Molecular characterization of *idiA* and adjacent genes in the cyanobacteria *Synechococcus* sp. strains PCC 6301 and PCC 7942

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IdiA (iron-deficiency-induced protein A) is a protein expressed at highly elevated levels in *Synechococcus* sp. strains PCC 6301 and PCC 7942 under Fe- or Mn-limiting growth conditions. Besides being similar to two bacterial Fe-binding proteins, SfuA and FbpA, IdiA shows similarity to two ORFs (*slr0513* and *slr1295*) of *Synechocystis* sp. PCC 6803. Northern blot analysis detected one transcript of about 1300 nt in RNA extracted from *Synechococcus* sp. PCC 6301 and PCC 7942 grown under Fe deficiency. The intensity of this transcript was considerably reduced in Fe-sufficient culture. It could be further shown that the regulation of IdiA expression is at the transcriptional level and that transcription and translation of IdiA are closely linked. Primer extension analysis indicated a single transcriptional start site 193 nt upstream of the first presumed translational start codon. Moreover, molecular characterization of the entire 5.8 kb chromosomal *Hind*III DNA fragment carrying the *idiA* gene from *Synechococcus* sp. PCC 6301 led to the identification of six long ORFs in addition to *idiA*. The two genes adjacent to *idiA*, and *dpsA* located 2018 nt downstream of *idiA*, were insertionally inactivated in *Synechococcus* sp. PCC 7942 and the corresponding mutants were partially characterized. These experiments provide evidence that the gene products of *idiB*, located immediately downstream of *idiA*, and of *dpsA* are involved in the activation of IdiA expression, since the absence of each of these two gene products prevents the greatly elevated expression of IdiA under nutrient deficiency.

Keywords: cyanobacteria, *Synechococcus* sp., *idiA*, transcript analysis, transcription factor

INTRODUCTION

Iron is an essential redox component in a great number of enzymes and protein complexes and thus iron deficiency has a profound effect on the physiology of cyanobacteria and other organisms (for reviews, see: Straus, 1994; Ferreira & Straus, 1994; Carr & Mann, 1994). It is well documented that in cyanobacteria Fe deficiency causes substantial changes in the ultra-structure of the thylakoid membranes, a decrease of

pigment content and a reduction of the photosynthetic activities. Under Fe limitation a new chlorophyll protein complex, called CPVI-4, is formed in *Synechococcus* PCC 7942 (Riethman & Sherman, 1988a, b). CPVI-4 might function in optimizing light energy transfer to the reaction centres or serve as a chlorophyll sink under Fe deficiency when other major chlorophyll-protein complexes are greatly degraded. It has also been shown for several cyanobacteria that the Fe-repressible *isiAB* operon is expressed, encoding a CP43-like chlorophyll-binding protein (IsiA) and a replacement for ferredoxin called flavodoxin (IsiB) (Laudenbach & Straus, 1988; Leonhardt & Straus, 1992, 1994). Subsequently, IsiA was identified to be a component of CPVI-4 in *Synechococcus* PCC 7942 (Burnap *et al.*, 1993). In addition, two more Fe-regulated genes have been identified in *Synecho-*

This paper is dedicated to Professor Dr Birgit Vennesland on the occasion of her 85th birthday.

Abbreviations: DIG, digoxigenin; PS I and II, photosystem I and II.

The GenBank accession number for the sequence of the region containing *idiA* is Z48754.

coccus PCC 7942: *irpA* encoding a protein located in the cytoplasmic membrane and presumably involved in Fe acquisition or storage (Reddy *et al.*, 1988), and *mapA* encoding a protein of unknown function (Webb *et al.*, 1994). Based on a nucleotide sequence evaluation by Straus (1994), it seems that all cyanobacterial Fe-stress-induced genes investigated so far contain operator sequences related to the Fur binding consensus sequence. Moreover, the search for a corresponding gene has led to the identification of a homologue of the 'ferric iron uptake regulation gene', *fur*, in *Synechococcus* PCC 7942 (Ghassemian & Straus, 1996).

Besides Fe, oxygenic photosynthetic organisms have an absolute requirement for manganese, an essential component in the water-oxidizing complex of photosystem II (PS II). Although substantial information exists in the literature on how Mn is incorporated into the water-oxidizing complex of PS II by a light-driven process (for review, see Debus, 1992), very little information is available on Mn metabolism in the oxygenic photosynthetic cell. Bartsevich & Pakrasi (1995, 1996) identified genes encoding an ABC transporter complex involved in Mn uptake in the cyanobacterium *Synechocystis* PCC 6803. However, it is unclear whether the transporter is located in the cytoplasm and/or the thylakoid membrane. Another problem that has also received little attention is related to the question of how the intrathylakoid space of a cyanobacterium is sufficiently supplied with Mn under conditions of Fe limitation. Under such conditions, elevated expression of Fe-binding proteins in the cytoplasm might lead to trapping of Mn in the cytoplasm, since practically all Fe-binding proteins characterized so far also bind Mn. Thus, a rather complex interrelationship must exist between Fe and Mn metabolism, especially in an oxygenic prokaryotic cell with no separate organelles.

