Title:

The two-component response regulator OrrA confers dehydration tolerance by regulating *anaKa* expression in the cyanobacterium *Anabaena* sp. strain PCC 7120

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Running title:

Dehydration tolerance in *Anabaena*

Summary

The aquatic cyanobacterium *Anabaena* sp. strain PCC 7120 exhibits dehydration tolerance. The regulation of gene expression in response to dehydration is crucial for the acquisition of dehydration tolerance, but the molecular mechanisms underlying dehydration responses remain unknown. In this study, the functions of the response regulator OrrA in the regulation of salt and dehydration responses were investigated. Disruption of *orrA* abolished or diminished the induction of hundreds of genes in response to salt stress and dehydration. Thus, OrrA is a principal regulator of both stress responses. In particular, OrrA plays a crucial role in dehydration tolerance because an *orrA* disruptant completely lost the ability to regrow after dehydration. Moreover, in the OrrA regulon, *anaKa* encoding a protein of unknown function was revealed to be indispensable for dehydration tolerance. OrrA and AnaK are conserved among the terrestrial cyanobacteria, suggesting their conserved functions in dehydration tolerance in cyanobacteria.

INTRODUCTION

Cyanobacteria comprise a diverse group of bacteria characterized by oxygen-evolving photosynthesis. Their photosynthetic ability enables them to inhabit almost all illuminated environments. The habitat of cyanobacteria is not limited to aquatic ecosystems, but it extends to terrestrial ecosystems including extremely arid environments such as deserts (Pointing and Belnap, 2012). Some cyanobacterial species belonging to the genera *Nostoc* and *Chroococcidiopsis* are present in dry areas, and they exhibit high tolerance to desiccation (Singh, 2018). For example, *Chroococcidiopsis* strains isolated from deserts could form colonies after 4 years of storage in a dry state, and the terrestrial strain *Nostoc commune* was revived via inoculation into medium after storage in a desiccated state for approximately 90 years (Lipman, 1941; Fagliarone *et al.*, 2017).

Loss of water from cells induces various stresses and damages various cellular components including proteins, membrane lipids, and DNA (Potts, 2001; Singh, 2018).

Desiccation-tolerant cyanobacteria adopt multiple strategies to survive harsh environments during desiccation. *N. commune* cells are embedded in extracellular polysaccharide (EPS), which protects cells from desiccation (Tamaru *et al.*, 2005). In the aquatic cyanobacterium *Anabaena* sp. strain PCC 7120 (hereafter *Anabaena*), EPS excretion was increased by overexpression of *sigJ*, which encodes the sigma factor of RNA polymerase, and the mutant displays higher dehydration tolerance than the wild-type strain (WT) (Yoshimura *et al.*, 2007). The accumulation of compatible solutes such as trehalose and sucrose, which stabilize the proteins and cellular membranes of dehydrated cells (Tapia and Koshland, 2014), represents another strategy of desiccation tolerance. *N. commune* and a *Chroococcidiopsis* strain accumulate trehalose and sucrose in response to dehydration, salt and osmotic stress (HersHKovitz *et al.*, 1991; Sakamoto *et al.*, 2009). *Anabaena* also utilizes both sugars as compatible solutes, and disruption of trehalose synthesis genes decreases dehydration tolerance (Higo *et al.*, 2006). Moreover, *Escherichia coli* expressing the sucrose synthesis gene *spsA* from the cyanobacterium *Synechocystis* sp. strain PCC 6803 (*Synechocystis* PCC 6803) accumulates sucrose within cells and exhibits a drastic increase in survival rate after dehydration (Billi *et al.*, 2002).

Oxidative damage caused by reactive oxygen species (ROS) is one of the most deleterious effects of dehydration (França *et al.*, 2007). ROS generation is increased by dehydration, resulting in lipid peroxidation, denaturation of proteins through oxidative modifications, and DNA double-strand breakage. Antioxidant defense systems that suppress ROS generation and scavenge generated ROS protect cellular components from oxidation (Singh, 2018). Because most antioxidant defense systems are proteinous, avoidance of protein oxidation is a key factor for desiccation tolerance (Daly, 2009). Desiccation-resistant bacteria display lower oxidative modification of proteins than desiccation-sensitive bacteria during dehydration (Fredrickson *et al.*, 2008). *Chroococcidiopsis* sp. strain CCME 029 is highly tolerant to desiccation, and it does not undergo protein oxidation after 1 year of incubation under dry conditions. Contrarily, *Synechocystis* PCC 6803 exhibits substantial protein oxidation, and it is

incapable of surviving under the same conditions (Fagliarone *et al.*, 2017).

Anabaena is a model organism of bacterial cellular differentiation (Flores *et al.*, 2019).

It has been also used to study the molecular mechanisms of responses to stresses including dehydration (Ohmori *et al.*, 2001; Wang *et al.*, 2002). Close relatives of *Anabaena* such as the terrestrial strain *Nostoc* sp. HK-01 exhibit high desiccation tolerance, and *Anabaena* itself displays moderate dehydration tolerance (Kato *et al.*, 2003; Singh *et al.*, 2013). Because genetic engineering cannot be performed in terrestrial *Nostoc* strains, *Anabaena* serves as a model organism for investigating the molecular mechanisms of desiccation tolerance in the terrestrial strains (Yoshimura *et al.*, 2007; Xu *et al.*, 2020). In *Anabaena*, *orrA*, which encodes a response regulator of the bacterial two-component regulatory system, was identified as a regulator of *lti2* in response to salt and osmotic stresses (Schwartz *et al.*, 1998). We previously indicated that OrrA regulates the expression of *spsA*, *susA*, and *susB*, which participate sucrose synthesis, in response to salt stress (Ehira *et al.*, 2014). Sucrose accumulation under salt stress conditions is lowered by the *orrA* disruption, and intracellular sucrose levels are increased by *orrA* overexpression (Ehira *et al.*, 2014). In this study, we analyzed the global effects of *orrA* disruption on gene expression, observing that OrrA is a master regulator of dehydration and salt stress responses in *Anabaena*. Moreover, among hundreds of genes regulated by OrrA, *anaKa*, which encodes a protein of unknown function, proved essential for dehydration tolerance in *Anabaena*.

