IdiA, a 34 kDa protein in the cyanobacteria Synechococcus sp. strains PCC 6301 and PCC 7942, is required for growth under iron and manganese limitations

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In the cyanobacteria Synechococcus PCC 6301 and PCC 7942 a protein with an apparent molecular mass of about 34 kDa (called IdiA for iron-deficiency-induced protein A) accumulates under iron and manganese limitation. IdiA from Synechococcus PCC 6301 was partially sequenced, showing that the N-terminal amino acid is an alanine. Moreover, the gene encoding this protein in Synechococcus PCC 6301 has been identified and completely sequenced. The idiA gene codes for a protein starting with valine and consisting of 330 amino acid residues. Thus, IdiA is apparently synthesized as a precursor protein of 36-17 kDa and cleaved to its mature form of 35.01 kDa between two alanine residues at positions 9 and 10. IdiA is a highly basic protein having an isoelectric point of 10.55 (mature protein). Comparison of the amino acid sequence of IdiA with protein sequences in the database revealed that IdiA has similarities to two basic bacterial iron-binding proteins, SfuA from Serratia marcescens and Fbp from Neisseria gonorrhoeae. Insertional inactivation of the idiA gene in Synechococcus PCC 7942 resulted in a mutant which was unable to grow under iron- or manganese-limiting conditions. Manganese limitation of the mutant strain led to a drastic reduction of photosystem II activity (O2 evolution) within less than 48 h, while wild-type cells required a prolonged cultivation in Mn-deficient medium before an effect on photosystem II was observed. Thus, IdiA is a protein involved in the process of providing photosystem II with manganese.

**Keywords:** Synechococcus PCC 6301 and PCC 7942, IdiA, idiA gene, iron and manganese limitations, photosystem I and II

**INTRODUCTION**

Iron is an essential redox component in a number of enzymes and in protein complexes of respiratory and photosynthetic electron transport. Although iron is the fourth most abundant element by weight in the earth’s crust, its biological availability is strongly reduced because at physiological pH Fe3+ forms insoluble hydroxides. This limitation in iron availability has led to several strategies for iron acquisition involving iron-binding molecules (siderophores) and the capability of storing iron during times of relative abundance. As Straus (1994) recently stated, the responses of many micro-organisms, including cyanobacteria, to limited iron availability can be divided into three categories: retrenchment (alterations and reduction in cellular structures and physiological activities), compensation (production of new proteins) and acquisition (enhancement of ability to scavenge iron from the environment) (see recent reviews by Straus, 1994; Ferreira & Straus, 1994; Carr & Mann, 1994).

Iron deficiency causes substantial changes in the ultrastructure of thylakoid membranes including a decrease of phycobilisomes, as well as of the chlorophyll protein complexes of PS I and PS II in Synechococcus PCC 7942 (Guikema & Sherman, 1984; Pakrasi et al., 1985a, b). Parallel to the reduction of the major chlorophyll protein

**Abbreviations:** Ap, ampicillin; Chl, chlorophyll; IdiA, iron deficiency induced protein A; Km, kanamycin; MSP, manganese stabilizing protein; PS I, photosystem I; PS II, photosystem II.

The GenBank accession number for the sequence reported in this paper is Z48754.
complexes, a substantial increase in a new chlorophyll protein complex called CPVI-4 was observed. This complex, which copurifies with PS II, consists of polypeptides of 36, 34 and 12 kDa, and is the major pigment-protein complex in *Synechococcus* PCC 7942 cells under conditions of iron limitation (Riehman & Sherman, 1988a, b). It has been suggested that CPVI-4 may function as an auxiliary light-harvesting complex that compensates for the loss of phycobilisomes during stress and may also function as a chlorophyll reservoir that contributes to the assembly of reaction centre complexes in the early stages of recovery from iron deficiency (Riehman & Sherman, 1988b). Further investigations with *Synechococcus* PCC 7942 resulted in the identification of two iron-regulated genes: *irp*-A, encoding a protein located in the cytoplasmic membrane and presumably involved in iron acquisition or storage (Reddy et al., 1988); and *map*-A, encoding a protein of unknown function (Webb et al., 1994). Irp-A was shown to be essential for growth under Fe-deficient conditions, while Map-A was shown to be not essential. In the marine cyanobacterium *Synechococcus* PCC 7002 an iron-repressible operon, *isi*AB, was identified that contains a gene predicted to encode a chlorophyll-binding protein and a flavodoxin (Leonhardt & Strauss, 1992). IsiA was also shown to be expressed under iron deficiency in the freshwater cyanobacterium *Synechococcus* PCC 7942 (the calculated molecular mass of the gene product was given as 36/8 kDa) (Laudenbach & Strauss, 1988) and in the nitrogen-fixing cyanobacterium *Anaabaena* sp. PCC 7120 (Leonhardt & Strauss, 1994). Insertional inactivation of the *isi*AB operon in *Synechococcus* PCC 7942 resulted in loss of CPVI-4 expression, implying that *isi*A encodes a component of the CPVI-4 complex (Burnap et al., 1993).

