Isolation and Characterization of a Drought-Tolerant Cyanobacterium, \textit{Nostoc} sp. HK-01

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A cyanobacterial strain was isolated from cyanobacterial crusts taken from soil in Hyogo Prefecture, Japan and identified as a \textit{Nostoc} species. This \textit{Nostoc} strain is drought-tolerant, and its cells differentiated into a particular type, hormogonia, under dark and nutrient-poor conditions. The strain grows well in a liquid medium with a simple nutrient composition that was originally developed for the cultivation of symbiotic \textit{Nostoc}. The maximum growth rate was attained at pH 8. The DNA sequences of the 16S rRNA region and \textit{trnL}(UAA)-intron region were determined to classify this \textit{Nostoc} species. The \textit{trnL}(UAA)-intron sequence was homologous to a symbiotic \textit{Nostoc}, and also to some extent to \textit{Anabaena} sp. PCC 7120. This cyanobacterium, named \textit{Nostoc} sp. HK-01, is promising as an improver of arid and nutrient-poor soils.

Key words: cyanobacterium, drought-tolerance, hormogonia, \textit{Nostoc}

Cyanobacteria of the genus \textit{Nostoc} are found on every continent on earth in a wide range of terrestrial and aquatic ecosystems. Although numerous strains of \textit{Nostoc} have been identified, only a few \textit{Nostoc} species have been characterized in detail\textsuperscript{3,4}. \textit{Nostoc commune} is a well-known, terrestrial and highly drought-tolerant cyanobacterium\textsuperscript{5,6,7}, and desiccated \textit{N. commune} has been shown to survive for almost 100 years\textsuperscript{8}. Desiccated \textit{N. commune} exhibits a rapid recovery of photosynthetic activity, attaining almost half of the maximum level within 1 h of rewetting\textsuperscript{9}. The \textit{Nostoc} species are thought to be very useful for agricultural applications, their N\textsubscript{2}-fixing activity in particular contributing greatly to improving the quality of nutrient-poor soils. However, isolation and cultivation techniques for terrestrial cyanobacteria are poorly established.

Sequence analysis of genes encoding the small-subunit of ribosomal RNA (16S rDNA) is currently the most promising approach to the phylogenetic classification of cyanobacteria\textsuperscript{20}. The sequence of the \textit{trnL}(UAA) intron (group I intron) is also well used for identifying cyanobacterial taxonomic levels\textsuperscript{11,20,21}. Combining these two methods of sequence analysis, cyanobacteria are classified with high confidence.

In this study, we succeeded in isolating a drought-tolerant \textit{Nostoc} sp. and cultivated it axenically. The phylogenetic position of the isolated \textit{Nostoc} was determined from the sequences of the 16S rDNA and \textit{trnL}(UAA) intron.

Materials and Methods

Cultivation media

For isolation, an agar containing a 20-fold dilution of DTN medium which was developed by Castenholz and modified later by Mühlenhoff and Chauvat was used\textsuperscript{2,9}. The DTN medium contains in one liter; 0.19 g Na\textsubscript{2}EDTA, 52.5 mg MgSO\textsubscript{4}, 0.1 g KNO\textsubscript{3}, 0.7 g NaNO\textsubscript{3}, 0.122 g NaH\textsubscript{2}PO\textsubscript{4}·2H\textsubscript{2}O, 42 mg CaCl\textsubscript{2}, 1 mg FeCl\textsubscript{3}·6H\textsubscript{2}O, 5 mg NH\textsubscript{4}Cl, 0.1 g Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}·5H\textsubscript{2}O, 83 mg NaHSO\textsubscript{3}, 0.42 g NaHCO\textsubscript{3}, and 0.5 ml trace elements (0.5 ml H\textsubscript{2}SO\textsubscript{4}, 0.5 g H\textsubscript{3}BO\textsubscript{4}, 2.28 g MnSO\textsubscript{4}·5H\textsubscript{2}O, 0.5 g ZnSO\textsubscript{4}·7H\textsubscript{2}O, 25 mg CuSO\textsubscript{4}·5H\textsubscript{2}O, 25 mg Na\textsubscript{2}MoO\textsubscript{4}·2H\textsubscript{2}O, 45 mg CoCl\textsubscript{2}·6H\textsubscript{2}O, 19 mg NiSO\textsubscript{4}(NH\textsubscript{4})SO\textsubscript{4}·6H\textsubscript{2}O and 4 mg NaSeO\textsubscript{4} in one liter\textsuperscript{2} and 8 mM Tricine-NaOH (pH 8.0). A medium developed by Watanabe and Kiyohara\textsuperscript{19} was used for the liquid culture. The medium contains in one liter; 0.125 g K\textsubscript{2}HPO\textsubscript{4},
Isolation of Drought-Tolerant Nostoc sp.

