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Prominent role of the three *Synechocystis* PchR-like regulators in the defense against metal and oxidative stresses

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ABSTRACT

The interplay between iron homeostasis and the defense to metal and oxidative stresses is crucial to the life of cyanobacteria, which perform the iron-requiring oxidative stress-generating photosynthetic process that supports a large part of the biosphere. Thus, we have investigated the role of the three PchR-like regulators of the model cyanobacterium Synechocystis PCC6803, which we previously found to be regulated by iron, cadmium and H_2O_2 stresses. Consistently, we presently report that the three PchR regulators are pleiotropic in operating in the tolerance to metals (Fe, Cd and Co) and oxidative (hydrogen peroxide, menadione and methylene blue) stresses. We also studied in detail the promoter of the pchR3 gene through transcriptional fusion to the cat reporter gene of our replicative promoter probe vector. We show that the pchR3 promoter is complex in comprising in that order (i) a positive element (PE); (ii) a negative element (NEu) upstream of (iii) the basic promoter (BP) which harbors a canonical –10 promoter box but no –35 box; and (iv) a negative element (NEd) downstream of BP. Interestingly, we found that the PE and NEu elements and part of BP are all located in the coding sequence of the gene (sll1488) upstream of pchR3, while the NEd element occurs in the pchR3 untranslated leader region, emphasizing that such regions can influence gene expression in cyanobacteria.

INTRODUCTION

Although iron is the fourth most plentiful element in the Earth's crust, it is frequently a growth-limiting nutrient [1, 2]. Indeed, the oxygenic photosynthesis, which emerged in early cyanobacterial cells approximately 2.8 billion years ago, raised the di-oxygen levels [3] that oxidized the soluble ferrous ions (Fe^{2+}) to insoluble ferric ions (Fe^{3+}). Bacterial cells utilize multiple strategies to maintain iron levels within a desired range, including (i) synthesis, export and re-import of powerful ferric ion chelators called siderophores; (ii) dedicated energy-consuming uptake systems;

(iii) sequestration in intracellular stores (ferritins); and (iv) releasing iron from iron-containing proteins, which are degraded in response to iron-starvation, and subsequent incorporation of the released iron atoms into a wealth of iron-requiring enzymes crucial to cell metabolism [4]. These processes are controlled by the iron-containing FUR protein that regulates more than 90 iron-responsive genes [5]. In *Pseudomonas aeruginosa*, the synthesis of the pyochelin siderophore, is also regulated by the FUR-controlled transcription activator PchR. In the presence of pyochelin, PchR induces the FUR-regulated pyochelin receptor gene ftpA and the two FUR-regulated pyochelin biosynthetic operon *pchDCBA* and *pchEFGHI*. PchR belongs to the family of AraC-type regulators, and accordingly negatively regulates its own expression [6-8].

In the oxidative environment of Earth, biological organisms must also protect themselves against the toxic oxidant radicals, superoxide anion ($O2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$), generated by di-oxygen and iron through the Fenton reaction. Consequently, these organisms have evolved complex systems to efficiently regulate iron homeostasis and oxidative stress responses, and their crucial crosstalk [9, 10]. These processes are most important in cyanobacteria [11] because they perform the two main iron-utilizing oxidant-generating processes respiration and photosynthesis [12], and the latter iron-rich machinery [13] imposes strong Fe requirements [14]. Some cyanobacteria produce siderophores while others can opportunistically use those produced by other bacteria [2]. However, very little is known concerning the interplay between the responses to iron and oxidative stresses in cyanobacteria, in spite of their environmental importance [3] and promising biotechnological potentials [15-17].

In this study, we have initiated the analysis of the three regulators of the unicellular model cyanobacterium *Synechocystis* PCC6803 (hereafter *Synechocystis*), because they are referred to as PchR-like regulators in Cyanobase (http://genome.kazusa.or.jp/cyanobase/). Furthermore we previously showed that these genes (sll1205, sll1408 and slr1489 we named *pchR1*, *pchR2* and *pchR3*) are regulated by iron (and cadmium) and hydrogen peroxide stresses [18]. In agreement with these findings, we presently report that the three PchR regulators play a prominent role in the protection against metal (iron, cadmium and cobalt) and oxidative (hydrogen peroxide, methylene blue and menadione) stresses, thereby emphasizing the interplay between these defense processes. Furthermore, we have characterized the complex organization of the promoter of the *pchR3* gene. The present mutants and data are of value to decipher the regulatory network underlying the crucial interplay between iron homeostasis and tolerance to metal and oxidative stresses in cyanobacteria [11, 12, 14].

MATERIALS AND METHODOLOGY

Bacterial strains, growth, and gene transfer procedures.

Synechocystis PCC6803 was grown under continuous white light (2,500 lux; 31.25 µE m^{-2} s^{-1}) at 30°C on BG11 medium [19] enriched with 3.78 mM Na_2CO_3 [20] hereafter referred to as MM for standard mineral medium. *E. coli* strains used for gene manipulation (TOP10; Invitrogen) or conjugative transfer (CM404) of replicative plasmids (Table 1) to *Synechocystis* [21] were grown on LB at 37°C (TOP10) or 30°C (CM404). Antibiotic selection was kanamycin (Km) 50 µg ml^{-1}, streptomycin (Sm) 5 µg ml^{-1} and spectinomycin (Sp) 5 µg ml^{-1} for *Synechocystis*; and ampicillin (Amp) 100 µg ml^{-1}, Km 50 µg ml^{-1} and Sp 100 µg ml^{-1} for *E. coli*.

The influence of the indicated agents on *Synechocystis* growth was assayed on cells grown three times in liquid cultures up to mid log phase (OD_{580} = 0.5 units, i.e. $2.5x10^7$ cells.ml^{-1}). Cells inoculated into liquid media with or without the tested agents, or spotted on the same solid media as 10 µL aliquots of four-fold serial dilutions were timely examined through OD_{580} measurements (liquid cultures) or scanning of the plates (solid cultures).