In previous publications (Michel & Pistorius, 1992; Michel *et al.*, 1996) we reported a protein called IdiA (iron-deficiency-induced protein A) that becomes expressed in greatly elevated levels in *Synechococcus* PCC 6301 and PCC 7942 under Fe deficiency and under Mn deficiency. On the basis of the derived amino acid sequence, processed IdiA has a calculated molecular mass of 35009 Da that agrees well with the published apparent molecular mass of 34 kDa for the isolated protein. Biochemical fractionation procedures and immunocytochemical investigations provided evidence that IdiA is located intracellularly and mainly associated with the cytoplasmic side of thylakoid membranes (Michel *et al.*, 1998). The gene encoding IdiA in *Synechococcus* PCC 6301 was identified, sequenced and partially analysed. Subsequent construction of an IdiA-free *Synechococcus* PCC 7942 mutant revealed that in the absence of IdiA the cells could not tolerate mild Fe or Mn deficiency as wild-type cells could. Measurements of PS II and PS I activities in the IdiA-free mutant provided evidence that mainly PS II was damaged under nutrient-deficient conditions. Thus, IdiA is obviously a protein optimizing and/or protecting PS II under Fe and/or Mn deficiency in a direct or indirect fashion.

In this study, an extended characterization of *idiA* in *Synechococcus* PCC 6301 was performed and the DNA region surrounding *idiA* in *Synechococcus* PCC 6301 and PCC 7942 was analysed.

METHODS

Origin of cyanobacteria, growth conditions and French press treatment. *Synechococcus* sp. strain PCC 6301 (SAUG B1402-1) 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 (growth for 2 d, inoculum 60 µl cells). *Synechococcus* PCC 6301 was grown at 30 °C in the medium of Kratz & Myers (1955) with slight modifications as described by Pistorius *et al.* (1989). *Synechococcus* PCC 7942 was grown in BG11 medium (Rippka, 1988) at 30 °C. For growth under nutrient-deficient conditions, cells were grown for several cycles under regular growth conditions, then harvested and, after washing with distilled water, were subsequently transferred into medium from which Mn or Fe was omitted. Growth of both *Synechococcus* strains on agar plates was achieved in BG11 medium containing 1% (w/v) agar (bacteriological grade; Gibco-BRL). Where indicated, antibiotics were added at the following concentrations (mg l⁻¹): kanamycin, 25; ampicillin (Ap), 10; spectinomycin (Sp), 25.

For preparation of French press extracts used for Western blotting experiments, *Synechococcus* cells were harvested by centrifugation, washed once with 10 mM sodium phosphate buffer, pH 7.0, and then resuspended in the same buffer to give a cell density of 100 µl cells ml⁻¹. This cell suspension was passed twice through a pre-chilled French press at 137.9 MPa. Unbroken cells were removed by centrifugation at 4000 g for 5 min. French press extracts for PS I and PS II activity measurements were made in a buffer containing 50 mM HEPES/NaOH, pH 6.5, 50 mM CaCl₂ and 400 mM sucrose.

Escherichia coli strains DH5αmc^r (Grant *et al.*, 1990) and SCS110 (Stratagene) were cultivated at 37 °C in LBG medium (Sambrook *et al.*, 1989) or on PA agar plates containing 17.5 g antibiotic medium no. 3 (assay broth, Oxoid) and 16 g bacteriological agar (Oxoid) in 1 l distilled water. Where indicated, antibiotics were added at the following concentrations (mg l⁻¹): ampicillin, 150; kanamycin, 50; spectinomycin, 100.

Biochemical determinations. PS I and PS II activities were determined as described by Michel *et al.*, 1996. Chlorophyll was estimated according to Grimme & Boardman (1972), phycocyanin and allophycocyanin according to Tandeau de Marsac & Houmard (1988) and protein according to Smith *et al.* (1985). SDS-PAGE was performed according to Laemmli (1970) or Schägger & von Jagow (1987). Protein samples for SDS-PAGE (10% polyacrylamide gel) were denatured either at 70 °C for 15 min (Laemmli system) or 5 min at 100 °C (Tris-Tricine system). Protein (15 or 30 µg) was applied in each lane (Figs 3 or 7, respectively). Immunoblotting was done as described by Michel & Pistorius (1992) by transferring proteins to nitrocellulose (Schleicher & Schüll, BA 85) and using previously raised anti-IdiA antiserum at a dilution of 1:15000. (Michel & Pistorius, 1992).

DNA isolation, cloning and sequencing. Plasmids pSVB30 (Arnold & Pühler, 1988), pNEB193 (New England Biolabs)

and pKPM24 (Michel *et al.*, 1996) were used as basic tools for genetic engineering. pKPM24 is a derivative of pSVB30 containing a 5.8 kb *Hind*III fragment of genomic *Synechococcus* PCC 6301 DNA carrying *idiA* (Michel *et al.*, 1996).

Total DNA from *Synechococcus* PCC 7942 and from the corresponding mutants was isolated by the Sarkosyl method and purified by the phenol extraction procedure (William, 1988; Sambrook *et al.*, 1989).

Cloning procedures, Southern blotting and other recombinant DNA techniques were done as described in Sambrook *et al.* (1989). Sequencing of the pKPM24 insert was performed by constructing corresponding overlapping subclones using the Pharmacia *double-stranded* Nested Deletion Kit and a Pharmacia ALFexpress DNA Sequencer. The nucleotide sequence of the 5.8 kb DNA fragment was determined by the dideoxy chain-termination method. The enzymes used in this study were purchased from Boehringer Mannheim, Pharmacia, Amersham and USB.

Computer analysis. Nucleic acid sequences were analysed using the Staden software package (Staden, 1986). The deduced amino acid sequences were analysed using two different programs: PC/GENE (IntelliGenetics Release 6.8, 1993) and XPIP (Staden, 1986). Homology searches were done with the BLAST algorithm by Altschul *et al.* (1990) in the SWISS-PROT, EMBL, PIR, GenBank and CyanoBase databases.

