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REVIEW

Function, regulation and distribution of IsiA, a membrane-bound chlorophyll *a*-antenna protein in cyanobacteria

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Abstract

IsiA is a membrane-bound Chl *a*-antenna protein synthesized in cyanobacteria under iron deficiency. Since iron deficiency is a common nutrient stress in significant fractions of cyanobacterial habitats, IsiA is likely to be essential for some cyanobacteria. However, the role it plays in cyanobacteria is not fully understood. In this review paper, we summarize the research efforts directed towards characterizing IsiA over the past three decades and attempt to bring all the pieces of the puzzle together to get a more comprehensive understanding of the function of this protein. Moreover, we analyzed the genomes of over 390 cyanobacterial strains available in the JGI/IMG database to assess the distribution of IsiA across the cyanobacterial kingdom. Our study revealed that only 125 such strains have an IsiA homolog, suggesting that the presence of this protein is a niche specific requirement, and cyanobacterial strains that lack IsiA might have developed other mechanisms to survive iron deficiency.

Additional key words: environmental stress; excitation energy transfer; gene regulation; photoprotection; photosynthesis; phylogenetic analysis.

Introduction

The Earth's atmosphere has undergone a gradual transformation into an oxidative environment since the great oxidation event in which photosynthetic organisms like cyanobacteria are thought to have played a major role (Holland 2006). As the oxygen concentration increased, Fe(II) was oxidized into Fe(III), resulting in the formation of water-insoluble oxides of iron. This led to the low bioavailability of iron in aquatic environments despite it being the fourth most abundant element in the Earth's crust. The low bioavailability of iron in aquatic ecosystems (Martin and Fitzwater 1988, Vrede and Tranvik 2006, North *et al.* 2007, Moore *et al.* 2013) has been a challenge for cyanobacteria. Pronounced effects of iron stress in cyanobacteria are the decreased contents of chlorophyll (Chl)-binding proteins, phycobilisomes (PBS), cytochromes, and ferredoxins (Fitzgerald *et al.* 1977, Sherman and Sherman 1983, Guikema and Sherman 1984). A strategy, which cyanobacteria have evolved to overcome such negative effects, is replacing these proteins with functional homologs which demand no iron. For instance, a flavodoxin, encoded by *isiB* (iron stress-induced) gene, is synthesized by many cyanobacteria under iron-stress conditions. This flavodoxin acts as a functional homolog of ferredoxin and compensates for its loss (Fitzgerald

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Abbreviations: CBP – chlorophyll-binding proteins; Chl *a* – chlorophyll *a*; EET – excitation energy transfer; FMO – Fenna-Mathews-Olson protein; Fur – ferric uptake regulator; F_v/F_m – maximal photochemical efficiency of PSII; GFP – green fluorescent protein; Hilp – high light-inducible proteins; HNLC – high-nitrate low-chlorophyll; IR – inverted repeat; IsiA – iron-stress-induced protein A; IsiB – iron-stress-induced protein B; IdiA – iron-deficiency-induced protein A; IdiB – iron-deficiency-induced protein B; IsrR – iron stress-repressed RNA; NPQ – nonphotochemical quenching; PBS – phycobilisome; Pcb – prochlorophyte chlorophyll *a*/*b* protein; PerR – peroxide operon regulator; ROS – reactive oxygen species; σ_{PSI} – effective cross-section of the photosynthetic reaction center PSI; UCYN-A – noncultivated unicellular cyanobacteria of group A.
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et al. 1977, Guikema and Sherman 1984). Other remarkable changes observed in iron-starved cyanobacterial cells were the decrease of PSI and PSII contents, the increase of PSII/PSI ratios as well as the synthesis of a Chl-binding protein with the molecular mass of about 36 kD called CPVI-4 (Pakrasi *et al.* 1985a,b). With sufficient iron, PSI and PSII are the major Chl-binding protein complexes in cyanobacteria (Pakrasi *et al.* 1985a,b). Under iron-deficient conditions, CPVI-4 protein becomes the dominant Chl-binding protein (Burnap *et al.* 1993), which implies that the synthesis of CPVI-4 may be produced to compensate for the loss of photosystems, especially for PSI. CPVI-4 is a product of an iron-induced gene, *isiA* (Burnap *et al.* 1993). This gene was initially discovered as part of an iron-stress-induced operon that includes a flavodoxin gene, *isiB* (Laudenbach and Straus 1988). It was later shown that *isiA* can be elsewhere in the genome and expressed independently of *isiB* (Leonhardt and Straus 1994).

Since iron deficiency is a common nutrient stress in notable portions of cyanobacterial habitats (Martin and Fitzwater 1988, Vrede and Tranvik 2006, North *et al.* 2007, Bibby *et al.* 2009, Moore *et al.* 2013) and the production of IsiA is one of the most noticeable responses to iron deficiency, it is reasonable to consider that the IsiA content in thylakoid membranes of cyanobacteria in certain environments is always maintained at high levels. In fact, it has been reported that *isiA* was found in iron-limited oceanic environments (Behrenfeld *et al.* 2006, Bibby *et al.* 2009, Schrader *et al.* 2011), which demonstrates the significant role that IsiA plays in helping cyanobacteria survive in iron-deficient environments. The importance of IsiA in stressed cyanobacteria has drawn the attention of researchers over the past decades, and numerous hypotheses about the functions of IsiA have been proposed. Despite all the endeavor, the pieces of the puzzle are yet to come together. In this paper, we review the efforts directed towards elucidating the attributes of the IsiA protein over the past decades. We have also attempted to analyze the currently rich repertoire of sequenced cyanobacterial strains to assess the distribution of this protein across the cyanobacterial kingdom and hypothesize plausible roles for it in these organisms.