RESULTS

OrrA is a major regulator of salt stress response

Sucrose synthesis under salt stress conditions is regulated by OrrA in *Anabaena* (Ehira *et al.*, 2014). The functions of OrrA in global salt stress response regulation were investigated by transcriptome analysis. The gene expression profiles of WT and the *orrA* mutant strain DRorrAS were compared between cells subjected to salt stress (50 mM NaCl) for 3 h and cells before stress treatment. The transcript levels of 298 genes were increased at least twofold by salt stress in WT,

while those of another 1,358 genes were decreased (Table S1). Three genes involved in sucrose metabolism, namely *spsA*, *susA* and *susB*, were upregulated by salt stress as previously reported (Ehira *et al.*, 2014). The upregulated genes included *mth* encoding an enzyme for trehalose synthesis (Higo *et al.*, 2006) and *anaK* genes (all4050, all4051, and all5315), which are predominantly expressed in cyanobacterial resting cells called akinetes in *Anabaena variabilis* (Zhou and Wolk, 2002). Genes encoding proteins that prevent ROS generation, such as *hli/scp* genes (asl0514, asl0873, asr3042, and asr3043) and *dps* genes (all0458 and all1173), and genes that detoxify ROS, such as *katA* and *katB*, were also upregulated (Howe *et al.*, 2018; Tibiletti *et al.*, 2018). The downregulated genes included genes involved in photosynthesis (*psa* and *psb* genes) and carbon fixation (*rbcLXS*, *prk*, *ccmNMLK*, *ecaA*, and *cmpABCD*), and genes encoding components of the phycobilisome (*apc*, *cpc*, and *pec* genes). Moreover, the transcript levels of genes involved in transcription and translation (*rpl* and *rps* genes, *rpoA*, *rpoC1*, and *rpoC2*), and ATP synthesis (*atp* genes) were decreased. The suppression of cell growth under salt stress conditions leads to downregulation of these genes (Ehira *et al.*, 2014).

In DRorrAS, the numbers of genes upregulated and downregulated by salt stress were decreased to 71 and 51, respectively (Table S1). Meanwhile, 37 genes were upregulated in both WT and DRorrAS (Fig. 1), but the degree of upregulation was higher in WT than in DRorrAS. Temporal changes in the expression of salt-induced genes were examined by quantitative reverse transcription (qRT)-PCR (Fig. 2). In WT, *mth* expression was induced within 30 min after NaCl addition, with the *mth* transcript level increasing approximately 800-fold after 60 min, but its induction was completely abolished in DRorrAS (Fig. 2A). The induction patterns of two *dps* genes (all0458 and all1173) differed from each other, but both genes lost salt-responsive induction by *orrA* disruption (Fig. 2B). There are four paralogs of *anaK* (*anaKa*, *anaKb*, *anaKc*, and *anaKd*) in *Anabaena* (Zhou and Wolk, 2002). All *anaK* genes were induced by salt stress, which depended on *orrA* (Fig. 2C). These results indicate that OrrA is a major regulator of salt stress response in *Anabaena*.

orrA is necessary for dehydration tolerance

OrrA regulates the expression of most salt-responsive genes, whereas its disruption does not affect growth under salt stress conditions (Ehira *et al.*, 2014). Expression of *orrA* is induced by not only salt stress, but also dehydration (Higo *et al.*, 2006). Moreover, many genes upregulated by salt stress, such as *mth*, *hli/scp* genes, and *dps* genes, are also upregulated by dehydration (Higo *et al.*, 2006). To investigate the effect of the *orrA* disruption on gene expression in response to dehydration, gene expression profiles were compared between WT and DRorrAS after 3 h of dehydration. In WT, 493 genes were upregulated by dehydration, while 1,307 genes were downregulated (Table S1). Among the upregulated genes in WT, 156 genes were also upregulated in DRorrAS, but the extent of upregulation was significantly lower in DRorrAS than in WT (Fig. 1 and Table S1). Salt stress and dehydration increased the expression of the same 239 genes, only 23 of which were upregulated by both stresses in DRorrS (Fig. 1). *spsA*, *susA*, and *susB*, which are involved in sucrose metabolism, were also induced by dehydration under the control of OrrA (Table S1). The intracellular sucrose level in WT was increased more than 80-fold after 9 h of dehydration, whereas the level was twofold lower in DRorrAS than in WT (Table 1). Thus, OrrA plays a principal role in regulating gene expression during dehydration in *Anabaena*.

