Role of iron uptake systems in coordinating iron homeostasis in NaCl-resistant mutant of the cyanobacterium *Nostoc muscorum*

Abstract

Aims: To investigate the effect of NaCl stress on parent *Nostoc muscorum* and its spontaneously occurring mutant clone showing resistance to growth inhibitory action of NaCl in terms of various physiological parameters. We have further analyzed the role of iron uptake systems in providing a resistant phenotype.

Place and Duration of Study: Division of Microbiology, Department of Botany Government Motilal Science College, Bhopal 462008 (M.P.) India. This work was carried out between August 2021 to May 2022.

Methodology: We have examined the various physiological parameters *viz.* growth, specific growth rate, photosynthetic O_2 evolution, and nitrogenase activity as per the prescribed protocol. Further, DNA microarray analysis was carried out using the Agilent platform.

Results: NaCl stress adversely affected growth, photosynthetic O_2 evolution, and nitrogenase activity of the wild-type *Nostoc muscorum*, while NaCl-resistant mutant remains unaffected under a given stress. Microarray data analysis identified 24 ORF related to the uptake of iron with fold regulation ≥ 2 in the mutant strain. These ORFs belonging to the ABC-type ferric iron transporter that plays a significant role in the iron acquisition were identified in the mutant strain.

Conclusion: The mechanism of iron homeostasis in the NaCl-resistant mutant has been explained. The results presented are essential to explain the regulatory role of the iron uptake system in stressed conditions.

Keywords: iron uptake, NaCl-resistant mutant, *Nostoc muscorum*

Introduction

Cyanobacteria are photosynthetic O_2 evolving Gram-negative prokaryotes and some of them are diazotrophic. They grow and survive in a variety of natural habitats and are considered an important player in biogeochemical cycles [1,2,3]. According to an estimate, about 30% of global primary productivity is the outcome of cyanobacterial carbon fixation. In addition to carbon fixation, their nitrogen-fixing ability also contributes immensely to the nitrogen budget in aquatic ecosystems [4]. The fluctuating environmental parameters directly or indirectly influence cyanobacterial distribution and productivity. Any upshift or downshift in environmental parameters resulted in up-regulation and downregulation of specific sets of genes. The perception and subsequent transduction of the environmental stresses are the initial events occurring in cyanobacteria to cope with the changes in the surroundings [5,6].

Iron is an essential micronutrient for almost all living organisms, despite that low iron bioavailability has been the major factor that determines the primary productivity of various aquatic ecosystems [7]. In autotrophs, iron is present as a metalloprotein which is necessary for photosynthesis, respiration, nitrogen fixation, cytochrome, and iron-sulfur proteins [8,9]. The reduction of ferric iron (FeIII) to the ferrous state (FeII) requires a high redox potential, suggesting its ability to govern various cellular redox processes. In photosynthetic organisms like cyanobacteria, the photosynthetic apparatus mainly consists of the plastoquinone pool, cytochrome b_6f complex, plastocyanin, and cytochrome. Iron has an indispensable role in the synthesis of photosynthetic pigments like chlorophyll *a* and c-phycocyanin [10]. Apart from this iron also has a major role in enzymatic reaction; it affects the synthesis and function of all cytochromes, the cytochrome oxidase complex, catalase, peroxidase, and ferredoxin [11]. The diazotrophic cyanobacteria fix atmospheric nitrogen using nitrogenase- a Fe_7Mo- containing enzyme; indicating that iron homeostasis is also regulated by nitrogen metabolism [12,6]. In cyanobacteria, two iron uptake strategies have been documented these are: siderophore-mediated transport and reductive iron uptake. The iron transport is mediated by a three-component system- i) a periplasmic binding protein e. g. FhuD/FutA/FecA, ii) a membrane-embedded permease e. g. FhuB/FutB/FecCD and iii) an ATP-binding protein e. g. FhuC/FutC/FecB [13].

Cyanobacterial photosynthesis is vulnerable to salt stress [14]. Under salt stress the ratio of PSI/PSII increased fuel P-type ATPase or other energy-demanding processes during salt stress [15]. The genome of many cyanobacterial species contains *isiA* gene its expression is induced by

iron starvation [16]. It is known to associate either with PSI or PSII [17]. In addition, the *isiA* gene is also transcribed under oxidative stress [18]. Under iron limitation, another protein known as iron deficiency-induced protein IdiA is also induced in cyanobacteria [19,20]. This protein provides protection to PSII against oxidative damage [21]. The sequence similarity of this protein with the family of bacterial periplasmic ABC transporter complex identifies these iron importers as FutA, SfuA, FbpA, and HitA. In order to understand the resistance mechanism of the mutant phenotype, the mutant for growth, photosynthetic O_2 evolution, and nitrogenase activity in the diazotrophic growth medium are analyzed. Further analysis of mutants enabled us to identify genes essential to iron uptake.

