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Uncovering the Unusual Long Chains of Vegetative Cells within Single Colonies of the Dryland Nitrogen-Fixing Cyanobacterium *Nostoc flagelliforme*

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Abstract: Heterocyst-forming cyanobacteria that colonize the drylands contribute to carbon and nitrogen supplies in nutrient-poor soils. As one of the representative cyanobacteria, *Nostoc flagelliforme* adapts well to the arid environment in the form of filamentous colonies (or filaments). To date, the adaptive changes, either genetic or micromorphological, that occur within single colonies of dryland cyanobacteria remain largely unclear. In this study, unusual long chains or trichomes of vegetative cells (not containing heterocysts) were observed within *N. flagelliforme* filaments. And the overall heterocyst frequency in the trichomes was counted to be 1.3–2.7%, different from the usually observed 5–10% heterocyst frequency in model *Nostoc* strains when grown in nitrogen-deprived medium. Thus, these phenomena seem contradictory to our usual recognition of *Nostoc* strains. Related transcriptional and heterocyst frequency analyses suggested no genetically significant alteration in heterocyst formation and nitrogen fixation in this strain. Also, the amounts of nitrogen sources in the extracellular polysaccharide (EPS) matrix released by *N. flagelliforme* cells that may cause the low heterocyst frequency were assessed to be equivalent to 0.28–1.10 mM $NaNO_3$. When combining these findings with the habitat characters, it can be envisaged that the released nitrogen sources from cells are confined, accumulated, and re-utilized in the EPS matrix, thereby leading to the formation of reduced heterocyst frequency and long-chained vegetative cells. This study will contribute to our understanding of the distinctive adaptation properties of colonial cyanobacteria in dryland areas.

Keywords: *Nostoc* colonies; environmental adaptation; heterocyst frequency; nitrogen source

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1. Introduction

Drylands account for approximately 40% of the terrestrial area and are typically characterized by organic matter deficiency and water scarcity. Cyanobacteria are pioneer organisms that colonize dryland soils and contribute to carbon and nitrogen supplies, as well as water redistribution and soil rehabilitation [1–4]. The predominant forms of cyanobacteria surviving the drylands are cyanobacterial biocrusts and free-living colonies. The representative species of the latter are *Nostoc flagelliforme* (filamentous colony) and *Nostoc commune* (lamellate colony) [5,6]. Two species are genetically close relatives. However, *N. flagelliforme* solely grows in arid regions (annual precipitation of <400 mm) and is arising as a terrestrial model species for scientific research [7,8]. The free-living *Nostoc* colony consists of numerous trichomes (chains of cells) encased by the common extracellular polysaccharide (EPS) matrix. Apart from facilitating the establishment of defined colony shapes, the EPS matrix and its accommodated compounds provide crucial protection for *Nostoc* cells against environmental (abiotic) stresses [9–11]. Thus, the EPS matrix plays a critical role in the adaptation and evolution of colonial *Nostoc* in the dryland environment.

In single *Nostoc* colonies, the EPS matrix usually accommodates ultraviolet-absorbing molecules (scytonemin and mycosporine-like amino acids) and exoproteins [11–14].

Other small molecules typically secreted by cyanobacteria, such as amino acids, phytohormones, and vitamins, are also supposed to be first confined in the EPS matrix. Unusual water uptake and loss (the kinetics are dependent on multiple factors) and hydrolysis of exoproteins also take place in this matrix [9,11,15]. In addition, we have recently uncovered a high level of genetic diversity within single colonies (or filaments) of *N. flagelliforme* based on single nucleotide polymorphism analysis [12]. Despite the above progress, distinctive adaptive features evolved in the EPS matrix of terrestrial colonial cyanobacteria have not been fully revealed.

Many multicellular cyanobacteria produce terminally differentiated nitrogen-fixing cells called heterocysts. Heterocysts are placed at regular or semi-regular intervals in the trichomes, and heterocyst frequency is tightly associated with the availability of environmental combined nitrogen [16,17]. In two model cyanobacteria, *Nostoc* sp. PCC 7120 and *Nostoc punctiforme* PCC 73102, heterocysts are regularly separated by 10–20 vegetative cells (5–10% heterocyst frequency) in the absence of combined nitrogen [18,19]. In symbiosis with a photosynthetic eukaryotic partner, the heterocyst frequency of *N. punctiforme* can be increased to 25–60% [18]. Heterocyst frequency can serve as an excellent marker for assessing ambient nitrogen availability and associated impacts on *Nostoc* strains.