To investigate the physiological role of OrrA in dehydration tolerance, growth ability after dehydration was analyzed. *Anabaena* filaments grown in the liquid medium were collected by filtration, and then filaments on the filters were dried in an incubator. After the indicated times, filaments were resuspended in liquid medium and incubated for 3 days. The weight of the filters decreased during the first 8 h of incubation before reaching a plateau, indicating that all water had evaporated (Fig. 3A). In WT, filaments were capable of restarting growth even after 24 h of incubation on the filters (Fig. 3B). DRorrAS filaments were able to regrow after 6 h of incubation on the filters, but their growth was severely impaired after 7 and 8 h of incubation (Fig. 3B). Moreover, DRorrAS completely lost its ability to grow after more than 10 h of incubation on the

filters. Thus, *orrA* is necessary for survival under dehydration conditions.

orrA is also involved in cold acclimation

OrrA was originally identified as a regulator of the salt and osmolyte responses of *lti2* (Schwartz *et al.*, 1998). In this study, we confirmed the OrrA-regulated *lti2* expression in response to salt and dehydration (Table S1). *lti2* is also known to be upregulated under low temperatures (Ehira *et al.*, 2005). In addition, low temperature-responsive genes, such as *alr0169*, *alr0803*, *alr0804*, and *alr1819*, were under the control of OrrA (Table S1). Hence, we analyzed the effects of *orrA* disruption on cold acclimation. *orrA* expression increased within 30 min after the temperature was decreased from 30°C to 22°C (Fig. 4A). Growth of DRorrAS at 30°C is comparable to that of WT (Ehira *et al.*, 2014), but its growth at 22°C was slower than that of WT (Fig. 4B). Thus, OrrA also influences cold acclimation.

anaKa is a determinant of dehydration tolerance

To further reveal the mechanisms of dehydration tolerance, the involvement of genes regulated by OrrA in dehydration tolerance was investigated. We focused on *anaK* because this gene is highly expressed in akinetes (Zhou and Wolk, 2002), which are cells that are extremely resistant to stresses such as cold and desiccation (Adams and Duggan, 1999). Four *anaK* paralogs, namely *anaKa*, *anaKb*, *anaKc*, and *anaKd*, were induced by dehydration, and this induction depended on OrrA (Table S1). Each *anaK* paralog was inactivated, and the growth of the disruptants after dehydration was determined (Fig. 5). The *anaKa* disruptant DRanaKaK could not grow after incubation on the filters for 24 h, whereas the other *anaK* disruptants retained similar growth ability as WT (Fig. 5A). Time-course experiments illustrated that DRanaKaK lost growth ability at 8 h (Fig. 5B), when water had completely evaporated from filters (Fig. 3A). This indicated that *anaKa* disruption has a fatal effect on survival under dehydration.

Disruption of *anaKa* alone resulted in the loss of dehydration tolerance. To examine

the involvement of other genes regulated by OrrA in dehydration tolerance, damage of DRorrAS and DRanaKaK cell after dehydration was evaluated by quantifying phycocyanin excretion from cells (Fig. 6). Phycocyanin is a water-soluble pigment protein and is released extracellularly upon cell lysis (Arii *et al.*, 2015). In WT, phycocyanin excretion was not enhanced by dehydration. In DRorrAS and DRanaKaK, excreted phycocyanin levels were comparable to WT levels before dehydration, but phycocyanin excretion was increased by dehydration. Phycocyanin excretion was more than twofold higher in DRorrAS than DRanaKaK, indicating greater cell membrane damage in DRorrAS. Thus, the *orrA* disruption is more detrimental than the *anaKa* disruption.

DISCUSSION

In this study, we revealed that the two-component response regulator OrrA played principal roles in the regulation of dehydration response and tolerance in *Anabaena*. *orrA* disruption abolished or diminished the induction of approximately 400 genes by dehydration (Fig.1 and Table S1). As previously indicated in response to salt stress (Ehira *et al.*, 2014), OrrA regulated sucrose synthesis under dehydration conditions (Table 1). In addition, the trehalose synthesis genes *mtH* and *mts*, which encode maltooligosyl trehalose hydrolase and maltooligosyl trehalose synthase, respectively, were regulated by OrrA (Table S1). Because disruption of *mtH* and *mts* results in decreased dehydration tolerance (Higo *et al.*, 2006), the depression of compatible solutes synthesis partly accounts for the susceptibility of the *orrA* disruptant to dehydration. In the OrrA regulon identified this study, *anaKa* was indispensable for dehydration tolerance in *Anabaena* (Fig. 5). *anaKa* encodes a protein of unknown function with a PRC-barrel domain at its N-terminus and a DUF2382 domain at its C-terminus. Although *anaKb*, *anaKc*, and *anaKd* were also induced by dehydration in an OrrA-dependent manner, their disruption did not affect dehydration tolerance. *anaKa* was more strongly induced than the other examined *anaK* genes (Fig. 2, Table S1). Differences in expression levels of AnaK proteins could explain the different phenotypes among the *anaK* mutants. OrrA and AnaK homologs are present in the genome of the

terrestrial cyanobacteria, such as *Nostoc* sp. HK-01, *N. flagelliforme*, and *Chroococcidiopsis thermalis* PCC 7203. OrrA and AnaK homologs are highly expressed in desiccated samples in *N. flagelliforme* (Shang *et al.*, 2019), and an AnaK homolog is induced by dehydration in *Nostoc* sp. HK-01 (Yoshimura *et al.*, 2006), suggesting their conserved functions in desiccation tolerance.

The function of the PRC-barrel domain is unknown. It is found in the H subunit of photosystem reaction center (PRC) of the purple bacteria (Anantharaman and Aravind, 2002). The H subunit of PRC is necessary for functional PRC assembly, and it regulates electron transfer in PRC (Takahashi and Wraight, 1996; Lupo and Ghosh, 2004). The PRC-barrel domain is also found at the C-terminus of RimM, which is involved in RNA processing and ribosome assembly (Anantharaman and Aravind, 2002). The PRC-barrel domain of AnaKa is phylogenetically most closely related to that of the H subunit of PRC (Anantharaman and Aravind, 2002). AnaKa homologs are present in the highly radiation- and desiccation-resistant bacterium *Deinococcus radiodurans* and some species of the genus *Psychrobacter*, which are psychro- and osmo-tolerant bacteria (Kim *et al.*, 2012). In *D. radiodurans*, the expression of the *anaKa* homolog DR1314 is upregulated by heat shock, and its induction is regulated by the extracytoplasmic function sigma factor Sig1 (Schmid *et al.*, 2005). DR1314 is also regulated by the response regulator DrRRA, which is essential for radioresistance in *D. radiodurans* (Wang *et al.*, 2016). The DR1314 disruptant is sensitive to hydrogen peroxide (H_2O_2) exposure and heat shock (Schmid *et al.*, 2005; Wang *et al.*, 2016). Thus, AnaK homologs play important roles in the stress resistance of extremophiles.

