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Outer membrane proteins induced by iron deficiency in *Anabaena* sp. PCC 7120

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Abstract

Iron deficiency can induce cyanobacteria to synthesize siderophore receptor proteins on the outer membrane to enhance the uptake of iron. In this study, an outer membrane of high purity was prepared from *Anabaena* sp. PCC 7120 based on aqueous polymer two-phase partitioning and discontinuous sucrose density ultra-centrifugation, and the induction of outer membrane proteins by iron deficiency was investigated using 2-D gel electrophoresis. At least five outer membrane proteins were newly synthesized or significantly up-regulated in cells transferred to iron-deficient conditions, which were all identified to be siderophore receptor proteins according to MALDI-TOF-MS analyses. Bacterial luciferase reporter genes *luxAB* were employed to monitor the transcription of the encoding genes. The genes were induced by iron deficiency at the transcriptional level in different responsive modes. Luciferase activity expressed from an iron-regulated promoter may be used as a bioreporter for utilizable iron in natural water samples.
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Keywords: *Anabaena*; Outer membrane; Siderophore receptors; Iron deficiency

1. Introduction

Iron is one of the essential nutrient elements for organisms. Iron-containing proteins are involved in important metabolic processes such as oxygen transport, electron transfer, oxidative phosphorylation and many oxido-reductive reactions. Iron is one of the most abundant elements on the surface of the earth, and usually exists in the form of Fe^{2+} or Fe^{3+} in aquatic environments. Fe^{2+} can be very easily oxidized into Fe^{3+}, transformed into iron hydroxide, which is insoluble and inaccessible to organisms. Microbes can synthesize siderophores, which are high-affinity Fe^{3+} chelators, and secrete them to the extracellular environment to form the siderophore–Fe^{3+} complex [1]. Gram-negative bacteria, including cyanobacteria, possess outer membrane receptors for the siderophore–Fe^{3+} complex [1,2]. These receptors in the outer membrane depend on the TonB–complex in the cytoplasmic membrane to transport the siderophore–Fe^{3+} complex to the periplasmic space. TonB traverses the periplasmic space and interacts with the outer membrane receptors, energizing the transport by outer membrane receptors with the proton gradient across the cytoplasmic membrane [1]. The siderophore–Fe^{3+} complexes are then transported into the cytoplasm by specific binding proteins in the periplasmic space and ABC (ATP-binding cassette) permeases on the cytoplasmic membrane [1].

Anabaena species are filamentous nitrogen-fixing cyanobacteria, which synthesize siderophores such as schizokinen and anachelin [3–5]. Iron starvation leads to oxidative stress in *Anabaena* [6]. A large gene cluster involved in siderophore synthesis was identified in *Anabaena* sp. PCC 7120 (hereafter referred to as *Anabaena* 7120) [7]. A proteomic study of the outer membrane in this cyanobacterium also revealed the presence of pre-

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dicted receptors for the siderophore–Fe^{3+} complex [8]. One of the proteins Alr0397 (SchT) was shown to be the TonB-dependent schizokinen transporter up-regulated under iron-deficient conditions [9]. In this study, we isolated the outer membrane of high purity from *Anabaena* 7120 grown in iron-deficient medium, analyzed the outer membrane proteins and found five such receptor proteins induced by iron deficiency, of which three proteins were identified for the first time. Bacterial luciferase genes *luxAB* were employed to show the transcription of the encoding genes, and all the genes were found induced at the transcriptional level with differences at time points and amplitudes.

2. Materials and methods

2.1. Strains, culture and conjugation

Anabaena 7120 was from the Freshwater Algal Culture Collection at the Institute of Hydrobiology of the Chinese Academy of Sciences, cultured in BG11 medium at 28–30 °C in the light of 50–70 $\mu E\ m^{-2}\ s^{-1}$ with aeration. *Anabaena* strains with *luxAB*-Ω were cultured in BG11 with spectinomycin (Sp, 10 mg/ml). Growth was monitored based on turbidity (OD730) and chlorophyll *a* (μg/ml) measurements. Chlorophyll *a* (μg/ml) was extracted with methanol and calculated as $13.34 \times OD_{664}$. For the induction by iron deficiency, *Anabaena* cells were washed twice with BG11 minus ferrous ammonium citrate (referred to as BG11-Fe) and re-suspended in the same medium. Conjugation between *Escherichia coli* and the cyanobacterium was performed according to Elhai et al. [10].