Characteristics of IsiA protein

The discovery of the IsiA protein dates back to the early 1970's when Öquist reported altered spectral properties in iron-deficient cyanobacterial cells (Öquist 1971). Following this, in the early 80's, Sherman's lab demonstrated that cyanobacterial cells subjected to iron starvation for a prolonged period can undergo severe structural and functional alterations (Guikema and Sherman 1983a, Sherman and Sherman 1983). A blue shift of Chl *a* absorbance from 685 nm to 673 nm and the presence of a sharp peak at ~685 nm in the 77K fluorescence emission spectrum of cyanobacterial cells were observed in iron-starved cyanobacteria (Öquist 1971, Burnap *et al.* 1993, Falk *et al.* 1995). In addition, a significant decrease in the PSI and PSII contents of the cells and synthesis of CPVI-4 protein were observed under these conditions (Guikema and Sherman 1983b, 1984, Pakrasi *et al.* 1985a, Burnap *et al.* 1993). It was later demonstrated that the spectral changes in iron-starved cells was also associated with the presence of CPVI-4 protein (Guikema and Sherman 1983a, Pakrasi *et al.* 1985a,b). The nucleotide sequence analysis in *Anacystis nidulans* R2 (*Synechococcus sp*. PCC7942) revealed that a gene, *isiA*, was located in the same operon as *isiB* (which encodes for flavodoxin), right upstream of it (Laudenbach *et al.* 1988, Leonhardt and Straus 1992). A later report demonstrated that the CPVI-4 protein synthesized in iron-starved cells was encoded by *isiA* gene (Burnap *et al.* 1993). The nucleotide sequence analysis showed that *isiA* is highly homologous to CP43 (Laudenbach and Straus 1988), a Chl-binding membrane protein which is a core antenna of PSII. Crystallographic analysis of PSII showed that CP43 has six transmembrane helices and binds to 13 Chl *a* (Barber *et al.* 2000, Ferreira *et al.* 2004, Umena *et al.* 2011). The folding diagram of IsiA based on its nucleotide sequence in *Synechocystis* PCC 6803 and the hydropathy analysis suggested that IsiA also had six transmembrane helices and the histidine residues were conserved, which implied that IsiA may bind to 13 Chl *a* (Bibby *et al.* 2001b, Feng *et al.* 2011). Later, a comparative study of the integrated absorption of PSI and IsiA, indicated that IsiA possesses 13~16 Chl *a* (Andrizhiyevskaya *et al.* 2002, Feng *et al.* 2011). Despite the similarity, a noticeable difference between CP43 and IsiA is that the large loop on the lumenal side joining helices V and VI in CP43 is missing in IsiA, thus resulting in the ~100 less amino acid residues in IsiA than in CP43 (Bibby *et al.* 2001b).

IsiA was found to form an antenna ring around PSI trimer under iron-deficient conditions (Bibby *et al.* 2001a, Boekema *et al.* 2001). Electron microscopy single-particle analysis revealed that the PSI–IsiA supercomplex consisted of 18 IsiA and a trimeric PSI (Bibby *et al.* 2001a, Boekema *et al.* 2001). Later reports showed that during prolonged iron starvation, the number of IsiA and PSI monomers varied in the PSI–IsiA supercomplexes (Yeremenko *et al.* 2004). At times, empty IsiA rings were also detected under such conditions (Yeremenko *et al.* 2004, Chauhan *et al.* 2011). While IsiA was considered to be mainly associated with PSI, it was originally proposed to serve as an antenna for PSII to compensate the loss of phycobilisomes during iron starvation (Pakrasi *et al.* 1985b). A recent study reported the formation of IsiA–PSI–PSII supercomplex under iron-deficient as well as under high-light conditions (Wang *et al.* 2010). In addition, the presence of IsiA in a complex containing PsaD, slr1128, and high light-inducible proteins (Hilps) under high-light conditions was also reported (Wang *et al.*

2008, Daddy *et al.* 2015). However, this Hlip-containing complex could not be detected when Komenda and Sobotka (2016) attempted to reproduce the above results. Therefore, whether IsiA is involved in the Hlip-containing complex remains unclear and further investigation is needed.

Unlike *isiB* gene (Kutzki *et al.* 1998), *isiA* was shown to be an essential gene in iron-starved cyanobacteria by insertional mutagenesis (Burnap *et al.* 1993). Since it was reported that iron deficiency is common in significant fractions of the habitats of cyanobacteria (Martin and Fitzwater 1988, Vrede and Tranvik 2006, North *et al.* 2007, Bibby *et al.* 2009, Moore *et al.* 2013) and *isiA* was detected in several model cyanobacteria, *isiA* was thought to be widespread in species across the cyanobacterial kingdom (Geiss *et al.* 2001a). However, later reports showed that some cyanobacterial strains including *Synechococcus* WH 8102 did not have *isiA* (Bailey *et al.* 2005). In addition, instead of IsiA, prochlorophytes including *Prochloron*, *Prochlorothrix*, and *Prochlorococcus* were found to have prochlorophyte Chl-*a*/*b* protein (Pcb), which is also a member of the six-transmembrane helices antenna superfamily (La Roche *et al.* 1996). The field measurements conducted in the global ocean demonstrated that *isiA* is more prevalent in some iron-limiting oceanic regions, such as equatorial Pacific and Atlantic (Schrader *et al.* 2011, Richier *et al.* 2012, Ryan-Keogh *et al.* 2012). These facts are in conflict with the idea that *isiA* gene is commonly distributed in most cyanobacterial strains. Therefore, the physiological significance of IsiA and the role it plays in cyanobacteria need to be revisited.

Expression of IsiA

The transcripts of *isiAB* (iron stress-inducible) operon, encoding for IsiA protein and flavodoxin, was first identified when *Anacystis nidulans* R2 (*Synechococcus sp*. PCC7942) was grown under iron-deficient conditions (Laudenbach *et al.* 1988, Laudenbach and Straus 1988). However, later reports showed that *isiAB* operon could also be transcribed under other stressful conditions including high salt, heat shock (Vinnemeier *et al.* 1998), high light (Havaux *et al.* 2005), limiting light (Foster *et al.* 2007, Sandrini *et al.* 2016), and oxidative stress (Yousef *et al.* 2003, Li *et al.* 2004) as well as in some mutants, such as *psaFJ*-null mutant (Jeanjean *et al.* 2003) and cytochrome c_6-deficient mutant (Ardelean *et al.* 2002). However, it should be noted that the synthesis and integration of IsiA in thylakoid membranes were not observed under some of the conditions mentioned above.