Although iron uptake has been extensively studied, especially in *Escherichia coli* (Braun & Hanke, 1991; Braun et al., 1987) and to some extent also in cyanobacteria (Straus, 1994), very little information exists in the literature about storage and transport of iron within the cyanobacterial cell and about Fe exchange between Fe storage proteins and the 'functional' Fe proteins/enzymes. Storage and buffering of iron in cells is mainly achieved by ferritins, which are widely distributed. A recent report (Laulhere et al., 1992) has shown their presence in the cyanobacterium *Synechocystis* PCC 6803 together with a pool of low-molecular-mass acidic proteins involved in Fe binding.

We have previously isolated a 34 kDa polypeptide from *Synechococcus* PCC 6301, which is closely related to *Synechococcus* PCC 7942 (Golden et al., 1989). After raising a polyclonal antiserum against this protein, it was shown that in *Synechococcus* PCC 6301 the 34 kDa protein is only weakly expressed under iron-sufficient conditions, but its expression is strongly enhanced under iron-deficient conditions (Michel & Pistorius, 1992). In the present work we have extended our investigations of the conditions leading to expression of this 34 kDa protein (called IdiA for iron deficiency induced protein A) and also investigated its location in the cell. Partial sequencing of IdiA from *Synechococcus* PCC 6301 allowed isolation and DNA sequencing of the gene encoding IdiA (*idi*A) and construction of an IdiA mutant of *Synechococcus* PCC 7942.

**METHODS**

**Bacterial strains and growth conditions.** *Synechococcus* sp. strain PCC 6301 (SAUG B 1402-1) and *Synechococcus* sp. strain PCC 7942 were obtained from the Sammlung von Algenkulturen der Universität Göttingen, Göttingen, Germany, and from the Pasteur Culture Collection of Cyanobacterial Strains, Paris, France, respectively.

For regular growth conditions, cyanobacteria were grown in gas wash bottles containing 250 ml medium in a stream of 2% (v/v) CO₂ in air (inoculum 50 μl cells). *Synechococcus* PCC 6301 was grown in the medium of Kratz & Myers (1955) with slight modifications as given by Pistorius et al. (1989) at 30 °C, and *Synechococcus* PCC 7942 was grown in BG11 medium (Rippka, 1988) at 30 °C. When cells were grown with ammonium as nitrogen source, nitrate was omitted and 7.5 mM NH₄Cl and 50 mM HEPES/NaOH, pH 7.6, were added to the medium. In micro-element solution Co(NO₃)₂ was replaced by CoSO₄. Growth of both *Synechococcus* strains on agar plates was in BG11 medium containing 1% (w/v) agar (Gibco BRL). Cells were cultivated under Fe-sufficient conditions (Kratz & Myers medium: 16 μM FeCl₃ and 14 μM FeSO₄; BG11 medium: 30 μM Fe-ferricate) or Fe-deficient conditions (the medium contained one tenth of the regular Fe concentration or no Fe, as indicated in the legends to the figures). Other stress conditions were achieved by the following modifications: Mg²⁺/Ca²⁺ deficiency: the medium contained one tenth of the regular MgCl₂ concentration and one half of the regular CaCl₂ concentration (Wälzlneik & Pistorius, 1991); Mn deficiency: the medium contained no manganese; nitrogen deficiency: the medium contained no nitrogen source; salt stress: the medium contained 0.68 M NaCl; heat stress: cells were grown in the regular medium for 2 d at 40 °C or for 2 d at 42 °C or 6 h at 45 °C after 2 d of regular growth at 30 °C. Where indicated, antibiotics were added at the following concentrations: 10 mg ampicillin l⁻¹ or 25 mg kanamycin l⁻¹.

Cyanobacterial growth was either determined as μ packed cell volume or as increase in optical density at 750 nm. *Escherichia coli* DH5α MCR (Grant et al., 1990) was cultivated at 37 °C in LB medium (Miller, 1972) or on PA plates containing, in 1 l distilled water, 17·5 g antibiotic medium no. 3 (assay broth, Oxoid) and 16·0 g bacteriological agar (Oxoid). Where indicated, antibiotics were added at the following concentrations: 150 mg ampicillin l⁻¹ and 50 mg kanamycin l⁻¹.

