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Two-component systems in *Prochlorococcus* MED4: Genomic analysis and differential expression under stress

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Abstract

Two-component signal transduction systems, composed of histidine sensory kinases and response regulators, constitute a key element of the mechanism by which bacteria sense and acclimatize to changes in their environment. The availability of whole genome sequences permits a detailed analysis of these genes in cyanobacteria. In the present paper, we focus mainly on *Prochlorococcus* MED4, a strain adapted to surface oceanic conditions, for which six putative response regulators (*rer*) and five putative histidine kinases (*hik*) were identified. These numbers are comparable to those found in the other marine picocyanobacteria but much lower than those found in freshwater cyanobacteria. Moreover, the diversity of these genes is low in *Prochlorococcus* since most histidine kinases are related to a single group (type I) and most response regulators to a single family (OmpR). Under standard conditions, quantitative reverse transcription polymerase chain reaction revealed that one *hik* (*hik03*) and two *rer* (*rer04* and *rer05*) genes were expressed at relatively high levels compared to the other two-component system genes. In response to high light exposure, a moderate increase (>5-fold) was observed in the expression of some putative *rer* genes (*rer01*, *rer04*, *rer05*, and *rer06*), whereas a smaller increase (<3-fold) in *hik03* and *hik04* mRNA levels was detected. In contrast, both cold and heat shocks decreased rather than increased the expression of most *hik* and *rer* genes.

Keywords: Cyanobacterium; Histidine kinase; Response regulator; Genome; Reverse transcription polymerase chain reaction

1. Introduction

Response to light and nutrient limitation is finely tuned in photosynthetic organisms so that they can sense and adapt to changes in their environment. These processes are essential for survival and growth in environments where these resources are limiting such as the open ocean, but have been little studied in marine microorganisms in spite of their ecological importance. When an environmental change occurs, many regulation mechanisms involved in controlling the expression of specific genes or the activation of their products are brought into play. One of these mechanisms involves two-component regulatory systems, which consist of two proteins, a histidine sensory kinase (Hik) and a response regulator (Rer). The sensor protein is responsible for detecting a change in the external environment and communicating the information to the Rer through phosphorylation/dephosphorylation of specific sites [1]. Once the Rer has been activated, it is able to control the expression of particular genes to appropriately respond to the external stimuli. Comparison of two-component regulatory systems in different bacterial species reveals that sensor–response regulatory pairs not only function similarly, but also display highly conserved amino acid domains [2].

Hik proteins contain an N-terminal signal input domain followed by a C-terminal transmitter domain about 240 amino acids long, with a His residue that can be autophosphorylated. Hik types I and II both possess orthodox kinase domains, while types III and IV possess so-called unorthodox kinase domains in which N1 and the N-box motif are either a glycine (type III) or a proline (type IV) residue, the F-box is absent, and the G2 motif is truncated [3]. Rer proteins contain a typical receiver regulatory domain of 120 residues with a conserved Asp residue that serves as the site of phosphorylation by the Hik, fused to a presumed signal output effector domain [4]. This effector

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domain is involved, for most Rer, in binding target DNA to trigger the cellular response [5]. Those domains are used to classify Rer. Members of the OmpR subfamily are DNA-binding transcriptional regulators with a typical receiver domain followed by a DNA-binding domain. The NarL subfamily also contains transcriptional regulators whose DNA-binding motif is postulated to be distinct from that of OmpR. In the PatA subfamily, the presumed signal output domain is located in front of the receiver. Finally, members of the CheY subfamily contain only a receiver domain and appear to lack any signal output domain.

The picocyanobacterium *Prochlorococcus* is the smallest known photosynthetic prokaryote and the most abundant oxyphototroph in the ocean [6]. It is ubiquitous within the 40°S to 40°N latitudinal band and can be found throughout a 100–200-m deep layer. Over this natural light gradient, ranging from 1500 μmol quanta m^{-2} s^{-1} near the surface to less than 1 μmol quanta m^{-2} s^{-1} below 150 m, cells of this genus are able to actively grow and photosynthesize. The ability of *Prochlorococcus* to tolerate such a wide range of light conditions results not only from its ability to photoacclimatize, but also from the occurrence of different *Prochlorococcus* ecotypes that differ in their pigment composition [7]. Biochemical studies suggest that different *Prochlorococcus* strains may have different antenna systems specifically adapted to the light environment from which they have been isolated [8].

In the present study, we analyzed the publicly available genome of *Prochlorococcus* MED4, a high light-adapted strain, to identify the genes encoding putative Hik and Rer, and to assess relationships between these genes and homologs from other bacteria and cyanobacteria. Based on this genomic information, we designed a set of primers specific for each *rer* and *hik* from *Prochlorococcus* MED4, and determined, using quantitative real-time reverse transcription polymerase chain reaction (RT-PCR), whether the expression of these genes was changed in response to high light exposure and temperature shock.

2. Materials and methods

2.1. Genomic analysis

The complete genomes of the marine *Prochlorococcus* spp. MED4 and MIT9313 and that of *Synechococcus* sp. WH8102 were downloaded from the Joint Genome Institute (JGI, Walnut Creek, CA, USA) web site at http://spider.jgi-psf.org/JGI_microbial/html/. The genome of *Prochlorococcus marinus* SS120 was retrieved from http://www.sb-roscoff.fr/Phyto/ProSS120. Genome sequences of *Synechocystis* sp. PCC 6803, *Anabaena* PCC 7120 and *Thermosynechococcus elongatus* BP-1 were obtained from the Cyanobase databank (http://www.kazusa.or.jp/cyano/index.html). The contig sequences of *Nostoc punctiforme* ATCC 29133 were retrieved from GenBank at http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/framik?-gi=5020&db=Genome.