Construction of the DNA cassette for targeted deletion of the *pchR1* (sll1205), *pchR2* (sll1408) and *pchR3* (slr1489) genes.

Each *pchR* gene, flanked by about 0.3 kb-long regions of its surrounding sequences to serve for targeted gene replacement through homologous recombinations [22], was PCR amplified with specific primers. After cloning in the pGEM-T

plasmid, each *pchR* coding sequence was deleted by either mutagenesis (Quick Change™ Mutagenesis kit, Stratagene) for both *pchR1* and *pchR3* or standard PCR-driven overlap extension for *pchR2* [23], and concomitantly replaced by a *Sma*I restriction site for the subsequent cloning of the transcription-terminator less antibiotic resistant gene (Table 1). The resulting deletion cassettes were verified by PCR and nucleotide sequencing (Big Dye kit, ABI Perking Elmer) to select those that carry the marker gene in the same orientation as the *pchR* gene it replaced.

Table 1. Characteristics of the plasmids used in this study

Plasmid	Relevant feature	Reference
pGEMT	AT overhang Amp^r cloning vector	Promega
pUC4K	Source of the Km^r marker gene	Pharmacia
pHPΩ45	Source of the Sm^{r}Sp^r marker gene	[39]
ppchR1	pGEMT with the *Synechocystis* pchR1 gene (sll1205) and flanking sequences, where the entire pchR1 coding sequence (CS) was replaced by a *Sma*I site	This study
pΔpchR1:: Sm^{r}Sp^r	ppchR1 with the Sm^{r}Sp^r marker inserted into its unique *Sma*I site	This study
ppchR2	pGEMT with the *Synechocystis* pchR2 gene (sll1408) and flanking sequences, where most of the pchR2 CS (from bp 14 to 999) was replaced by a *Sma*I site	This study
pΔpchR2:: Km^r	ppchR2 with the Km^r marker inserted into its unique *Sma*I site	This study
ppchR3	pGEMT with the *Synechocystis* pchR3 gene (slr1489) and flanking sequences where entire pchR3 CS was replaced by a *Sma*I site	This study
pΔpchR3:: Sm^{r}Sp^r	ppchR3 with the Sm^{r}Sp^r marker inserted into its unique *Sma*I site	This study
pSB2A	Replicative Km^r Sm^{r}Sp^r promoter-probe plasmid vector harboring a unique *SnaB*I site in front of its promoter-less *cat* reporter gene	[27]
pSB543	pchR3 PR (–386 to +157, relative to TSS) cloned at the *SnaB*I site of pSB2A	This study
pSB520	pchR3 PR (–386 to +134, relative to TSS) cloned at the *SnaB*I site of pSB2A	This study
pSB490	pchR3 PR (–386 to +104, relative to TSS) cloned at the *SnaB*I site of pSB2A	This study
pSB376	pchR3 PR (–242 to +134, relative to TSS) cloned at the *SnaB*I site of pSB2A	This study
pSB346	pchR3 PR (–242 to +104, relative to TSS) cloned at the *SnaB*I site of pSB2A	This study
pSB270	pchR3 PR (–242 to +28, relative to TSS) cloned at the *SnaB*I site of pSB2A	This study
pSB218	pchR3 PR (–104 to +104, relative to TSS) cloned at the *SnaB*I site of pSB2A	This study
pSB132	pchR3 PR (–104 to +28, relative to TSS) cloned at the *SnaB*I site of pSB2A	This study
pSB139	pchR3 PR (+28 to +157, relative to TSS) cloned at the *SnaB*I site of pSB2A	This study
pSB086	pchR3 PR (+28 to +104, relative to TSS) cloned at the *SnaB*I site of pSB2A	This study
pSB116	pchR3 PR (+28 to +134, relative to TSS) cloned at the *SnaB*I site of pSB2A	This study

aa, amino acid; CS, Protein Coding Sequence; Δ, deletion; *cat*, chloramphenicol acetyl transferase; PR, promoter region; TSS, transcription start site

Measurement of photosynthetic pigments and photosynthetic activity.

Absorption spectra of whole cell samples (adjusted to 0.5 unit of OD_{580} for equal scattering) were monitored with a $UVIKON_{XL}$ spectrophotometer (Secomam). Maximum light absorption (λ_{max}) are 630 nm for phycocyanin; 442 and 681 nm for chlorophyll *a*; and 350 to 540 nm for carotenoids. Phycocyanin content was estimated with the appropriate equation ($OD_{620} - 0.7 \times OD_{650}$) / 7.38 [24] and extinction coefficients [25]. For chlorophyll *a*, 0.5 ml of mid-log phase culture was extracted with 1 ml of methanol (90% v/v) for 60 min at 4°C in darkness, and the absorbance value at 665 nm was multiplied with the coefficient factor of 13.42 [26]. All assays were repeated three times.

Construction of transcriptional fusions to the *cat* reporter gene and CAT assay.

The *pchR3* promoter region and segments of thereof were amplified by PCR, using site-specific oligonucleotides that flanked the PCR DNA product with blunt-ended restriction sites in such

a way that all nucleotide substitutions were eliminated upon cleavage. The resulting blunt ended promoter fragments were cloned in the unique *Sna*BI site of the promoter probe vector, pSB2A [27], i.e. in front of its promoter-less *cat* reporter gene. The sequence of each promoter insert was verified (Big Dye kit; ABI Perkin-Elmer) before and after replication in *Synechocystis*. Then, 1-2 × 10^9 cells grown on standard plates up to mid-log phase culture were rapidly harvested and disrupted with a chilled Eaton press, prior to CAT assay [28]. CAT activities are the mean value of three measurements performed on two independent cellular extracts; 1 CAT unit = 1 nmol of chloramphenicol acetylated. min^{-1}. mg^{-1} of protein.