It was also indicated that OrrA regulates ROS defense systems (Table 2). ROS, which include singlet oxygen (1O_2), superoxide anion (O_2^-), H_2O_2 , and hydroxyl radical ($\bullet OH$), is the main damaging factor during dehydration (Singh, 2018). O_2^- , which is generated by the reduction of oxygen through photosynthetic electron transport, is detoxified by superoxide dismutase (SOD), which dismutates it into H_2O_2 and O_2 with. In *Anabaena*, *sodA* and *sodB* both encode SOD (Li *et al.*, 2002), but their expression was not upregulated by dehydration. H_2O_2 is removed

by catalases and peroxiredoxin (Prxs), which convert it into water. The catalase genes *katA* and *katB* were induced by dehydration; however, catalase activity is low in *Anabaena*, and Prxs play a more prominent role in H₂O₂ removal (Pascual *et al.*, 2010). Although alr4641 (encoding 2-Cys-Prx), the overexpression of which protects *Anabaena* cells from oxidative stress (Banerjee *et al.*, 2015), is induced by O₂⁻ and H₂O₂ (Hurtado-Gallego *et al.*, 2019), its expression was altered by dehydration. •OH is formed by the reaction of Fe²⁺ and H₂O₂, and Dps proteins sequester iron to prevent •OH formation (Howe *et al.*, 2018). *Anabaena* carries four *dps* genes (all0458, all1173, alr3808, and all4145), and all0458 and all1173 were induced by dehydration. Thus, some H₂O₂-detoxifying enzymes were upregulated during dehydration, but H₂O₂ generation during dehydration is obscure because H₂O₂-responsive genes such as alr4641 and *trxA2* were not induced (Ehira and Ohmori, 2012; Hurtado-Gallego *et al.*, 2019). Conversely, many genes encoding proteins involved in defense against ¹O₂ were induced by dehydration (Table 2). Excited chlorophylls can transfer excitation energy to oxygen, resulting in ¹O₂ formation (Latifi *et al.*, 2009). High-light-inducible proteins, which are encoded by the *hli/scp* genes, prevent ¹O₂ formation by consuming the excess of PSII-generated electrons (Sinha *et al.*, 2012). Orange carotenoid protein (OCP) is an efficient quencher of ¹O₂ (Sedoud *et al.*, 2014). Recent findings indicated that All3221 and Alr4783 proteins of *Anabaena*, which are homologous to the N-terminal domain (NTD) of OCP, are effective quenchers of ¹O₂ (López-Igual *et al.*, 2016). Moreover, in *N. flagelliforme*, a remarkable desiccation-tolerant terrestrial cyanobacterium, NTD-OCP proteins accumulated in cells collected from dried fields, suggesting its involvement in desiccation tolerance (Yang *et al.*, 2019). AstaP, another OCP, was originally found in a green alga isolated from the dried surface of heated asphalt in midsummer, and it also displays ¹O₂ quenching activity (Kawasaki *et al.*, 2013). *Anabaena* carries four genes encoding AstaP homologs (alr1819, all4647, all4894, and all5264), all of which were induced by dehydration. It is suggested that *N. commune* deactivates photosynthetic machinery in response to dehydration to protect photosystems from ¹O₂ generated through the imbalance between chlorophyll excitation

and photosynthetic electron transport (Hirai *et al.*, 2004). Thus, defense systems against $^1\text{O}_2$ are especially important in dehydration tolerance of *Anabaena*.

The OrrA regulon defined in this study contains a huge number of genes. A transcriptional network is likely to be involved in the dehydration response, because eight genes encoding transcriptional regulators, including *sigB2* (Ehira *et al.*, 2014), were upregulated by dehydration under the control of OrrA (Table S1). In addition, the expression of more than 10 genes encoding signal transduction proteins, such as sensor histidine kinase (Hik) and Ser/Thr kinases, increased in an OrrA-dependent manner. These regulators constitute a regulatory network of the dehydration response of *Anabaena*.

OrrA was identified as a master regulator of the response to salt stress and dehydration and was involved in the response to low temperature (Figs. 1 and 4). OrrA was originally identified as the regulator of *lti2* in response to salt and osmotic response (Schwartz *et al.*, 1998), implying that OrrA also plays an important role in the osmotic stress response. Evaporation of water from the medium surrounding cells increases external solute concentrations, resulting in salt and osmotic stresses. Therefore, a common signaling pathway could activate OrrA in response to dehydration, salt, and osmotic stresses. Because OrrA is a response regulator, its activity is regulated through the phosphorylation by a Hik. Three Hiks are located upstream of *orrA* in the genome of *Anabaena* (Ehira *et al.*, 2014). These Hiks are involved in dehydration response, but none of them is a cognate Hik for OrrA in *Anabaena* (K. Shimonaka & S. Ehira, unpublished data). Identification of a cognate Hik for OrrA would reveal the mechanisms by which dehydration signals are sensed.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions—*Anabaena* sp. strain PCC 7120 and its derivatives were grown at 30°C under continuous illumination provided by a fluorescent lamp at 30 $\mu\text{E m}^{-2} \text{s}^{-1}$ in the nitrogen-free modified Detmer's medium (MDM₀) (Watanabe, 1960). Liquid cultures were

bubbled with air containing 1% (v/v) CO₂. Salt stress conditions were generated by adding 50 mM NaCl to cultures in the late-logarithmic phase (optical density at 750 nm [OD₇₅₀] = 0.5-0.7). Dehydration stress was imposed on *Anabaena* cells as described previously (Higo *et al.*, 2006). A 25-ml portion of cultures in the late-logarithmic phase was filtered through 47-mm, 0.45-μm pore size mixed cellulose ester filters (Tokyo Roshi Kaisha, Tokyo, Japan) and dried for the indicated times at 30°C under continuous illumination provided by a fluorescent lamp at 10 μE m⁻² s⁻¹ in a Petri dish.