**Preparation of French press extracts, isolation of membranes, periplasm and spheroplast fraction.** Cells were grown for 2 d under regular growth conditions (nitrate as nitrogen source, cultivated in gas wash bottles containing 250 ml medium, see above), harvested by centrifugation, washed once either with phosphate buffer or with sucrose-containing medium (see below) and then resuspended to give a cell density of 100 μ cells ml⁻¹. Subsequently the cells were broken by treatment in a French press at 138 MPa. Unbroken cells were removed by centrifugation for 5 min at 4000 g (Sorvall SS34), and the supernatant was referred to as French press extract. When photosynthetic activities were measured, the cells were washed with 50 mM HEPES/NaOH, pH 6.3, containing 50 mM CaCl₂ and 400 mM sucrose, and resuspended in the same medium. Otherwise the cells were washed and resuspended in 10 mM sodium phosphate buffer, pH 7.0.

Isolation of the outer cell membrane, cytoplasmic membrane and thylakoid membrane of *Synechococcus* PCC 6301 was ac-
According to Omata & Murata (1984), and extraction of the soluble protein fraction from the periplasmic space and isolation of the spheroplast fraction was according to Block & Grossman (1988). The spheroplast fraction separated from the periplasmic fraction by centrifugation was broken by a French press treatment for the experiments shown in Fig. 2.

**Isolation of IdiA from Synechococcus PCC 6301.** IdiA used for sequencing was isolated as described previously (Michel & Pistorius, 1992) with minor modifications. As starting material, the thylakoid membrane fraction was used, which was obtained from Fe-starved Synechococcus PCC 6301 cells grown in medium containing one tenth of the regular Fe concentration (harvested after the second transfer into Fe-deficient medium). IdiA was solubilized from thylakoid membrane preparations by treatment with 1% N-dodecyl-N,N-dimethylammonio-3-propanesulphonate for 1 h on ice. Other conditions were as described by Michel & Pistorius (1992). Subsequently IdiA was purified on a DEAE-Sephacel column and on a MonoS column coupled to FPLC (Pharmacia) as described previously (Michel & Pistorius, 1992).

Since IdiA in *Synechococcus PCC 6301* is present in the membrane fraction as well as in the soluble protein fraction of total French press extracts, IdiA was also isolated from the latter fraction to allow comparison of the N-terminal amino acid sequence. For this purpose *Synechococcus PCC 6301* cells were grown for 2 d in Fe-deficient medium, harvested by centrifugation, washed and resuspended in 10 mM sodium phosphate buffer, pH 7.0. Cells were broken by treatment in a French press at 138 MPa (twice) and the membrane fraction was subsequently removed by centrifugation for 3 h at 160000 g (Beckman, Ti60). The soluble protein fraction obtained was dialysed against 25 mM Tris/HCl, pH 8.2, containing 10 mM NaCl and chromatographed on a DEAE Sephacel column. Elution of IdiA was achieved with a linear gradient of 10-500 mM NaCl in the equilibration buffer. After dialysing the fractions containing IdiA (detected by immunoblotting) against 25 mM MES/NaOH, pH 6.0, containing 10 mM NaCl, the sample was chromatographed on a MonoS column coupled to FPLC (Pharmacia). Elution of IdiA was achieved with a linear gradient of 10-500 mM NaCl in the equilibration buffer. IdiA eluted from this column at an NaCl concentration of 250 mM.

**Measurements of PS I and PS II activities.** PS I and PS II activities were determined by O2 exchange in French press extracts using a Clark-type electrode as described previously (Michel & Pistorius, 1992). For PS I measurements the reaction mixture contained, in a total volume of 1.9 ml, 54 mM HEPES/NaOH, pH 7.0, 0.4 mM KCN, 0.17 mM methylviologen, 3.3 mM sodium ascorbate, 80 mM 2,6-dichlorophenol-indophenol, 10 mM 3-(3,4-dichlorophenyl)-1,1-dimethyleane and French press extract corresponding to 10 μg Chl. For PS II measurements the reaction mixture contained, in a total volume of 1.9 ml, 54 mM HEPES/NaOH, pH 7.0, 33 mM CaCl₂, 167 mM sucrose, 1.7 mM potassium ferricyanide and French press extract corresponding to 10 μg Chl.