RNA isolation.

300 ml of mid-log phase culture was rapidly concentrated (40-fold) by centrifugation, spotted as 20 µl dots on solid medium, incubated for 2 h under standard condition, rapidly harvested and disrupted [18, 20]. Cell extracts were incubated with 25 U of RNase-free DNase I (Roche) for 15 min at 37°C before and after isolation of total RNA with the RNeasy (Qiagen). The RNA concentration and purity were determined by A_{260} and A_{280} measurements (A_{260}/A_{280} > 1.9), as well as by migration on agarose gels to verify the absence of RNA degradation.

Determination of transcription start site by 5' rapid amplification of cDNA ends.

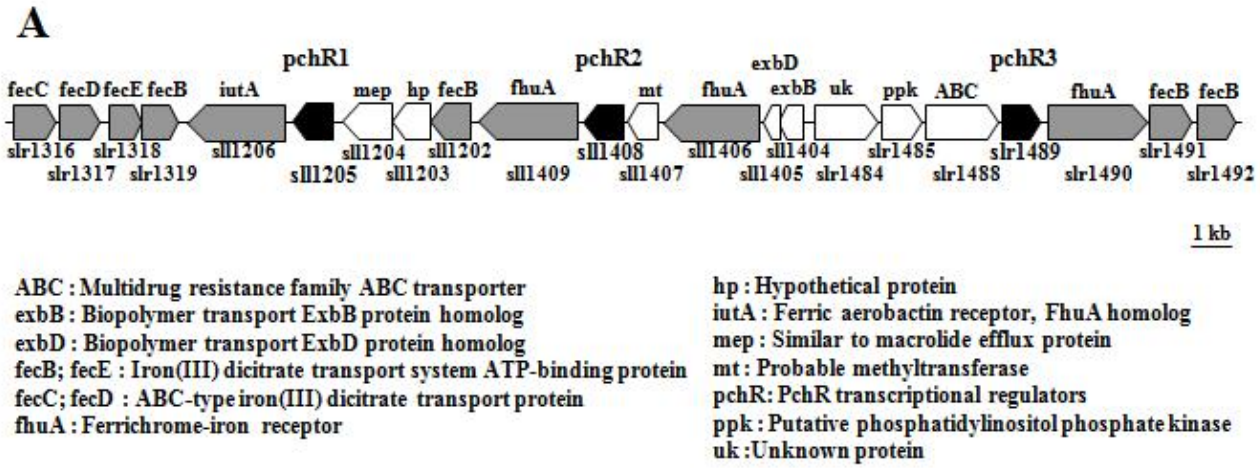
The 5' triphosphate ends of 20 µg total RNA was converted to 5' monophosphate by a 60 min treatment at 37°C with 5 U Tobacco acid pyrophosphatase (TAP, Epicentre) in 50 mM sodium acetate buffer (pH 6.0), 1 mM EDTA, 0.1% β-mercaptoethanol, 0.01% Triton X-100 and 20 U RNasin (Promega). After ethanol precipitation, washing and resuspension in 40 µl water, 10 µg of these RNA was incubated for 60 min at 37°C with 20 U T4 RNA ligase (Invitrogen) and 250 pM of an RNA adapter (5'-GUCCAUGUAGACACUCAGGUAAA-3') in 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT and 20 U RNasin. RNA were again precipitated with ethanol, washed and resuspensed in 20 µl water. 2 µg of RNA was incubated with 2 pM gene specific primers for 5 min at 65°C, cooled on ice, and incubated for 60 min at 42°C and 50°C for 10 min in RT-Buffer (Invitrogen) containing 0.5 mM dNTPs, 10 mM DTT, 8 U RNasin, and 200 U SuperScript II. The reaction was stopped by a 15 min incubation at 70°C, and 1 µl of the first-strand cDNA reaction was amplified by PCR in PCR Buffer (Invitrogen) containing dNTPs 0.2 µM, *pchR3*-specific primer 0.4 µM, the DNA version of the RNA anchor 0.4 µM, $MgCl_2$ 2 mM and 2.5 U of Taq polymerase. PCR conditions were: 5 min initial denaturation at 94°C, followed by 35 cycles of 90°C for 20 s, 55-49°C (touch down -0.3°C/cycle) for 30 s (annealing), 72°C for 30 s (extension), and a 10 min final extension at 72°C. The resulting DNA product was visualized on a 1% agarose gel from which the band of interest was excised, gel eluted with the NucleoSpin Extract II kit (Macherey-Nagel), cloned into pGEM-T, and sequenced using the vector-specific T7-forward and SP6-reverse primers. The nucleotide immediately downstream of the DNA anchor corresponds to the transcription start site of *pchR3*.

RESULTS AND OBSERVATIONS

The three *Synechocystis* PCC6803 proteins homologous to the bacterial iron regulator PchR are dispensable to cell viability.

The genome of *Synechocystis* PCC6803 (hereafter *Synechocystis*) possesses three genes, *pchR1* (sll1205), *pchR2* (sll1408) and *pchR3* (slr1489), encoding proteins homologous (http://genome.kazusa.or.jp/cyanobase and Fig. 1) to the bacterial iron regulator PchR [8]. Interestingly, we previously reported that the *Synechocystis pchR* genes are strongly regulated by iron availability (positively by iron starvation and negatively by iron excess), and that *pchR1* and *pchR3*, but not *pchR2*, are regulated (positively) by H_2O_2 [18]. These findings suggest that the PchR regulators might operate in the tolerance to iron and oxidative stresses, which are poorly characterized in cyanobacteria [12, 14]. To start investigating the role of the *Synechocystis* PchR regulators, we constructed the Δ*pchR1*::Sm^rSp^r, Δ*pchR2*::Km^r and Δ*pchR3*::Sm^rSp^r deletion cassettes (Table 1) harboring the transcription terminator-less Km^r or Sm^rSp^r marker in place of the indicated *pchR* protein-coding sequences. After transformation of *Synechocystis*, we verified through PCR and DNA-sequencing (data not shown) that the antibiotic resistant marker had

properly replaced the studied *pchR* gene in all 10 copies of the polyploid [22] chromosome. All three Δ*pchR* mutants were found to grow well under standard laboratory conditions (Fig. 2), showing that all three PchR proteins are dispensable to the viability of *Synechocystis*.