Mutant construction— All primers used in this study (Table S2) were designed based on genome data from CyanoBase (Fujisawa *et al.*, 2017). DRorrAS was used as an *orrA* disruptant in this study (Ehira *et al.*, 2014). DRanaKaK, DRanaKbK, DRanaKcS, and DRanaKdS, in which *anaKa* (*all4050*), *anaKb* (*all5315*), *anaKc* (*all4051*), and *anaKd* (*alr5332*), respectively, were disrupted, were constructed by replacing each gene with an antibiotic-resistant gene in the same manner as performed for DRorrAS using primer pairs specific for each gene (Ehira *et al.*, 2014).

DNA microarray analysis— Total RNA was extracted from whole filaments according to Pinto *et al.* (Pinto *et al.*, 2009) and was treated with DNase I (Takara Bio, Shiga, Japan). Global gene expression was analyzed using the *Anabaena* oligonucleotide microarray as described previously (Ehira and Ohmori, 2006). Microarray analyses were conducted using three sets of RNA samples isolated from independently grown cultures. Two hybridization reactions were performed with different combination of Cy-dyes for each set of RNA samples. Thus, six replicates were available per gene to determine changes in gene expression. Genes with at least twofold differences in transcript levels ($P < 0.05$; Student's *t*-test) were identified. The microarray data were deposited in the KEGG Expression Database (accession numbers ex0001979 to ex0002002).

qRT-PCR— cDNAs were synthesized from 1 μg of total RNA with random hexamer primers using a PrimeScript first strand cDNA synthesis kit (Takara Bio). qRT-PCR was performed using the Thermal Cycler Dice Real Time System II (TP900; Takara Bio) in a 20-μl reaction mixture

containing 10 μ l of THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan), 0.2 μ M each of gene-specific forward and reverse primers, and cDNA. Relative transcript levels were normalized to the value for 16S rRNA and represented as means of duplicate measurements. Experiments were repeated at least three times.

Dehydration tolerance assay– Cells dehydrated for the indicated times on filter paper were suspended in MDM₀. The suspended cultures were diluted with MDM₀ to OD₇₅₀ of 0.1 and incubated at 30°C at 10 μ E m⁻² s⁻¹. After 72 h, OD₇₅₀ of the cultures was measured.

Determination of sucrose contents– The low-molecular-mass compounds of cells dehydrated for the indicated time were extracted with 80% ethanol for 3 h at 65°C (Higo *et al.*, 2006). After centrifugation, supernatants were vacuum-dried, and the residue was dissolved in 0.5 ml of water. The samples were treated with 3 U of invertase (Sigma) in 50 mM sodium acetate (pH 4.5) for 2 h at 37°C, and the concentration of released glucose was determined using a Glucose Assay Kit (Biovision). The chlorophyll *a* content of cultures was determined as described previously (Mackinney, 1941).

Quantification of excreted phycobiliproteins– Dehydrated cells were resuspended in MDM₀ and statically incubated for 5 min at room temperature. Cells were collected by centrifugation, and the absorbance of the supernatants at 620 nm was measured. A₆₂₀ was normalized by OD₇₅₀ measured prior to centrifugation.

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Author contributions

M. Ohmori and S. Ehira conceived the study. S. Kimura and S. Ehira designed the experiments. S.

335 Kimura, M. Sato, X. Fan, and S. Ehira conducted the experiments and analyzed the data. S. Ehira
336 wrote the manuscript, and all authors edited and approved the final manuscript.

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338 **Conflict of Interest**

339 The authors declare that they have no conflicts of interest.

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References

- Adams, D.G., and Duggan, P.S. (1999) Heterocyst and akinete differentiation in cyanobacteria. *New Phytol* **144**: 3–33.
- Anantharaman, V., and Aravind, L. (2002) The PRC-barrel: a widespread, conserved domain shared by photosynthetic reaction center subunits and proteins of RNA metabolism. *Genome Biol* **3**: 1–9.
- Arii, S., Tsuji, K., Tomita, K., Hasegawa, M., Bober, B., and Harada, K.I. (2015) Cyanobacterial blue color formation during lysis under natural conditions. *Appl Environ Microbiol* **81**: 2667–2675.
- Banerjee, M., Chakravarty, D., and Ballal, A. (2015) Redox-dependent chaperone/peroxidase function of 2-Cys-Prx from the cyanobacterium *Anabaena* PCC7120: role in oxidative stress tolerance. *BMC Plant Biol* **15**: 60.
- Billi, D., Wright, D.J., Potts, M., Helm, R.F., Crowe, J.H., and Prickett, T. (2002) Engineering desiccation tolerance in *Escherichia coli*. *Appl Environ Microbiol* **66**: 1680–1684.
- Daly, M.J. (2009) A new perspective on radiation resistance based on *Deinococcus radiodurans*. *Nat Rev Microbiol* **7**: 237–245.
- Ehira, S., Kimura, S., Miyazaki, S., and Ohmori, M. (2014) Sucrose synthesis in the nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120 is controlled by the two-component response regulator OrrA. *Appl Environ Microbiol* **80**: 5672–5679.
- Ehira, S., and Ohmori, M. (2006) NrrA, a nitrogen-responsive response regulator facilitates heterocyst development in the cyanobacterium *Anabaena* sp. strain PCC 7120. *Mol Microbiol* **59**: 1692–1703.
- Ehira, S., and Ohmori, M. (2012) The redox-sensing transcriptional regulator RexT controls expression of thioredoxin A2 in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J Biol Chem* **287**: 40433–40440.
- Ehira, S., Ohmori, M., and Sato, N. (2005) Identification of low-temperature-regulated ORFs in

the cyanobacterium *Anabaena* sp. strain PCC 7120: distinguishing the effects of low temperature from the effects of photosystem II excitation pressure. *Plant Cell Physiol* **46**: 1237–1245.