In native habitats, *N. flagelliforme* undergoes periodic rehydration and dehydration, interrupted by either long- (monthly level) or short-term (daily level) dormancy. Most of the time, it is dew or moisture from the air (more than 50% relative humidity) that sustains its slow growth [20]. The soil is extremely nitrogen-deficient, containing less than 0.04% total nitrogen. Under these environmental conditions, this pioneer organism should possess sufficient nitrogen-fixing capacity by maintaining a high heterocyst frequency to sustain growth or fertilize the soil. However, because of the arid environmental character, organic nitrogen sources released from *N. flagelliforme* cells after nitrogen fixation may be confined and accumulated in the EPS matrix for a period of time (unless encountering intermittent rain), which might instead lead to a low heterocyst frequency in the trichomes of *N. flagelliforme*. In this study, we aimed to investigate this possibility to gain more insight into ecologically or genetically distinctive alterations in dryland colonial cyanobacteria.

2. Materials and Methods

2.1. Microscopic Observation

N. flagelliforme samples (filaments) were sourced from different regions in arid steppes of the west and west-northern parts of China [12]. The regions include Guyuan City (GY), Jiuquan City (JQ), Zhongwei City (ZW), Siziwangqi County (SZWQ), Huhehaote City (HHHT), Yinchuan City (YC), and Guide County (GD) [12]. The filaments were washed 3–5 times with sterilized water and kept rewetted in darkness for 2–3 days to allow the softening of the EPS matrix. Then, the filaments were slightly pressed on temporary microscope slides and subjected to observation under a light microscope (Axio Scope A1, Zeiss Inc., Oberkochen, Germany) with or without excitation of the fluorescence (450–490 nm). With the fluorescence excitation, heterocysts show no red color due to the lack of chlorophyll *a* [19]. The reliability of distinguishing vegetative cells and heterocysts under the microscope was also verified in this study (Figure S1). Heterocyst frequency was measured as the vegetative cell number between adjacent heterocysts (heterocyst interval) or expressed as the ratio of heterocysts to vegetative cells in the trichomes except hormogonia.

2.2. Transcriptional Analysis

N. flagelliforme samples were mixed and washed with sterilized water 5 times and then rehydrated on three conditions ($BG11_0$ solution, $BG11_0$ + 0.55 mM $NaNO_3$, $BG11_0$ + 0.55 mM NH_4Cl). $BG11_0$ is a widely used nitrogen-free algal medium [21]. Usually, the nitrogen fixation of *N. flagelliforme* could be detected at 1 h after rehydration and reached a maximum at 8 h [20]. After rehydration for 1 h on three conditions, the values of the photosystem II activity parameter Fv/Fm of three samples recovered from 0 to 0.13,

0.17, and 0.16, respectively, and at 4 h, they recovered to 0.24, 0.29, and 0.29, respectively. The 4 h rehydrated samples were subjected to RNA extraction and quantitative polymerase chain reaction (qPCR) analysis. The representative heterocyst development and nitrogen fixation-associated genes as well as their primers for qPCR analysis, are shown in Supplemental Table S1. Three independent replicates were conducted. Two reference genes, the 16S rRNA gene and *rnpB*, were used for normalization. The transcriptional levels of these genes in two nitrogen source-treated samples ($BG11_0$ + 0.55 mM $NaNO_3$; $BG11_0$ + 0.55 mM NH_4Cl) were compared against those in the nitrogen-free sample ($BG11_0$ solution), and their relative ratios were calculated (%).