The induction of *isiAB* operon as a function of the ammonium ferric citrate [Fe(NH_4) citrate] concentration in *Synechocystis* 6803 was determined by monitoring the fluorescence signal of green fluorescent protein (GFP) fused with *isiAB* promoter in iron-starved cells at different Fe(NH_4) citrate concentrations (Geiss *et al.* 2001a). The results showed a noticeable increase in GFP fluorescence signal when the Fe(NH_4) citrate concentration was below 0.77 μM (Geiss *et al.* 2001a), which again confirmed that the *isiAB* promoter is iron-responsive. Meanwhile, the mechanism of iron-responsive regulation controlling *isiAB* expression has been studied (Ghassemian and Straus 1996, Vinnemeier *et al.* 1998, Kunert *et al.* 2003). One of the hypotheses is that the *isiAB* expression is controlled by the ferric uptake regulator (Fur) (Ghassemian and Straus 1996, Vinnemeier *et al.* 1998, Kunert *et al.* 2003), a repressor binding to a Fur box under iron-replete conditions to repress gene expression, which is commonly found in prokaryotes (Stojiljkovic and Hantke 1995). Under nutrient-replete conditions, the Fur repressor binds to a Fur box upstream of *isiAB* operon and prevents the binding of RNA polymerase. On the other hand, under stressful conditions, the Fur repressor falls off and the expression of *isiAB* becomes possible (Vinnemeier *et al.* 1998, Kunert *et al.* 2003). As expected, the Fur box consensus sequences were found upstream of *isiA* gene in some cyanobacteria (Kunert *et al.* 2003), and the de-repression of *isiAB* operon was observed in the strains with insertional mutagenesis of *fur* gene (Ghassemian and Straus 1996). To further determine the regulatory elements controlling *isiAB* expression, GFP-containing strains were constructed in which GFP was fused with truncated *isiAB* promoter fragments containing different compositions of regulatory elements which include the A+T-rich region, inverted repeat (IR), and the Fur box (Kunert *et al.* 2003). Intriguingly, the truncation of the sequence between the A+T-rich and IR regions dramatically reduced the GFP fluorescence detected in iron-deficient conditions. This region was therefore considered to act as an unidentified positive regulatory element. Furthermore, a deletion of the Fur box also resulted in lower GFP fluorescence under iron-deficient conditions compared to iron-replete conditions, suggesting that an unrevealed mechanism is involved in repressing *isiAB* expression (Kunert *et al.* 2003). Besides the regulatory mechanism at transcriptional level, an antisense, IsrR (iron stress-repressed RNA), was identified that was involved in the posttranscriptional regulation of *isiA* expression (Dühring *et al.* 2006). IsrR is a cis-encoded antisense transcribed from the noncoding strand of *isiA* gene. It forms IsrR-*isiA* RNA heteroduplexes with *isiA* mRNA, and the heteroduplexes are targeted for selective degradation (Dühring *et al.* 2006). The inverse relationship between IsrR and *isiA* mRNA has been determined under oxidative stress, iron-deficient, and high-light conditions (Dühring *et al.* 2006). It is likely that IsrR is also present under the stressful conditions mentioned above, but the accumulation of *isiA* mRNA exceeds the amount of IsrR, thus resulting in the production of IsiA (Dühring *et al.* 2006). This regulatory machinery enables the cyanobacteria to instantly respond

to the environmental signals and control the expression of *isiA*. No *isiA* mRNA was detected from the IsrR knock-down strain in iron-replete conditions, which implied that IsrR regulatory mechanism was independent of the Fur mechanism (Dühring *et al.* 2006). However, this does not explain the expression/de-repression of *isiA* in other stressful conditions.

In order to elucidate the factors responsible for the induction of the *isiAB* operon, stress conditions other than iron-deficiency have been also studied, and the results suggested that cross-talks between multiple stress-induced genes existed (Jeanjean *et al.* 2003, Yousef *et al.* 2003, Michel and Pistorius 2004, Havaux *et al.* 2005). The production of reactive oxygen species (ROS) is inevitable in photosynthetic organisms, thus resulting in the oxidative stress in cells. A strong and rapid induction of *isiAB* operon was determined when *Synechocystis* PCC 6803 was treated with 75 μM H_2O_2 (Yousef *et al.* 2003, Li *et al.* 2004). In addition, studies focusing on the responses of cyanobacterial cells to oxidative stress conditions suggested that the ROS may interfere with the binding of Fur (ferric uptake regulator) or PerR (peroxide operon regulator) with the DNA as ROS was known to extract their metal cofactors, and thus de-repress the iron-inducible genes (Li *et al.* 2004). Interestingly, it was reported that no *isiA* transcript was detected in cells grown in iron and manganese co-limiting conditions (Salomon and Keren 2015). Since manganese depletion led to a decrease in the PSII content, which resulted in the limited production of ROS, the absence of *isiA* transcript in the cells under these conditions revealed the connection between *isiA* expression and oxidative stress, which appears to be a superior trigger for *isiA* expression (Salomon and Keren 2015). Besides *isiAB*, researchers have found that some other iron-inducible genes were also induced under oxidative stress conditions, such as *idiA* (iron-deficiency-induced), a gene encoding for a protein produced under stressful conditions involved in protecting PSII against the damage caused by ROS (Michel *et al.* 1996, Michel and Pistorius 2004). Even though the expression of *isiA* and *idiA* were thought to be independently controlled by Fur and IdiB (Michel *et al.* 1996), respectively, the *idiB* deletion strain showed a decreased IsiA content under iron-deficient conditions, which implied the cross-talk between the iron-inducible genes. Given that the stressful conditions inducing *isiAB* operon are either linked to oxidative stress or iron deficiency, a strong relationship between iron homeostasis and oxidative stress in cyanobacterial cells has been proposed (Yousef *et al.* 2003, Michel and Pistorius 2004). These findings explained the induction of *isiAB* operon under some stressful conditions other than iron stress, and supported the hypothesis that the cross-talk among stress-inducible genes are involved in *isiAB* expression. However, the expression of *isiA* has also been observed in the cells transitioning into stationary growth phase under normal physiological conditions without any stress imposed (Singh and Sherman 2006). Therefore, further investigation is needed to elucidate the entire story of the expression of *isiA*.

