Quantitative Comparison of Total RNA of the Cyanobacterium *Nostoc Muscorum* and its Various Spontaneously Occurring Mutants

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Abstract

Experiments were conducted to examine the impact of RNA integrity number on various physiological parameters such as growth, percent survival, heterocyst frequency, time for heterocyst differentiation, nitrogenase activity, and photosynthetic O_2 evolution, in wild type *Nostoc muscorum* and its various spontaneously occurring LiCl, NaCl, and sucrose-resistant mutants. We observed that the aforementioned physiological parameters of these mutants did not alter drastically under stress conditions. These results suggested that RNA integrity number is a prerequisite for the up regulation and down regulation of specific genes with resistant phenotypes.

Keywords: Mutants; *Nostoc muscorum*; RNA; RNA integrity number

Introduction

Cyanobacteria are Gram negative, oxygen-evolving photosynthetic prokaryotes [1] with long evolutionary history, thus making them the most successful organism on this planet [2,3]. They are widely distributed in almost all ecological niches [4]. Cyanobacterial distribution in various natural habitats is mainly determined by the salinity of the surrounding medium [5]. In nature, water availability and amount of dissolved ions are the key factors determining cyanobacterial distribution. In both the situations cells have to maintain their internal environment at a physiological functional range [6]. Because of their ability to grow and survive in stressful natural habitats, high photosynthetic efficiency, variety of metabolic pathways, and genetic manipulability, cyanobacteria are a potential source of biofuels and high value products [7,8]. The biotechnological potential of cyanobacteria can be exploited only after understanding cyanobacterial physiology and metabolism at a molecular level. In the modern era, gene expression data are the most reliable information for understanding cyanobacterial physiology and metabolism. Authenticity of the gene expression data mostly depends on the quality of extracted RNA [9].

Genes can be expressed as mRNA, which is a measure of the cellular activities occurring during various metabolic activities. The assessment of RNA integrity is crucial for establishing the validity and reproducibility of gene expression. RNA data is a fundamental element for successful microarray or RT-PCR analyses. Genetic information encoded in the genome is transferred to RNA molecules for producing different types of proteins, suggesting that RNA molecules provide direct information of cellular activities [10-13].

In this study, the spontaneously occurring LiCl, NaCl, and sucrose-resistant mutants of cyanobacterium *Nostoc muscorum* were analyzed in terms of their RNA integrity number (RIN).

Material and Methods

Organism and growth conditions

N. muscorum used in the present study is a filamentous, diazotrophic and heterocyst-forming species. *N. muscorum* and its spontaneously occurring mutants were routinely grown and maintained in the Chu. No 10 medium [14] without any combined nitrogen source. Wild type *N. muscorum* and its spontaneously occurring LiCl-, NaCl-, and sucrose-resistant mutants were grown and maintained as described previously [15-17].

Isolation of LiCl, NaCl and sucrose-resistant mutants

Thick cyanobacterial cultures were streaked on the nutrient plates containing lethal dose of LiCl. After 3 to 4 weeks

incubation, few pinhead colonies appeared on the nutrient plates. These colonies were transferred onto a fresh diazotrophic growth medium and allowed to grow for 3-4 generations and subsequently their mutant stability was evaluated. Mutants resistant to NaCl (100mM) and sucrose (250mM) were generated by the Li+-R mutants.

Growth, percent survival, heterocyst frequency, and time for heterocyst differentiation were measured as described previously [15]. Nitrogenase activity was determined by gas chromatography. Enzyme activity was measured using acetylene reduction technique as described previously [18]. Photosynthetic O_2 evolution of the cyanobacterial samples was measured by using an O_2 electrode (Hansatech Instrument Ltd. UK) and further analyzed using the Oxygraph Plus software (version 1.0).

RNA extraction

Wild type *N. muscorum* and its spontaneously occurring mutants were cultivated and the pellets were used for RNA extraction. Total RNA was isolated according to the genotypic protocol using a Qiagen RNeasy minikit with DNase treatment. mRNA quality assessed with Bioanalyzer 2100 (Agilent, CA, USA). RNA concentration and purity can be analyzed spectrophotometrically or by electrophoretically verifying ribosomal integrity. In spectrophotometric analysis, 230, 260, and 280 nm are the commonly used absorbance value. The ration of absorbance at 260/280 and 260/230 nm are used to evaluate the purity of the RNA and nucleic acid sample respectively.