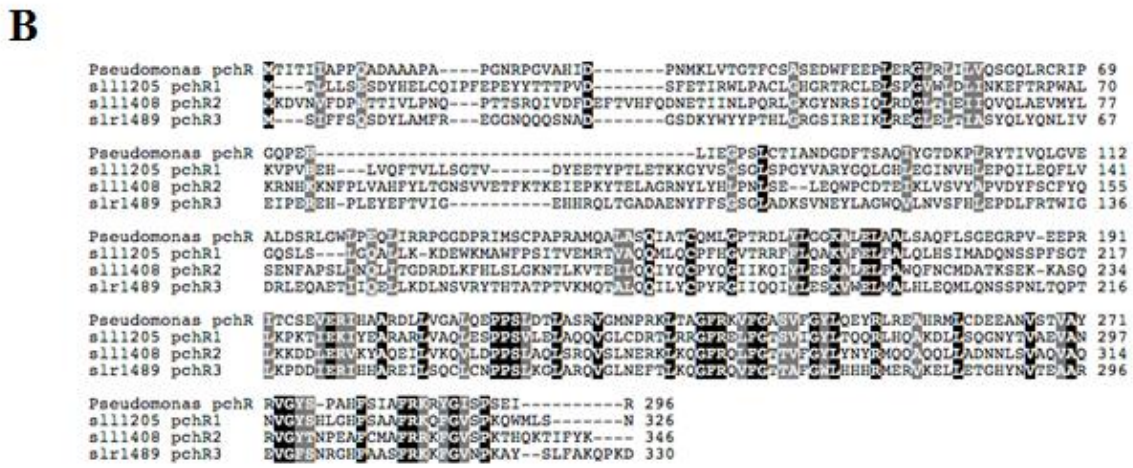


Fig. 1 Panel A genome organization around the three *Synechocystis pchR* genes. The genes are represented by boxes (black, *pchR*; grey genes involved iron homeostasis; and white hypothetical or unknown genes) pointing into the direction of their transcription. Panel B. BoxShade representation of the *Pseudomonas aeruginosa* PchR regulator (GenBank Accession Number P40883) amino acids sequence aligned against the relevant proteins PchR1 (sll1205), PchR2 (sll1408) and PchR3 (slr1489) from *Synechocystis* (http://genome.kazusa.or.jp/cyanobase/) with ClustalW2. Identical and conserved amino acids are shaded by black or grey background, respectively.

Both PchR1 and PchR3, but not PchR2, are required for normal abundance of the photosynthetic machinery.

Under standard growth conditions, both the WT strain and the Δ*pchR2* mutant displayed the normal blue-green color typical of cyanobacteria (Fig. 2). By contrast, both the Δ*pchR1* and Δ*pchR3* mutants were yellowish green indicating that they contained a smaller content of the photosynthetic pigments. Indeed, we found that Δ*pchR1* and Δ*pchR3* cells have a two- to four-fold reduced levels of phycocyanin and chlorophyll, as compared to WT and Δ*pchR2* cells (Fig. 2).

Influence of the three PchR regulators on the tolerance to metal and oxidative stress.

Having observed that all three *pchR* genes are regulated positively by iron starvation and negatively by iron excess [18], we anticipated the PchR regulators to operate in the protection against iron stresses. Indeed, all three Δ*pchR* mutants appeared to be more resistant to iron

excess than the WT strain, and slightly sensitive to iron limitation and (Fig. 3). These mutants were also challenged with H_2O_2, which was previously shown to induce both *pchR1* and *pchR3*, not *pchR2* [18]. The Δ*pchR1* and Δ*pchR3* mutants were found to be more resistant to H_2O_2 than the WT and Δ*pchR2* strains in that order (Fig. 3). Hence, both the PchR1 and PchR3 regulators, not pchR2, behave similarly as the PerR regulator of *B. subtilis* [29] and *Synechocystis* [18] in that they are induced by H_2O_2 and their absence increases the tolerance to H_2O_2. We also challenged the three Δ*pchR* mutants with methylene blue and menadione, which produce singlet oxygens ($^1O_2$) and superoxide anions ($O2^{\cdot-}$), respectively. Both the Δ*pchR1* and Δ*pchR3* mutants were found to be more resistant to methylene blue and menadione than the WT and Δ*pchR2* strains. These data are consistent with the lower abundance of photosynthetic pigments, which can generate oxidative stress [12], in the Δ*pchR1* and Δ*pchR3* mutants as compared to the WT and Δ*pchR2* strains.