The *hik* and *rer* genes were identified by similarity searches using the *rer* and *hik* sequences of *Synechocystis* PCC 6803 [9] against genome sequences of the four marine picocyanobacteria (*Prochlorococcus* MED4, *Prochlorococcus* MIT9313, *Prochlorococcus* SS120 and *Synechococcus* WH8102) using the (gapped) local tblastn program [10]. Multiple alignments of protein sequences were performed with the neighbor joining method implemented in the ClustalX program [11] with default parameters and manually improved. GeneDoc was used to prepare alignment figures (http://www.psc.edu/biomed/genedoc/). Protein secondary structures were predicted with the BCM Search Launcher programs (http://dot.imgen.bcm.tmc.edu:9331/pssprediction/pssp.html) and the DC-Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Transmembrane segments were predicted with the TMpredict algorithm (http://www.ch.embnet.org/software/TMPRED_zform.html). For identification of the PAS/PAC domain, the program SMART was used (http://smart.embl-heidelberg.de/). The genomic regions surrounding the Hik and Rer genes was examined using the Artemis software package (http://www.sanger.ac.uk/Software/Artemis/).

Histidine kinases and response regulators of *Prochlorococcus* MED4 were used to search the other cyanobacterial genomes for homologs using the blastp program. We consider two sequences homologs if their full-length sequences share at least 30% amino acid sequence identity.

2.2. Culturing and stress experiments

Prochlorococcus PCC 9511, an axenic strain equivalent to MED4 (the 16S rRNA sequences of these two strains are 100% identical [12]) was grown in PCR-S11 medium [6] at 20°C under low light (20 μE m^{-2} s^{-1}). For the high light stress experiment, low light-adapted cells were grown to a density of 6×10^7 cells ml^{-1} and transferred to high light (200 μE m^{-2} s^{-1}). Cells were harvested 30 min, 1 h, 3 h, 6 h and 12 h after the initial exposure to high light for RNA isolation. For the heat shock experiments, cells (density = 6×10^7 cells ml^{-1}) adapted to 20°C were shifted to 35°C, whereas for the cold shock experiments, they were transferred to either 13°C or 4°C. Cells were harvested 30 min, 1 h, 3 h, 6 h and 12 h after the initial exposure to heat shock or cold shock for RNA isolation.

2.3. RNA isolation

Cells were immediately pelleted (a process that took less than 5 min) at the end of the stress exposure. RNA was isolated from pelleted cells frozen at −80°C through a slight modification of the method of de Saizieu et al. [13] as described by Bhaya et al. [14]. Briefly, 500 μl of acidi-

Table 1
Primers specific for the response regulators (*rer*) and histidine kinases (*hik*) of *Prochlorococcus* MED4

Name	Primer forward 5′-3′	T_m (°C)	Primer reverse 5′-3′	T_m (°C)	Fragment length (bp)
hik01	GAAAACGATATCCTACTTGGTGTCC	61.3	TCCAATCTCTATTCTCTAAGTTCGC	59.7	496
hik02	TGAGGGAACATCAGGATTTGG	58.4	TCCCTCCATGAACTTCAACAATT	58.5	127
hik03	ATTCGTGACAATGGGGTAGG	57.3	AGTTTCTATAGTCGCCCCGC	59.4	201
hik04	TGAGAAACATGGTGGGCAAA	58.5	AAACCAAAATGTTGTGCCGATT	59.2	63
hik05	GAGATGCGATGAAAGTAGCC	57.3	AACGGTTTCTCATTGATTCG	53.2	359
rer01	CTGCCGTGAGACTTACCCAT	58.4	GCAATATCCCGCTCCATAAA	59.5	224
rer02	TGATCGTTACCGCATTAGGG	60	TCAGGATTTGCAGGATCAGC	60	423
rer03	AGAGGATGGATGGGAAAAAGC	58.5	TCTCGGCATCATAATATCGCTAATAA	59.1	69
rer04	AGGCAAGTTTCTAGGGCTGG	58.7	CAGGATTTCTTGGATCTGGC	59.3	217
rer05	GAGTTTGCATTGCTTTGTGCTT	58.2	TCTTGGCGACTTCGAACCA	58.9	58
rer06	GCTTAGAAGTAGGAGCTGATGATTACC	58.3	TAAGGCTCTGCACCTAGCAATTAA	58.4	76
psbA	AATGATTCCATGCCTCTTGCA	62.7	GCTGCGATGAAAGCAACGA	63.5	76
rnpB	GAGGATAGTGCCACAGAAACATACC	59	TAAGATGGGCAATCATCTATCTAGG	56.7	257

fied phenol and 500 μl of NAES (50 mM sodium acetate pH 5.1, 10 mM EDTA, 1% sodium dodecyl sulfate) were added to cell pellets from a 150-ml culture. After the addition of 100 mg of glass beads (0.1 μm, average diameter, Bio-Rad, Hercules, CA, USA), the suspension was vortexed three times for 20 s each. This was followed by two phenol–chloroform (1:1) and one chloroform extraction. The RNA preparations were treated for 1 h at room temperature with DNase I (20 U DNase I FPLC pure, Amersham Biosciences) followed by phenol–chloroform extraction (1:1) and precipitation in 2 volumes of ethanol. The final pellet was dissolved into 50 μl of 10 mM Tris (pH 8.0) with 1 mM EDTA and stored at −80°C for the RT-PCR.