Oligonucleotide synthesis and PCR. The following oligonucleotides used for primer extension assays were designed on the basis of certain DNA regions upstream and downstream of the *idiA* start codon using the selection criteria of the program Primer Design version 2.2 (Scientific & Educational Software): KPM1 (20mer), Cy5'-GATTGTCGGTGTGTAGTGC-3' (2307–2288); KPM2 (21mer), Cy5'-GACTTCACCTTCAGC-AGCTTC-3' (2270–2250); KPM3 (23mer), Cy5'-GCGGCTG-CCTTCACTCTTGAGAC-3' (2408–2386); KPM4 (20mer), Cy5'-CACTCTTGTATCGGCGCCT-3' (2078–2059); KPM5 (20mer), Cy5'-TAATAGCGTTCCGGCGAGAG-3' (2216–2197); KPM6 (17mer), Cy5'-CGCACCGCGATAACT-CA-3' (2031–2015) (Cy, indodicarbocyanine).

The oligonucleotides used to generate a PCR-derived *idiA* fragment (2256–3241) for all types of labelling experiments were designed using the program Oligo version 4.0 (Rychlik & Rhoads, 1989): IdiA00A (22mer), P5'-GCTGAAGGTGAA-GTCAATTTGT-3' (plus strand: 2256–2277); IdiA00B (21mer), P5'-TGAAGTGATCCAATAACGTCT-3' (minus strand: 3221–3241). All oligonucleotides were manufactured by Pharmacia Biotech.

PCR was performed with pKPM24 as template using *Pwo* DNA polymerase (Boehringer Mannheim) according to the manufacturer's recommendations.

RNA isolation, Northern blotting and primer extension assay. RNA was isolated from *Synechococcus* cultures at various growth phases using a modified hot-phenol method according to Reddy *et al.* (1990). For hybridization experiments, the RNA was denatured for 10 min at 68 °C with a buffer containing formamide and formaldehyde [20 mM MOPS/NaOH, pH 7.0, 5 mM sodium acetate, 1 mM disodium EDTA, 50% (v/v) formamide, 16% (v/v) formaldehyde]. After adding stop solution [20 mM disodium EDTA, 95% (v/v) formamide, 0.1% bromophenol blue, 0.1% xylene cyanole], the RNA (10 µg) was separated in a formaldehyde agarose gel system (1.3 g agarose in 73 ml dimethyl-dicarbonate-treated double-distilled H₂O, 10 ml 200 mM

MOPS/NaOH, pH 7.0, 50 mM sodium acetate, 10 mM disodium EDTA, 17 ml formaldehyde). After transfer to a nylon membrane (Hybond-N+; Amersham), the probes were hybridized with an [α -³²P]dATP-labelled 986 bp *idiA* PCR fragment (2256–3241) in Church buffer [7% (w/v) SDS, 50% (v/v) formamide, 5 × SSC (75 mM sodium citrate, pH 7.0, 750 mM NaCl), 2% (w/v) blocking reagent (Boehringer Mannheim), 50 mM sodium phosphate buffer, pH 7.0, 0.1% (v/v) laurylsarcosine]. The labelling reaction was done with the Random Primed DNA Labelling Kit (Boehringer Mannheim), according to the manufacturer's recommendation. Labelled fragments were cleaned by centrifugation through a Sephadex G25 column. Alternatively, the *idiA* PCR fragment was labelled with digoxigenin (DIG)-dUTP using the DIG DNA Labelling and Detection Kit (Boehringer Mannheim). RNA markers were from New England Biolabs.

The primer extension assay was carried out according to Katzen *et al.* (1996). The annealing mix contained 10 µg RNA in a volume of 6 µl, 20 pmol Cy5' labelled primer (Pharmacia Biotech) in 1 µl dimethyldicarbonate-treated double-distilled H₂O, 2 µl 5 × annealing buffer (10 mM Tris/HCl, pH 7.9, 1.25 M KCl, 1 mM EDTA) and 1 µl RNase inhibitor (1 unit µl⁻¹; Pharmacia). The annealing reaction was carried out for 2 h at 37, 42 and 50 °C. For the extension reaction, 10 µl annealing mix, 23 µl PE mix (1 µl 1 M dithiothreitol and 6.5 µl 10 mM dNTP mix in 192.5 µl 20 mM Tris/HCl, pH 8.3, 10 mM MgCl₂) and 1 µl SuperScript RNase H⁻ reverse transcriptase (200 units µl⁻¹; Gibco-BRL) were incubated for 45 min at 37 °C. The products were pelleted by centrifugation for 15 min at 4 °C after precipitation by adding 300 µl ethanol and storing for 20 min at -70 °C. The resulting pellet was resuspended in 7.5 µl TE buffer (10 mM Tris/HCl, pH 7.5, 1 mM disodium EDTA) and 6 µl stop solution from the AutoRead Sequencing Kit (Pharmacia).

Construction of mutants in *Synechococcus* PCC 7942. The construction of mutants was performed by transforming wild-type cells of *Synechococcus* PCC 7942 according to Laudenbach & Straus (1988) with the corresponding plasmids for insertional gene inactivation. All blunt-ended DNA fragments needed for this purpose were made using T4 DNA polymerase or Klenow enzyme (Boehringer Mannheim). Mutants K9 (VORF2), K10 (VORF4) and K11 (VORF7) were constructed by transforming wild-type cells with pKPM227, pKPM229 and pKPM230 (all derivatives of pKPM24), respectively. pKPM227 was constructed by incorporation of a *Sma*I fragment from pHP450 (Fellay *et al.*, 1987), carrying a spectinomycin resistance cassette, into the blunt-ended single *Kpn*I site of pKPM24 at position 1397. pKPM229 carries the *Sma*I fragment cloned into the blunt-ended single *Apa*I site of pKPM24 at position 3882. pKPM230 also contains the *Sma*I fragment cloned into the blunt-ended singular *Bst*XI site of pKPM24 at position 5282. All other conditions were as described by Michel *et al.* (1996).