The time-course studies on synthesis and integration of IsiA protein in thylakoid membranes under iron-deficient conditions (Pakrasi *et al.* 1985a, Yeremenko *et al.* 2004, Ryan-Keogh *et al.* 2012, Fraser *et al.* 2013, Ma *et al.* 2017) provided another aspect for understanding the dynamic changes of *isiA* expression. IsiA was first identified as CPVI-4 protein (Pakrasi *et al.* 1985b), isolated from *Synechococcus sp*. PCC7942 cells iron-starved for 4 to 5 d. In addition, the PSI–IsiA supercomplex with 18 IsiA and 1 trimeric PSI was also isolated and visualized from *Synechocystis* PCC 6803 and *Synechococcus sp*. PCC7942 cells after short-term iron starvation (Bibby *et al.* 2001a, Boekema *et al.* 2001). It was later revealed that various other IsiA-associated supercomplexes can be formed with a few additional days of iron starvation (Yeremenko *et al.* 2004, Kouril *et al.* 2005). Yeremenko *et al.* (2004) performed electron microscopy followed by particle analysis on protein complexes isolated from *Synechocystis* PCC 6803 cells grown under conditions of prolonged iron deficiency, and visualized the IsiA ring structures with various compositions of IsiA and PSI (Yeremenko *et al.* 2004). Although the majority of PSI–IsiA supercomplex found was PSI_3IsA_{18}, with longer period of iron deficiency, smaller ring structures consisting of 12~14 IsiA and 1 PSI monomer, larger ones comprised of double IsiA rings with PSI at the center, IsiA double rings without PSI, as well as partial ring structures were observed (Yeremenko *et al.* 2004). However, it is difficult to monitor the exact amount of all these supercomplexes during iron starvation. To understand the dynamic changes of IsiA-associated ring structures during iron starvation, others have attempted to track the amount of bound and unbound IsiA at different time points (Ryan-Keogh *et al.* 2012, Fraser *et al.* 2013). It was found that the amount of unbound IsiA increased as the iron starvation prolonged and the growth of cells slowed after 72 h (Ryan-Keogh *et al.* 2012, Fraser *et al.* 2013). Nevertheless, the spectroscopic results showed fluorescence quenching caused by unbound IsiA at the early iron-starvation stage (van der Weij-de Wit *et al.* 2007). A recent study showed the complexities of IsiA-associated supercomplexes when they separated the thylakoid protein complexes by sucrose gradient ultracentrifugation from cells after 1–15 d of iron starvation (Ma *et al.* 2017). The protein fractions isolated from thylakoid membranes became more complicated as the iron starvation prolonged, and revealed distinct fluorescence properties (Ma *et al.* 2017). These findings suggested that IsiA proteins in various ring structures assembled during iron starvation, probably served distinct purposes to meet the need at different levels of iron deficiency.

The nutrient availabilities and the expression of IsiA in the world oceans have been also studied. In oligotrophic water and high-nitrate low-Chl (HNLC) regions, bioavailability

of iron is the main factor that limits the growth of phytoplankton (Martin and Fitzwater 1988, Coale *et al.* 1996, Tsuda *et al.* 2003, North *et al.* 2007). The analysis of dataset, obtained from the Global Ocean Sampling Project, has revealed the environmental diversity of Chl-binding protein complexes (Bibby *et al.* 2009). The *pcb*/*isiA* gene family had a higher genetic diversity in the open-ocean regions, and *isiA*-like gene was found predominately at the interface of two geographically defined ocean regions which are dominated by *pcb*-type and PBS-type light-harvesting systems, respectively. It was suggested that *isiA*-like gene was restricted to a defined oceanic region, so that the detection of *isiA*-like gene could be used as a biomarker of iron limitation in the ocean. Other studies suggested that 30% of the ocean is HNLC region, in which the low F_v/F_m value was detected and attributed to the presence of uncoupled IsiA rings (Behrenfeld *et al.* 2006, Moore *et al.* 2013). Schrader *et al.* (2011) have investigated *isiA* expression in *Synechocystis* PCC 6803 under iron and nitrogen co-limiting as well as high-nitrate low-iron conditions to mimic the natural environments (Schrader *et al.* 2011). Interestingly, the cells grown in co-limiting conditions showed low fluorescence emission and high F_v/F_m value similar to that of cells grown under nutrient-replete conditions (Schrader *et al.* 2011). On the other hand, cells grown in high-nitrate and low-iron, or HNLC, conditions showed the high fluorescence emission and low energy transfer efficiency (Schrader *et al.* 2011). In addition, the cells grown under co-limiting conditions had limited IsiA, while under HNLC conditions, the cells possessed a huge IsiA pool, which was not coupled to PSI (Schrader *et al.* 2011). These results suggested that the majority of IsiA was produced under co-limiting conditions was well-coupled to PSI and served as an accessory antenna of PSI. The energy transfer from IsiA to PSI was efficient so that the IsiA did not contribute to the fluorescence emission. Under high-nitrate low-iron conditions, a huge amount of IsiA antenna decoupled from PSI reaction center was produced, thus leading to the high fluorescence emission and low energy transfer efficiency (Schrader *et al.* 2011). The field studies conducted by collecting and analyzing the phytoplankton populations from HNLC, co-limiting and nutrient-replete regions showed the spectroscopic properties that were in agreement with laboratory experimental data (Schrader *et al.* 2011). The field data revealed the possible composition of photosynthetic proteins in cyanobacteria living in aquatic habitats with different nutrient availabilities. In nutrient-replete environments, cyanobacteria have PSI, PSII with phycobilisome, and no IsiA; in co-limiting regions, cyanobacteria have decreased PSI, limited IsiA coupled with PSI, and PSII with no phycobilisome; in HNLC environments, cyanobacteria have decreased PSI, IsiA coupled with PSI, IsiA rings decoupled from PSI and PSII with phycobilisome attached (Fig. 1). Besides, the results from field studies and laboratory experiments supported that up to half of total Chl in HNLC existed in uncoupled IsiA complexes and remained photo-synthetically inactive (Behrenfeld *et al.* 2006, Schrader *et al.* 2011).