2.4. Real-time quantitative RT-PCR analysis of gene expression

Specific primers were designed using the Primer Express™ software (Applied Biosystems) for *Prochlorococcus* MED4 *hik*, *rer*, *psbA*, and *rnpB* genes (Table 1). Primer specificity and efficiency were checked by PCR amplification on *Prochlorococcus* MED4 genomic DNA. For the RT reaction, about 200 ng of RNA was incubated with a mix using random hexamer primers (50 ng for 20 μl of final volume, Promega) for 10 min at 70°C prior to adding 100 U of Superscript II RT (Gibco BRL, Grand Island, NY, USA). RNA was reverse transcribed for 1 h at 42°C followed by an incubation at 72°C for 10 min. 0.1 μl of the

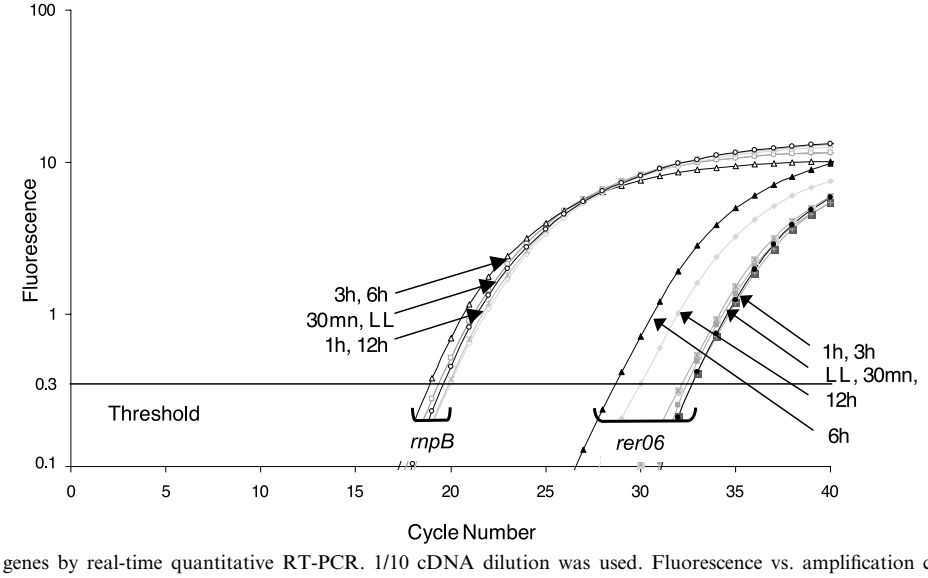

Fig. 1. Analysis of *rer* genes by real-time quantitative RT-PCR. 1/10 cDNA dilution was used. Fluorescence vs. amplification cycle for *rer06* and *rnpB* (control) cDNA during high light stress experiment. The threshold is the numerical value assigned (0.3) for each run, which reflects a statistically significant point above the calculated baseline.

RT reaction was used for PCR. Samples for which the RT step was omitted were used as negative controls to check that the extracted RNA was not contaminated with DNA. 12.5 μl of SYBR Green PCR Master mix (Applied Biosystems) containing *Taq* DNA polymerase, dNTP, $MgCl_2$, and SYBR Green I dye was used. Each reaction (25 μl) contained 1 μl of the respective cDNA dilution 1/10, primers at 0.4 μmol l^{-1}. Real-time quantitative PCR reactions were performed on the ABI Prism™ 5700 sequence detection system using the SYBR Green kit as recommended by the manufacturer (Applied Biosystems). The amplification program consisted of 1 cycle of 95°C with 60-s hold, followed by 40 cycles of 95°C with 15-s hold, specified annealing temperature with 20-s hold, 60°C with 40-s hold. The generation of specific PCR products was confirmed by melting curve analysis (one cycle at 95°C with 0-s hold, 65°C with 10-s hold, and 95°C with 0-s hold) and gel electrophoresis. Each quantitative RT-PCR experiment was performed on two replicates for two distinct biological samples using separate cultures grown under identical conditions ($n = 4$). *rnpB* (encoding RNase P) was used as a reference gene because it is known to be a stable housekeeping gene [15]. It was detected at the same level in all the samples by quantitative RT-PCR (Fig. 1). *psbA* (encoding the reaction center II subunit D1) was used as a positive control since it has been shown to be induced by high light exposure [16]. The ΔΔCt method was used to calculate gene expression changes relative to the non-stressed condition, normalized against *rnpB* (User Bulletin #2, ABI Prism 7700 sequence detection system, Applied Biosystems: http://dna-9.int-med.uiowa.edu/RealtimePCRdocs/Compar_Anal_Bulletin2.pdf) using the equation:

$$\text{Relative change} = 2^{-[(C_{T\ \text{target gene}} - C_{T\ rnpB})S - (C_{T\ \text{target gene}} - C_{T\ rnpB})NS]}$$

where C_T is the threshold cycle for gene amplification (see Fig. 1), S = stressed sample and NS = non-stressed sample.

Table 2
List of the two-component system genes of *Prochlorococcus* MED4

Gene	Type/Subfamily	Length (aa)	Accession number
hik01	Type I	379	ZP_00104274
hik02	Type I	373	CAB61760
hik03	Type I	383	ZP_00104692
hik04	Type I	690	ZP_00105296
hik05	–	456	ZP_00105522
rer01	Subfamily OmpR	252	ZP_00104138
rer02	Subfamily OmpR	249	ZP_00104144
rer03	Subfamily NarL	243	ZP_00104179
rer04	Subfamily OmpR	249	ZP_00105558
rer05	Subfamily OmpR	246	ZP_00105081
rer06	Subfamily OmpR	243	ZP_00104691

3. Results

3.1. Identification and analysis of the two-component member genes in Prochlorococcus MED4

Analysis of the *Prochlorococcus* MED4 genome allowed us to identify five *hik* and six *rer* putative coding sequences that we designated *hik01–hik05* and *rer01–rer06*, respectively (Table 2). Each predicted peptide sequence was inspected to check whether it contained (1) the stretches of amino acids that are conserved in most *hik* or *rer* sequences known to date and (2) the invariant phosphorylated residues (His or Asp) described by Stock et al. [1].