**Protein determination, SDS-PAGE and immunoblotting.** Protein was determined according to Smith et al. (1985). SDS-PAGE was performed according to Laemmli (1970) or Schägger & von Jagow (1987). Protein samples for SDS-PAGE were denatured either at 70 °C for 15 min (Laemmli system) or at 100 °C for 5 min (Schägger & von Jagow system). Immunoblotting was done as described by Michel & Pistorius (1992) by transferring proteins to nitrocellulose (Schleicher and Schüll, BA85) and with the following previously raised antibodies: anti-IdiA antisera (Michel & Pistorius, 1992), anti-D1 and anti-MSP antisera (Engels et al., 1992). As the second antiseraum, peroxidase-coupled anti-rabbit immunoglobulins/HRP (DAKO Chemicals; dilution 1:500), was used.

**Protein sequencing.** For N-terminal sequencing, IdiA was concentrated in a Centricon 3 tube (Amicon) at 4 °C (coated with 0.1% Tween 20; Pierce, sequencing grade). Subsequently, 0.2 nmol of the protein were sequenced on a pulsed liquid phase sequenator model 477 A with HPLC system 120A (Applied Biosystems) as described by Bökenkamp et al. (1994). To obtain additional endogenous amino acid sequences, IdiA was treated with endopeptidase Lys-C (Achromobacter lyticus, Wako Chemicals) or with endopeptidase Glu C (Staphylococcus aureus V8, sequencing grade, Bohringer Mannheim). For Lys-C cleavage, 2.75 μg Lys-C and 55 μg IdiA were incubated in 50 μl 0.1 M Tris/HCl, pH 9.0, 1 mM EDTA and 0.1% Triton X-100 (hydrogenated, Calbiochem). After incubation for 15 h at 30 °C, 400 μl 10 mM Tris/HCl, pH 8.0, containing 6 M guanidino-hydrochloride, 6 μl 88% (v/v) formic acid and 2.5 μl 1 M dithiothreitol were added, and the sample was chromatographed on an Aquapore PR300 (C8) reverse-phase column (30 x 21 mm, Brownlee/Applied Biosystems) using a microbore 140A HPLC system (Applied Biosystems) with on-line UV detection at 215 nm. Peptides were separated at 100 μl min⁻¹ using (argon-purged) solvents A (0.1% trifluoroacetic acid in H₂O) and B (60% acetonitrile and 20% 2-propano and 0.085% trifluoroacetic acid in H₂O) as follows: linear gradient 1-10% B for 1 min, linear gradient 10-20% B for 9 min, linear gradient 20-50% B for 45 min, linear gradient 50-80% B for 15 min, linear gradient 80-99% B for 5 min, and finally 99% B for 10 min. Peptides were sample manually, frozen and stored at -80 °C. For Glu-C cleavage, 1 nmol IdiA protein was buffer-exchanged with 100 mM (NH₄)₂CO₃, pH 7.8, and concentrated to 50 μl in a Centricon 3. The sample was made 0.1% in SDS and heated to 95 °C for 5 min. Endopeptidase Glu-C was added at a ratio of 1:20 (Glu-C sample), and the sample was digested at room temperature for 2 hr. SDS-PAGE and immunoblotting to ProBlott PVDF membranes, as well as sequencing from the membranes were performed as described by Bökenkamp et al. (1994).

**Oligonucleotide synthesis.** The following oligonucleotide was designed on the basis of a partial amino acid sequence of IdiA from the N-terminal region utilizing a codon usage dictionary of *Synechococcus PCC 6301* (1989) with on-line UV detection at 215 nm. Peptides were separated at 100 μl min⁻¹ using (argon-purged) solvents A (0.1% trifluoroacetic acid in H₂O) and B (60% acetonitrile and 20% 2-propano and 0.085% trifluoroacetic acid in H₂O) as follows: linear gradient 1-10% B for 1 min, linear gradient 10-20% B for 9 min, linear gradient 20-50% B for 45 min, linear gradient 50-80% B for 15 min, linear gradient 80-99% B for 5 min, and finally 99% B for 10 min. Peptides were sample manually, frozen and stored at -80 °C. For Glu-C cleavage, 1 nmol IdiA protein was buffer-exchanged with 100 mM (NH₄)₂CO₃, pH 7.8, and concentrated to 50 μl in a Centricon 3. The sample was made 0.1% in SDS and heated to 95 °C for 5 min. Endopeptidase Glu-C was added at a ratio of 1:20 (Glu-C sample), and the sample was digested at room temperature for 2 hr. SDS-PAGE and immunoblotting to ProBlott PVDF membranes, as well as sequencing from the membranes were performed as described by Bökenkamp et al. (1994).