Distribution and phylogeny of IsiA

Earlier studies focused on IsiA have relied on molecular techniques as well as genome sequence data to evaluate its distribution across the cyanobacterial kingdom (Geiss *et al.* 2001a, Bibby *et al.* 2009, Shih *et al.* 2013). Though initially thought to be widespread among cyanobacteria (Geiss *et al.* 2001a), later studies with marine microbes indicated that the gene is not ubiquitous in cyanobacteria (Bailey *et al.* 2005, Bibby *et al.* 2009). Shih *et al.* (2013) studied the distribution of Chl-binding proteins (CBP's) and found them to be widely distributed (84 of the 126 strains studied) across the cyanobacterial phylum. In this analysis, *isiA*-containing strains formed the largest clade of the CBP's (CBPIII). In the recent past, there has been an upsurge in the cyanobacterial sequenced database, with the sequences of many ecologically and physiologically diverse strains becoming available. This provides us with a unique opportunity to assess the relevance of this protein across the cyanobacterial phylum. We analyzed the genomes of ~390 cyanobacterial strains currently available in the *JGI/IMG* database for the presence of the *isiA* gene. A *blastp* search for IsiA across the available strains was performed, using *Synechocystis* 6803 IsiA, a 342-amino acid protein, as the template, and hits with ≥ 70% identity were designated as homologs of IsiA. In addition, some hits with lower percent identity but with proximity to the *isiB* gene in the genome and/or presence of a corresponding antisense RNA were also included as IsiA homologs for our analysis. The length of the protein identified in the above searches was also monitored to rule out nonspecific selections. In total, 125 cyanobacterial strains were found to contain IsiA that complied with one or more of the above criteria. These included unicellular as well as filamentous cyanobacteria from diverse ecological niches, with distinct physiological traits. Prominent among the ~265 strains, which lack *isiA*, are the *Prochlorococcus* and the marine *Synechococcus* strains (~150 strains). In addition, *Planktothrix, Tolypothrix*, and the thylakoid-less *Gloeobacter* strains do not appear to have *isiA*. Several cyanobacterial genera include strains that do not harbor *isiA*. Examples are the bloom-forming filamentous strains of *Microcystis* and *Anabaena* and unicellular diazotrophic strains like *Cyanothece* among others. The rationale for this variability among strains within a genus remains unclear. However, the variability is likely to be attributed to the presence of other low-iron-responsive proteins or to the differences in their niches which in turn determines their exposure to different environmental stresses. In accordance with the findings of Shih *et al.* (2013), we also

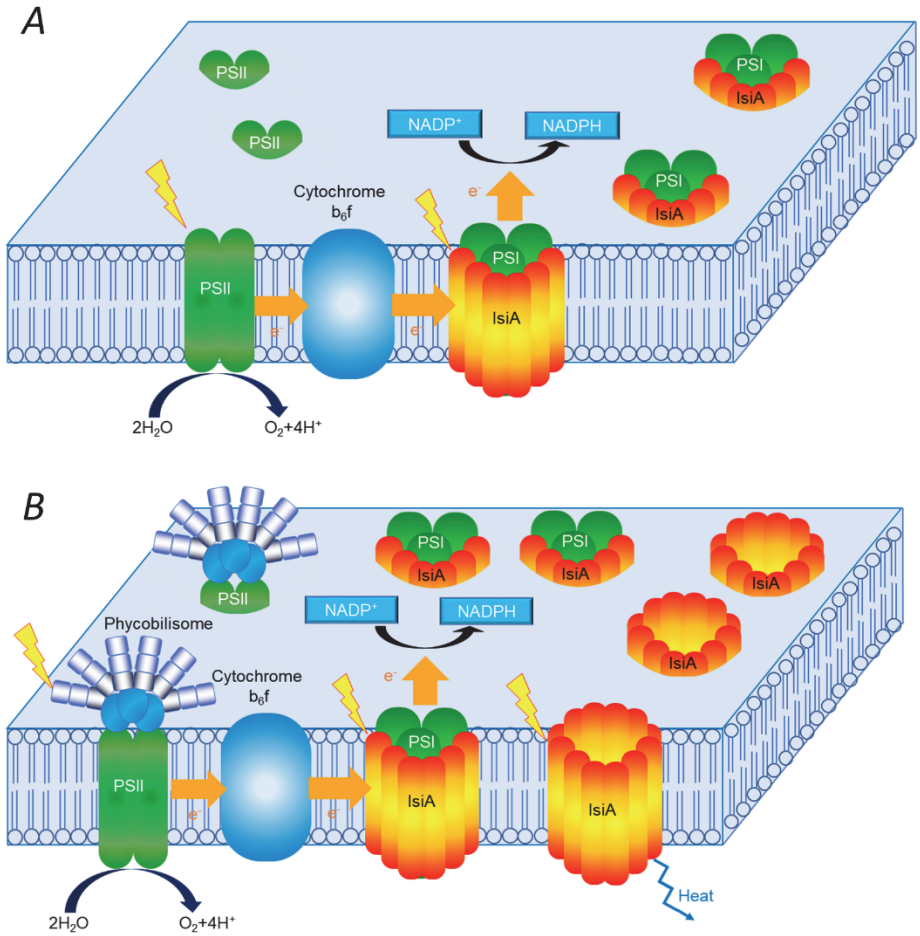

Fig. 1. A schematic model of thylakoid membranes of cyanobacteria grown under (*A*) iron and nitrogen co-limiting and (*B*) HNLC conditions. Under co-limiting conditions, cyanobacterial thylakoid membranes contain PSI–IsiA and PSII without phycobilisomes, while under HNLC conditions, cyanobacterial thylakoid membranes have PSI–IsiA, IsiA rings, and PSII with phycobilisomes attached.