2.3. Testing of Heterocyst Frequency upon Nitrogen Availability

The relationship between the combined nitrogen level and the frequency of heterocyst formation was tested using a cell suspension culture of *N. flagelliforme* developed from natural sample [22]. The cell suspension was inoculated into $BG11_0$ solution supplemented with various concentrations of $NaNO_3$. The final $NaNO_3$ concentrations were respectively 0.07, 0.14, 0.28, 0.55, 0.73, and 1.10 mM, which correspond to 1/256, 1/128, 1/64, 1/32, 1/24, and 1/16 of the $NaNO_3$ level (1/256 N–1/16 N) in BG11 solution [21]. The cultures were statically cultivated at 25 °C under continuous illumination of 20 µmol photons m^{-2} s^{-1} for two weeks. The model cyanobacterium *Nostoc* sp. PCC 7120, which was cultivated in $BG11_0$ solution at 30 °C, was used as a control. For a long-term experiment, cell suspension of *N. flagelliforme* was cultivated for 30 days in $BG11_0$ solution. These cultures were gently shaken two times per day. After cell collection, heterocyst frequencies were counted.

3. Results and Discussion

Native habitat and *N. flagelliforme* filaments are shown in Figure 1A. A single filament can be recognized as an EPS-encased colony of *Nostoc* cells. The trichomes within single colonies of *N. flagelliforme* were observed under a light microscope (Figure 2A). Vegetative cells appeared red under the excitation of fluorescence. Mature heterocysts were obviously larger than vegetative cells and showed no red color under the fluorescence (Figure S1). As shown in Figure 2A, a few long trichomes of vegetative cells (not containing heterocysts) were observed within a colony, and one had more than 100 vegetative cells. Further, heterocyst frequencies of trichomes in various *N. flagelliforme* filaments sourced from different regions were counted (Figure 2B). A large variation in average heterocyst interval was observed among these filaments, ranging from 37.1 to 79.2 vegetative cells, corresponding to 1.3–2.7% heterocyst frequency. Compared to *Nostoc* sp. PCC 7120 and *N. punctiforme* PCC 73102 (5–10% heterocyst frequency), this heterocyst frequency is much lower upon nitrogen-deficient conditions. These results demonstrate the existence of long-chained vegetative cells and reduced heterocyst frequency in *N. flagelliforme* colonies.

Figure 1. A native habit (**A**) and filamentous colonies (**B**) of *N. flagelliforme*.