RESULTS AND DISCUSSION

Nucleotide sequence analysis of a 5.8 kb *Hind*III chromosomal DNA fragment from *Synechococcus* PCC 6301 carrying *idiA*

Plasmid pKPM24 (Michel *et al.*, 1996) carrying *idiA* was derived from plasmid pSVB30 (Arnold & Pühler, 1988) and contains a 5.8 kb chromosomal *Hind*III DNA fragment from *Synechococcus* PCC 6301. Previously,

Fig. 1. Physical map of the 5763 bp *Hind*III DNA fragment of *Synechococcus* PCC 6301 carrying *idiA*. pKPM24 is a derivative of pSVB30. Besides *idiA* (ORF3, 2121–3221), six additional long ORFs are located on this DNA fragment: ORF1, incomplete, 807–1; ORF2, 872–2017; ORF4 (*idiB*), 4090–3464; ORF5, 4747–4187; ORF6, 5004–4744; ORF7 (*dpsA*), incomplete, 5239–5763. The size and orientation of the genes are shown by the shaded arrows.

2273 bp had been sequenced. In this study the complete nucleotide sequence of both strands of the entire 5763 bp DNA fragment (GC content 55.08 mol%) was determined and the physical map with the relevant restriction sites is shown in Fig. 1. Besides *idiA* (ORF3, 2121–3221), the 5.8 kb DNA fragment contains six additional long ORFs: two located upstream (ORFs 1 and 2) and four located downstream of *idiA* (ORFs 4–7).

Homology analysis of the identified ORFs revealed that the ORF1 (807–1) gene product shows extensive homology to LivH from *E. coli* (accession no. P08340; 33.09% identity, 21.56% conservative changes) (Landick & Oxender, 1985; Nazos *et al.*, 1986) and to BraD from *Pseudomonas aeruginosa* (accession no. P21627; 30.48% identity, 22.68% conservative changes) (Hoshino & Kose, 1990). LivH (307 aa) as well as BraD (308 aa) are components required for the high-affinity branched chain amino acid transport system in the respective species. Both are hydrophobic proteins with six predicted transmembrane helices. In addition, ORF1 of *Synechococcus* PCC 6301 shows similarity to *slr0949* of *Synechocystis* PCC 6803 which encodes a protein of 286 aa with a calculated molecular mass of 31 133 Da (55.02% identity, 17.84% conservative changes) (CyanoBase). Like LivH, BraD and Slr0949, the ORF1 product of *Synechococcus* PCC 6301 is a hydrophobic protein and contains six transmembrane helices. It has a potential cleavage site between aa 26 and 27 with either an ALA motif or an AA motif (Proteome Project, Cyano2Dbase; Sazuka & Ohara, 1996) and is preceded by a possible ribosome-binding site (Shine–Dalgarno sequence) beginning at position 833 (GGAG) (Sazuka & Ohara, 1996). ORF1 on pKPM24 is incomplete; the sequenced part encodes 269 aa. The hydrophobic nature of the ORF1 gene product in *Synechococcus* PCC 6301 clearly classifies this protein as an integral membrane protein. However, it is unknown whether this protein is located in the cytoplasmic membrane or in the thylakoid membrane.

ORF2 (872–2017) has a potential Shine–Dalgarno sequence at position 859 (GAGG), shows homology to *slr1025* from *Synechocystis* PCC 6803 (33.86% identity, 13.65% conservative changes) (CyanoBase) and encodes a protein of unknown function. Further evaluation of the derived amino acid sequence provides evidence that the N-terminal region (890–906) of ORF2 has homology to a dinucleotide-binding domain with a GXGXXG motif (Wierenga *et al.*, 1986). The highest level of homology was found to exist with the FAD-containing sarcosine oxidase from *Bacillus* sp. (accession no. D16521; Suzuki *et al.*, 1994).

ORF4 (subsequently called *idiB*; 4090–3464) has a possible Shine–Dalgarno sequence at position 4102 (AGGA) and encodes a protein of 208 aa with a calculated molecular mass of 23 237 Da which has similarity to CysR from *Synechococcus* PCC 7942 (39.32% identity and 12.14% conservative changes) (Nicholson & Laudenbach, 1995). CysR in PCC 7942 encodes a polypeptide which is sulfur-regulated and which has homology to the DNA-binding regulatory proteins Fnr and Crp from *E. coli* (Unden & Guest, 1985; Pastan & Adhya, 1976) and FixK from *Sinorhizobium meliloti* (Batut *et al.*, 1989). The greatest similarity between CysR and the regulatory proteins from enteric bacteria is in the helix–turn–helix motif which is directly involved in DNA binding (Brennan & Matthews, 1989). ORF4 contains such a helix–turn–helix motif (A/G-X₃-G-X₅-I/V), has high similarity to CysR over the whole sequence and preferentially resembles NtcA in its helix–turn–helix motif (Vega-Palas *et al.*, 1992; Frias *et al.*, 1993; Wei *et al.*, 1993; Bradley & Reddy, 1997). Thus, ORF4 might also be a DNA-binding protein (see below). The alignment of conserved sequences of this motif (A, G and I/V) is shown in Fig. 2. In addition ORF4 possesses a motif of the type SPXX at the N terminus (residues 23–26: SPTT). A similar motif is also found in CysR. Such a motif is common among transcriptional regulators of the helix–turn–helix type (Suzuki, 1989). The variable residues are most often S, T, A, L or P and the motif never lies in the helix–turn–helix domain.

