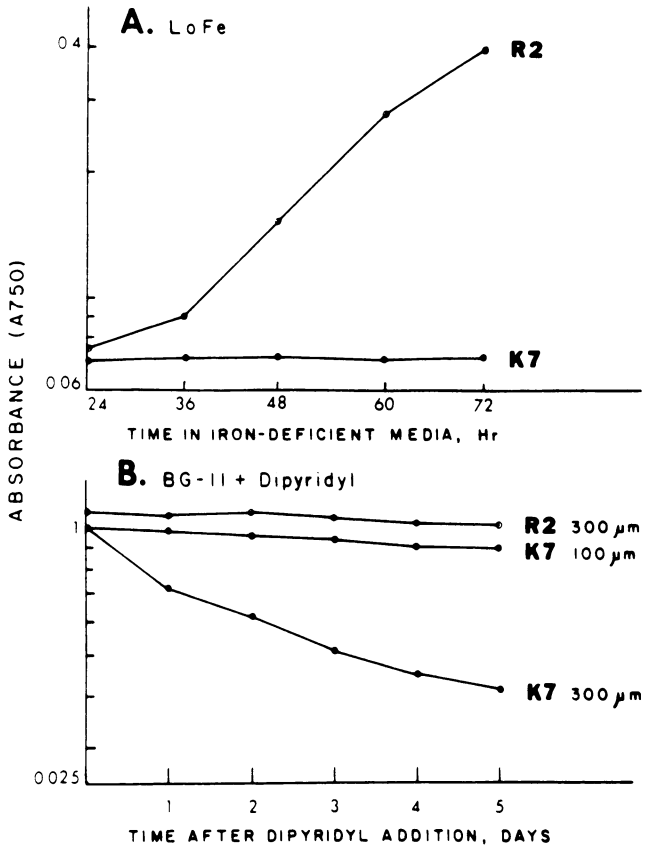

FIG. 9. (A) Growth characteristics of *Synechococcus* sp. strain PCC7942 and the *irpA*::Tn*5* derivative, K7. *Synechococcus* sp. strain PCC7942 (R2) and K7 cells grown in iron-containing medium were diluted 1:10,000 into iron-deficient BG-11 medium, subcultured once more at a 1:1,000 dilution, and monitored for growth by light scattering at 750 nm. (B) Sensitivity of *Synechococcus* sp. strain PCC7942 (R2) and K7 to 2,2'-dipyridyl. Log-phase cells grown in iron-containing medium were treated with dipyridyl at the indicated concentrations. Note that dipyridyl inhibits growth of the wild-type cells; however, only the K7 culture is killed at the 300 μM concentration.

Spectroscopic studies. Dipyridyl-treated *Synechococcus* sp. strain PCC7942 and K7 cultures were examined for spectral characteristics diagnostic for iron stress. As mentioned in the Introduction, low-iron-grown *Synechococcus* sp. strain PCC7942 exhibits altered absorbance spectra; i.e., the long-wavelength absorbance peak is blue shifted 5 to 7 nm (14), compared with spectra of BG-11-grown cells. Additionally, the low-temperature (77 K) chlorophyll fluorescence emission spectrum of iron-starved *Synechococcus* sp. strain PCC7942 yields a major emission peak at 682 to 684 nm which is absent in cells provided with sufficient iron (14, 28); similarly, the iron-deficient cells are missing the main 716-nm fluorescence peak. *Synechococcus* sp. strain PCC7942 and K7 have identical spectra when grown in BG-11 medium, but when absorbance and fluorescence emission spectra were obtained from K7 and wild-type (R2) cultures treated with dipyridyl, only K7 exhibited the signals associated with iron starvation (Fig. 10). The absorbance spectrum of K7 was blue shifted 6 nm compared with that of *Synechococcus* sp. strain PCC7942 (Fig. 10) after dipyridyl treatment for 20 h. In contrast, the wild-type spectrum was identical to that of BG-11-grown cells, which have an absorbance maximum at 680 to 681 nm (14). The 77 K fluorescence emission spectrum of K7 resembled closely the spectrum of low-iron-grown cells (14, 28), whereas R2 exhibited the 715-nm peak (Fig. 10) attributed to photosystem I (24). Last, these effects of dipyridyl on K7 occurred at 100 μM, whereas the spectra of wild-type cells were unaffected at 300 μM dipyridyl.

DISCUSSION

In this study, we identified an iron-regulated gene from the cyanobacterium *Synechococcus* sp. strain PCC7942. The *irpA* gene product is a 36-kDa envelope membrane protein which is induced under iron-limited conditions and which disappears in cells recovering from iron starvation. We base our contention in regard to the regulation of *irpA* on the sequence of the promoter region and on the properties of the mutant K7. The *irpA* DNA sequence and its derived amino acid sequence were used in a search for homologous sequences in the Genbank and PIR (Protein Identification Resource) data bases. There were no sequences with significant sequence homology at either the nucleic acid or amino