**DNA isolation, cloning, sequencing and analysis.** Plasmid pSVB30 was used in this study (Arnold & Pühler, 1988). The details of the pSVB30 derivatives constructed in this work are given below and in the legend to Fig. 5. Total DNA of *Synechococcus PCC 6301* was isolated by the Sarkosyl method and purified by the phenol extraction procedure (William, 1988; Sambrook et al., 1989). DNA was digested with HindIII and size fractionated on agarose gels. The fragments were recovered by using the "Jetsorb gel extraction kit" from Genomed. A 0.7 kb EcoRI PvuII fragment and a 2.2 kb BglII fragment of the 5.4 kb HindIII fragment were cloned into the linearized vector plasmid pSVB30 (Arnold & Pühler, 1988). Cloning procedures, Southern blotting, and other recombinant DNA methods were performed using established techniques (Sambrook et al., 1989). Subclones of the resulting hybrid.
plasmid were made by using the Pharmacia Double stranded nested deletion kit. Overlapping subclones were used for sequencing on an ALF Sequencer (Pharmacia). The enzymes used in this study were purchased from Boehringer, Bethesda Research Laboratories or Pharmacia. All reactions were performed following the recommendations of the corresponding manufacturer.

Nucleic acid sequences were analysed using the Staden Software package (Staden, 1986). Analysis of the deduced protein sequence was performed with the program 'PC/Gene' (Rel. 6.80, 1993, IntelliGenetics). Related protein sequences were searched in the SwissProt and GenBank databases using the 'BLAST' software (Altschul et al., 1990).

**Construction of an IdiA-free Synechococcus PCC 7942 mutant.** A 5.4 kb HindIII DNA fragment of Synechococcus PCC 6301 carrying the *idiA* gene cloned into vector plasmid pSVB30 (plasmid pKPM24) was chosen for gene interruption with a kanamycin resistance gene. A 2.2 kb BamHI fragment from plasmid pH1P50Km² (Fellay et al., 1987) carrying the kanamycin resistance gene was cloned into the unique BamHI site of the *idiA* gene 309 bp downstream from the putative starting codon. Insertion of the kanamycin resistance gene into the *idiA* gene resulted in two additional HindIII cleavage sites. This construction was transferred to *E. coli* DH5α MCR, and clones with the interrupted *idiA* gene were identified by screening for double resistance (Ap resistance encoded by the vector, Km resistance encoded by the Tn5 fragment) and subsequent restriction analysis. Detection was achieved with the synthetic oligonucleotide. The protocol for construction of the IdiA-free mutant of *Synechococcus* PCC 7942 was basically the same as described for construction of an MSP-free *Synechococcus* PCC 7942 mutant (Boeckholt et al., 1991).

**RESULTS AND DISCUSSION**

**Growth conditions leading to expression of IdiA**

For these experiments *Synechococcus* PCC 6301 was grown under regular growth conditions for 2 d, then washed with distilled water, and transferred into either a medium depleted of Fe or Mn, containing a reduced Mg²⁺/Ca²⁺ concentration, or containing ammonium instead of nitrate as nitrogen source, or no nitrogen source. The cells were also exposed to elevated temperatures or elevated NaCl concentrations (salt stress) (see Methods). After a growth period of 2 d or several 2 d periods under the respective conditions (see legend of Fig. 1), the cells were harvested, washed and resuspended in 10 mM sodium phosphate buffer, pH 7, broken by treatment in a French press, and the extract was submitted to SDS-PAGE and immunoblotting. IdiA was hardly detectable when cells were grown on nitrate under regular growth conditions (Fig. 1, lane A). These cells were taken from stock cultures growing slowly in test tubes under low light intensities. When these cells were grown over a prolonged period under regular experimental growth conditions, a small amount of IdiA was observed (lane B). The highest expression of IdiA was observed under iron deficiency (Fig. 1, lane C; see also Fig. 4 of Michel & Pistorius, 1992). Besides Fe deficiency, substantial accumulation of IdiA was obtained when manganese was omitted from the medium or when the Mg²⁺/Ca²⁺ concentration in the medium was reduced (lanes D, E, F). Growth of the cells under iron-sufficient conditions with ammonium replacing nitrate as nitrogen source also caused accumulation of IdiA (lane G), while no accumulation was observed under nitrogen-deficient conditions (not shown). Heat or salt stress did not, or only very slightly, increase the expression of IdiA (data not shown), indicating that IdiA is not a general heat or salt stress protein. The results indicate that IdiA mainly accumulates under iron limitation and other conditions leading to damage of PS II, such as manganese limitation (Mn is a cofactor of photosynthetic water oxidation; see Debus, 1992) or Mg²⁺/Ca²⁺ deficiency, which also leads to substantial reduction of PS II activity (Wälzlein & Pistorius, 1991). Small amounts of IdiA also accumulate in fast-growing cultures (with nitrate or ammonium as nitrogen source) under iron-sufficient conditions (Fig. 1, lanes B and G). This is most likely due to a kinetically limited Fe uptake from BG 11 medium in fast growing cultures resulting in a lowered internal level of Fe and thus causing partial derepression of *idiA*.