No amino acid sequence significantly similar to the proteins encoded by ORF5 (4747–4187) or ORF6 (5004–4744) was found in the NBRF or GenBank databases. ORF7 (5239–5763) from *Synechococcus* PCC 6301, with a possible Shine–Dalgarno sequence at 5230, is identical to *dpsA* from *Synechococcus* PCC 7942 sequenced and characterized by Pena & Bullerjahn (1995). The codon for the last amino acid of DpsA from PCC 6301 is not located on pKPM24. As stated by Pena & Bullerjahn (1995), the Dps family of proteins are a diverse group of bacterial stress-induced polypeptides that bind DNA and likely confer resistance to peroxide damage during periods of oxidative stress and long-term nutrient limitation. The C terminus of DpsA from PCC 7942 has about 60% similarity to the C terminus of bacterioferritin. It has been shown that the DpsA complex contains haem and has weak catalase activity *in vitro*. Moreover, it has been suggested that the protein

ORF4 <i>Syn.</i> PCC 6301	RFGLNTEHGWMIDLH---LTHQVLADIVSSTRVTITPKLINQFETEGRLIRLSGQR	191
CysR <i>Syn.</i> PCC 7942	RFGQPATGWEIDLRRIPILTHQVIAELSSSTRVTITRLLGFEFRQAGRIHR-RDRA	189
NtcA <i>Syn.</i> PCC 6803	DFGVPAAPDGIRIDLK---LSHQAIIEAIGSTRVTITRLLGDLREG-NMISITKKK	211
NtcA <i>Cya.</i> ATCC 51142	DFGVPTPEGIRIELK---LSHQAIIEAIGSTRVTITRLLGDLRQE-EMISIHKKK	212
NtcA <i>Ana.</i> PCC 7120	DFGVPCADGITIDLK---LSHQAIIEAIGSTRVTITRLLGDLREK-KMISIHKKK	209
NtcA <i>Syn.</i> PCC 7942	DFGIPSPDGITIDLK---LSHQAIIEAIGSTRVTITRLLGDLRES-KLIAIHKKR	208
		
	Helix Turn Helix	

Fig. 2. Comparison of the ORF4 gene product (IdiB) from *Synechococcus* PCC 6301 with the regulatory and DNA-binding proteins CysR from *Synechococcus* PCC 7942 (accession no. P27369), NtcA from *Synechocystis* PCC 6803 (P33779), NtcA from *Cyanobacteria* ATCC 51142 (U80855), NtcA from *Anabaena* PCC 7120 (Q05061) and NtcA from *Synechococcus* PCC 7942 (P29283). The helix–turn–helix and putative DNA binding motif (underlined) is indicated. The typical, highly conserved amino acid residues of the helix–turn–helix motif are boxed. ‘|’ indicates identical amino acids; ‘.’ indicates conservatively exchanged amino acids (A/S/T, D/E, N/Q, R/K, I/L/M/V and F/Y/W). Gaps were introduced into the sequences to maintain optimal alignment.

Fig. 3. Time course of *idiA* mRNA expression (a) and IdiA protein expression (b) under Fe deficiency in *Synechococcus* PCC 6301. *idiA* mRNA was detected by Northern blotting and IdiA protein by Western blotting. *Synechococcus* PCC 6301 cells were grown under Fe-sufficient growth conditions (Lanes 1) and then transferred with dilution (60 µl cells per 250 ml medium) into medium containing no Fe. Cell samples were taken at different growth stages: OD₇₅₀ of 0.8 (lanes 2), 1.2 (lanes 3), 1.6 (lanes 4), and 2.0 (lanes 5). *Synechococcus* cells grown under Fe-deficient conditions (OD₇₅₀ of 2.0) in the presence of rifampicin (lane 6) or chloramphenicol (lane 7) [200 µg (ml medium)⁻¹ each] were chosen as control.

may have a peroxide-consuming function when located on the chromosome and that this activity may be a necessary feature to handle the endogenous oxidative stresses associated with oxygenic photosynthesis.

Nucleotide sequence analysis and extended homology analysis of *idiA*

Previously, we assumed that *idiA* encoded a protein of 330 aa with a predicted molecular mass of 36 167 Da (Michel *et al.*, 1996). Only 163 aa derived from the nucleotide sequence were verified by protein sequencing. On the basis of N-terminal sequencing of the isolated IdiA protein, it could be concluded that the mature IdiA is a processed protein consisting of 321 aa with a

calculated molecular mass of 35 009 Da (processing between two alanine residues). The calculated molecular mass of the processed protein agrees quite well with the apparent molecular mass of 34 kDa determined by SDS-PAGE. Due to the processing of IdiA, an uncertainty remained concerning the translational start of *idiA*. For this reason the 5' region of *idiA* was again analysed. This additional sequencing provided evidence that from the previously published sequence (Michel *et al.*, 1996) one nucleotide had to be omitted (2240). Evaluation of the newly determined 5' terminal nucleotide region revealed that three ATG codons are in the correct reading frame, corresponding to positions 2121–2123 (Met-1), 2148–2150 (Met-10) and 2160–2162 (Met-14). Each could serve as initiator codon. Thus, it remains uncertain