Fagliarone, C., Mosca, C., Ubaldi, I., Verseux, C., Baqué, M., Wilmotte, A., and Billi, D. (2017) Avoidance of protein oxidation correlates with the desiccation and radiation resistance of hot and cold desert strains of the cyanobacterium *Chroococcidiopsis*. *Extremophiles* **21**: 981–991.

Flores, E., Picossi, S., Valladares, A., and Herrero, A. (2019) Transcriptional regulation of development in heterocyst-forming cyanobacteria. *Biochim Biophys Acta Gene Regul Mech* **1862**: 673–684.

França, M.B., Panek, A.D., and Eleutherio, E.C. (2007) Oxidative stress and its effects during dehydration. *Comp Biochem Physiol A Mol Integr Physiol* **146**: 621–631.

Fredrickson, J.K., Li, S.M., Gaidamakova, E.K., Matrosova, V.Y., Zhai, M., Sulloway, H.M., et al. (2008) Protein oxidation: key to bacterial desiccation resistance? *ISME J* **2**: 393–403.

Fujisawa, T., Narikawa, R., Maeda, S.I., Watanabe, S., Kanesaki, Y.Y., Kobayashi, K., et al. (2017) CyanoBase: a large-scale update on its 20th anniversary. *Nucleic Acids Res* **45**: D551–D554.

Hershkovitz, N., Oren, A., and Cohen, Y. (1991) Accumulation of trehalose and sucrose in cyanobacteria exposed to matric water stress. *Appl Environ Microbiol* **57**: 645–648.

Higo, A., Katoh, H., Ohmori, K., Ikeuchi, M., and Ohmori, M. (2006) The role of a gene cluster for trehalose metabolism in dehydration tolerance of the filamentous cyanobacterium *Anabaena* sp. PCC 7120. *Microbiology* **152**: 979–987.

Hirai, M., Yamakawa, R., Nishio, J., Yamaji, T., Kashino, Y., Koike, H., and Satoh, K. (2004) Deactivation of photosynthetic activities is triggered by loss of a small amount of water in a desiccation-tolerant cyanobacterium, *Nostoc commune*. *Plant Cell Physiol* **45**: 872–878.

Howe, C., Ho, F., Nenninger, A., Raleiras, P., and Stensjö, K. (2018) Differential biochemical properties of three canonical Dps proteins from the cyanobacterium *Nostoc punctiforme* suggest

distinct cellular functions. *J Biol Chem* **293**: 16635–16646.

Hurtado-Gallego, J., Redondo-López, A., Leganés, F., Rosal, R., and Fernández-Piñas, F. (2019) Peroxiredoxin (*2-cys-prx*) and catalase (*kataA*) cyanobacterial-based bioluminescent bioreporters to detect oxidative stress in the aquatic environment. *Chemosphere* **236**: 124395.

Kato, H., Shiga, Y., Nakahira, Y., and Ohmori, M. (2003) Isolation and characterization of a drought-tolerant cyanobacterium, *Nostoc* sp. HK-01. *Microbes Environ* **18**: 82–88.

Kawasaki, S., Mizuguchi, K., Sato, M., Kono, T., and Shimizu, H. (2013) A novel astaxanthin-binding photooxidative stress-inducible aqueous carotenoprotein from a eukaryotic microalga isolated from asphalt in midsummer. *Plant Cell Physiol* **54**: 1027–1040.

Kim, S.J., Shin, S.C., Hong, S.G., Lee, Y.M., Choi, I.G., and Park, H. (2012) Genome sequence of a novel member of the genus *Psychrobacter* isolated from Antarctic soil. *J Bacteriol* **194**: 2403–2403.

Latifi, A., Ruiz, M., and Zhang, C.C. (2009) Oxidative stress in cyanobacteria. *FEMS Microbiol Rev* **33**: 258–278.

Li, T., Huang, X., Zhou, R., Liu, Y., Li, B., Nomura, C., and Zhao, J. (2002) Differential expression and localization of Mn and Fe superoxide dismutases in the heterocystous cyanobacterium *Anabaena* sp. strain PCC 7120. *J Bacteriol* **184**: 5096–5103.

Lipman, C.B. (1941) The successful revival of *Nostoc commune* from a herbarium specimen eighty- seven years old. *Bull Torrey Bot Club* **68**: 664.

López-Igual, R., Wilson, A., Leverenz, R.L., Melnicki, M.R., Bourcier de Carbon, C., Sutter, M., et al. (2016) Different functions of the paralogs to the N-terminal domain of the orange carotenoid protein in the cyanobacterium *Anabaena* sp. PCC 7120. *Plant Physiol* **171**: 1852–1866.

Lupo, D., and Ghosh, R. (2004) The reaction center H subunit is not required for high levels of light-harvesting complex 1 in *Rhodospirillum rubrum* mutants. *J Bacteriol* **186**: 5585–5595.

Mackinney, G. (1941) Absorption of light by chlorophyll solutions. *J Biol Chem* **140**: 315–322.