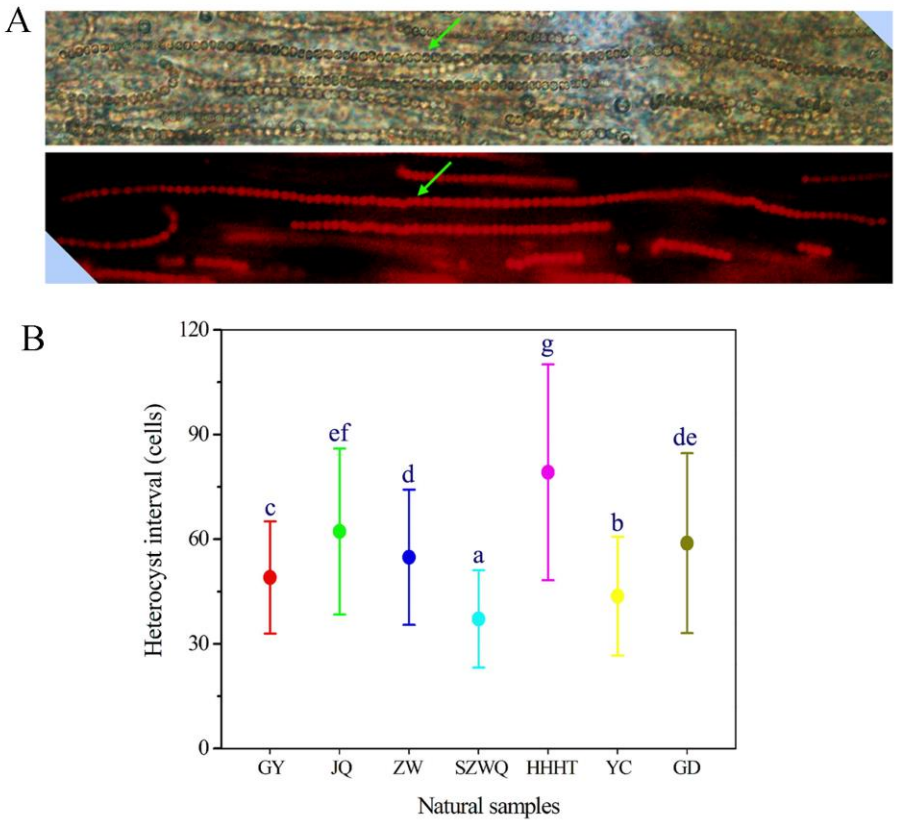

Figure 2. Microscopic observation of the trichomes within a single colony of *N. flagelliforme* (**A**) and the measurement of heterocyst intervals in various natural colonies sourced from different regions (**B**). The green arrow points to a very long trichome consisting of only vegetative cells. Observation was conducted under white light (upper panel in (**A**)) or under fluorescence (lower panel in (**A**)). Vegetative cell size, 4−6 μm. Data shown in (**B**) are the mean ± SD (n = 116–149). Letters a–g represent the significant difference at $p < 0.05$ (Tukey's multiple comparison test).

Further, the potential molecular or genetic alteration in heterocyst formation and nitrogen fixation in *N. flagelliforme* samples was assessed via transcriptional analysis (Figure 3). Important marker genes associated with heterocyst development (*hetR*, *patA*, and *hepA*) and nitrogen fixation (*nifH*) were used (Table S1). Generally, the transcription of these genes will be repressed in heterocyst-forming cyanobacteria when they are exposed to combined nitrogen-replete conditions [16]. Water-washed *N. flagelliforme* filaments were treated with nitrate and ammonium solutions for 4 h. As shown in Figure 3, both treatments led to a reduced transcription of these genes compared to the control (100%). This result indicates a genetically normal response of *N. flagelliforme* in response to external nitrogen addition.

The frequency of heterocyst formation in relation to ambient nitrogen availability was further assessed using a cell suspension culture of *N. flagelliforme* (Figure 4). In the $BG11_0$ solution, *N. flagelliforme* trichomes had a heterocyst interval of approximately 15 vegetative cells at the rapid growth phase, similar to that in *Nostoc* sp. PCC 7120. With the addition of 1/256–1/64 N levels in $BG11_0$ solution, the heterocyst intervals were enlarged to be approximately 30 vegetative cells. With the addition of 1/32–1/24 N levels, the heterocyst intervals were enlarged to be more than 60 vegetative cells. At the 1/16 N level, most of the trichomes had very few or occasional heterocysts, and long trichomes consisting of more than 100 vegetative cells occurred. Thus, like *Nostoc* sp. PCC 7120, heterocyst frequency in *N. flagelliforme* was still correlated with nitrogen availability, which is also consistent with the transcriptional analysis result (Figure 3). However, it is worth noting that in long-term cultivation, *N. flagelliforme* trichomes had a heterocyst interval of approximately 30 vegetative cells (3.3% heterocyst frequency) (Figure 4). Relative to 5–10%

heterocyst frequency in model *Nostoc* species, this reduced heterocyst frequency might imply a genetically subtle alteration in nitrogen fixation and homeostasis.