The Agilent 2100 Expert Software automatically provides RIN value for assessing total RNA quality. RIN provides a quantitative value for RNA integrity that facilitates the standardization of quality interpretation. Analysis of single stranded RNA provides information on its size distribution and concentration and allows the relative quantification of fragments within a size range. The resulting electropherogram should have at least 2 distinct peaks representing the 16s and 23s ribosomal RNA. Additional bands represent the lower marker and potentially, the 5s RNA. Presence or absence of 5s RNA depends on the purification method, 5s RNA generally abundantly present in column-purified total RNA.

Results and Discussion

RNA integrity is a prerequisite for obtaining gene expression data. In this study, we compared the different spontaneously occurring mutants in terms of their RNA integrity. In the first series of experiments, RNA samples were electrophoretically (Bioanalyzer 2100, Agilent) separated on a micro fabricated chip and subsequently analyzed by laser-induced fluorescence detection. UV/VIS spectrophotometer was used to assess the quality and quantity of the RNA at multiple wavelengths (260/280 and 260/230nm). Bioanalyzer profiles of RNA are given in (Table 1). The absorbance were considered to be an as an acceptable indicator of high RNA quality [19,20].

Table 1: RNA concentration and purity of RNA samples of the cyanobacterium *N. muscorum* and its various mutant clones estimated using Nanodrop Spectrophotometer.

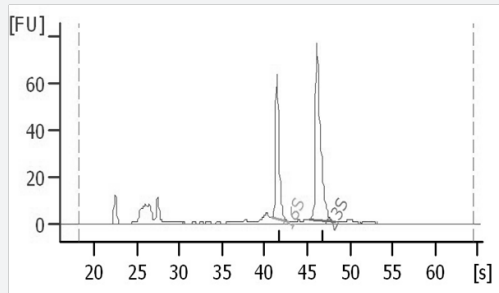| Sample Name	Absorbance Value 260/280	Absorbance Value 260/230	RNA Con. ng/µl	Total Yield in ng	QC Purity	QC Concentration/ Yield	QC Integrity
Wild	2.1	1.03	355.11	8877.75	Optimal	Optimal	Good
Li+-R	2.13	2.64	1879.95	46998.75	Optimal	Optimal	Optimal
Na+-R	2.13	2.64	1863.69	46592.25	Optimal	Optimal	Optimal
Su-R	2.16	0.99	754.95	18873.75	Optimal	Optimal	Optimal

Figure 1(A): Electropherogram of BA Reference.

Figure 1(B): Electropherogram of Ladder.

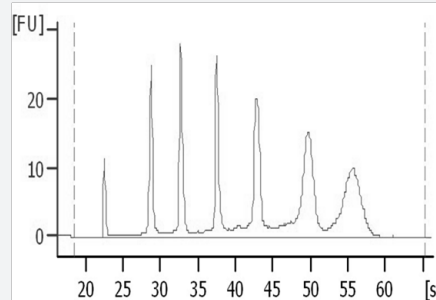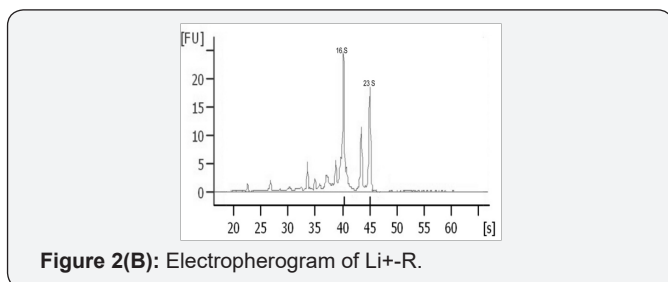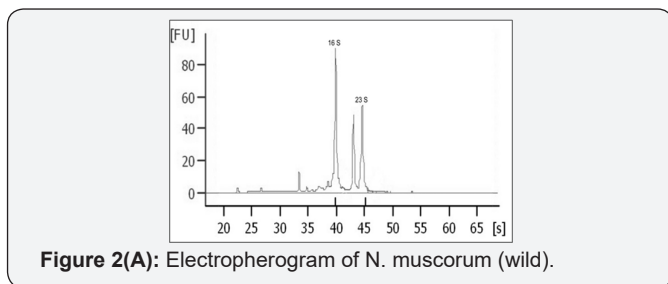**Figure 2(A):** Electropherogram of N. muscorum (wild).