Materials and Methods

Organism and growth conditions

In the present investigation cyanobacterium, *Nostoc muscorum* was used as a model organism to understand salinity stress response at the genomic level. The cultures were grown in bulk in the Chu No. 10 medium [22] in the growth chamber, with light intensity at a photon fluence rate of 50 µmol m^{-2} s^{-1} and a temperature of 28 ± 2°C.

Isolation of NaCl-resistant (NaCl-R) mutant, measurement of growth and specific growth rate, photosynthetic O_2 evolution, and nitrogenase activity

NaCl-R mutants were isolated and maintained as described previously [23,24]. Growth, photosynthetic O_2 evolution, and nitrogenase activity of the wild-type *N. muscorum* and its mutant were measured as described previously [25]. The specific growth rate constant (k) was calculated by the formula given by [26].

$K = 2.303 (\log N_2 – \log N_1)/ (T_2 – T_1)$

Where,

N_1 = Initial cell density at time T_1

N_2 = The final cell density at time T_2

DNA microarray

In DNA microarray technique mRNA or total RNA from the sample is used to generate a labeled sample which is hybridized in parallel with a large DNA sequence immobilized on a solid surface in an ordered array. We used a customized 8×15k Array (AMADID: 050709) with 60mer oligonucleotide probes which have been in situ spotted on a glass slide by Agilent technology (USA) as described by [27].

Sample preparation- Preparation of Target DNA

The sample preparation includes total RNA extraction from cyanobacterial cells (wild type and both mutants) and its conversion to cRNA, labeling, and purification. The complete protocol was followed as SOP described by Agilent.

RNA isolation: The procedure as described by Agilent has been followed to isolate total RNA from the experimental samples. The method of extraction was Qiagen RNeasy Mini kit with DNase treatment. RNA concentration and purity of samples were estimated using NanoDrop Spectrophotometer and were analyzed by Bioanalyzer 2100 (Agilent) prior to GeneChip hybridization. RNA was considered to be of good/optimal purity (OD 260/280 >1.8 and <2.2; OD 260/230 >0.5 and <2.4) with optimal concentration (>50 ng/µl and <2500 ng/µl) and an RNA integrity number (RIN) was ≥7.0.

RNA labeling and amplification: RNA labeling and amplification were done by using Agilent Quick-Amp Kit, USA P/No. 5190-0442 (USA).

cRNA Purification: The complementary RNA generated was purified by using Qiagen's RNeasy Minikit (Cat#74106).

cRNA Quantification: The cRNA purified was quantified and labeling QC was performed by using NanoDrop Spectrophotometer. The Nanodrop analysis of labeled cRNA with Cy3 dye showing Specific activity >8.0 was considered good and was suitable for hybridization.

Hybridization:

Hybridization was done using Agilent's In situ Hybridization kit (P/No. 5190-0404) as per the manufacturer's instructions. Hybridization was carried out in Agilent's surehyb chambers at 65 °C for 16 h.

Washing and Scanning

After hybridization, washing was done with wash buffers (P/No. 5188-5327) which eliminated all the unbound labeled target cRNAs. For scanning Agilent G4900DA SureScan Microarray Scanner was used as per the manufacturer's instructions.

Data analysis

Hybridization was analyzed based on "g(r) is PosAndSignif". Normalization was done using GeneSpring GX vr.12.0 Software. Feature extracted data were analyzed using GeneSpring GX vr.12.0 software from Agilent (USA).

Statistical analysis

The statistical significance of the gene expression was calculated by using a Student's t-test analysis. Only genes with high levels of significance ($P < 0.05$) and a minimum absolute value of $\log_2 > 1$ were systematically considered in this study [28].

Results

Cyanobacteria require iron as an essential micronutrient for the synthesis of chlorophyll pigment, photosynthesis, and nitrogen metabolism. Cyanobacteria grow and thrive in various ecological niches with varying concentrations of this micronutrient. Instead of those cyanobacteria adopted different strategies to compensate for the iron imbalance. These strategies are mainly related to the synthesis of chlorophyll pigment, regulation of photosynthesis, and nitrogen metabolism. To understand the molecular basis of iron homeostasis in the mutant strain, we performed some physiological experiments under stress conditions in both strains.