Conserved motifs in the 240-aa typical transmitter domain of Hik, H1 (the autophosphorylated histidine residue), N, G1, F and G2 (Fig. 2), were found in the five putative *Prochlorococcus* Hik. *Prochlorococcus* MED4 contains only type I Hik, the one most commonly found among eubacteria [3].

Conserved motifs in the 120-aa typical receiver domain of Rer are DD, D1 (the phosphorylated Asp residue) and K (Fig. 3). Five out of six putative Rer of *Prochlorococcus* MED4 (Rer01–04 and Rer06) contain the four expected

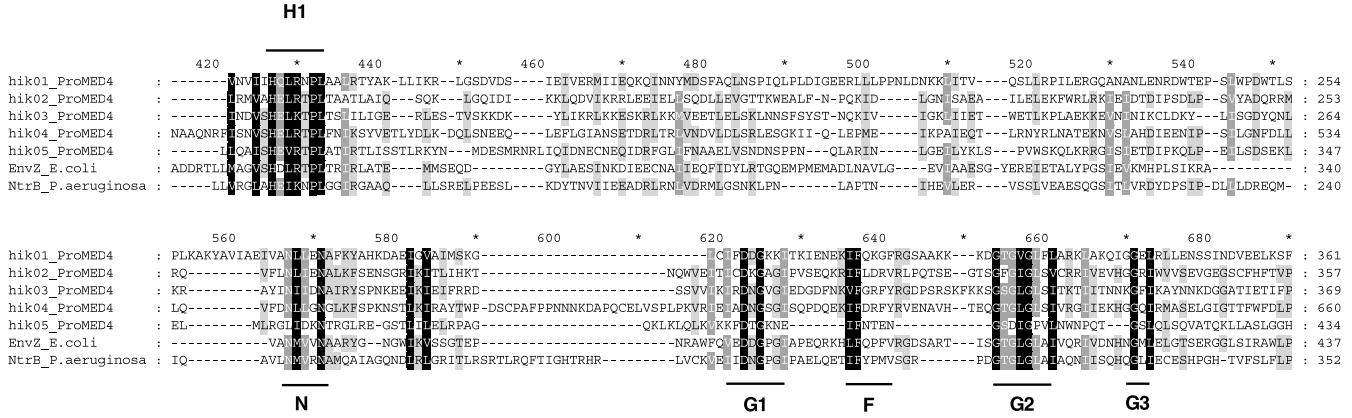

Fig. 2. Alignment of the conserved region of *Prochlorococcus* MED4 Hik amino acids with *E. coli* EnvZ and *Pseudomonas aeruginosa* NtrB. Identical or similar residues in at least two of the sequences are displayed on a black or gray background, respectively. The conserved motifs H, N, G1, F, G2, G3 of the 240-aa typical transmitter domain of Hik [1] are annotated.

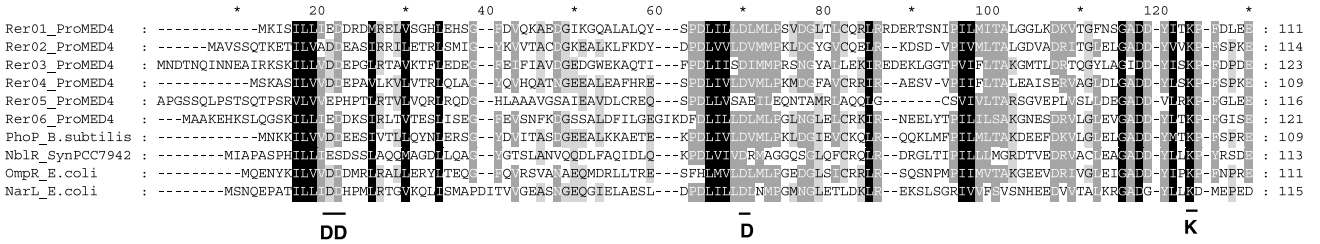

Fig. 3. Alignment of the conserved region of *Prochlorococcus* MED4 Rer amino acids with *B. subtilis* PhoP, *Synechococcus* PCC 7942 NblR and *E. coli* NarL and OmpR. Identical or similar residues in at least two of the sequences are displayed on a black or gray background, respectively. The conserved residues DD, D, K of the 120-aa typical receiver domain of Rer [1] are annotated.

conserved residues (three Asp, one Lys). In Rer05, the three Asp residues are replaced by Glu, Pro and Ala suggesting that the activity of Rer05 may be phosphorylation-independent. Phylogenetic analyses (not shown) revealed that Rer01–02 and Rer04–06 show good similarity to typical bacterial OmpR-class response regulators, possessing a motif called the HTH (helix-turn-helix) LuxR motif that may be important for DNA binding and interaction with the α-subunit of RNA polymerase [1]. Rer03 has a best hit to proteins belonging to the NarL class [17].