Figure 2(B): Electropherogram of Li+-R.

degradation of the RNA sample leads to a shift in the RNA size distribution toward smaller fragments and a decrease in fluorescence signal.
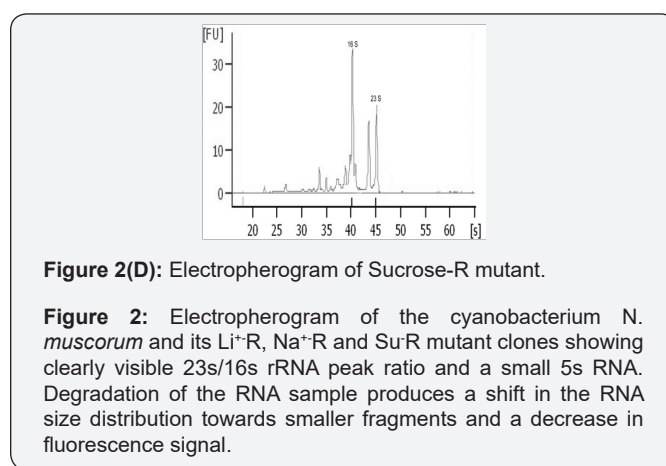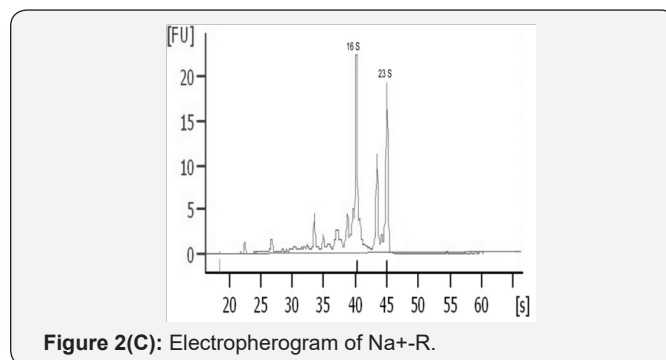

Figure 2(C): Electropherogram of Na+-R.

Figure 2(D): Electropherogram of Sucrose-R mutant.

Figure 2: Electropherogram of the cyanobacterium N. *muscorum* and its Li+-R, Na+-R and Su-R mutant clones showing clearly visible 23s/16s rRNA peak ratio and a small 5s RNA. Degradation of the RNA sample produces a shift in the RNA size distribution towards smaller fragments and a decrease in fluorescence signal.

Another parameter for the standardization of RNA quality assessment is the ratio of the 16s and 23s ribosomes. During electrophoresis an RNA ladder was used as a mass and size standard for estimating the RNA band size. Furthermore, the electropherogram of BA reference compared the chromatograms of different samples (Figure 1). The results indicated that compared with wild type *N. muscorum*, the rRNA ratio of the mutants decreased (Table 2), suggesting that mutational acquisition is an indicator of rRNA ratio degradation. Similar results on the RNA status were also reported in human brain tissues [21]. As shown in the electropherogram (Figure 2)

Table 2: Table showing RIN visualization in terms of RNA area, concentration, rRNA ratio [23s/16s] and RIN value of the wild type *N. muscorum* and its various mutants clones (the Agilent 2100 bioanalyzer expert software).