Wild type and its NaCl-R mutant both were cultivated in a diazotrophic growth medium. Growth and specific growth rate of the wild-type *N. muscorum* were severely inhibited under the graded concentration of NaCl, unlike wild type, growth of the NaCl-R mutant was largely unaffected under the graded concentration of NaCl. Further, in order to investigate the effect of NaCl on photosynthetic O_2 evolution and nitrogenase activity, both *N. muscorum* and its NaCl-R mutant were examined for the aforementioned parameters under NaCl stress. Both the examined parameters were adversely affected under the graded concentration of NaCl in the wild type; on the contrary same parameters remained unaffected in the NaCl-R mutant (Table 1 & 2). This indicates that mutation leading to NaCl resistance provides protection under a given stress.

In the next series of experiments the transcriptome analysis of the wild type and its mutant strain was carried and this allowed insight into the mechanism that provides resistant phenotype. DNA microarray technology is used to survey a large number of up-regulated and down-regulated genes in the present investigation. In this study, we used the same technology to survey differential expression of up-regulated genes in the mutant (increase in fold value compared with the wild-type strain (expression level {0}). We surveyed upregulated genes belonging to the functional category- iron transport and binding proteins in the mutant strain. The acceptable validation results with fold change ≥2 magnitude were considered in the present study [28].

In the transport and binding protein category, a large number of genes were found to be upregulated suggesting their role in iron uptake and protection against given stress. An overview of the number of differentially regulated genes and their transcript abundance is given in table 3. In total, we have identified 24 ORF with fold regulation ≥2 in the mutant strain. The highest increase in transcript level was observed for ORF *alr2176*, this ORF is code for Fe(III) dicitrate-binding protein of ABC transporter. The biological role of this ORF is to transport iron from one side of a membrane to the other by means of some agent such as a transporter or pore. Another ORF identified in this study with a fold value of 4.0 is *alr1382*, this ORF codes for ABC transporter ferric iron-binding periplasmic protein and transcript into *futA*, *idiA*, and *sfuA* genes. Another ORF with fold value 3.42 (*alr1383*) is also upregulated and it is transcripts into *futB* and *sfuB*.

In *Anabaena* sp. PCC 7120, five gene clusters with similarities to the Fut-, Fec- and Fhu-system are annotated. In this study, we have identified up-regulation of *futA* and *futB* in the mutant strain. These transporters are known to be involved in Fe(III) transport. Likewise, the expression level of *fecC1*, *fecD1* and *fecB2* were upregulated compared with wild-type. These genes are known to encode for iron ABC transporter permease and ferrichrome ABC transporter substrate-binding protein. The overexpression of *fec*-gene clusters is reported to occur during nitrogen-limiting conditions. In the present study, the overexpression of these genes could be justified on the basis of the fact that all the experiments were performed in diazotrophic growth conditions.

In addition to IsiA some cyanobacterial strains synthesize the iron deficiency-induced protein IdiA [19]. In cyanobacteria, IdiA is mainly associated with the thylakoid membrane and exposed more under iron limiting conditions owing to phycobilisomes degradation. The photosynthetic O_2 evolution of the mutant strain was unaffected under stress conditions, therefore; the overexpression of IdiA protein in this study suggested its role in protecting the acceptor side of PSII against oxidative damage, especially under NaCl-stress conditions. Other genes identified in this study, code for ferric iron transporter (*sfuA* and *sfuB*) known to involve in ferric iron transport. These transporters delivered iron by transferring and transporting ferric ion across the inner membrane.

In addition to ORF mentioned above, a large gene cluster involved in siderophore synthesis was also identified in the present study suggesting their role in iron acquisition and uptake. These ORF identified as *alr2176*, *alr2588*, *all2674*, *all1101*, *alr2209*, *alr2185*, *alr2213*, *alr2590*, *all2158*, *alr2592*, *all2148*, *all1100*, *alr2593*, *alr2179*, *all2609*, *alr2596*, and *alr2210*. These ORF were annotated for iron(III) dicitrate-binding protein of ABC transporter, ferrichrome-iron receptor, ferric aerobactin receptor, and iron ABC transporter permease (Table 3). In the present study only outer membrane receptors for the siderophore Fe(III) i. e. dicitrate-binding protein showed overexpression. These receptors depend on the Ton-B complex present in the cytoplasmic membrane to transport the siderophore-Fe(III) complex to the periplasmic space through the proton gradient across the membrane. Based on the above facts, the overexpression of iron ABC transporter permease has been justified, because the siderophore-Fe(III) complexes are transported to the cytoplasm through iron permease.