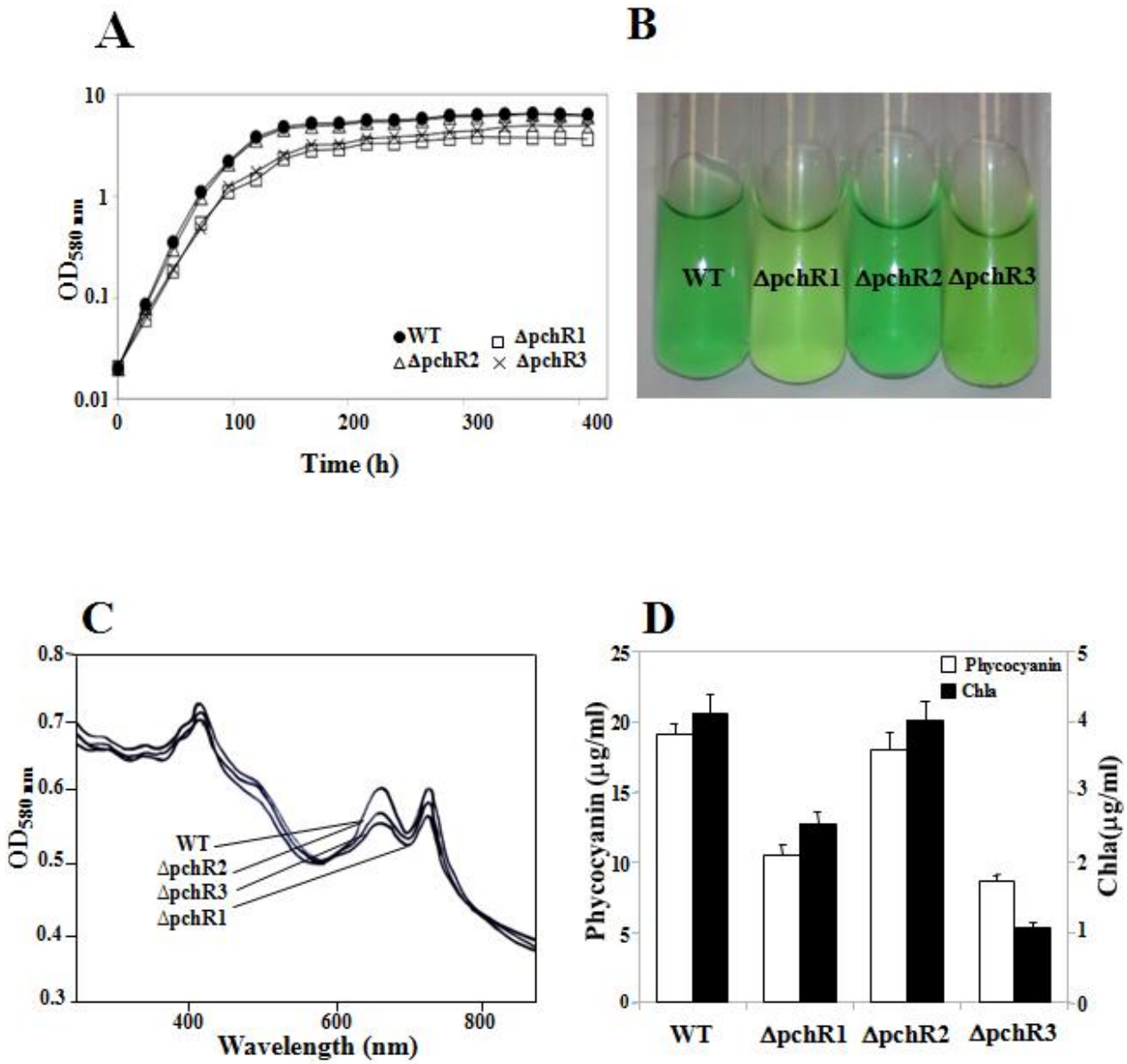


Fig. 2 Influence of the PchR regulators on the growth and photosynthetic pigments of *Synechocystis* growing under standard conditions. Panel A. Typical growth curves of WT cells (black circles) and mutants Δ*pchR1* (open squares), Δ*pchR2* (open triangles) and Δ*pchR3* (crosses). Panel B. Typical photographs of the corresponding cultures observed at mid-log phase (OD_{580} = 0.5) after transfer to 1-cm large glass tubes. Panel C. Absorption spectra of the corresponding cultures adjusted to equal light scattering at 580 nm. Panel D. Chlorophyll a (Chl a) and phycocyanin contents (mg.ml^{-}

[1]) of the WT and Δ*pchR* mutants. All experiments were repeated three times.