located *isiA* in the same gene cluster with other CBP proteins in several strains. This might be indicative of the parallel functions of these light-harvesting proteins in some common pathways and these genes might have been included in specific gene islands by horizontal transfer as an adaptive strategy to specific environmental needs.

The distribution of IsiA varied among symbiotic strains. Uncultivated unicellular N_2-fixing cyanobacteria of group A (UCYN-A) are known to be endosymbionts of prymnesiophytes. Both *UCYNA-1* and *UCYNA-2* contain genes for PSI but lack genes for PSII resulting in the loss of photosynthetic ability. Our analysis revealed that these strain lacks IsiA. On the other hand, a photosynthetic symbiont of tunicates, *Prochloron didemni*, has the *isiA* gene (Fig. 2) (Zehr *et al.* 2008, Donia *et al.* 2011). This suggests that the IsiA machinery is likely to be maintained when there is a need for increasing photosynthetic efficiency and/or for dissipation of excess light energy, both presumably unnecessary in UCYN-A.

Of the 125 strains containing *isiA*, which were identified, 61 strains representing different cyanobacterial genera were selected for a phylogenetic analysis (Fig. 2). In this study the selection of the strains was not based on their ecology. Instead, strains representative of the diverse cyanobacterial genera, which are commonly studied for their interesting physiology or ecology and are currently present in the sequenced database, were selected. The tree revealed that the IsiA of *Synechocystis* 6803 is closely related to that of *Spirulina major* 6313 and they both share a common ancestor with *Thermosynechococcus elongatus*, *Cyanothece* 7425, and *Synechococcus* 6312. The IsiA in the marine *Cyanothece* strains 51142 and 0110 grouped separately from the terrestrial strains *Cyanothece* 7425 and 7424 and appeared to have co-evolved with the closely related marine strain *Crocosphaera watsonii*. Interestingly, the IsiA of 16 heterocystous cyanobacteria, which were included in this study, grouped together in a clade (highlighted in blue), implying the co-evolution of this gene in these members of the specialized group of cyanobacteria. Some of these strains were found to have *isiA* in the same operon as *isiB* as opposed to *isiB* being separately expressed as reported for some heterocystous