3.2. Comparison with other cyanobacteria

All genomes of the marine picocyanobacteria available to date harbor a very low number of *hik* and *rer* genes (Table 3) as compared to freshwater strains [18–23]. Most of the regulators and sensors found in MED4 have homologs in the other marine strains as well as in the freshwater *Synechocystis* PCC 6803. This suggests that some regulatory components have been conserved during evolution among marine and freshwater strains. However, the degree of conservation of these genes is variable (Table 4): *hik02*, *hik04* and *rer01–05* are better conserved than are *hik01*, *hik03*, *hik05*, and *rer06*. The *hik03*/*rer06* system, which we have identified as a putative *phoR*/*phoB* system, is one of the least conserved. In the low light-adapted strain *Prochlorococcus* MIT9313, *hik03* (i.e. *phoR*) is present, but displays a stop codon followed downstream by one or possibly two additional frameshifts within the reading frame. The occurrence of these frameshifts has been confirmed by independent gene sequencing (D.J. Scanlan, personal communication). So in MIT9313, *hik03* is clearly non-functional. In the other low light-adapted strain *Prochlorococcus* SS120, which has a more compact genome than MIT9313 (1.75 Mb vs. 2.43 Mb), it is even completely absent. However, SS120 possesses an operon encoding one putative two-component system that has no homolog in the other picocyanobacterial genomes. This operon is part of a group of four genes whose function is unknown (data not shown).

3.3. Analysis of the expression of the Prochlorococcus hik and rer genes under different stresses

Real-time quantitative RT-PCR was used to analyze changes in the levels of *hik* and *rer* gene transcripts induced by stresses. These consisted of exposure to high light of cultures of the axenic *Prochlorococcus* strain PCC 9511 over a period of 12 h, as well as heat and cold shock over a period of 4 h (Table 5).

To determine the reliability of these data, each experiment was performed on two biological samples with two replicates ($n = 4$). Overall, quantitative RT-PCR data were reproducible, although in some cases standard deviations were relatively high (maximum 12.83 ± 3.01). Primer efficiencies were about the same for *rnpB* and *hik*/*rer* genes ranging from 97% to 100% (data not shown). Relative levels of *hik*/*rer* gene expression estimated in the non-stressed samples indicated that three genes were relatively highly expressed compared to the others: *hik03*, *rer04* and

Table 3
Number of putative two-component system genes in cyanobacteria and other bacteria

Organism	Histidine kinase	Response regulator	Reference
Prochlorococcus MED4	5	6	This study
Prochlorococcus SS120	5	6	This study
Prochlorococcus MIT9313	8	12	This study
Synechococcus WH8102	7	14	This study
Thermosynechococcus elongatus BP-1	18	23	[18]
Synechocystis PCC 6803	42	38	[7]
Anabaena PCC 7120	131	80	[19]
Nostoc punctiforme	146	168	[20]
Escherichia coli	29	34	[21]
Bacillus subtilis	36	34	[22]
Streptococcus pneumoniae	13	14	[23]

Table 4
Genes homologous to MED4 two-component system genes in other cyanobacteria

Gene name	ProMED4	ProSS120		ProMIT9313		SynWH8102		Syn6803		Thermosyn		AnabPCC7120		NostPunc	
	Ref	Ref	% identity	Ref	% identity	Ref	% identity	Ref	% identity	Ref	% identity	Ref	% identity	Ref	% identity
hik01	ZP_00104274	pro0301	39	ZP_00114092	39	ZP_00114701	34	–	–	–	–	–	–	–	–
hik02	CAB61760	pro1121	66	ZP_00113429	64	ZP_00115115	64	sll0750	37	tlr0029	35	all3600	37	ZP_00109603	36
hik03 phoR	ZP_00104692	–	–	ZP_00113347	40	ZP_00115287	35	sll0337	35	tll0925	31	all4502	31	–	–
hik04 dspA	ZP_00105296	pro1422	71	ZP_00113711	72	ZP_00114938	70	sll0698	50	tlr0437	49	alr3511	48	ZP_00111644	51
hik05	ZP_00105522	pro1734	39	ZP_00113953	45	ZP_00116205	42	slr1285	33	tlr1565	35	alr2572	36	–	–
hik06	–	pro1543	–	–	–	–	–	sll1475	32	–	–	–	–	–	–
rer01	ZP_00104138	pro0150	87	ZP_00114203	82	ZP_00104138	82	slr0115	72	tlr2423	66	all0129	72	ZP_00111698	72
rer02	ZP_00104144	pro0156	86	ZP_00114209	85	ZP_00116387	85	slr0947	83	tll2364	81	all3822	85	ZP_00109580	84
rer03	ZP_00104179	pro0194	70	ZP_00114257	71	ZP_00116429	74	slr1783	50	tlr1263	55	all1736	55	ZP_00107435	55
rer04	ZP_00105558	pro1780	85	ZP_00112646	83	ZP_00114589	79	slr0947	54	tll2364	58	all3822	56	ZP_00109580	55
rer05	ZP_00105081	pro1083	81	ZP_00113428	77	ZP_00115244	85	sll1330	36	tlr1330	35	all4312	34	ZP_00107745	33
rer06 phoB	ZP_00104691	–	–	ZP_00113345	46	ZP_00115286	53	slr0081	51	tlr0589	50	all4503	52	ZP_00108178	48
rer07	–	pro1542	–	–	–	–	–	–	–	–	–	alr9013	32	–	–

Percentages of amino acid identity were determined using BLAST. ProMED4: *Prochlorococcus* MED4; ProSS120: *Prochlorococcus* SS120; ProMIT9313: *Prochlorococcus* MIT9313; SynWH8102: *Synechococcus* WH8102; Syn6803: *Synechocystis* PCC 6803; Thermosyn: *Thermosynechococcus elongatus* BP-1; AnabPCC7120: *Anabaena* PCC 7120; NostPunc: *Nostoc punctiforme*. Gene reference (Ref) is either from GenBank (ProMED4, SynWH8102, NostPunc), Cyanobase (Syn6803, Thermosyn, AnabPPC7120), or the ProSS120 web site.