Discussion

The bioavailability of iron is the major factor that determines the primary productivity of both fresh water and marine ecosystems [7]. The bioavailability of iron depends upon its oxidative state (FeII and FeIII), pH and availability of other ions in the external environment [29]. Under these conditions, cyanobacteria have evolved different strategies to efficiently scavenge, incorporate and store this micronutrient in the cell [30,31]. Another strategy adopted by cyanobacteria is to store iron within a protein cage and to protect the cell from oxidative damage, a process in which ferritin family proteins play an important role [32].

In the present investigation, *futA* and *futB* (*alr1382* and *alr1383*) encode an ABC-type Fe(III) uptake system induced in the mutant strain suggesting their role in the iron transport system. These ORFs are annotated as periplasmic iron transport proteins in the cyanobacterium *Anabaena* sp. PCC 7120 [33].

The outer membrane receptor protein i. e. FecA in *E. coli* undergoes major conformational changes to utilize iron dicitrate. This conformational change induced FecR, so that FecI in tern activate RNA polymerase which leads to the transcription of *fecABCDE* genes encoding the ferric citrate transport system [34]. Our results showed that ferrichrome-iron receptor genes were all induced in the mutant strain, therefore; these genes must be induced by the siderophore-Fe(III) receptor present on the outer membrane so that primary producers like cyanobacteria may also utilize heme as a source of iron in aquatic habitats.

An iron-deficiency induction of the *idiA* gene was reported in cyanobacteria [35], the present study is in harmony with the above finding. This gene and its product are specific for cyanobacteria and cannot be found in green algae or higher plants [36]. This protein is known to induce iron deficiency and play an important role in the photosynthetic electron transport chain in cyanobacteria. In addition, *sfu* genes were also identified in this study. In bacterial system, iron is delivered as transferrin by the transport system such as Sfu [37]. This system is involved in transporting ferric ions across the inner membrane. The Sfu proteins are classified as a typical ABC transporter and located in the periplasm. Out of these SfuB is a cytoplasmic-membrane protein and SfuC is a membrane-bound protein carrying a nucleotide-binding motif. The up-regulation of these proteins benefits the cells exposed to salt that can adversely affect photosynthetic efficiency.

The electron microscopic analysis revealed that IdiA directly interacts with CP43 and D1 protein of the PSII system [38]. This interaction protects the cytosolically exposed acceptor side of PSII exposed after phycobilisome degradation [39]. Phycobilisome degradation is known to occur when cyanobacterial cells are exposed to salt stress [11].

In *Anabaena* sp. PCC 7120 TonB-dependent receptor is known to transport a particular siderophore. This schizokinen transporter, SchT is encoded by *alr0379* in this species [40]. It is homologous to the hydroxyl-carboxylate siderophore transporter IutA, identified as the ferric

aerobactin transporter in *E. coli* [31]. The uptake of scizokinen is greatly reduced in the *schT* mutant [40], two additional transporters of the IutA type are encoded in the genome of *Anabaena* sp. PCC 7120, they are characterized as *alr2209* and *alr2581* [31]. In the present study iron transporter encoded by *alr2209* was found to over expressed, suggesting its role in transporting siderophore in the mutant strain. In *Anabaena* sp. PCC 7120 *fhu*-cluster is related to periplasmic iron-siderophore uptake. The expression of the *fhu* genes is regulated by ExbB3/D3 and TonB3. These genes were upregulated under iron-limiting conditions [33].

The *fec*-gene clusters are known to express differentially under nitrogen-limiting conditions and deviant copper. On the other hand, *futB* showed altered expression under elevated copper concentration [33]. The overexpression of *fecC1*, *fecD1*, *fecB2* and *fecB1* are known to be expressed under deviant concentrations of iron and copper [29]. Their role in resistant phenotype is unique and at this stage, it is not possible to express their role in resistant phenotype. However, it could be speculated that in the examined cyanobacterium above mentioned gene clusters support copper uptake under iron-limiting conditions. Since the *futA2* mutant accumulated iron and copper in the periplasm but not in the copper containing plastocyanin leading to the suggestion that FutA2 is involved in copper homeostasis in addition to its role in iron transport [41].

Siderophore-mediated iron uptake cannot account for iron uptake in all cyanobacteria. Both non-ribosomal peptide synthetases and polyketide synthases genes were prevalent among filamentous and heterocystous strains of cyanobacteria [42]. In the cyanobacterium *Synechocystis*, PCC 6803 a reductive iron uptake strategy was documented [29]. Further, the iron uptake system *viz.* FutABC and FeoB were identified which encode for Fe(III) and Fe(II) transporter [43] (Katoh et al., 2001). In the siderophore-producing cyanobacterium *Anabaena flos-aquae* siderophore-independent uptake of inorganic iron was reported under iron limiting growth conditions [44].