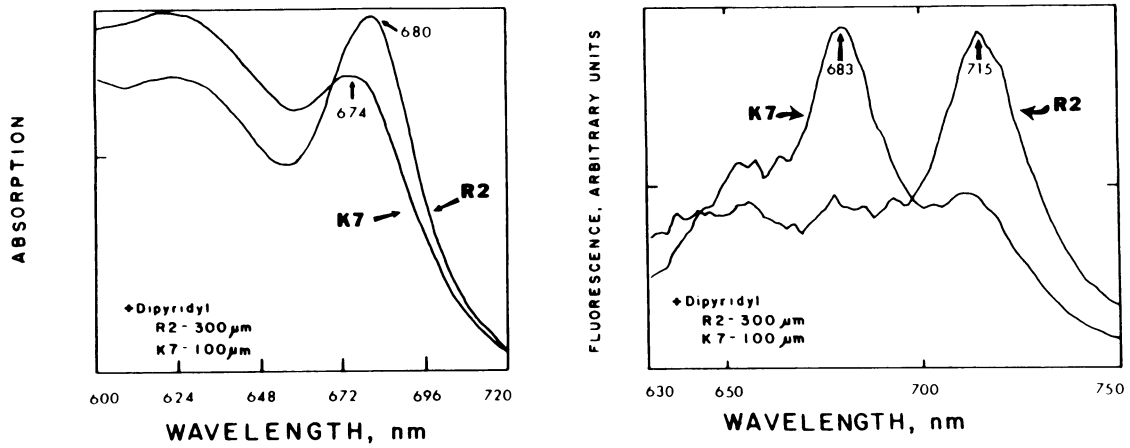

FIG. 10. Spectroscopic properties of dipyridyl-treated *Synechococcus* sp. strain PCC7942 (R2) and K7. For these experiments, the *Synechococcus* sp. strain PCC7942 culture was treated with 300 μM dipyridyl and the K7 culture contained 100 μM dipyridyl. Left panel: Absorption spectra of the R2 and K7 cultures; right panel: low-temperature (77 K) fluorescence emission spectra of dipyridyl-treated R2 and K7.

acid level. However, there was an interesting similarity between a sequence 200 bp upstream from the *irpA* gene (Fig. 6) and an *E. coli* consensus sequence in the aerobactin operon (9). This operon is negatively regulated by a Fur repressor protein (3), and the Fur-binding site was recently determined to be within an inverted repeat (9). The 9-bp inverted repeat 5′ to the *irpA* coding sequence contains TCATT, which is identical to the *E. coli* consensus sequence. The major difference between the *E. coli* and *Synechococcus* sp. strain PCC7942 sequences was the length of DNA separating the dyads. In addition, transposon-induced mutagenesis of *irpA* by Tn*5* and the subsequent transformation of the mutated gene back into the *Synechococcus* sp. strain PCC7942 chromosome yielded a mutant strain that cannot grow in low-iron media. These findings suggest strongly that the *irpA* gene product is an essential protein required for growth of *Synechococcus* sp. strain PCC7942 under iron-deficient conditions.

The hydropathy plot of the 36-kDa protein indicates the possibility of a 44-amino-acid signal sequence and two transmembrane helices. The structural features of the amino-terminal portion indicate a charged segment followed by a hydrophobic core. In general, Pro or Gly residues are expected to be present within the hydrophobic core to destabilize the helix (26); indeed, four glycine residues are present within the predicted hydrophobic core of the presumptive signal. The processing site is predicted to be between Ala-44 and Leu-45 by comparison with some known processing sites (26). The position of Ala at the −1 location has been found in many proteins carrying signal sequences (41). The first signal sequence identified in *Synechococcus* sp. strain PCC7942 was for the Mn-stabilizing protein (20), and this 28-amino-acid signal sequence contained helix-destabilizing residues Pro and Gly at the −3 and −5 positions. In the case of *irpA*, Gly residues are present at −5 and −14 positions from the predicted processing site.

The *irpA* open reading frame had two potential initiation codons upstream from the λgtAN26 fusion joint, a GTG at 96 bp and an ATG at 159 bp. The possibility of GTG acting as the start codon was based on the following reasons. (i) It was the first initiator codon in the open reading frame before the λgt11 fusion joint; (ii) a plasmid clone which produced the *lacZ-irpA* fusion protein and the original λgtAN26 fusion supported the same frame; and (iii) the other fusion plasmids in the same frame as ATG yielded no immunoreactive hybrid protein. The double-stranded DNA sequencing of *lacZ-irpA* fusion plasmids allowed us to check for potential sequencing errors and arrive at the most probable start codon in the *irpA* gene.

The Tn*5* insertion into *irpA* may exert polar effects on other genes, as DNA sequence analysis 3′ to *irpA* has revealed a putative ribosome-binding site and open reading frame (K. J. Reddy and L. A. Sherman, unpublished data). Since it is possible that *irpA* is the promoter-proximal gene in a large *irp* operon, we cannot determine whether *irpA* is the only gene affected by the mutation. Nevertheless, the behavior of the K7 mutation suggests that the *irpA* region encodes a protein(s) involved in iron transport. Our reasons for proposing this are twofold. First, the *irpA* polypeptide is likely a component of the gram-negative envelope, a location consistent with an uptake function. Second, addition of dipyridyl to K7 results in the cultures exhibiting the spectral characteristics diagnostic for the thylakoid reorganization events associated with iron starvation. This suggests that the pathway controlling iron-stress-mediated thylakoid organization is not altered in K7 but rather that K7 is impaired in the ability to compete with dipyridyl for iron. The overall result is the depletion of the intracellular pool of iron in dipyridyl-treated K7, resulting in the iron-stressed phenotype at a comparatively low dipyridyl concentration. To help test this hypothesis, we are currently investigating whether K7 is defective in siderophore synthesis or uptake or both.

A feature of the *irpA* gene which has attracted our attention is the structural similarity of the 5′ upstream region of *irpA* to the iron-regulated operator of *E. coli* (Fig. 6); this is the sequence which is recognized by the Fur repressor-Fe(II) complex (3, 9). This region of homology may represent a conserved sequence involved in iron regulation in bacteria. We will soon begin functional studies to examine whether this sequence confers iron-regulated negative control of transcription on a reporter gene. If so, this will be the first report of an operator sequence in a cyanobacterium. The large amount of data obtained from the *E. coli* Fe(III) uptake system will present a useful base of information to guide future studies of iron metabolism in cyanobacteria.

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