rer05 (Table 5). This analysis also demonstrated the accumulation of *rer01*, *rer04*, *rer05* and *rer06* transcripts in response to high light exposure. However, the kinetics of transcript accumulation differed. The level of *rer01* mRNA increased after 3 h with a maximum at 6 h. The levels of *rer04* and *rer05* mRNA increased transiently after 1 h with a maximum level at 6 h, but the increase was more marked for *rer05* ($\sim$12-fold). *rer06* mRNA level did not increase during the first 3 h but after 6 h of high light exposure the level was $\sim$10-fold higher than that observed for control cells at low light (Fig. 1, Table 5). On the other hand, the level of *hik05* mRNA did not vary significantly and the levels of *hik01*, *hik02*, *rer02* and *rer03* declined. Finally, the levels of the *hik03* and *hik04* transcripts also seemed to increase transiently but the increase was less marked ($<$3-fold after 30 min, 6 h and 1 h respectively).

A moderate increase ($\sim$4-fold) in the levels of *rer03* and *hik01* mRNAs was detected after 4 h of moderate cold shock (13°C) whereas *rer06* transcripts accumulated to a lesser extent ($<$3-fold) and the levels of other transcripts did not vary significantly. A decrease was observed in mRNA accumulation for *hik01*, *hik02*, *hik04* and *rer06* after stronger cold shock (4°C) whereas the other transcripts did not change significantly, except *rer03* and *rer04* that increased by $\sim$2-fold after 30 min and 4 h of cold shock, respectively. Finally, most of the genes seemed to be severely down-regulated after heat shock, except *hik01* which showed a moderate increase ($<$3-fold).

4. Discussion

4.1. Genomic analysis of the two-component system members

Sequence analysis of the *Prochlorococcus* MED4 genome revealed the presence of only 11 putative genes encoding two-component systems, whereas approximately 80 have been detected in the freshwater cyanobacterium *Synechocystis* PCC 6803 [9], and 314 are annotated in the genome of *N. punctiforme* (Table 3). This results in part from the strong genome compaction of MED4, which possesses the smallest known genome of all oxygen-evolving prokaryotes (1.66 Mb). However, genome reduction is unlikely to be the sole cause of the occurrence of such a small number of genes encoding two-component systems. Indeed, the proportion of these genes is very low relative to the genome size compared to freshwater strains (3.66 Mb for *Synechocystis*, 9.2 Mb for *Nostoc*). Furthermore, all other marine picocyanobacteria sequenced to date (*P. marinus* SS120, *Prochlorococcus* sp. MIT 9313, and *Synechococcus* sp. WH8102) share with MED4 the presence of a very limited set of *rer* and *hik* genes (Table 3) despite their somewhat larger genomes (1.75, 2.41 and 2.43 Mb, respectively). This suggests that marine picocyanobacteria, in general, may require fewer regulatory sys-

Table 5
Real-time quantitative RT-PCR estimates of mRNA levels of histidine kinases and response regulators

Gene	Relative level $\times 10^{-2}$ at $t=0$	Induction ratio									
		High light stress				Cold shock 4°C		Cold shock 13°C		Heat shock 35°C	
		30 min	1 h	3 h	6 h	12 h	30 min	4 h	30 min	4 h	30 min	4 h
hik01	1.7	0.80 ± 0.09	1.54 ± 0.23	0.99 ± 0.21	1.27 ± 0.12	*0.35 ± 0.10*	0.36 ± 0.09	0.37 ± 0.07	1.27 ± 0.18	**3.81 ± 0.78**	1.45 ± 0.20	2.77 ± 0.24
hik02	0.28	1.82 ± 0.27	1.53 ± 0.46	0.82 ± 0.14	*0.15 ± 0.03*	*0.06 ± 0.02*	1.22 ± 0.13	0.66 ± 0.20	1.11 ± 0.31	0.90 ± 0.21	1.45 ± 0.24	1.31 ± 0.23
hik03	8.04	2.09 ± 0.30	0.84 ± 0.16	0.82 ± 0.07	0.80 ± 0.14	1.15 ± 0.21	1.21 ± 0.24	0.96 ± 0.25	1.96 ± 0.14	1.47 ± 0.31	*0.13 ± 0.03*	*0.18 ± 0.04*
hik04	0.98	1.99 ± 0.12	2.54 ± 0.34	1.21 ± 0.14	2.29 ± 0.34	0.69 ± 0.19	1.00 ± 0.30	0.50 ± 0.21	1.04 ± 0.17	0.91 ± 0.18	0.50 ± 0.09	1.00 ± 0.13
hik05	0.41	0.91 ± 0.13	1.49 ± 0.17	1.48 ± 0.23	1.24 ± 0.08	1.42 ± 0.23	0.74 ± 0.12	1.13 ± 0.13	1.16 ± 0.18	0.83 ± 0.26	*0.32 ± 0.08*	*0.36 ± 0.09*
rer01	0.68	0.78 ± 0.04	0.89 ± 0.04	**3.96 ± 0.61**	**5.94 ± 1.25**	1.43 ± 0.19	1.19 ± 0.25	0.86 ± 0.08	0.60 ± 0.23	1.06 ± 0.11	*0.12 ± 0.02*	*0.10 ± 0.02*
rer02	0.34	1.30 ± 0.09	1.23 ± 0.13	1.47 ± 0.13	0.59 ± 0.24	*0.24 ± 0.06*	1.05 ± 0.14	1.57 ± 0.40	0.80 ± 0.21	1.04 ± 0.13	*0.28 ± 0.08*	*0.08 ± 0.03*
rer03	0.29	0.50 ± 0.10	1.11 ± 0.09	0.60 ± 0.21	*0.33 ± 0.08*	0.80 ± 0.14	2.11 ± 0.49	2.04 ± 0.42	1.73 ± 0.46	**4.23 ± 0.66**	*0.37 ± 0.04*	*0.33 ± 0.14*
rer04	8.26	1.62 ± 0.12	2.88 ± 0.35	**5.78 ± 0.99**	**5.17 ± 1.12**	1.15 ± 0.21	1.24 ± 0.18	2.25 ± 0.53	1.75 ± 0.57	0.66 ± 0.10	*0.37 ± 0.10*	*0.34 ± 0.09*
rer05	12.5	**4.13 ± 0.45**	**3.93 ± 0.58**	**6.41 ± 1.01**	**12.83 ± 3.01**	**4.32 ± 0.99**	1.48 ± 0.27	1.90 ± 0.21	1.62 ± 0.25	0.90 ± 0.09	*0.13 ± 0.02*	*0.12 ± 0.04*
rer06	2.19	0.93 ± 0.14	0.67 ± 0.15	0.66 ± 0.20	**10.67 ± 2.57**	**4.84 ± 1.58**	1.13 ± 0.11	*0.48 ± 0.11*	2.31 ± 0.39	1.36 ± 0.24	*0.04 ± 0.01*	*0.29 ± 0.11*