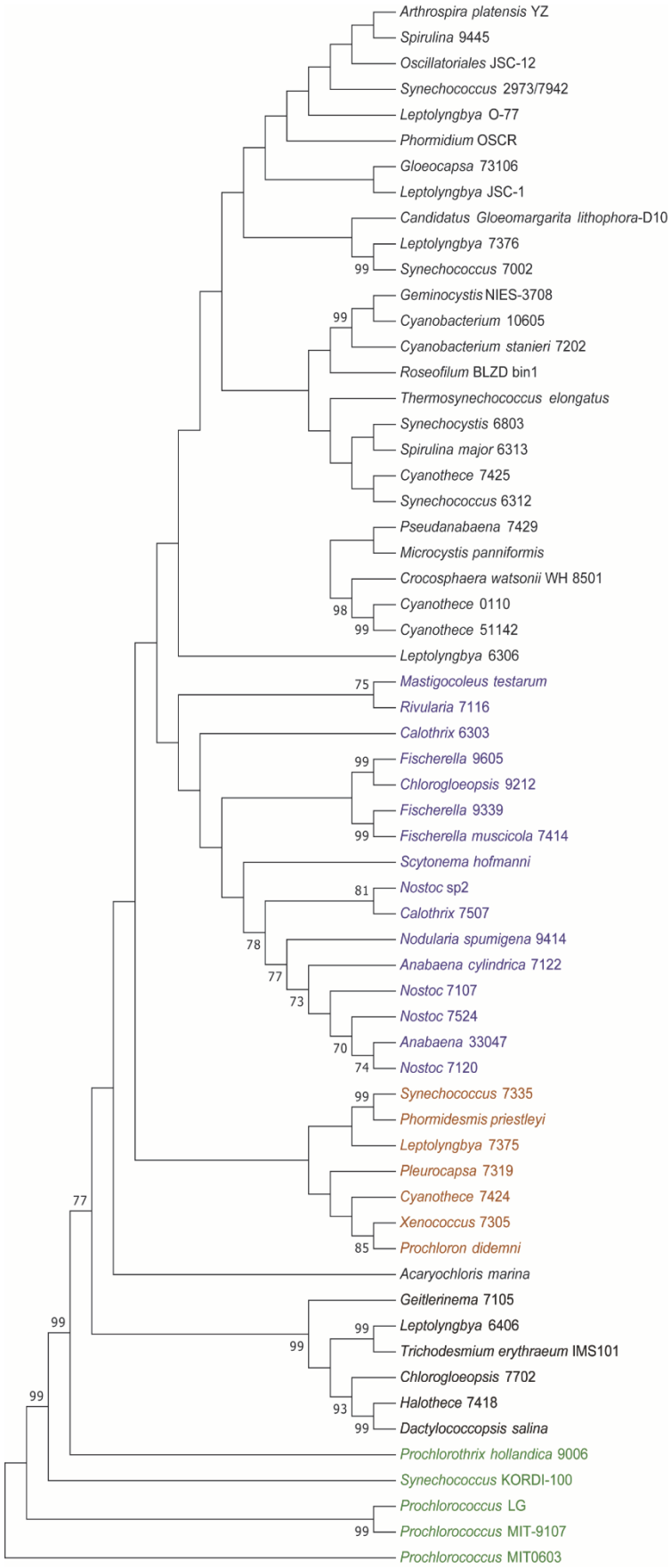

Fig. 2. Phylogenetic tree of the IsiA protein from 61 sequenced representatives of diverse cyanobacterial species. IsiA protein sequences were obtained from the *JGI/IMG* microbial database and aligned with *ClustalW* within *MEGA 7*. The phylogenetic tree was generated using *MEGA 7* (neighbor-joining method) (Saitou and Nei 1987) . The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). Only the nodes supported with a bootstrap of ≥ 70% are shown. All positions containing gaps and missing data were eliminated. *Prochlorococcus* strains containing the *pcb* genes were used to root the tree (green). The filamentous heterocystous cyanobacteria are shown in blue. The marine *Synechococcus* Kordi-100 strain and *Prochlorothrix hollandica*, which also contain the *pcb* gene, grouped together with the *Prochlorococcus*. Some anaerobic nitrogen fixers, which contain the pigment phycoerythrin, formed a distinct clade in the tree and are shown in orange color.

cyanobacteria (Geiss *et al.* 2001a,b). Some nonheterocystous anaerobic nitrogen fixers [except *Cyanothece* 7424, which has been reported as both an anaerobic (Turner *et al.* 2001) and aerobic (Bandyopadhyay *et al.* 2011)] grouped together (highlighted in brown) and appear to have evolved from a common ancestor. The *pcb*-containing *Prochlorothrix* and marine *Synechococcus* KORDI-100 grouped together with the *Prochlorococcus* strains (which also contain *pcb* genes) which were used as an outlier in this study (highlighted in green).

Discovery of IsiA functions

Although IsiA has been intensively studied for more than three decades, its functions are not fully understood yet. To elucidate the functions of IsiA, a comprehensive understanding of the factors, which induce its expression and the physiological changes under these conditions, is needed. As discussed in the previous section, most of the stressful conditions inducing *isiA* expression can be linked to oxidative stress or iron deficiency. In iron-deficient conditions, the decrease of PSI contents and the loss of thylakoid membranes are two of the significant changes (Sherman and Sherman 1983, Guikema and Sherman 1984). Since IsiA protein is the major Chl-binding protein produced under iron-deficient conditions, it is likely that the production of IsiA can compensate for the loss of the pigment-binding proteins. Therefore, the hypotheses for IsiA functions proposed are: (*1*) as a Chl-storage protein (Riethman and Sherman 1988); (*2*) as an accessary antenna for PSI (Burnap *et al.* 1993); and (*3*) as a dissipater to quench light energy (Park *et al.* 1999).

The analysis of Chl-binding proteins in thylakoid membranes of *Synechococcus sp*. PCC 7942 during the recovery from iron starvation showed a decrease in the IsiA content and a recovery of PSI and PSII contents within 24 h after the addition of iron (Pakrasi *et al.* 1985a). Moreover, experimental data showed that the addition of gabaculine, a Chl-synthesis inhibitor, did not affect the spectral change at the early stage of recovery for iron starvation (Guikema 1985). Therefore, IsiA has been thought to serve as a Chl *a*-storage protein that maintains the Chl *a* content in cells under iron-deficient conditions, and releases Chl *a* for the synthesis of other Chl-binding proteins, such as PSI, when iron concentration gets back to normal levels. Additionally, unlike PSII, IsiA is mobile in thylakoid membranes (Sarcina and Mullineaux 2004) probably because of the loss of the huge loop on the lumenal side, which is the main difference between IsiA and CP43 (Burnap *et al.* 1993). Furthermore, the binding of Chl *a* to IsiA was considered unstable (Riethman and Sherman 1988), which suggested that IsiA is able to deliver Chl *a* during the recovery from iron starvation. In HNLC environments, a huge pool of IsiA complexes were observed, which were unlikely to serve the purpose of photoprotection (Yeremenko *et al.* 2004, Ihalainen *et al.* 2005, Behrenfeld *et al.* 2006, Schrader *et al.* 2011). Instead, the IsiA complexes may be produced to maintain the Chl *a* content. It needs to be noted that atmospheric deposition of iron is an important iron source that episodically provides soluble iron to phytoplankton. The Chl *a* in IsiA can be rapidly released and used to produce PSI and PSII once their living environments receive iron pulses (Krishnamurthy *et al.* 2010, Schrader *et al.* 2011).

When IsiA was first identified in iron-starved *Synechococcus sp*. PCC 7942, it was thought as an intermediate antenna complex of PSII that absorbs light energy to compensate for the loss of phycobilisomes (Pakrasi *et al.* 1985b). The image of PSI–IsiA supercomplex obtained by electron microscopy single-particle analysis provided abundant structural information which showed that IsiA was a peripheral membrane antenna associated with PSI (Bibby *et al.* 2001a, Boekema *et al.* 2001). Since a PSI trimer has 288 Chl *a* (Jordan *et al.* 2001) and an IsiA has 13 Chl *a*, according to the latest report (Feng *et al.* 2011), the IsiA ring surrounding the PSI trimer in a PSI_3IsiA_{18} supercomplex increases the theoretical absorption cross-section by 81%, which suggests a great potential of IsiA for improving the light absorption capacities of PSI. The spectroscopic data showed multiple energy transfer stages after the excitation of Chl *a* in PSI–IsiA supercomplexes, indicating the fast and efficient energy transfer between IsiA, within IsiA, and from IsiA to PSI (Melkozernov *et al.* 2003, Andrizhiyevskaya *et al.* 2004). In addition, a recent report showed that the electron throughput in PSI was enhanced while PSI was coupled to an IsiA ring (Sun and Golbeck 2015). Moreover, the mere 16% increase in exciton trapping time in $PSI–IsiA_{DR}$, the largest PSI–IsiA supercomplex isolated, which has an IsiA double ring and a PSI trimer, compared to that in PSI timer, showed the well-coupled pigment network in PSI–IsiA supercomplex (Chauhan *et al.* 2011). The effective absorption cross-section of PSI (σ_{PSI}) in iron-starved *Synechocystis* sp. PCC6803 was measured *in vivo* and a 60% increase in σ_{PSI} was observed with the accumulation of IsiA (Ryan-Keogh *et al.* 2012). Given the experimental data mentioned above, it was demonstrated that IsiA serves as a peripheral membrane antenna of PSI. However, since the available PSI–IsiA crystal structure is only at the resolution of ~20 Å (Nield *et al.* 2003), it is impossible to accurately simulate the excitation energy transfer (EET) within the IsiA ring or from IsiA ring to PSI. The models of the EET in PSI–IsiA reported were constructed based on the positions of Chl *a* in CP43 and the relative positions between CP43 and PSII reaction center (Nield *et al.* 2003, Riley *et al.* 2006, Feng *et al.* 2011). It was proposed that the helices 5 and 6 of IsiA are facing the PSI trimer, and a well-defined path for EET from IsiA ring to PSI exists (Nield *et al.* 2003, Riley *et al.* 2006). Additionally, similar to the two lowest energy states observed in CP43, which