2.2. Isolation of the outer membrane

Anabaena 7120 was grown in BG11 with aeration to the exponential phase, harvested by centrifugation and immediately frozen in liquid nitrogen for use in the isolation of the outer membrane. The purification of the outer membrane was performed according to Norling et al. [11] and Huang et al. [12] with modifications. Total membranes were subjected to separation with the 6.6% two-phase system twice followed by separation with the 7.2% system twice. Membranes in the upper phase were collected by ultra-centrifugation (103,000*g*), used for preparation of the discontinuous sucrose density gradient: 2.0 ml of 60%, 3.0 ml of 50% (with the collected membrane sample), 3.0 ml of 40%, 2.0 ml of 30%, and 2.0 ml of 10% sucrose, and further purified by ultra-centrifugation (197,000*g*) for 4 h. Membranes from fractions 50% to 60% were collected and precipitated by ultra-centrifugation, stored at −70 °C. In order to prevent protein degradation, all steps including centrifugation were performed at 4 °C and the proteinase inhibitor PMSF was added prior to cell breakage and during the membrane preparations.

2.3. Protein analyses

Outer membranes were extracted twice with methanol/chloroform according to Wessel et al. [13]. The resulted protein precipitate was solubilized in re-hydration solution (8 M urea, 2% CHAPS, 15 mM DTT, 0.5% IPG buffer) and applied to the first-dimension protein separation on an IPGphor Electrophoresis System (Amersham Biosciences) using a 13-cm linear IPG (immobilized pH gradient) strip (pH 4–7, Amersham Biosciences). Isoelectric focusing was performed until a total of 130,000 Vh was achieved. For the second dimensional separation, gel strips were equilibrated as described by Cordwell et al. [14] and applied onto 12% polyacrylamide gels for SDS–PAGE. Proteins were visualized by staining with Coomassie brilliant blue G-250. SDS–PAGE and Western blot analysis were performed as standard methods [15]. Rabbit antisera were prepared in this laboratory. Protein spots were analyzed by the MALDI-TOF mass spectrum, which was performed at the Health Science Center of Peking University.

2.4. Assays of luciferase activity

Luciferase activity was measured according to Elhai and Wolk [16]. One milliliter of cyanobacterial cells was supplemented with 0.1% (v/v) *n*-decanol, thoroughly mixed and measured in a tube luminometer (Berthold Biotechnologies). Luciferase activity was calculated as relative luminescence units per microgram of chlorophyll *a*.

2.5. Construction of plasmids

Upstream sequences of outer membrane protein genes were generated by PCR amplifications using primers 1 and 2 listed in Table 1, cloned in the T-vector pMD18-T (Takara) and confirmed by sequencing. The plasmids were restricted with *Xho*I and *Sma*I, blunted with T4 DNA polymerase, inserted with the *luxAB*-Ω fragment excised with *Sma*I from pRL58 [17]. From the resulted plasmids, the promoter-*luxAB*-Ω structure was excised with *Nhe*I and *Kpn*I, blunted with T4 DNA polymerase and cloned into *Xba*I-cut and T4-DNA polymerase blunted pRL278 [18]. The plasmids used to construct single-crossover homologous recombinants of *Anabaena* 7120 are listed in Table 1.

2.6. Bioinformatics

MALDI-TOF-MS analysis data were processed using MASCOT (http://www.matrixscience.com) to identify genes (or ORFs) corresponding to the protein spots. Amino acid sequences of the proteins were retrieved from the Cyanobase (bacteria.kazusa.or.jp/cyanobase/). Signal P3.0 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict signal peptide sequences, TMHMM 2.0.1 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used to predict transmembrane regions, phyre version 0.2

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Table 1
Anabaena strains, plasmids and primers used in this study.