which of the three ATG codons might be the actual initiator codon for *idiA*. Assuming that the first ATG is the initiator codon, then *idiA* would consist of 1101 bp (start codon ATG at 2121 and stop codon TAA at 3221), encoding a protein of 366 aa with a calculated molecular mass 40167 Da and an isoelectric point of 10.54. If one of the other two ATG codons is the correct start codon, then a protein of 39035 Da (pI 10.68) or 38601 Da (pI 10.74), respectively, would result. In the new numbering system, based on the longest possible IdiA protein, the processing site of IdiA lies between Ala-45 and Ala-46. This cleavage site corresponds to a cleavage site as described for *slr1725* from *Synechocystis* PCC 6803 by Sazuka & Ohara (1996) (Proteome Project, Cyano-2Dbase). However, the removed peptide of 5158 Da does not possess the characteristic properties of a prokaryotic signal sequence according to the evaluation with PCGENE. Although the real translational start site of *idiA* remains uncertain, the presence of a possible assumed Shine–Dalgarno sequence at position 2104 (GGGG) would favour the first ATG codon in position 2121 as the most likely candidate. However, the GGGG sequence does not correspond to the three possible ribosome-binding sequences (GAGG, AGGA, GGAG) described for *Synechocystis* PCC 6803 by Sazuka & Ohara (1996). There is a putative large hairpin of 35 bp (3243–3277), in close proximity to the stop codon of *idiA*, that may terminate transcription of *idiA*.

Besides showing homology to two bacterial Fe-binding proteins, SfuA from *Serratia marcescens* (Mietzner *et al.*, 1987; Angerer *et al.*, 1990) and FbpA from *Neisseria* sp. (Berish *et al.*, 1990a, b) as previously reported (Michel *et al.*, 1996), IdiA also shows homology to two ORFs from *Synechocystis* PCC 6803 (CyanoBase). These are *slr0513* (52.02% identity and 9.83% conservative changes) and *slr1295* (48.89% identity and 11.39% conservative changes) encoding proteins of 38155 Da (pI 5.74) and 39370 Da (pI 4.63), respectively. These two *Synechocystis* proteins have also been classified as Fe-binding proteins on the basis of their similarity to SfuA and FbpA.

Transcript and primer extension analysis of *idiA*

Fig. 3 shows the expression of IdiA at the protein level (Western blot) in comparison to mRNA expression (Northern blot). For these experiments *Synechococcus* PCC 6301 was grown in Fe-sufficient medium for 2 d and then cells were transferred into Fe-deficient medium (60 µl cells ml⁻¹), and grown for 2–4 d. Samples were taken at the times indicated in Fig. 3. For Western blot analysis the corresponding French press extracts of cell suspensions were used and for Northern blot analysis mRNA was extracted from cell suspensions. A 986 kb PCR *idiA* fragment labelled with DIG-dUTP or [³²P]dATP was used as a probe for identification of the *idiA* transcript. Hardly any *idiA* transcript was detectable in *Synechococcus* PCC 6301 cultures grown in Fe- and Mn-supplemented BG11 medium. The *idiA* tran-

Fig. 4. Comparison of *idiA* transcript size in *Synechococcus* PCC 6301 (a) and PCC 7942 (b). Total RNA from the two strains was submitted to Northern blot analysis. The transcript size of the *idiA* mRNA corresponds to about 1300 nt. Lane M, RNA markers. The position of the 1280 nt fragment (barely visible) is marked by a triangle. In each of the lanes A–C, 10 µg total RNA extracted from *Synechococcus* cells was applied. Lanes: A, RNA from cultures grown for 2 d under Fe-sufficient conditions; B, RNA from cultures grown for 2 d in medium with one-tenth of the regular Fe concentration; C, RNA from cultures grown for 2 d in medium without Fe.

script was greatly enhanced when cells were exposed to Fe deficiency. Only one transcript of about 1300 nt in length was detected, suggesting that *idiA* is transcribed monocistronically (Fig. 4). The high level of *idiA* transcript continued for at least 48 h, but was significantly diminished after cells were exposed for more than 48 h to Fe-deficient growth. The results also show that synthesis of IdiA parallels synthesis of *idiA*-specific mRNA, indicating that synthesis of IdiA starts without delay when the *idiA*-specific mRNA level goes up (Fig. 3). The high level of IdiA remains for at least 72 h growth under Fe deficiency (not shown), even though reduction of the *idiA* transcript was observed to have started after 48 h under the chosen growth conditions. Comparable results concerning IdiA expression at the transcriptional and translational level (not shown) and transcript size (Fig. 4) were also obtained with *Synechococcus* PCC 7942.

Primer extension analysis was undertaken to determine the transcriptional start and to possibly identify a promoter region upstream of *idiA* (Fig. 5) that should be

Fig. 5. Primer extension analysis of the primary transcript of *idiA* from *Synechococcus* PCC 6301. (a) Fluorogram obtained from a primer extension assay with Cy5'-labelled oligonucleotide primer KPM2 corresponding to positions 2270–2250 on pKPM24. The peak represents cDNA obtained after retrotranscription at 37 °C for 1 h. Annealing was carried out at 37, 42 and 50 °C for 2 h. The arrow indicates the direction of transcription. The transcriptional start is marked with +1. (b) DNA sequencing of the DNA region upstream of *idiA* was done with the same primer. The sequence represents the non-coding strand. (c) Partial nucleotide sequence of the *idiA* region between positions 1905 and 2504. The first nucleotide is boxed and the forward arrow indicates the transcriptional start site and the direction of transcription. The three possible translational start codons of *idiA* are underlined. The position of primer KPM2 is boxed.