The second column displays levels relative to *rnpB* at $t=0$. The following columns provide induction ratios ± S.D. relative to the levels at $t=0$ following light and temperature changes. Values in bold are >3, values in italic are <0.5. Experiments were performed on two replicates for two separate samples ($n=4$).

tems than freshwater ones. Indeed, marine microorganisms are exposed to a narrower spectrum of potential environmental changes than those concentrated in habitats such as temporary ponds or soil that undergo unpredictable and rapid changes. This fact could also explain the low diversity of Hik and Rer found in marine picocyanobacteria, each mostly belonging to a single protein group (Hik type I and OmpR, respectively).

In *Escherichia coli*, *hik/rer* gene pairs are, in general, located within the same transcriptional unit or operon, and function together in a specific signaling pathway. However, for both *Synechocystis* PCC 6803 and *Prochlorococcus* MED4, only a small fraction of signal transducers, 14 for *Synechocystis* [9] and one for *Prochlorococcus* (*hik03/rer06*), are located in close proximity. Sequence analysis of Hik03/Rer06 reveals that this regulatory pair shares considerable homology with the PhoR/B homologs of *Bacillus subtilis*. PhoR/B regulate the expression of a series of genes important for continued growth and survival during phosphate limitation [24]. Examination of the genome vicinity of *hik03* (Fig. 4) reveals the presence of one gene homolog to the *B. subtilis pstS* gene, which encodes part of a high-affinity phosphate uptake system [25]. PstS, expressed only under P depletion, was detected in various *Prochlorococcus* strains [26] and may play a role in adaptation of these strains to oligotrophy. The presence of the *hik03/rer06* gene pair in the *Prochlorococcus* MED4

Fig. 4. Comparison of the genetic maps of the *phoRB* regions (*hik03/rer06*) from *Prochlorococcus* MED4, *Prochlorococcus* MIT9313, *Prochlorococcus* SS120 and *Synechococcus* WH8102 containing *pst* genes involved in phosphate assimilation mechanisms. Open reading frames are indicated by boxes: black boxes correspond to *phoRB* genes, dark gray boxes correspond to *pst* genes and light gray boxes correspond to the *envZ/ompR* system. Top boxes correspond to forward strand and bottom boxes correspond to reverse strand. Boxes without a name correspond to genes whose putative function has not been identified.

genome and its absence or non-functionality in SS120 and MIT9313 (Fig. 4), respectively, is interesting because MED4 is characteristic of populations living in the surface layer of the ocean where it has recently been demonstrated that phosphorus can be limiting [27], while the two other strains correspond to populations located at the bottom of the euphotic zone where phosphate is available through upward diffusion.

Prochlorococcus MED4 Hik04 may play a key role in its capacity to acclimatize to its specific environment. Hik04 shows high similarity with DspA of *Synechocystis* sp. PCC 6803 which was first identified as a factor that confers resistance to a variety of herbicides and calmodulin antagonists [28]. More recently, DspA (also called Hik33) was hypothesized to sense decreases in temperature and to regulate the expression of some cold-inducible genes [29]. Furthermore, NblS, the DspA homolog in *Synechococcus* PCC 7942, is involved in controlling photosynthesis-related gene expression during high light and nutrient stress [30]. The structure of Hik04 is basically the same as that of its *Synechococcus* PCC 7942 homolog, NblS [30]. It is the only histidine kinase containing a putative membrane-spanning domain or a PAS domain in *Prochlorococcus* MED4. Originally identified as components of circadian clock regulators in eukaryotes, these domains have recently been found in a variety of sensor proteins from both prokaryotes and eukaryotes [31,32]. Analysis of the predicted Hik04 protein sequence in *Prochlorococcus* MED4 also showed the presence of a HAMP domain, involved in intramolecular interactions and signal transduction, transmitting conformational changes from the periplasmic region to the cytoplasmic region of the molecules [33]. Moreover, *hik04* (*dspA*) is located close to the clock genes *kaiB* and *kaiC* [34], but transcribed in the opposite direction, and gene order is highly conserved in *Prochlorococcus* and *Synechococcus* strains (Fig. 5). The *kaiABC* gene cluster is essential for generation of circadian rhythms in the cyanobacterium *Synechococcus* sp. strain PCC 7942 [34]. In contrast to *Synechococcus* PCC 7942 or *Synechocystis* PCC 6803, *Prochlorococcus* MED4 and MIT9313 do not possess the *kaiA* gene encoding a positive enhancer of *kaiB* and *kaiC* [34]. Recently, it has been shown that the *kaiABC* operon in *Synechococcus* sp. strain PCC 7942 can also be controlled by a histidine sensor kinase, SasA [35], whose homolog in MED4 is Hik02.