Strains, plasmids or primers	Derivation, relevant characteristics or sequence
Anabaena sp. PCC 7120	
SR2508	Nm^rSp^r, resulted from single crossover with pHB2508, to report the transcription of P*alr0397* with *luxAB*
SR2509	Nm^rSp^r, resulted from single crossover with pHB2509, to report the transcription of P*all1101* with *luxAB*
SR2510	Nm^rSp^r, resulted from single crossover with pHB2510, to report the transcription of P*alr2153* with *luxAB*
SR2511	Nm^rSp^r, resulted from single crossover with pHB2511, to report the transcription of P*alr2581* with *luxAB*
SR2579	Nm^rSp^r, resulted from single crossover with pHB2579, to report the transcription of P*alr2588* with *luxAB*
SR2564	Nm^rSp^r, resulted from single crossover with pHB2564, to report the transcription of P*all2674* with *luxAB*
SR2512	Nm^rSp^r, resulted from single crossover with pHB2512, to report the transcription of P*all3310* with *luxAB*
SR2513	Nm^rSp^r, resulted from single crossover with pHB2513, to report the transcription of P*all4026* with *luxAB*
Plasmids	
pHB2508	$Km^r(Nm^r)Sp^r$; P*alr0397-luxAB*-Ω
pHB2509	$Km^r(Nm^r)Sp^r$; P*all1101-luxAB*-Ω
pHB2510	$Km^r(Nm^r)Sp^r$; P*alr2153-luxAB*-Ω
pHB2511	$Km^r(Nm^r)Sp^r$; P*alr2581-luxAB*-Ω
pHB2579	$Km^r(Nm^r)Sp^r$; P*alr2588-luxAB*-Ω
pHB2564	$Km^r(Nm^r)Sp^r$; P*all2674-luxAB*-Ω
pHB2512	$Km^r(Nm^r)Sp^r$; P*all3310-luxAB*-Ω
pHB2513	$Km^r(Nm^r)Sp^r$; P*all4026-luxAB*-Ω
pHB2508	$Km^r(Nm^r)Sp^r$; P*alr0397-luxAB*-Ω
Primers	
Palr0397-1	5′-GCTAGCGAGCCTCACTAATGGCAATCC-3′
Palr0397-2	5′-CTCGAGGTTGCGACTGGATTATGGCT-3′
Palr0397-3	5′-GCTACTGGGTGATTGTGCC-3′
Pall1101-1	5′-GCTAGCTGAAACACCCTCTTACACCC-3′
Pall1101-2	5′-CTCGAGCCATTGGACGCTTTTGGAG-3′
Pall1101-3	5′-CCAAGATGCTGTGCGTTTG-3′
Palr2153-1	5′-GCCAGTCAAGATTGCTAGCC-3′
Palr2153-2	5′-CTCGAGCAGCAGCGAATACCGCAG-3′
Palr2153-3	5′-GTGGACGGGTGTCTTTACTATC-3′
Palr2581-1	5′-GCTAGCCTTGACGATAACCTTCACCC-3′
Palr2581-2	5′-CTCGAGCCGCTCACTCTTCTCACTATC-3′
Palr2581-3	5′-GTTGAATCCCACCACCAC-3′
Palr2588-1	5′-GCTAGCGGATTACCACCCACGAAATG-3′
Palr2588-2	5′-CTCGAGACCGTCGCCGCCAAAC-3′
Palr2588-3	5′-CTGCTCCTAACTGTCTGTGG-3′
Pall2674-1	5′-GCTAGCGACAGCCAACTCAGTCAACC-3′
Pall2674-2	5′-CTCGAGAACACCAATGGCGGGTTGCC-3′
Pall2674-3	5′-CCTCAACCAGCAGCAGGAAG-3′
Pall3310-1	5′-CGCGCTAGCCTTTACTATTGG-3′
Pall3310-2	5′-CTCGAGTCAGCACTGGTGGTGGG-3′
Pall3310-3	5′-CCTTGATGCTGATGAGAAGTTG-3′
Pall4026-1	5′-GCTAGCGGCAGATTGGGTTTACCGT-3′
Pall4026-2	5′-GCTAGCGGCAGATTGGGTTTACCGT-3′
Pall4026-3	5′-GGGCATTCTGACTTTGAGG-3′
luxAB-1	5′-AATGGGTCTCGCACTTTCGCC-3′

(http://www.sbg.bio.ic.ac.uk/phyre/) was used to predict the 3-dimensional structure of proteins.