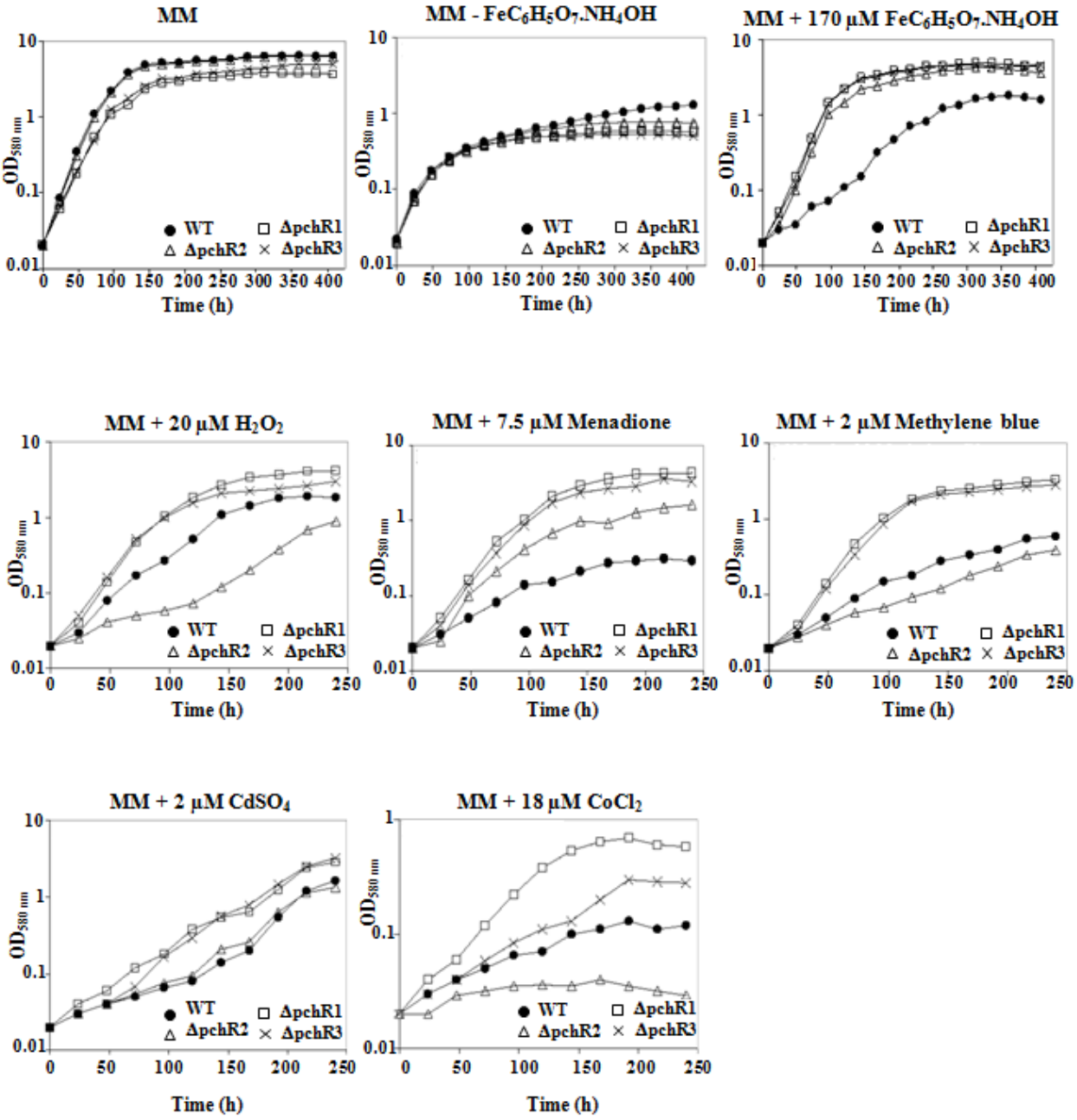


Fig. 3 Influence of the three *Synechocystis* PchR regulators on the tolerance to metal and oxidative stresses. Cells were incubated in standard mineral medium (MM) with or without the indicated metals or oxidative agents. These experiments were repeated three times.

We also investigated the influence of the PchR regulators on the tolerance to cadmium and cobalt, two toxic metals that impair iron homeostasis and generate oxidative stress [18, 30]. The Δ*pchR1* and Δ*pchR3* mutants were found to be more resistant to Cd than both the WT and Δ*pchR2* strains, in agreement with the Cd down-regulation of both the *pchR1* and *pchR3* genes [18]. The same results were observed in the case of the Co stress. Interestingly, cobalt is the first toxic agent to which Δ*pchR1* cells resist differently (i.e. better) than Δ*pchR3* cells. As the Δ*pchR2* mutant is extremely sensitive to cobalt, it will be very interesting in the future to characterize and compare the global transcriptome responses to cobalt of the three Δ*pchR* mutants, to decipher the selectivity redundancy of the PchR1, PchR2 and PchR3 regulators. Collectively, our findings,

which were confirmed by similar tests performed on solid medium (data not shown), indicate that the PchR regulators operate in the coordination of the defenses against metal and oxidative stresses, and that the Δ*pchR* mutants will be useful to decipher the underlying processes.

Analysis of the *pchR3* promoter region through transcriptional fusion to the *cat* reporter gene of a replicative promoter probe vector.

In the frame of our long-term interest in transcription we started the analysis of the *pchR3* promoter. Thus, we cloned the *pchR3* promoter region in front of the promoterless *cat* reporter gene of our pSB2A promoter probe vector, which replicates autonomously in *Synechocystis* at one copy per copy of the polyploid chromosome [27]. To avoid missing an important promoter element, we cloned a large part (543 bp) of the *pchR3* regulatory region in pSB2A. This 543 bp *pchR3* DNA region encompasses in that order (i) a part (386 bp) of the upstream slr1488 coding sequence (http://genome.kazusa.or.jp/cyanobase/), because a coding sequence may influence the transcription of a downstream gene [31]; (ii) the untranslated leader region (100 bp) of *pchR3*, which may influence transcription [32]; and (iii) the beginning (57 bp) of the coding sequence of *pchR3*, which may influence transcription [20, 28, 33]. The resulting *pchR3-cat* reporter plasmid we constructed (pSB543, Fig. 4) directed a good level of *cat* expression (CAT activity 112 units), indicating that the *pchR3* promoter is as active as the other *Synechocystis* promoters we previously studied with pSB2A: namely *secA* (secretion; [28]), *gap2* (glycolysis; [33]), *fed1* (photosynthesis; [34]), *lexA* (regulation; [20]) and *recA* (DNA repair; [20]). As the usual control we have verified that the empty pSB2A vector directed no *cat* expression.

Expression of the *pchR3* gene is controlled by sequences both upstream and downstream of its basic promoter.

To localize the *pchR3* promoter and identify possible *cis*-acting regulatory element, we first mapped transcription start site (TSS) of *pchR3* with the "5' Race" technique [35] that works well in *Synechocystis* [31]. The *pchR3* TSS was found to be the G nucleotide located at 100 bp upstream of the ATG start codon (Fig. 4). Then we performed a deletion analysis of the *pchR3* regulatory region cloned in the pSB2A promoter probe vector (Fig. 4). The smallest *pchR3* fragment exhibiting a promoter activity was found to extend from –104 to +28 (See the pSB132 plasmid) relative to the TSS site taken as the origin of distance (noted as +1). Consistently, the *pchR3* DNA segments lacking the basal promoter (BP) exhibited no promoter activity (See the three reporter plasmids pSB086, pSB116 and pSB139; Fig. 4). The *pchR3* BP promoter contains a –10 hexanucleotides (5'-TAACCT-3') matching both the sequence (5'-TATaaT-3') and position (7±1 nucleotides upstream of the TSS) of the canonical Pribnow box of σ^{70}-type *E. coli* promoters [36]. By contrast, no –35-like sequence (5'-TTGaca-3') was found in *pchR3* BP, neither at 17 bp upstream of the –10 box (the *E. coli* canonical distance [37]) nor at 30 bp (the spacing length sometime encountered in *Synechocystis* [28, 38]. Hence, the *pchR3* BP promoter resembles the BP promoters of the *Synechocystis* genes *recA* and *lexA*, which have no –35-like promoter box [20].