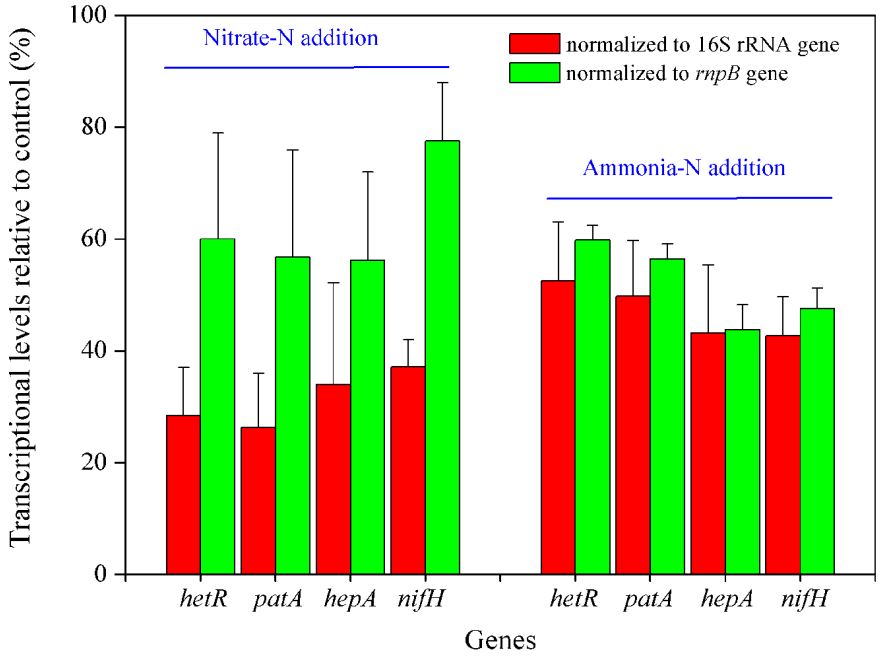

Figure 3. Relative transcriptional levels of heterocyst development and nitrogen fixation-associated marker genes in natural colonies of *N. flagelliforme* upon nitrate and ammonium addition. The transcriptional levels on the nitrogen-free condition were set as 100%. Data shown are the mean ± SD (n = 3).

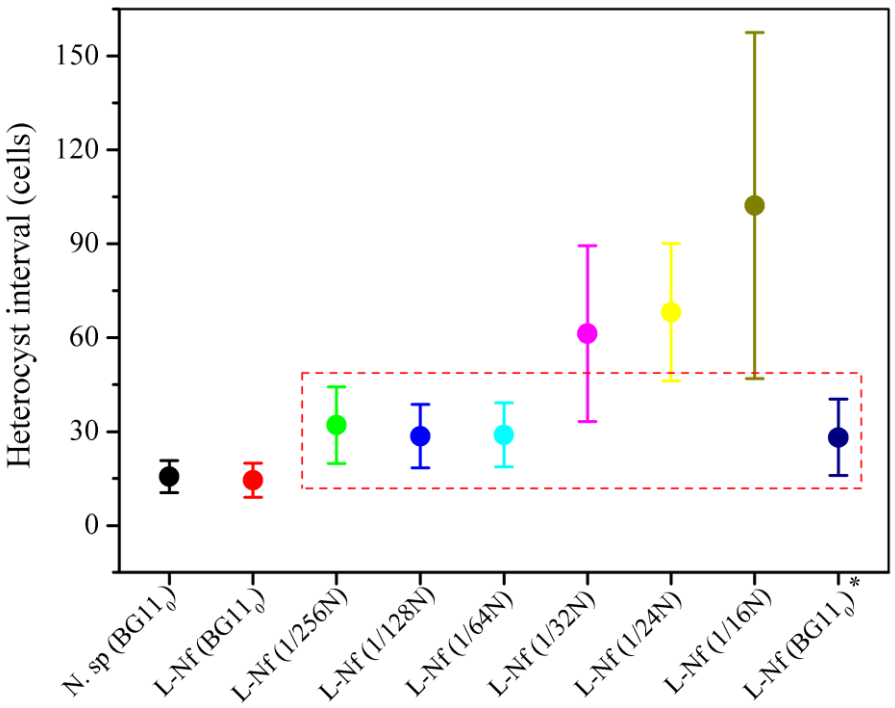

Figure 4. The frequencies of heterocyst formation in the trichomes of liquid suspension culture of *N. flagelliforme* under different nitrate levels. * one-month cultivation. *N.* sp, *Nostoc* sp. PCC 7120, as a control. L-Nf, liquid suspension culture of *N. flagelliforme*. For 1/16–1/32 N cultures, the data shown are the mean ± SD (n = 28–30); for others, the data shown are the mean ± SD (n = 46–71). The rectangle highlights similar heterocyst intervals among the four tests.