Crusts of cyanobacteria were collected at the Harima Science Garden City Campus of Himeji Institute of Technology, Hyogo Prefecture, Japan. Isolation of Drought-Tolerant Nostoc sp. HK-01 was successful. Hormogonia differentiation encompassed the Nostoc, they were washed with sterile water and then cultured using kanamycin (5 μg ml⁻¹) to reduce bacterial contaminants. Finally, the pure Nostoc sp. named Nostoc sp. strain HK-01 was successfully isolated. Strain HK-01 was kept under a 16 h light (20 to 50 μE m⁻² s⁻¹)-8 h dark cycle at 22°C.

A test for purity of the culture was carried out as described by Rippka(13). An aliquot of cultured cells was grown in DTN medium supplemented with 0.5% (w/v) glucose at 37°C for 3 days in room light, and then at 30°C for 5 days in the dark. An aliquot of this culture was propagated on an M9 plate containing 0.5% (w/v) glucose and 0.05% (w/v) casamino acid at 30°C for 2 weeks in the dark, and then contamination by other bacteria was checked with a microscope.

Drought-tolerant test

Nostoc sp. HK-01 was grown on the plate containing 1/20 DTN under light (40 μE m⁻² s⁻¹) at 30°C for 2 weeks. Then it was dried for a week under room lights at 25°C and 30% relative humidity, and stored for 3 months under room lights at 25°C. The dried strain was soaked with sterile water for 10 min, streaked on a 1/20 DTN plate and incubated under light (40 μE m⁻² s⁻¹) at 30°C.

Hormogonia differentiation

Nostoc sp. HK-01 was streaked on DTN and 1/20 DTN plates and cultivated under continuous light (40 to 50 μE m⁻² s⁻¹) at 30°C for 4 days. These plates were then placed in the dark for 2 days, and hormogonia were found during the dark period. They were cultivated under continuous light again.

Growth of Nostoc sp. HK-01 in liquid medium

To find an adequate medium for optimum growth, Nostoc sp. HK-01 was grown in DTN medium, 4-fold and 20-fold diluted DTN medium, and WK medium under continuous light (40 μE m⁻² s⁻¹) at 30°C by shaking at 120 rpm. To examine the optimum pH for growth, the pH of the WK medium was adjusted with 5 mM 2-morpholinoethanesulfonic acid (MES)-NaOH (pH 6.0), N-2-hydroxyethylpiperezine-N’-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.0), N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH (pH 8.0) or N-cyclohexyl-2-aminoethanesulfonic acid (CHES)-NaOH (pH 9.0, 10.0). The chlorophyll concentration extracted by methanol was measured at 665 nm and then calculated with A₆₆₅ 1=13.42 μg Chl/ml.

DNA sequencing

DNA was extracted from Nostoc sp. HK-01 according to the method of Murray and Thompson(10) using cetlytrimethylammonium bromide (CTAB) to reduce contamination by polysaccharide. PCR amplifications were performed with a Gene Amp® PCR System 9700 (Applied Biosystems, USA) using primers for 16S rDNA (5’-AGAGTTTGTATCCTGGCTC-3’ and 5’-AAAGGAGGTGATCCAGCC-3’), tRNA-Leu (UAA) (5’-TGTGCCGGAATGTAGACGCTAC-3’, and 5’-GGGTGAGGGACTTGA-3’) (21). The 1.4 kbp (16S rDNA) and 0.3 kbp (trnL(UAA)-intron) nucleotide sequences were directly determined with a capillary DNA sequencer (model 310S, PE-biosystems, USA) using a Big Dye terminator DNA sequencing kit (PE-biosystems). The primers 5’-AACCTCTTTTCTCAGGGA-3’ and 5’-GTCTCCTCTAGAGTGCCCA-3’ were used to determine the inner sequence of the 16S rRNA.