Very interestingly, the activity of the *pchR3* BP promoter was found to be 2.5 fold higher than that of the full regulatory region (Fig. 4, compare pSB132 with pSB543), suggesting that the BP promoter might be negatively regulated by one or several *cis*-acting element(s) occurring in BP-flanking sequences. Indeed, we found two *cis*-acting negative elements (NE) lying both downstream (NEd, +28 to + 134) and upstream (NEu, –242 to –104) of BP (–104 to + 28). The existence of the downstream NEd element was inferred from the three pair-wise comparisons of the CAT activities driven by (i) pSB132 (BP; –104 to +28; 286 CAT units) and pSB218 (–104 to +104; 62 CAT units); (ii) pSB346 (–242 to +104; 59 CAT units) and pSB270 (–242 to +28; 148 CAT units); and (iii) pSB376 (–242 to +134; 52 CAT units) and pSB270 (–242 to +28; 148 CAT units). Similarly, the existence of the upstream NEu element was deduced from the comparison of the CAT activities driven by the plasmids pSB132 (BP; –104 to + 28; 286 CAT units) and pSB270 (–242 to +28; 148 CAT units). Also interestingly, we found a positive element (PE) in the most upstream part (–386 to –242) of the *pchR3* regulatory region, as deduced by the comparisons of pSB520 (–386 to +134; 116 CAT units) with pSB376 (–242 to +134; 52 CAT units) on one hand, and pSB490 (–386 to +104;

114 CAT units) and pSB346 (–242 to +104; 59 CAT units) on the other hand. Furthermore, the *pchR3* PE and NEu regulatory elements and part of the BP promoter are all located in the coding region of the upstream gene slr1488, while the *pchR3* NEd element occurs in the *pchR3* untranslated leader region. These findings are consistent with previous analyses of other cyanobacterial genes, which showed that *cis*-acting regulatory DNA elements may be present in untranslated leader regions [28, 32, 33] or in upstream coding sequences [31].