3. Results and discussion

Anabaena 7120 can utilize the residual iron in cells and nutrient salts to support its growth in BG11 without ferrous ammonium citrate (BG11-Fe) for a limited period of time. Under the condition of aeration, compared to its growth in BG11 with ferrous ammonium citrate (BG11+Fe), *Anabaena* 7120 showed significantly reduced growth in BG11-Fe on the 4th day and gradually ceased its propagation (Fig. 1). The outer membrane was isolated from *Anabaena* 7120 induced in BG11-Fe for 48 h and subjected to 2-D gel electrophoresis protein analysis and compared with that from cells grown in BG11+Fe. As seen with absorption spectra and Western blot detection of marker proteins specific to three different membranes, the outer membrane isolated by aqueous polymer two-phase partitioning and discontinuous sucrose density ultra-centrifugation showed high purity, and no contamination by cytoplasmic and thylakoid membranes was found (data not shown). At least seven newly synthesized or significantly up-regulated protein spots were found on the 2-D gel with the outer membrane of cells induced by iron deficiency (Fig. 2). Three independent experiments showed

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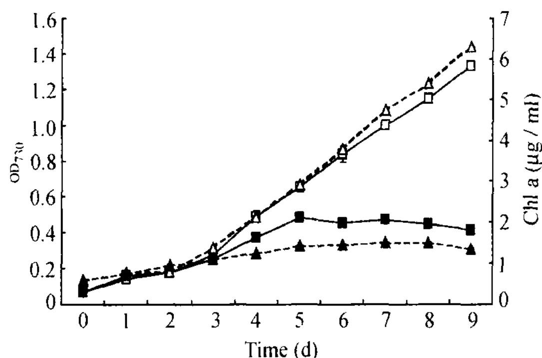

Fig. 1. Effects of iron on the growth of *Anabaena* 7120 bubbled with air. Empty and solid triangles stand for chlorophyll *a* contents in BG11 with (+Fe) and without (−Fe) ferrous ammonium citrate, respectively; empty and solid squares stand for turbidity (OD_{730}) in BG11+Fe and BG11-Fe, respectively.

consistent results. MALDI-TOF-MS analysis showed that these seven spots were from five different proteins (Table 2). Protein spot 1 was All1101, spot 2 was Alr2581, spots 3 and 4 were Alr0397, spots 5 and 7 were All2674, and spot 6 was Alr2153. The presence of two spots for the same protein suggested post-translational modification(s) of the protein.

Signal P3.0 and TMHMM 2.0.1 predict that all these five outer membrane proteins carry signal peptide sequences and transmembrane regions (Table 2). Similarity search results indicate that All1101 (Expect = 3e-73) and All2674 (Expect = 2e-68) are highly similar to the ferrichrome-iron receptor FhuA in *E. coli*, Alr2581 (Expect = 2e-60) and Alr0397 (Expect = 2e-73) similar to the ferric aerobactin receptor IutA, Alr2153 (Expect = 7e-29) similar to the heme/hemoglobin receptor ChuA. As shown by the 3-D structure predicted with phyre version 0.2, all these proteins possess β-barrel and the inner plug-like domain as the siderophore receptors in *E. coli* [2]. Of 48 cyanobacterial strains found in the NCBI GenBank, only several species possess homologues. For example, Alr2153 finds counterparts of high similarity in strains *Synechococcus* sp. JA-3-3Ab, *Synechococcus* sp. PCC 7002 and *Acaryochloris marina* MBIC11017. The high similarity of this protein to the heme/hemoglobin receptor in *E. coli* sug-

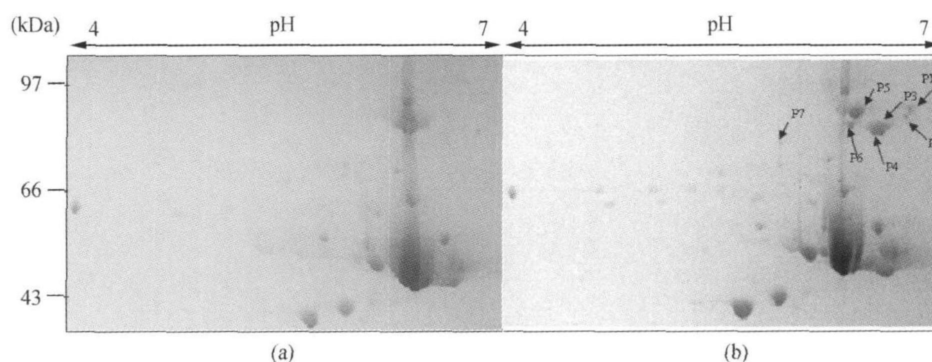

Fig. 2. The 2-D gel electrophoretogram of outer membrane proteins in *Anabaena* 7120 cultured in BG-11+Fe (a) and BG11-Fe (b). Spots 1–7 in (b) are newly synthesized or up-regulated proteins. Identifications of these proteins are given in Table 2.