419 Ohmori, M., Ikeuchi, M., Sato, N., Wolk, P., Kaneko, T., Ogawa, T., *et al.* (2001)
420 Characterization of genes encoding multi-domain proteins in the genome of the filamentous
421 nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. *DNA Res* **8**: 271–284.
422 Pascual, M.B., Mata-Cabana, A., Florencio, F.J., Lindahl, M., and Cejudo, F.J. (2010)
423 Overoxidation of 2-Cys peroxiredoxin in prokaryotes: cyanobacterial 2-Cys peroxiredoxins
424 sensitive to oxidative stress. *J Biol Chem* **285**: 34485–34492.
425 Pinto, F.L., Thapper, A., Sontheim, W., and Lindblad, P. (2009) Analysis of current and
426 alternative phenol based RNA extraction methodologies for cyanobacteria. *BMC Mol Biol* **10**:
427 79.
428 Pointing, S.B., and Belnap, J. (2012) Microbial colonization and controls in dryland systems. *Nat*
429 *Rev Microbiol* **10**: 551–562.
430 Potts, M. (2001) Desiccation tolerance: a simple process? *Trends Microbiol* **9**: 553–559.
431 Sakamoto, T., Yoshida, T., Arima, H., Hatanaka, Y., Takani, Y., and Tamaru, Y. (2009)
432 Accumulation of trehalose in response to desiccation and salt stress in the terrestrial
433 cyanobacterium *Nostoc commune*. *Phycol Res* **57**: 66–73.
434 Schmid, A.K., Howell, H.A., Battista, J.R., Peterson, S.N., and Lidstrom, M.E. (2005) Global
435 transcriptional and proteomic analysis of the Sig1 heat shock regulon of *Deinococcus*
436 *radiodurans*. *J Bacteriol* **187**: 3339–3351.
437 Schwartz, S.H., Black, T.A., Jäger, K., Panoff, J.M., and Wolk, C.P. (1998) Regulation of an
438 osmoticum-responsive gene in *Anabaena* sp. strain PCC 7120. *J Bacteriol* **180**: 6332–6337.
439 Sedoud, A., López-Igual, R., Ur Rehman, A., Wilson, A., Perreau, F., Boulay, C., *et al.* (2014)
440 The cyanobacterial photoactive orange carotenoid protein is an excellent singlet oxygen
441 quencher. *Plant Cell* **26**: 1781–1791.
442 Shang, J.L., Chen, M., Hou, S., Li, T., Yang, Y.W., Li, Q., *et al.* (2019) Genomic and
443 transcriptomic insights into the survival of the subaerial cyanobacterium *Nostoc flagelliforme* in
444 arid and exposed habitats. *Environ Microbiol* **21**: 845–863.

445 Singh, H. (2018) Desiccation and radiation stress tolerance in cyanobacteria. *J Basic Microbiol*
446 **58**: 813–826.

447 Singh, H., Anurag, K., and Apte, S.K. (2013) High radiation and desiccation tolerance of
448 nitrogen-fixing cultures of the cyanobacterium *Anabaena* sp. strain PCC 7120 emanates from
449 genome/proteome repair capabilities. *Photosynth Res*.

450 Sinha, R.K., Komenda, J., Knoppová, J., Sedlářová, M., and Pospíšil, P. (2012) Small CAB-like
451 proteins prevent formation of singlet oxygen in the damaged photosystem II complex of the
452 cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Cell Environ* **35**: 806–818.

453 Takahashi, E., and Wraight, C.A. (1996) Potentiation of proton transfer function by electrostatic
454 interactions in photosynthetic reaction centers from *Rhodobacter sphaeroides*: first results from
455 site-directed mutation of the H subunit. *Proc Natl Acad Sci U S A* **93**: 2640–2645.

456 Tamaru, Y., Takani, Y., Yoshida, T., and Sakamoto, T. (2005) Crucial role of extracellular
457 polysaccharides in desiccation and freezing tolerance in the terrestrial cyanobacterium *Nostoc*
458 *commune*. *Appl Environ Microbiol* **71**: 7327–7333.

459 Tapia, H., and Koshland, D.E. (2014) Trehalose is a versatile and long-lived chaperone for
460 desiccation tolerance. *Curr Biol* **24**: 2758–2766.

461 Tibiletti, T., Rehman, A.U., Vass, I., and Funk, C. (2018) The stress-induced SCP/HLIP family of
462 small light-harvesting-like proteins (ScpABCDE) protects photosystem II from photoinhibitory
463 damages in the cyanobacterium *Synechocystis* sp. PCC 6803. *Photosynth Res* **135**: 103–114.

464 Wang, L., Hu, J., Liu, M., Yang, S., Zhao, Y., Cheng, K., *et al.* (2016) Proteomic insights into the
465 functional basis for the response regulator DrRRA of *Deinococcus radiodurans*. *Int J Radiat*
466 *Biol* **92**: 273–280.

467 Wang, L., Sun, Y.P., Chen, W.L., Li, J.H., and Zhang, C.C. (2002) Genomic analysis of protein
468 kinases, protein phosphatases and two-component regulatory systems of the cyanobacterium
469 *Anabaena* sp. strain PCC 7120. *FEMS Microbiol Lett* **217**: 155–165.

470 Watanabe, A. (1960) List of algal strains in collection at the Institute of Applied Microbiology,

471 University of Tokyo. *J Gen Appl Microbiol* **6**: 283–292.

472 Xu, H.F., Dai, G.Z., Ye, D.M., Shang, J.L., Song, W.Y., Shi, H., and Qiu, B.S. (2020)

473 Dehydration-induced DnaK2 chaperone is involved in PSII repair of a desiccation-tolerant

474 cyanobacterium. *Plant Physiol* **182**: 1991–2005.