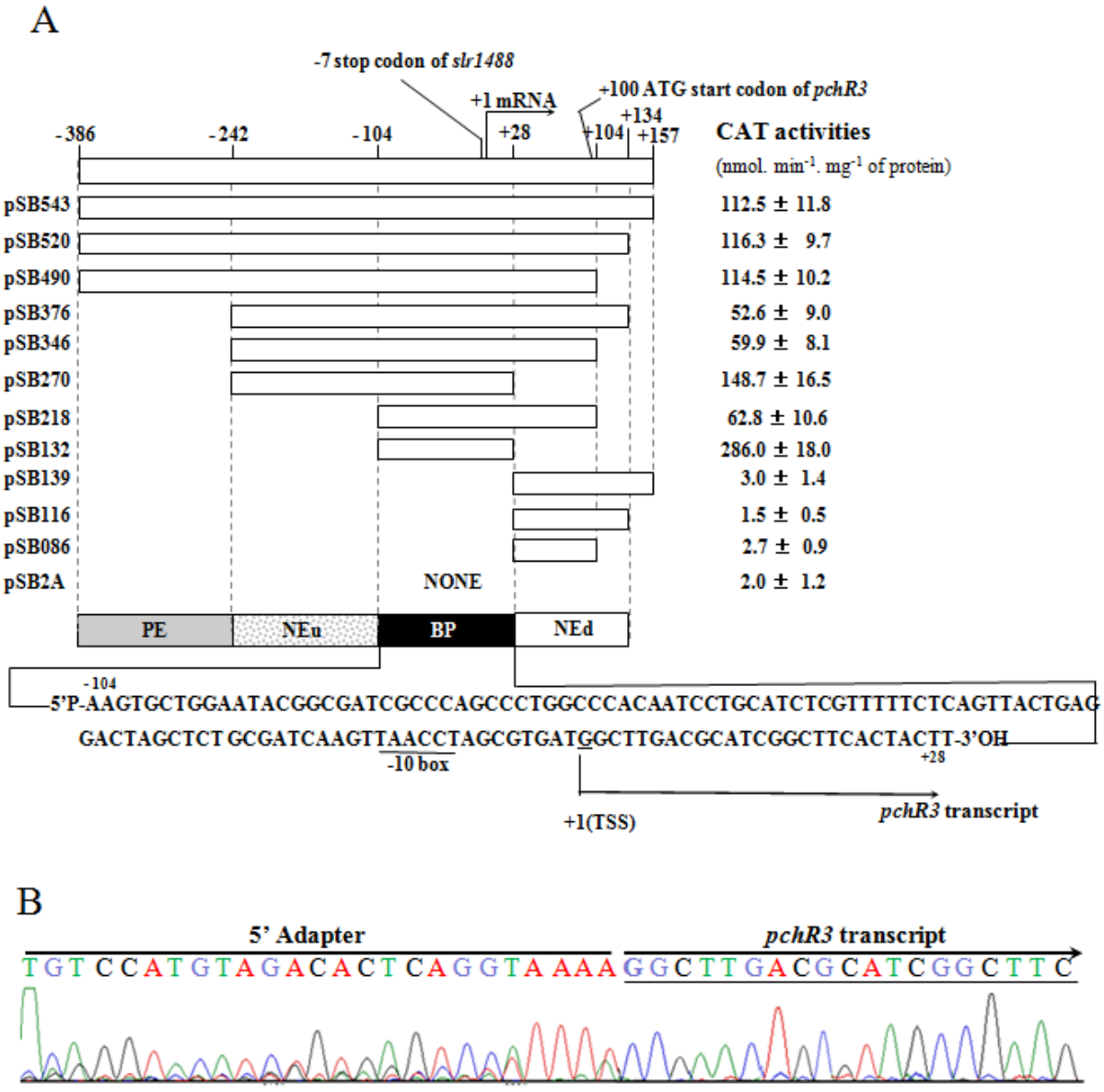


Fig. 4 Panel A. Deletion analysis of the *pchR3* promoter region transcriptionally fused to the *cat* reporter gene of the pSB2A promoter-probe plasmid. CAT specific activities driven by the resulting plasmids replicating in *Synechocystis* growing under standard conditions are expressed in nmol of chloramphenicol acetylated. min^{-1}. mg^{-1} of protein. The nucleotide positions within the *pchR3* gene are indicated relative to its transcription start site (bent arrow) noted as +1. The absence of promoter insert in the empty pSB2A vector is indicated as "None". The basic promoter (BP) and *cis*-acting regulatory elements are boxed, and indicated as PE for positive element; and NEu and NEd for the negative elements lying upstream and downstream of BP. The expended view of BP shows its nucleotide sequence spanning its transcription start site (the underlined G nucleotide highlighted with a bent arrow), and its presumptive –10 promoter box. Panel B. Determination of the *pchR3* transcription start site with the 5'-RACE technique. The top part shows the nucleotide sequence of the RT-PCR cDNA products amplified with both the anchor (5' adapter) and the *pchR3* specific primer. The first nucleotide (G) of the underlined sequence downstream of the anchor is the transcription start site of *pchR3* gene.

DISCUSSION

The ability to survive metal and oxidative stresses is central to the lifestyle of cyanobacteria, which colonize most aquatic environments [3, 12, 14] and have promising biotechnological potentials [15-17]. Therefore, we have investigated the cyanobacterial proteins resembling the bacterial iron regulator PchR (Fig. 1), in the widely-used unicellular cyanobacterium *Synechocystis* PCC6803 (*Synechocystis*). We show that all three *Synechocystis* PchR regulators are dispensable to cell growth under standard photoautotrophic conditions (Fig. 2), even though both PchR1 and PchR3 (not PchR2) are required for normal abundance of the iron-rich photosynthetic machinery. As expected, we found that all three PchR regulators play a prominent role in the protection against metals (iron, cadmium and cobalt) and oxidative (hydrogen peroxide, menadione and methylene blue) stresses (Fig. 3). These findings are consistent with our previous observations [18] that the *pchR* genes respond to Fe, Cd and H_2O_2 stresses (regulation by Co, menadione and methylene blue were not tested in our previous work).

In the frame of our long-term interest in gene expression [20, 28, 33, 34] we thoroughly analyzed the promoter region of the *pchR3* gene. For this purpose, we cloned and sub-cloned the *pchR3* promoter region in front of the promoter-less *cat* reporter gene of our replicative promoter probe vector pSB2A [27], and introduced the resulting reporter plasmids (Fig. 4) in *Synechocystis*. The longest *pchR3* promoter region we tested (pSB543 plasmid; –386 to + 157 relative to the transcription start site we mapped with the 5' Race technique (Fig. 4) directed a good level of *cat* expression (CAT activity 112 units; Fig. 4), indicating that *pchR3* is expressed to similar levels as the other genes we previously studied with pSB2A, namely: *secA* (secretion; [28]), *gap2* (glycolysis; [33]), *fed1* (photosynthesis; [34]), *lexA* (regulation; [20]) and *recA* (DNA repair; [20]). The basic promoter (BP) of *pchR3*, i.e. the smallest *pchR3* fragment exhibiting a promoter activity, was found to extend from –104 to +28 (pSB132 plasmid, Fig. 4). The *pchR3* BP promoter contains a hexanucleotides matching both the sequence (5'-TAACCT-3') and position (8 nucleotides upstream of the TSS) of the canonical –10 box of σ70-type *E. coli* promoters [36, 37]. By contrast, we found no –35-like element (5'-TTGACA-3') in BP, neither at 17 bp upstream of the –10 box (the canonical position in *E. coli*) nor at 30 bp (the distance sometime encountered in *Synechocystis* [28, 34]. Thus, the *pchR3* genes resembles the recA and lexA genes which have no –35 promoter box [20]. Very interestingly, the *pchR3* promoter activity was found to be regulated by three regulatory elements flanking BP (Fig. 4). The first two elements that increase (PE, for positive element; –386 to –242) or decrease (NEu, for negative element upstream; –242 to –104) BP activity are located in the protein coding sequence of the upstream gene slr1488. Furthermore, slr1488 appeared to contain a large part of the *pchR3* BP promoter (Fig. 4), a finding similar to a recent observation that the promoter of the salt-responsive gene sll1566 is located in the upstream coding sequence ssl3076 [31]. Finally, we showed that the downstream negative NE element of *pchR3* (NEd; +28 to + 104; Fig. 4) is located in the untranslated leader region of *pchR3*. This finding confirm earlier reports that untranslated regions can influence gene expression in cyanobacteria [28, 32, 33].

CONCLUSION

Collectively, the present data indicate that the PchR regulators play prominent roles in the protection against metal and oxidative stresses, which are often encountered by cyanobacteria [11, 12, 14, 18]. These findings are consistent with the fact that most cyanobacteria possess several *pchR* genes (data not shown), to the noticeable exception of the *Prochlorococcus* species, which live in open ocean where metal availability is rather constant. We believe that the presently reported Δ*pchR* mutants of *Synechocystis* will be useful tools to decipher the regulatory network underlying the crosstalk processes between iron homeostasis and the protection against metal and oxidative stresses.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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