Nitrogen-fixing cyanobacteria can release ammonia, amino acids, or proteins into the extracellular environment and re-utilize them [11,23–26]. Cyanobacterial extracellular enzymes with a capacity for EPS degradation have also been identified, and extracellular organic carbon can be re-utilized [27]. Organic matter excretion is crucial to carbon and nitrogen cycling in cyanobacterial communities. In native habitats, desert cyanobacteria utilize early-morning dew and rely on dawn illumination or weak red light for growth [28,29]. After that, they rapidly enter a state of dormancy along with rising temperature and declining relative humidity. Thus, the time for photosynthesis and nitrogen fixation is very limited, which may transiently result in relatively excess nitrogen. The dynamics of the main exoprotein, WspA, in natural *N. flagelliforme* were analyzed [11]. The dehydration process induces its biosynthesis, and the rehydration process leads to its secretion and then hydrolysis. Since the EPS matrix is not an aqueous environment and has an acid-base buffering capability [30], it is difficult to precisely correlate the amount of EPS matrix-containing nitrogen sources with the heterocyst frequency of trichomes. Nevertheless, as implied in Figures 2B and 4, the nitrogen levels in natural filaments (outside of cells) that may cause the low heterocyst frequency were equivalent to 0.28–1.10 mM $NaNO_3$ (1/64–1/16 N levels). A high EPS matrix-containing nitrogen level (e.g., equivalent to 1/16 N level) will surely lead to the occurrence of long trichomes consisting of only vegetative cells. Of course, considering potentially uneven moisture absorption in the arid environment, an uneven allocation of released nitrogen sources in the EPS matrix may also possibly lead to the occurrence of long-chained vegetative cells.

Environmental abiotic factors such as temperature, light intensity, and mineral nutrients can also exert an influence on heterocyst formation or affect its nitrogenase activity [31–33]. Such impacts were also implied in the fluctuating carbon/nitrogen ratios of *N. flagelliforme* cultures under simulated conditions (Figure S2). Studies from biological soil crusts have shown that the nitrogenase activity varied widely in response to changing environmental conditions [34,35]. Thus, it cannot simply attribute the nitrogenase activity or the amount of fixed nitrogen to the number or frequency of heterocyst. Despite all this, viewed from the perspective of its origin, the dominant factor for determining proper heterocyst frequency in nitrogen-fixing heterocystous cyanobacteria should still be nitrogen availability or nitrogen demand. Notably, atmospheric nitrogen deposition is rising as a new threat to biodiversity and ecosystem function in drylands [36]. The potential impact or impact extent from this nitrogen input in *N. flagelliforme* filaments needs to be assessed in the future.

4. Conclusions

N. flagelliforme is a representative member of dryland soil surface-dwelling cyanobacteria. Beyond expectation, unusually long trichomes of vegetative cells as well as overall low heterocyst frequency, were observed in its natural colonies. The transcriptional response of heterocyst development and nitrogen fixation-associated genes upon nitrogen addition was in line with expectations. The frequency of heterocyst formation was tested to be proportional to the concentration of external combined nitrogen. Therefore, it is not a genetically significant alteration in heterocyst formation and nitrogen fixation that results in these unusual phenomena. It should be attributed to the EPS matrix of *N. flagelliforme* that hold-ups released nitrogen sources in the specific terrestrial environment. The re-utilization of extracellular combined nitrogen will inhibit heterocyst formation and thus lead to the occurrence of long-chained vegetative cells. The nitrogen levels in the EPS matrix were estimated to be equivalent to 0.28–1.10 mM $NaNO_3$. Particularly, a downward tendency in heterocyst frequency was traced in this species, implying a genetically subtle alteration. Thus, the establishment of the defined colony form strengthens cyanobacterial adaptation to arid environments, and in turn, it facilitates the genetic alteration of cells in a subtle way. In the next step, the relationship between heterocyst frequency and nitrogenase activity in the native environment, the nitrogen cycling and homeostasis in single cyanobacterial colonies, and even the impact of excessive nitrogen deposition need to be explored. We

hope this communication will expand our understanding of the special environmental adaptability of colonial cyanobacteria in dryland areas.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nitrogen5010009/s1, Figure S1: The discrimination of proheterocyst, mature heterocyst, and vegetative cell in a trichome; Table S1: The genes and their primers used for transcriptional analysis; Figure S2: The C/N ratios in natural colonies and liquid suspension cultures of *N. flagelliforme*.

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Data Availability Statement: The data presented in this study are available in the main text and Supplementary Files.

Conflicts of Interest: The authors declare no conflict of interest.

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