lying about 50 bp in front of the transcriptional start (Curtis & Martin, 1994). The sequence of the primers used was that of the non-coding strand. Four different primers (KPM2, KPM4, KPM5 and KPM6; see Methods) complementary to sequences downstream from the assumed translational start codon for *idiA* were successfully used. The experiments gave evidence that the transcriptional start lies at position 1929. Thus, the transcriptional start lies 193 bp before the assumed translational start (assuming the longest possible protein, i.e. ATG at position 2121). Based on primer extension analysis and on the assumption that the transcription ends at the hairpin at 3243–3277, the transcript of *idiA* from *Synechococcus* PCC 6301 consists of 1349 nt and this value is in close agreement with the transcript size estimated from Northern blot analysis

(about 1300 nt for *Synechococcus* PCC 6301 and PCC 7942; Fig. 4). The DNA region upstream of the transcriptional start does not display nucleotide sequences similar to –10 or –35 promoter sequences of *E. coli* (Curtis & Martin, 1994), nor to typical Fur-binding sequences (Straus, 1994). However, there is evidence that a likely Fur-binding sequence might be present in the 5' untranslated region at position 1974–1992.

Insertional inactivation of ORF2, ORF4 and ORF7 and preliminary characterization of the corresponding *Synechococcus* PCC 7942 mutants

Synechococcus PCC 6301 and 7942 have been shown to be highly similar (Golden *et al.*, 1989). However, since *Synechococcus* PCC 7942 is better than PCC 6301 for

Fig. 6. Physical map of plasmids pKPM227, pKPM229 and pKPM230 used for construction of *Synechococcus* PCC 7942 mutants K9, K10 and K11 (a) and the corresponding Southern blots (b). All three plasmids are derivatives of plasmid pKPM24 and were constructed by insertion of a spectinomycin resistance cassette. The size and orientation of the three selected genes and the orientation of the resistance cassettes are shown by arrows. The restriction sites removed by insertion of the resistance cassette are indicated with outlined letters. The derived mutants were named *Synechococcus* PCC 7942 K9 (insertional inactivation of ORF2), K10 [insertional inactivation of ORF4 (*idiB*)] and K11 [insertional inactivation of ORF7 (*dpsA*)]. (b) Southern blots of genomic DNA from wild-type *Synechococcus* PCC 6301 (lane A) and PCC 7942 (B), and from *Synechococcus* PCC 7942 mutants K9 (C), K10 (D) and K11 (E) digested with *Hind*III, *Hind*III/*Eco*RI or *Hind*III/*Bam*HI as indicated. DNA was detected with the DIG-labelled 5.8 kb *Hind*III insert from pKPM24. The DNA fragments obtained with all three restriction enzyme combinations are identical for *Synechococcus* PCC 6301 and PCC 7942. Lanes M, DIG-labelled DNA markers III (Boehringer Mannheim).

Table 1. Growth, pigment content and photosynthetic activities of *Synechococcus* PCC 7942

Cells were grown in Fe-sufficient (+Fe) or Fe-deficient (−Fe) medium under standard conditions. After 2 d growth, cells were harvested, resuspended in 50 mM HEPES/NaOH, pH 6.5, containing 50 mM CaCl₂ and 400 mM sucrose, to give a cell density of 100 µl cells ml^{−1}. Subsequently, the cell suspensions were broken by French press treatment. All measurements were performed with this French press extract (FPE). Details are given in Methods. The data are representative of three to five independent growth experiments.

Strain	Medium	Growth (OD ₇₅₀)	Chlorophyll [mg (ml FPE) ^{−1}]	Phycocyanin [mg (ml FPE) ^{−1}]	Allophycocyanin [mg (ml FPE) ^{−1}]	PS II activity [µmol O ₂ evolved (mg Chl) ^{−1} h ^{−1}]	PS I activity [µmol O ₂ consumed (mg Chl) ^{−1} h ^{−1}]
Wild-type	+Fe	2.3	0.72	4.8	2.5	127	124
	−Fe	1.6	0.41	2.9	2.1	108	123
Mutant I4 (VORF3 = <i>VidiA</i>)	+Fe	1.4	0.33	2.4	1.5	79	133
	−Fe	0.4	0.21	1.8	1.3	21	124
Mutant K9 (VORF2)	+Fe	2.3	0.62	4.3	1.8	104	102
	−Fe	1.4	0.39	2.6	1.4	86	112
Mutant K10 (VORF4 = <i>VidiB</i>)	+Fe	2.3	0.66	4.1	2.5	102	73
	−Fe	1.7	0.29	0.8	0.6	75	74
Mutant K11 (VORF7 = <i>VdpsA</i>)	+Fe	2.3	0.66	4.7	3.1	104	131
	−Fe	1.6	0.45	2.0	3.4	47	152

transformation, we utilized strain PCC 7942 for mutant studies, as in a previous paper (Michel *et al.*, 1996). The comparative restriction analysis of the respective DNA region in *Synechococcus* PCC 6301 and 7942 is presented in Fig. 6(b), showing that on this basis no differences in this DNA region could be detected between these two *Synechococcus* strains.

For mutant studies, ORF2 (located immediately upstream of *idiA*), ORF4 (*idiB* – located immediately downstream of *idiA*) and ORF7 (*dpsA* – located 2018 bp downstream of *idiA*) were interrupted by insertion of a spectinomycin resistance cassette (Fig. 6a). In all three cases the resistance cassette was in the same orientation as the corresponding gene. Transformation of *Synechococcus* PCC 7942 cells with the corresponding plasmids pKPM227, pKPM229 or pKPM230 (see Methods and Fig. 6a) resulted in Sp^R and Ap^S transformants, implying that the desired double recombination event had occurred. This was verified by Southern analysis (Fig. 6b). The obtained mutants were called *Synechococcus* PCC 7942 K9 (inactivation of ORF2), K10 [inactivation of ORF4 (*idiB*)] and K11 [inactivation of ORF7 (*dpsA*)].