Table 2
Proteins identified in the outer membrane of *Anabaena* 7120.

Spot No. in Fig. 1	ORF	Gene product	MOWSE score	Masses matched	Seq. cov. (%)	MW, kDa/pI	Signal P 3.0	Position of transmembrane helices
1	all1101	Ferrichrome-iron receptor	295	28/40(70%)	38	98.1/4.6	LIA-PT(31)	17–33
2	alr2581	Ferric aerobactin receptor	110	18/40(45%)	25	96.4/4.7	AVA-ET(34)	14–34
3	alr0397	Ferric aerobactin receptor	158	19/40(48%)	26	94.3/4.8	VWS-LI(36)	24–40 608–632
4	alr0397	Ferric aerobactin receptor	262	29/40(73%)	30	94.3/4.8	VWS-LI(36)	24–40 608–632
5	all2674	Ferrichrome-iron receptor	271	29/40(73%)	39	95.5/4.7	VWA-AP(26)	6–27
6	alr2153	Heme/hemoglobin receptor	153	20/40(50%)	23	89.5/4.8	VFA-AE(23)	6–22
7	all2674	Ferrichrome-iron receptor	275	26/40(65%)	37	95.4/4.7	VWA-AP(26)	6–27

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gests that photoautotrophs like cyanobacteria may also utilize heme as a source of iron in water bodies.

To see if these genes are regulated at the transcriptional level, we cloned their promoter regions, positioned bacterial luciferase genes *luxAB* downstream of the cloned promoter regions, introduced the resulted plasmids into *Anabaena* 7120 through conjugation and obtained single-crossover homologous recombinants for each gene. The homologous recombination of these plasmids into the *Anabaena* genome was confirmed with polymerase chain reactions using primer 3 and luxAB-1 (Fig. 3). Of the two primers, primer 3 is located upstream of the cloned promoter region, and luxAB-1 is located at the 5′ end of *luxA*. According to Ref. [8], the other three proteins involved in iron transport were identified in the outer membrane of *Anabaena* cells grown under iron-sufficient conditions. We also included these three encoding genes as controls for the *luxAB*-based transcriptional study. *Anabaena* strains harboring promoter-*luxAB*-Ω are listed in Table 1.

The cyanobacterial strains were transferred into BG11-Fe to induce with iron deficiency, and LuxAB activity was followed within 48 h. Of the eight genes, some showed significant up-regulation with different extents at the transcriptional level, while the rest showed almost no up-regulation. In Fig. 4, *all4026* showed almost no transcriptional regulation, *alr2588*, *all3310* and *all2674* showed low level up-regulation with peaks at 12 h, 24 h and 36 h, respectively. The rest of the four genes were all greatly up-regulated, but *all1101*, *alr2153* and *alr2581* showed up-regulation within 12 h post-induction with iron deficiency, while *alr0397* showed up-regulation after 12 h. Among these genes, *alr2588*, *all3310* and *all4026* encode proteins of low levels, whose spots were hardly visible on the Coomassie-stained 2-D gel. *alr0397*, *all1101*, *alr2153* and *alr2581* showed great up-regulation at both transcriptional and protein levels. *all2674* was significantly up-regulated at the protein level, but less up-regulated at the transcriptional level.