475 Yang, Y.W., Yin, Y.C., Li, Z.K., Huang, D., Shang, J.L., Chen, M., and Qiu, B.S. (2019) Orange

476 and red carotenoid proteins are involved in the adaptation of the terrestrial cyanobacterium

477 *Nostoc flagelliforme* to desiccation. *Photosynth Res* **140**: 103–113.

478 Yoshimura, H., Ikeuchi, M., and Ohmori, M. (2006) Up-regulated gene expression during

479 dehydration in a terrestrial cyanobacterium, *Nostoc* sp. strain HK-01. *Microbes Environ* **21**:

480 129–133.

481 Yoshimura, H., Okamoto, S., Tsumuraya, Y., and Ohmori, M. (2007) Group 3 sigma factor gene,

482 *sigJ*, a key regulator of desiccation tolerance, regulates the synthesis of extracellular

483 polysaccharide in cyanobacterium *Anabaena* sp. strain PCC 7120. *DNA Res* **14**: 13–24.

484 Zhou, R., and Wolk, C.P. (2002) Identification of an akinete marker gene in *Anabaena variabilis*.

485 *J Bacteriol* **184**: 2529–2532.

486

487

488 Table 1. Sucrose contents after dehydration

Strain	Sucrose ($\mu\text{mol/ mg chl}a$)	
	0 h	9 h
WT	0.06 ± 0.06	5.00 ± 0.39
DRorrAS	0.04 ± 0.04	2.26 ± 0.29

489 WT, wild-type *Anabaena* sp. strain PCC 7120; DRorrAS, *orrA* mutant *Anabaena* sp. strain PCC

490 7120; chl a , chlorophyll a

491

492

493 Table 2. OrrA-regulated genes that are related to ROS defense

ORF No.	Product
Defense against H ₂ O ₂	
alr0998 (<i>katA</i>)	Fe catalase
alr3090 (<i>katB</i>)	Mn catalase
all0458 (<i>dps</i>)	Dps
all1173 (<i>dps</i>)	Dps
Defense against ¹ O ₂	
asl0514 (<i>hli/scp</i>)	High-light-inducible/small CAB-like protein
asl0873 (<i>hli/scp</i>)	High-light-inducible/small CAB-like protein
asr3042 (<i>hli/scp</i>)	High-light-inducible/small CAB-like protein
asr3043 (<i>hli/scp</i>)	High-light-inducible/small CAB-like protein
all3221	Homolog of the N-terminal domain of OCP
alr4783	Homolog of the N-terminal domain of OCP
alr1819	AstaP
all4647	AstaP
all4894	AstaP
all5264	AstaP

494 ROS, reactive oxygen species; ORF, open-reading frame; H₂O₂, hydrogen peroxide; ¹O₂, singlet

495 oxygen; OCP, orange carotenoid protein

496

Figure legends

Fig 1. Venn diagrams of genes upregulated by salt stress and dehydration in wild-type *Anabaena* sp. strain PCC 7120 (WT) and the *orrA* disruptant DRorrAS. The numbers in parenthesis indicate genes upregulated in both WT and DRorrAS.

Fig 2. Temporal changes in the transcript levels of genes upregulated by salt stress. The transcript levels of *mth* (A), *dps* (B), and *anaK* (C) after the addition of 50 mM NaCl were determined by qRT-PCR in WT (black circles) and the *orrA* disruptant (white circles). The transcript levels were determined in duplicated measurements using three independently grown cultures. The transcript level at 0 min in WT was taken as 1. Data are presented as the mean $\pm$ SD.

Fig 3. Dehydration tolerance of the *orrA* disruptant.

A. Changes in weight of filters during 24h of incubation. Filaments of WT (black circles) and the *orrA* disruptant (white circles) were collected on filters and the filters dried for 24 h. The weight of each filter was measured during the 24-h incubation and presented as a ratio to the control (0 h).
B. Growth ability of the *orrA* disruptant after dehydration. Filaments on the filters were resuspended in liquid medium at the indicated time, the optical density at 750 nm (OD_{750}) of the suspension was adjusted to 0.1, and then filaments were incubated for 3 days in liquid medium. OD_{750} after the 3-day incubation was indicated for WT (black circles) and the *orrA* disruptant (white circles). The experiments were repeated using three independently grown cultures. Data are presented as the mean $\pm$ SD.

Fig 4. Involvement of OrrA in cold acclimation.

A. Changes in the *orrA* transcript levels after a shift to low temperature (22°C). The transcript level at 0 h was taken as 1.
B. Growth of WT (black circles) and the *orrA* disruptant (white circles) at 22°C. Growth was

monitored by measuring OD₇₅₀. The experiments were repeated with three independently grown cultures. Data are presented as the mean ± SD.

Fig 5. Dehydration tolerance of disruptants of the *anaK* genes. Filaments of disruptants of *anaKa* (ΔK_a), *anaKb* (ΔK_b), *anaKc* (ΔK_c), *anaKd* (ΔK_d), and *orrA* ($\Delta orrA$) were subjected to dehydration for 0 (white bars) or 24 h (gray bars), and then their growth ability was evaluated as described in Fig 3 (A). Temporal changes of the growth ability of WT (black circles) and the *anaKa* disruptant (white circles) during 24 h of incubation were determined (B). The experiments were repeated using three independently grown cultures. Data are presented as the mean ± SD.

Fig 6. Excretion of phycocyanin after dehydration. Phycocyanin excreted in the medium from the cells of WT (black bars), the *orrA* disruptant (gray bars), and the *anaKa* disruptant (white bars) that were dehydrated on filters for 0 or 24 h was determined by measuring the absorbance at 620 nm.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6