Comparative analysis of the three *Synechococcus* mutants with the wild-type showed that there was no significant reduction in growth of the mutants in comparison to the wild-type when cells were grown for 48 h in Fe-sufficient or Fe-deficient medium, while the previously constructed *IdiA*-free mutant, *Synechococcus* PCC 7942 I4 (Michel *et al.*, 1996), showed significant reduction in growth under the latter conditions (Table 1). On the basis of tested parameters, mutant K9 did not significantly deviate from the wild-type in contrast to mutants K10 and K11. Comparative Western blot analysis with anti-*IdiA* antiserum gave clear evidence that in mutants K10 and K11 lacking *IdiB* or *DpsA*, respectively, the expression of *IdiA* was greatly reduced

compared to wild-type cells, an observation most clearly seen under Fe deficiency (Fig. 7). As expected, PS II activity in mutants K10 and K11 was lower than in wild-type cells but not as low as in the completely *IdiA*-free mutant I4. Absence of *IdiB* under Fe-deficient conditions led to a significantly higher reduction of chlorophyll, phycocyanin and allophycocyanin, and also to a reduction of PS I activity in addition to a reduction of PS II activity compared to wild-type cells. In contrast, the absence of *DpsA* led only to a minor deviation of chlorophyll and phycocyanin content in comparison to wild-type but caused a significant increase in the allophycocyanin content. Moreover, PS I activity remained very stable under Fe-deficient growth conditions. Obviously, in mutant K11 the large reduction of PS II activity under Fe deficiency, in part due to the reduced *IdiA* level, is compensated by alterations in the pigment content and by a process leading to stabilization of PS I (Table 1). As a consequence, this mutant can grow quite well under mild Fe deficiency in contrast to the completely *IdiA*-free mutant I4 with a greatly reduced pigment content under Fe deficiency.

Concluding remarks

Analysis of the DNA region close to *idiA* revealed that in the region downstream of *idiA* two genes (*idiB* and *dpsA*) are located whose gene products function either directly or indirectly in activating *IdiA* expression. A direct regulation of *IdiA* expression on transcriptional level due to binding of these two proteins to the promoter region of *idiA* seems likely since *IdiB* has similarity to DNA-binding proteins with a helix–turn–helix and a SPXX motif, typical for this type of transcriptional factor (see Fig. 2), and *DpsA* belongs to a group of stress-induced polypeptides that presumably bind DNA as stated by Pena & Bullerjahn (1995). This

Fig. 7. Analysis of IdiA protein expression in *Synechococcus* PCC 7942 wild-type (a) and mutants K9 (c; VORF2), K10 (d; VORF4) and K11 (e; VORF7). Cultures were grown for 2 d in Fe-sufficient (+Fe) or Fe-deficient (-Fe) medium. The previously constructed mutant I4 (b; completely IdiA-free) is included for comparison. After preparation of French press extracts (see legend to Table 1), samples were submitted to SDS-PAGE and after transfer of proteins to nitrocellulose immunostaining was performed with the anti-IdiA antiserum.

implies that regulation of IdiA expression is rather complex. Whether IdiA expression is additionally regulated by the Fur protein, shown to be also present in *Synechococcus* PCC 7942 (Ghassemian & Straus, 1996), is presently unknown. A likely Fur-binding consensus sequence (see Straus, 1994), with 11/19 nt similar to this sequence, lies in the 5' untranslated region of *idiA* (see also Vinnemeier *et al.*, 1998).

It should also be pointed out that although IdiB and DpsA are functional in activating IdiA expression, these presumed DNA-binding proteins seem to have a regulatory function, not only on IdiA expression but also on expression of other photosynthesis-related proteins. The phenotype of mutants K10 and K11 are similar with respect to the reduced IdiA expression under Fe deficiency leading to reduced PS II activity, but differ in pigment content and PS I activity (see above).

IdiA has homology to two bacterial Fe-binding proteins, SfuA and FbpA, that function as the periplasmic-binding protein component of a high-affinity transporter (ABC type) for Fe from human transferrin (Chen *et al.*, 1993; Adhikari *et al.*, 1996; Forng *et al.*, 1997). In contrast to these proteins, IdiA is located intracellularly, mainly associated with the cytoplasmic side of the thylakoid membrane (Michel *et al.*, 1998). Thus in spite of some similarity to SfuA and FbpA, neither the cellular location nor the neighbouring genes suggest that IdiA is part of a classical periplasmic ABC transporter for Fe. On the basis of the available data, it can be concluded that the main function of IdiA is in protecting and/or optimizing PS II activity under nutrient deficiency. The complex regulation of IdiA expression emphasizes the importance of IdiA in optimizing PS II activity. Because of its similarity to bacterial Fe- (and Mn-) binding proteins and its location on the cytoplasmic side of the thylakoid membrane, IdiA might function either as a Fe-binding protein that specifically provides PS II with the Fe located on the acceptor side (Askwith & Kaplan, 1998; Merchant & Dreyfuss, 1998) or it might be a component of a Mn transporter via the thylakoid membrane. Although the complex compensating reactions occur-

ring in the photosynthetic reaction centres and in the pigment complexes in response to Fe deficiency are far from fully understood, it is quite obvious that the chlorophyll-protein complex CPVI-4 (containing a CP43 homologue protein, IsiA) (Riethman & Sherman, 1988a, b; Laudenbach & Straus 1988; Burnap *et al.*, 1993) and, as our results show, IdiA have an important function in this process.

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