**Localization of IdiA in the Synechococcus PCC 6301 cell**

To obtain information about the localization of IdiA in the Fe-depleted cell, extensive subcellular fractionations were performed. The soluble periplasmic proteins were
IdiA protein in cyanobacteria

Investigation of the presence or absence of IdiA in the soluble protein fraction extracted from the periplasmic space of Synechococcus PCC 6301. Cells were grown for 2 d under Fe sufficient conditions (lanes A and B) and then cultivated for 2 d in a medium containing one tenth of the regular Fe concentration (lanes C and D) or lacking Fe (lanes E and F). Lanes A, C and E show the French press extracts of the spheroplast fraction (acting as controls to document IdiA accumulation in the cell) and lanes B, D and F show the corresponding soluble protein fractions extracted from the periplasmic space. Samples were submitted to SDS-PAGE (40 μg protein in French press extract and 20 μg protein in the protein fraction from periplasm) and, after transfer to nitrocellulose, immunostaining was performed with the anti-IdiA antiserum (dilution 1:4000).

IdiA was purified from thylakoid membrane preparations of Synechococcus PCC 6301 grown under Fe-deficient conditions. N-terminal sequencing yielded an amino acid sequence of 18 amino acid residues with alanine as the N-terminal amino acid residue (Table 1). In addition, IdiA was submitted to proteolysis with the endopeptidases Lys-C and Glu-C. The N-terminal amino acid sequence of these proteolytic fragments of IdiA are summarized in Table 1. N-terminal sequencing of IdiA isolated from the soluble protein fraction gave a contiguous amino acid sequence of 12 residues which was identical to that for the thylakoid-membrane-associated IdiA. Thus, both purifications gave a protein which starts with an alanine.

Characterization of the gene encoding IdiA from Synechococcus PCC 6301

The presence of a single copy of the putative idiA gene of Synechococcus PCC 6301 was detected using an oligonucleotide designed on the basis of an amino acid sequence from the N-terminal region of IdiA (see Methods) (Fig. 4). The oligonucleotide hybridized with a 5.4 kb HindIII fragment, which was isolated from a size-fractionated gene bank based on vector plasmid pSVB30. The physical map of this 5.4 kb fragment in plasmid pKPM24 is shown in Fig. 5(a).

Fragments of pKPM24 obtained by partial EcoRI/PstI and BglII digestion were subcloned and used for DNA sequence analysis (see Methods). The sequenced 2273 bp

Fig. 2. Investigation of the presence or absence of IdiA in the soluble protein fraction extracted from the periplasmic space of Synechococcus PCC 6301. Cells were grown for 2 d under Fe sufficient conditions (lanes A and B) and then cultivated for 2 d in a medium containing one tenth of the regular Fe concentration (lanes C and D) or lacking Fe (lanes E and F). Lanes A, C and E show the French press extracts of the spheroplast fraction (acting as controls to document IdiA accumulation in the cell) and lanes B, D and F show the corresponding soluble protein fractions extracted from the periplasmic space. Samples were submitted to SDS-PAGE (40 μg protein in French press extract and 20 μg protein in the protein fraction from periplasm) and, after transfer to nitrocellulose, immunostaining was performed with the anti-IdiA antiserum (dilution 1:4000).

Fig. 3. Subcellular fractionation for isolation of various membranes from Synechococcus PCC 6301 grown under Fe limiting conditions. Membranes from Synechococcus PCC 6301 cells grown for 2 d in medium without Fe were separated on a sucrose gradient according to the procedure described by Omata & Murata (1984). Lanes 1, cytoplasmic membrane; lanes 2, thylakoid membrane; lanes 3, phycobilisomes contaminated with thylakoid membranes; lanes 4, outer membrane. Samples were submitted to SDS-PAGE and, after transfer to nitrocellulose, immunostained with (a) the anti-IdiA antiserum (dilution 1:5000), (b) anti-D1 antiserum (dilution 1:2000) or (c) anti-MSP antiserum (dilution 1:2000).
Table 1. Partial amino acid sequence of several proteolytic peptides of IdiA isolated from thylakoid membrane preparations of *Synechococcus* PCC 6301 cells grown under iron-deficient conditions

Details of experimental procedures are given in Methods.