Conclusion

Iron acquisition and uptake systems of *N. muscorum* were strongly induced in the mutant strain. Further, it is also reported that out of all iron uptake systems the transcripts of *fut*/*fec*/*fhu*/*sfu*/*idi* were increased manyfold in the mutant strain. Thus, it is concluded that under

such stressful situations iron uptake genes are induced to avoid oxidative stress as much as possible.

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Table 1

Showing NaCl tolerance characteristic of the wild type *N. muscorum* in terms of growth (OD change at 663nm), photosynthetic O_2 evolution (m mol O_2 evolved g^{-1} Chl *a*) and nitrogenase activity (m mol C_2H_4 formed g^{-1} Chl *a* h^{-1}).

Parameters	Control	+30mM NaCl	+60mM NaCl	+90mM NaCl
Growth	0.82±0.02	0.58±0.02	0.36±0.01	0.12±0.01
Specific growth rate	0.78±0.02	0.60±0.02	0.38±0.01	0.14±0.01
Photosynthetic O_2 evolution	540±22.1	372±18.6	320±20.8	108±6.1
Nitrogenase activity	12.28±1.5	8.2±1.3	7.3±1.1	1.2±0.07

Non-hererocystous NH_4^+-grown cultures (1mM) were stressed with NaCl at different concentration for 12h, later washed and used as inoculums for incubation on diazotrophic growth medium and then examined for their respective characteristic.

Each reading is an average (±SEM) of three independent experimental determinations.

Table 2

Showing NaCl tolerance characteristic of the NaCl-R mutant in terms of growth (OD change at 663nm), photosynthetic O_2 evolution (m mol O_2 evolved g^{-1} Chl *a*) and nitrogenase activity (m mol C_2H_4 formed g^{-1} Chl *a* h^{-1}).

Parameters	Control	+30mM NaCl	+60mM NaCl	+90mM NaCl
Growth	0.78±0.02	0.78±0.02	0.78±0.02	0.77±0.02
Specific growth rate	0.76±0.02	0.77±0.02	0.75±0.02	0.76±0.02
Photosynthetic O_2 evolution	527±18.5	527±18.5	526±17.3	526±17.6
Nitrogenase activity	11.50±2.2	11.5±1.3	11.48±1.1	11.48±1.2

Non-hererocystous NH_4^+-grown cultures (1mM) were stressed with NaCl at different concentration for 12h, later washed and used as inoculums for incubation on diazotrophic growth medium and then examined for their respective characteristic.

Each reading is an average (±SEM) of three independent experimental determinations.

Table 3

ORF related to the uptake of iron in the NaCl-R mutant of the cyanobacterium *N. muscorum*.

ORF	Gene	Annotation	Fold change
alr2176		iron(III) dicitrate-binding protein of ABC transporter	4.31
alr1382	*futA, idiA, sfuA*	ABC transporter ferric iron-binding periplasmic protein	4.00
alr2588		ferrichrome-iron receptor	3.67
all2674		ferrichrome-iron receptor	3.64
all1101		ferrichrome-iron receptor	3.54
all2586	*fecC1*	iron ABC transporter permease	3.42
alr1383	*futB, sfuB*	permease of iron(III) ABC transporter	3.42
all0388	*fhuD*	periplasmic iron-compound-binding protein of iron(III) ABC transporter	3.41
alr2209		ferric aerobactin receptor	3.07
all2585	*fecD1*	iron ABC transporter permease	3.03

alr2185		ferrichrome-iron receptor	3.02
alr3243	*fecB2*	ferrichrome ABC transporter substrate-binding protein	2.91
alr2213		iron(III) dicitrate ABC transporter substrate-binding protein	2.81
alr2590		iron(III) dicitrate-binding periplasmic protein	2.65
all2158		ferrichrome-iron receptor	2.61
alr2592		ferrichrome-iron receptor	2.55
all2148		ferrichrome-iron receptor	2.52
all1100		iron(III) dicitrate-binding periplasmic protein	2.45
alr2593		iron(III) dicitrate-binding periplasmic protein	2.45
alr2179		ferrichrome-iron receptor	2.36
all2609		iron(III) dicitrate-binding periplasmic protein	2.23
alr2583	*fecB1*	iron(III) dicitrate-binding periplasmic	2.20

	protein	
alr2596	ferrichrome-iron receptor	2.14
alr2210	iron(III) dicitrate ABC transporter substrate-binding protein	2.02