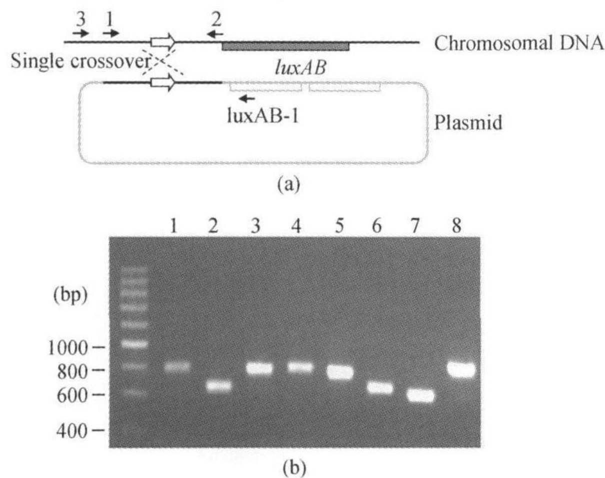

Fig. 3. Detection of single-crossover homologous recombinations in *Anabaena* 7120. (a) A diagram showing single-crossover homologous recombination. Short lines with arrowheads stand for PCR primers. Primers 1 and 2 were used in cloning of the promoter regions, primers 3 and luxAB-1 were used in verification of single-crossover homologous recombinations. Primers 1, 2 and 3 for each gene and luxAB-1 are listed in Table 1. (b) The electrophoretogram of DNA fragments generated from polymerase chain reactions detecting homologous single recombination in *Anabaena* 7120. 1, *Anabaena* 7120::$P_{alr0397}$-*luxAB*-Ω; 2, *Anabaena* 7120::$P_{all1101}$-*luxAB*-Ω; 3, *Anabaena* 7120::$P_{alr2153}$-*luxAB*-Ω; 4, *Anabaena* 7120::$P_{alr2581}$-*luxAB*-Ω; 5, *Anabaena* 7120::$P_{alr2588}$-*luxAB*-Ω; 6, *Anabaena* 7120::$P_{all2674}$-*luxAB*-Ω; 7, *Anabaena* 7120::$P_{all3310}$-*luxAB*-Ω; and 8 *Anabaena* 7120::$P_{all4026}$-*luxAB*-Ω.

In this study, analysis of outer membrane proteins in *Anabaena* revealed the presence of at least five siderophore receptors significantly up-regulated under the condition of iron deficiency, and the encoding genes were shown to be induced at the transcriptional level. Moslavac et al. used HPLC-MS analysis to perform proteomic analysis of outer membrane proteins under iron-sufficient conditions and found four siderophore receptors including Alr0397 [8]. We showed that the other three receptors they identified are basically not regulated at the transcriptional level. In *Synechocystis* sp. PCC 6803, microarray analysis was employed to investigate iron-deficiency-induced genes, and only one gene encoding the ferrichrome-Fe^{3+} receptor was identified [19]. Such differences may be due to the variation in iron-uptake of the outer membrane proteins in different cyanobacterial species, or, on the other hand, indicate that protein analysis is more effective than the DNA microarray in identifying regulated expression of outer membrane protein genes. In *E. coli*, the outer membrane receptor protein FecA undergoes major conformational changes upon occupancy by diferric dicitrate, affecting FecR in the cytoplasmic membrane, so that FecI on the plasma membrane is released to the cytoplasm and promotes the transcriptional expression of genes *fecABCDE* encoding the ferric citrate transport system [20]. In contrast, our results showed that *alr0397*, *all1101*, *alr2153* and *alr2581* were all significantly induced by iron deficiency. Therefore, these genes must not be induced by siderophore–Fe^{3+} occupancy of receptors on the surface. In *Anabaena* 7120, the uptake of iron is regulated by FurA, and the binding of FurA to the promoter of *isiB*, a gene encoding flavodoxin and up-regulated by iron deficiency, has been shown [21]. However, the iron-deficiency-induced siderophore receptor genes found in this study are not in the list of genes with predicted recognition sites for FurA [22]. Protein kinase genes *pkn41* and *pkn42* are both induced by iron deficiency, and affect the uptake of iron by *Anabaena* cells [23]. Whether *pkn41* and *pkn42* affect the expression of these five outer membrane siderophore receptor genes should merit further investigations. Among these five genes, the induction of *alr0397* by iron deficiency was delayed relative to other genes. Such a delay may be due to the difference in the level of utilizable iron required to initiate the gene expression; alternatively, *alr0397* is induced by iron-deficiency caused physiological stress [6] rather than the availability of utilizable iron. It is also noteworthy that relative to its up-regulation at the protein level,