<table>
<thead>
<tr>
<th>N-terminal sequence of IdiA</th>
<th>AEGEVNLYSGHRHTDNQ</th>
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<td>VNL1EGEATALLLARL</td>
<td>MIAADGAATKEAWR</td>
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</tbody>
</table>

Fig. 4. Southern blot of total DNA from *Synechococcus* PCC 6301 digested with various endonucleases. Genomic DNA from *Synechococcus* PCC 6301 was digested with BamHI (lane A), EcoRI (lane B), HindIII (lane C), PstI (lane D), KpnI (lane E), XbaI (lane F) and Xhol (lane G). Detection of the idiA-carrying DNA fragment was performed with a DIG-labelled synthetic oligonucleotide synthesized according to the amino acid sequence of the N-terminal region of the isolated IdiA from *Synechococcus* PCC 6301.

IdiA has no typical prokaryotic signal sequence, although it is apparently synthesized as a precursor protein. The isoelectric point is 10.68 and 10.55 for the unprocessed and processed forms, respectively, which is consistent with the behaviour of IdiA during purification; it bound more tightly to cation- than to anion-exchange columns. IdiA is classified as a peripheral protein having no...
transmembrane helices according to the criteria of Kyte & Doolittle (1982), Klein et al. (1985) and Eisenberg et al. (1984), while one potential transmembrane helix is predicted (in position 195–210 of the unprocessed protein) according to Rhao & Argos (1986).

Analysis of the DNA sequence upstream of the idiA gene (Fig. 6) revealed two similarities with similarity to sequences upstream of irpA and the operon iriAB. The sequence 5'-cAgtcTGATACaTGTTc-3' between nucleotides -65 and -83 corresponds in 11 of 19 bases (nucleotides in capital letters) to a sequence upstream of the coding region of the irpA gene of Synechococcus PCC 7942 (Reddy et al., 1988) and the sequence 5'-AgTcgTcGgGCttTGT-3' between nucleotides -45 and -64 corresponds in 12 of 20 bases (nucleotides in capital letters) to a sequence upstream of the coding

Fig. 6. Complete nucleotide sequence of the idiA gene from Synechococcus PCC 6301 and its derived amino acid sequence and the nucleotide sequence of three additional ORFs: ORF 1, bp 38–208; ORF 2, bp 312–605; idiA, bp 723–1712; and ORF 3, bp 2148– (incomplete). The amino acid residues verified by sequencing the idiA protein are underlined. The position of the oligonucleotide binding site is double-underlined. The unique BamHI site in the idiA coding region used for cassette mutagenesis is boxed. Two sequences (bp -65 to -83 and bp -45 to -64) with homologies to upstream regions of iron-regulated cyanobacterial genes are also boxed.
region of the isiA operon (Laudenbach & Straus, 1988; Straus, 1994). This might imply that idiA, irpA and isi/AB have regulatory sites in common although belonging to different regulons.

Comparison of the IdiA sequence with sequences in the PIR database (not shown) revealed similarities to two basic proteins with iron-binding properties located in the periplasm: SfuA from Serratia marcescens (molecular mass of precursor and mature protein 36.15 and 33.5 kDa, respectively, and isoelectric point of the precursor protein 9.52; Mietzner et al., 1987; Angerer et al., 1990); and Fbp from Neisseria gonorrhoeae (molecular mass 33.5 kDa and isoelectric point 10.1; Berish et al., 1990a) or from N. meningitidis (Berish et al., 1990b). These two bacterial proteins belong to a class of basic proteins which can bind iron reversibly similar to the basic iron-binding protein lactoferrin (Montreuil et al., 1960; Lippard & Berg, 1994). In addition to sequence homology SfuA and IdiA are both synthesized as precursors and are processed between two alanine residues (Angerer et al., 1990). However, SfuA has a typical prokaryotic signal sequence, which is presumably cleaved when the protein is transported into the periplasm, while IdiA has no typical signal sequence (only nine amino acid residues are removed), and it is located intracellularly, being mainly associated with the thylakoid membrane. Whether IdiA might be transported into the lumen of the thylakoid, cannot be answered at present.