may play a role in the photoinhibitory and light-harvesting processes (Reppert *et al.* 2008), the analogous energy states identified in IsiA are likely to facilitate energy transfer from IsiA to PSI (Feng *et al.* 2011). Furthermore, Chl *a* 44 and 37, located in the proximity of PSI, were proposed to be the Chls contributing to the lowest-energy states A and B (Feng *et al.* 2011), which is in agreement with the structural model proposed by Nield *et al.* (2003).

While IsiA was considered to improve the absorption cross-section of PSI and help capture light energy, some proposed that IsiA also functions as a nonphotochemical quencher that protects PSII from photodamage (Park *et al.* 1999). This hypothesis was supported by the fact that the strain with the nonfunctional *isiA* gene (*isiA*$^-$), had higher rate of oxygen evolution under modest illumination and was more sensitive to light intensity (Park *et al.* 1999). By overexpressing *isiA* in a *Synechococcus* sp. PCC 7942 strain, the photoinhibition of photosynthesis under high-light conditions was eliminated, which again showed that IsiA was involved in photoprotection (Sandström *et al.* 2001). Moreover, a blue light-induced fluorescence quenching was observed in iron-starved cells (Cadoret *et al.* 2004). However, it was not clear how a protein is able to function as a light-harvesting antenna as well as a nonphotochemical quencher. This question was addressed by later studies in which the various IsiA-associated ring structures were identified and experimentally proved to play distinct roles in iron-starved cells (Yeremenko *et al.* 2004, Ihalainen *et al.* 2005). Intriguingly, later reports showed that the blue light-induced fluorescence quenching in the IsiA-deletion strain was similar to that in wild type, and instead, the orange carotenoid protein played the central role in this process, suggesting that IsiA is not involved in the blue light-induced nonphotochemical quenching (NPQ) process (Wilson *et al.* 2006, Karapetyan 2007). This again questioned the mechanism of IsiA-mediated dissipation of light energy in iron-starved cells. Berera *et al.*(2009) proposed that energy in the Chl *a* pool was ultimately transferred to a quenching site, a carotenoid in IsiA (Berera *et al.* 2009, 2010), which is the same mechanism as the light-harvesting complex II in green plant and Hilp utilizes for energy dissipation (Ruban *et al.* 2007, Niedzwiedzki *et al.* 2016). Nevertheless, the direct evidence of carotenoid involvement in this quenching process was missing. In addition, it has been determined that the lifetime of excited Chl *a* fluorescence in IsiA is highly dependent on temperature (Chen *et al.* 2017), which is not shown in Hilp (Niedzwiedzki *et al.* 2016). Additionally, our previously published spectroscopic results showed that EET between Chl *a* and carotenoids in IsiA was absent, suggesting a novel quenching mechanism other than carotenoid quenching process in IsiA (Chen *et al.* 2017). Based on the spectroscopic results, we proposed that the quenching process was completed by a cysteine-mediated protein–pigment interaction which was previously demonstrated in Fenna-Mathews-Olson (FMO) protein, a light-harvesting protein in green purple bacteria (Orf *et al.* 2016).

Conclusion

During the past decades, considerable efforts have been devoted towards understanding the role that IsiA plays in cyanobacteria. Previous reports have shown that IsiA is required for growth of cyanobacteria under iron-deficient conditions (Burnap *et al.* 1993, Park *et al.* 1999). The hypothesis that IsiA stores Chl *a* in iron-deficient conditions and assimilates into photosynthetic proteins is supported by previous studies. Besides, the *in vitro* and *in vivo* measurements demonstrated that IsiA serves as an accessary antenna of PSI to increase the absorption cross section of PSI in PSI–IsiA supercomplex. Furthermore, it has been determined that IsiA dissipates excess light energy to prevent photosynthetic proteins from photodamage when it is in an IsiA aggregate. However, the high-resolution crystal structure of IsiA is still unavailable, which makes it even more difficult to understand the processes and mechanisms of energy transfer from IsiA to PSI and the excited energy quenching in IsiA aggregate. In addition, the expression of *isiA* has been observed under various stressful conditions, although what IsiA does in these conditions is still unclear.

About 390 strains with available genomes in the *JGI/IMG* database have been analyzed in this study. Surprisingly, only about one-third of these strains were found to have the *isiA* gene. Habitat of the strain could be a determining factor for the presence or absence of the *isiA* gene. The chances are that the strains without IsiA either live in iron-replete habitats or have developed other approaches to survive in iron-deficient conditions. Our study shows that *isiA* is ubiquitous among aquatic cyanobacterial strains which are likely to be subjected to iron deficiency under their natural growth conditions.

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