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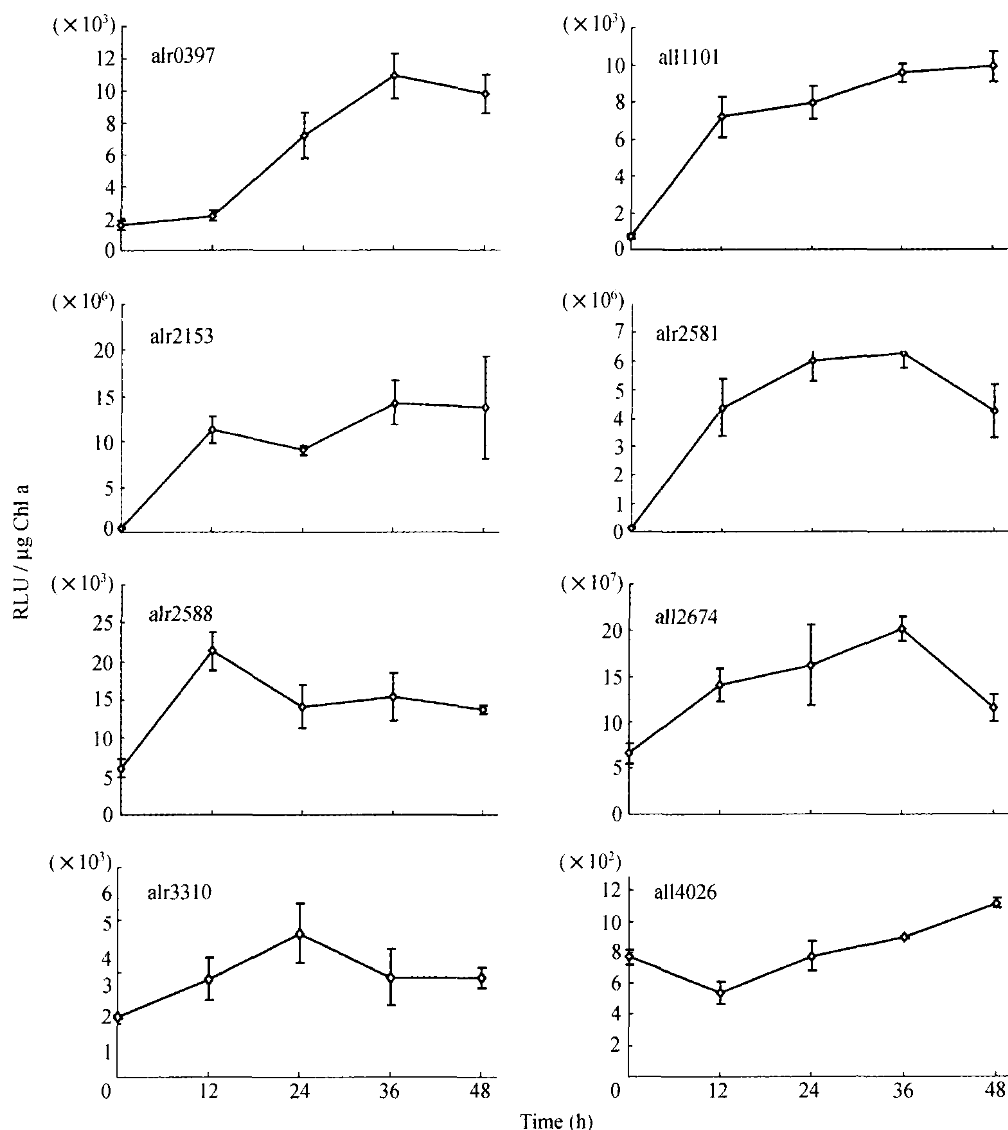

Fig. 4. LuxAB activity of *Anabaena* 7120 harboring promoter-*luxAB*-Ω upon transfer to BG-11-Fe showing transcriptional responses of indicated genes to iron deficiency. RLU, relative luminescence units.

all2674 showed less increase of transcriptional activity in response to iron deficiency. Post-transcriptional regulation remains to be tested for such a gene. Among the up-regulated genes found in this study, *all1101*, *alr2153* and *alr2581* showed low background of transcriptional activity and high amplitude of up-regulation. Their promoters may be used in the conditional expression of other genes, providing a new tool for the investigation of gene functions. In addition, luciferase activities expressed from the iron-deficiency-induced promoters may be applied to bioassays of utilizable iron in natural water samples [24].

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