Besides having sequence homology to SfuA and Fbp, IdiA has a slight similarity to a membrane-associated protein, MapA, of unknown function from Synechococcus PCC 7942 (Webb et al., 1994) (data not shown).

Insertional inactivation of the idiA gene from Synechococcus PCC 7942

To inactivate the idiA gene, plasmid pKPM24 was used for mutational gene interruption with a 2.2 kb BamHI fragment taken from plasmid pH455ΩKmR (Fellay et al., 1987). The resulting plasmid, pKPM224, is shown in Fig. 5c. The KmR gene in this plasmid is orientated in the same direction as the idiA gene.

Synechococcus PCC 7942 is closely related to Synechococcus PCC 6301 (Golden et al., 1989), but is more readily transformed than Synechococcus PCC 6301. Therefore, Synechococcus PCC 7942 was transformed with pKPM224 (Fig. 5c). More than 80% of the Km-resistant colonies had lost the vector-encoded Ap resistance, implying that the wild-type idiA gene was replaced by the inactivated gene. The replacement of the idiA gene in PCC 7942 was verified by Southern analysis of a Km-resistant and Ap-sensitive clone (Fig. 7) which was called Synechococcus PCC 7942 14 and used in the subsequent experiments.

Immunoblot analysis of Synechococcus PCC 7942 and mutant 14 cells with the anti-IdiA antiserum showed that the mutant cells contained no detectable IdiA either under Fe-sufficient or under Fe-deficient growth conditions, clearly demonstrating that the mutant 14 is indeed an IdiA-free mutant (Fig. 8).
IdiA protein in cyanobacteria

Comparative analysis of *Synechococcus* PCC 7942 and mutant I4

The IdiA mutant I4 of *Synechococcus* PCC 7942 was able to grow photoautotrophically. The growth rate in Fe- and Mn-sufficient medium was approximately 50–75% the rate of the wild-type (Fig. 9). The reduced growth rate of the mutant was observed in the presence or absence of kanamycin, indicating that the growth defect of the mutant under iron-sufficient conditions was not a consequence of the added antibiotic. When cells were transferred to a medium containing either no Fe or no Mn, wild-type cells grew only slightly slower for the first 3 d than cells grown in Fe- and Mn-sufficient medium. Omission of Fe from the medium affected wild-type cells after the second transfer in Fe deficient medium, while omission of Mn required several transfers (at least 6 × 2 d) before a reduction in the growth rate was detectable. In contrast, growth of mutant cells was immediately affected by omission of Fe or Mn, indicating that mutant cells lacking IdiA were unable to cope with a reduced level of either Fe or Mn.

A comparative analysis of PS I and PS II activities in *Synechococcus* PCC 7942 wild-type and IdiA mutant cells grown in Fe- and Mn-sufficient or -deficient medium is summarized in Fig. 10. Wild-type cells grown under Fe-deficient conditions have an approximately 30% lower PS I activity, while the PS II activity remained unchanged or even increased slightly within the first 2 d of Fe deficiency. When comparable measurements were performed with the IdiA mutant strain I4, PS I and PS II activity were only slightly lower in the mutant (10–20% for PS I activity and 15–30% for PS II activity) under Fe- and Mn-sufficient conditions as compared to wild-type. Under either Fe- or Mn-limited growth conditions, PS I activity in the mutant I4 dropped to about the same level as in wild-type. Thus, the absence of IdiA does not greatly affect PS I activity within the first 2 d of Fe or Mn deficiency. However, a substantial difference was observed in PS II activity in mutant cells grown either under Fe or Mn deficiency compared to wild-type. In the IdiA mutant I4, Fe as well as Mn limitation caused a 50–90% reduction of photosynthetic *O₂* evolution within 48 h. Thus, it can be concluded that IdiA is essential under iron as well as manganese limitation and that absence of IdiA affects activity of PS II significantly more than that of PS I, implying that IdiA has an essential role in the process of providing PS II with manganese.

So far only limited information exists in the literature about manganese metabolism in photosynthetic organisms (for a general review on Mn metabolism see Fälüas da Silva & Williams, 1993) and of how PS II is supplied with manganese (Debus, 1992). Whether IdiA is a general iron- and/or manganese-binding protein in *Synechococcus* PCC 6301 and PCC 7942, which is either loosely associated with thylakoid membranes or located in the lumen of the thylakoid remains to be determined. However, the results clearly show that IdiA is essential under iron as well as manganese limitation and that its absence affects PS II activity more than PS I activity suggesting that its main role is in manganese metabolism.
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