RIN visualization	BA Reference	Ladder	Wild Type	Li+-R	Na+-R	Sucrose-R
RNA Area	329.8	314.8	274.1	106.3	98.5	126.3
RNA Concentration	157 ng/μl	150 ng/μl	2057 ng/μl	330 ng/μl	305 ng/μl	392 ng/μl
rRNA Ratio [23s/16s]	1.7		0.6	0.5	0.7	0.5
RIN	9.6		8.6	7.4	7.2	7.5

In the next series of experiments, we compared the RIN values of the mutants with wild type *N. muscorum*. Data as shown in table 2 clearly indicated that RIN values of the mutants were lesser than those of wild type *N. muscorum*. However, the RIN values of these mutants were sufficient for microarray analysis [22,23] thus, suggesting that RIN is a powerful tool for RNA integrity assessment.

Wild type *N. muscorum* and its spontaneously occurring mutants were compared in terms of various physiological parameters, such as growth, percent survival, heterocyst frequency, time for heterocyst differentiation, nitrogenase activity and photosynthetic oxygen evolution under stress conditions (15mM LiCl, 100mM NaCl, and 250mM sucrose). Results suggested that the spontaneously occurring mutants were highly tolerant to the aformention stresses (Table 3) because their physiological parameters were not drastically altered. However, the physiological parameters of wild type *N. muscorum* were severely impaired under these stresses (Table 4).

Table 3: Table showing tolerance characteristic of Li$^+$-R, Na$^+$-R and sucrose resistant mutants in the presence of lethal dose of LiCl, NaCl and sucrose in terms of growth (OD change at 663nm), percent survival, heterocyst frequency (HF%), time for heterocyst differentiation in h (tHet), nitrogenase activity (m mol C_2H_2 formed g^{-1} Chl a h^{-1}), photosynthetic O_2 evolution (m mol O_2 evolved g^{-1} Chl a).Non-hetercystous NH4$^+$-grown cultures were stressed with respective stress for 12 h, later washed and used as inoculums for incubation on fresh diazotrophic growth medium and then examined for their respective characteristic. Each reading is an average (±SEM) of three independent experimental determinations.

Parameter	Wild Type	Li+-R	Na+-R	Sucrose
Growth	0.82±0.072	0.79±0.069	0.78±0.065	0.79±0.067
Percent Survival	100	100	100	100
HF%	7-8	7-8	7-8	7-8
tHet	24-30	25-31	24-30	24-31
Nitrogenase activity	12.28±1.16	12.11±1.09	12.07±1.04	12.14±1.11
Photosynthetic O2 evolution	540±38.4	538±32.6	536±36.3	540±40.1

Table 4: Table showing LiCl, NaCl and sucrose tolerance characteristic of the wild type *N. muscorum* in terms of growth (OD change at 663nm), percent survival, heterocyst frequency (HF%), time for heterocyst differentiation in h (tHet), nitrogenase activity (C_2H_2 formed g^{-1} Chl a h^{-1}), photosynthetic O_2 evolution (m mol O_2 evolved g^{-1} Chl a). Non-hetercystous NH4$^+$-grown cultures were stressed with respective stress for 12h, later washed and used as inoculums for incubation on fresh diazotrophic growth medium and then examined for their respective characteristic. Each reading is an average (±SEM) of three independent experimental determinations.

Parameter	Control	+LiCl	+NaCl	+Sucrose
Growth	0.82±0.072	0.0	0.0	0.0
Percent Survival	100	0.0	0.0	0.0
HF%	7-8	0-0	0-0	0-0
tHet	24-30	α	α	α
Nitrogenase activity	12.28±1.16	0.0	0.0	0.0
Photosynthetic O2 evolution	540±38.4	49±3.3	36±2.9	63±5.6

The results identified, various metabolic pathways as possible targets of resistance phenotypes (Table 4) suggesting that the RIN is a crucial parameter for assessing up regulation and down regulation of specific genes. This approach can be used to identify various metabolic changes that occur in resistant phenotypes, thus leading to the identification of metabolic pathways as possible targets, for generating cyanobacterial strains that can be used as biofertilizers in usar lands. Furthermore, this approach can also be used to improve the phenotypes of mutants for biotechnological applications.

Conclusion

In conclusion, use of the 23S to 16S rRNA ratio and RIN value would be a good measure to assess up regulation and down regulation of specific genes to identify specific physiological pathways alter under the growth inhibitory concentration of various structural analogs.

Acknowledgement

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