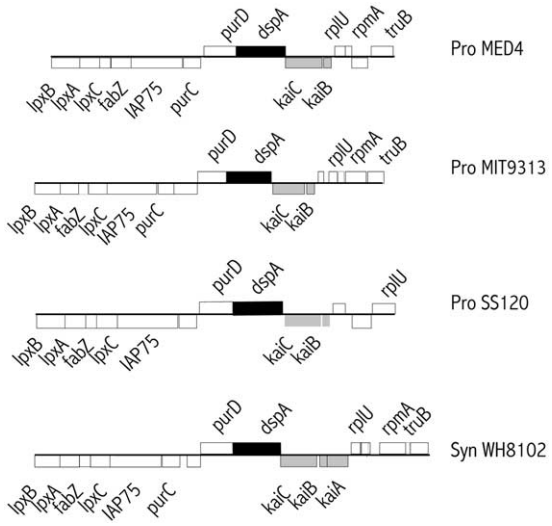

Fig. 5. Comparison of the genetic maps of the *dspA* regions from *Prochlorococcus* MED4, *Prochlorococcus* MIT9313, *Prochlorococcus* SS120 and *Synechococcus* WH8102 containing *kaiB* and *kaiC* clock genes. Open reading frames are indicated by boxes, black boxes correspond to *dspA* gene and gray boxes correspond to *kai* genes. Top boxes correspond to forward strand and bottom boxes correspond to reverse strand. Boxes without a name correspond to genes whose putative function has not been identified.

4.2. *Expression of* Prochlorococcus *MED4 two-component systems in response to stress*

The tiny size of the *Prochlorococcus* genome and its small number of genes make it a very interesting model to study and characterize the putative function of two-component systems. Although Rer are known to be affected at both the transcriptional and post-transcriptional levels [35], we examined here only the first process using quantitative RT-PCR. Under non-stressed conditions, expression levels of *hik03*, *rer04* and *rer05* are markedly higher than those of the other two-component system genes (Table 5), although still about 10 times lower than those of a housekeeping gene such as *rnpB*. In particular, *hik03* (*phoR*) was not found to be induced after stress exposure but its constitutive expression level could be sufficient for it to act with its putative associated response regulator, *rer06* (*phoB*), whose transcript level is increased more than 10 times upon transfer to high light. It may seem surprising that *rer06* is induced by high light because *phoB* typically responds to Pi-limiting conditions [24]. However, in *Synechocystis* sp. strain PCC 6803, the *pho* regulon also exhibits increased expression when the cells are exposed to high light [12]. This fact could have two possible explanations. Under high light, the growth rate of the organism could exceed its capacity to assimilate phosphate, triggering a phosphate starvation response and the activation of the *pho* regulon. Alternatively, the *phoR*/*phoB* system could respond to different stresses and therefore be directly activated by high light. It would be useful to characterize the responses of *hik03* and *rer06* to nutrient stresses, including phosphate limitation, to check the putative biological functions controlled by this system. Besides *rer06*, *rer01*, *rer04*, and *rer05* are also induced in response to changes in light intensity (Table 5). Rer01 and Rer04 are highly similar to members of the OmpR subfamily Rer of cyanobacteria, and particularly to the DNA-binding regulators of *Tolypothrix* PCC 7601, RpaA and RpaB [36], and *Synechocystis* PCC 6803 Rer, slr0115 and slr0947, respectively, which are known to influence the distribution of excitation energy absorbed by

the phycobilisomes [37]. Although *rer05* does not have strong homology to any previously well characterized gene, it could be one of the key response regulators in *Prochlorococcus* MED4 because its mRNA level after 6 h of light stress is very high, even higher than that of *rnpB*.

The levels of induction of *rer* genes in response to a shift to high light are moderate (5- or 10-fold increase) in comparison with the 25-fold increase observed for heat shock genes (such as *dnaK*) under the same stress conditions (Mary et al., in press). Moreover, the level of *rer* gene transcripts increased transiently, with a maximum level after 6 h of high light exposure, and no concomitant accumulation of *hik* mRNA was detected. Most histidine kinases are known to respond to stress by post-translational alterations (i.e. phosphorylations) [3]. Our results suggest that, in *Prochlorococcus* at least, this could also be the case. In contrast, an increase of *rer* gene transcription seems to be necessary to control the expression of specific stress response genes.

Temperature shock induced a decrease rather than an increase of most of the transcript levels of two-component genes in *Prochlorococcus* MED4. This could be explained either by the repression of transcription or by the rapid degradation of mRNAs. Previous studies showed that *Prochlorococcus* cannot be found in waters with temperature below 12°C [6] and culture experiments confirmed its sensitivity to low temperatures [38]. It is therefore possible that *Prochlorococcus* may not able to respond very well to temperature shocks. However, the levels of *hik01* and *rer03* mRNAs showed a moderate increase after the cold shock at 13°C whereas these gene transcripts did not accumulate after high light exposure.

This study is a first step in the understanding of the involvement of these systems in response to various stresses, especially those experienced in the natural habitat of these marine picocyanobacteria (high surface light, UV radiation, nutrient depletion). Future work is needed to elucidate how *rer* genes are activated and how sensors and regulators are coupled to each other.

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