16S rDNA phylogenetic analysis

DNA sequences corresponding to the complete or near complete 16S rRNA gene were aligned using the program CLUSTAL W(18), and finally refined visually. The positions with gaps and undetermined and ambiguous sequences were removed for subsequent phylogenetic analyses. Phylogenetic analyses were performed by the neighbor-joining (NJ)(14) and maximum-parsimony (MP) methods following Sano et al.(15). We used PAUP ver. 4(17) for MP analyses. The most parsimonious trees were found by the heuristic search option using 1,000 replications of random sequence addition with Tree Bisection-Reconnection (TBR) branch swa-
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Nucleotide sequence accession number

The nucleotide sequences of the trnL (UAA) intron and 16S rDNA for isolated Nostoc have been registered under DDBJ accession number AB085687 and AB085688, respectively. The accession numbers of nucleotide sequences used in this study are shown in Table 1.

Results

Isolation and purification of Nostoc sp. HK-01

The components of the culture medium, as well as temperature and light conditions, needed to isolate and cultivate the cyanobacterium were determined. After many trials, Nostoc sp. HK-01 was successfully isolated using a low-concentration inorganic-nutrient (1/20 DTN) plate. The isolated Nostoc strain grew well on a 1/20 DTN plate and dense colonies were obtained (Fig. 1A). On the other hand, the colonies grown on a high-concentration inorganic-nutrient (DTN) plate were sparse, and grew more slowly than those grown on the 1/20 DTN plate (Fig. 1B). These findings suggest that a low-concentration of inorganic-nutrient is desirable for the growth of strain HK-01 on the plate.

Drought-tolerance test

To investigate drought-tolerance, strain HK-01 was first subjected to dehydration for at least 3 months, and then rewetted and cultured on a 1/20 DTN plate. In terms of growth rate and the number of colonies, the rewetted HK-01

Table 1. Nucleotide sequences used in this study.

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<th>a) 16S rDNA sequence</th>
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Isolated Nostoc in this study

Fig. 1. Microphotographs of isolated Nostoc sp. HK-01 grown on 1/20 DTN (panel A) and DTN (panel B) plates. Scale bar=100 μm.

Fig. 2. Microphotographs of the isolated Nostoc sp. HK-01 cells. Panel A: vegetative cells and heterocysts (arrows), panel B: hormogonium, panel C: akinetes (arrowheads) and heterocyst (arrow). Scale bar=10 μm.
Isolation of Drought-Tolerant Nostoc 85 differed little from the non-dried, cultivated form (Fig. 3), indicating that the strain is drought resistant.

Hormogonia differentiation

Strain HK-01 cells differentiated into motile hormogonia under the previously mentioned nutrient and/or light conditions (Fig. 4). The newly streaked cells were pre-cultured under light for 4 days to exclude the influence of the transfer to the new medium, as reported previously4,12). On the plate with low-concentration inorganic-nutrient (1/20 DTN), more than 95% of strain HK-01 cells formed hormogonia within 12–24 h of cultivation in the dark. These hormogonia could not change into original vegetative cells in the dark, but could under light. On the other hand, this differentiation did not occur on the plate with high-concentration inorganic-nutrient (DTN). It was confirmed that the differentiation into hormogonia requires darkness and a low nutrient concentration.

Growth of Nostoc sp. HK-01 in liquid culture

To find a medium that sustains the optimum growth of strain HK-01 in the liquid medium, variously diluted DTN media (Fig. 5A) were investigated but this strain grew very slowly in all of them. Therefore, a medium with a simpler nutrient composition, i.e., WK medium originally devel-
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19), was used. The growth of strain HK-01 cells in the WK medium was obviously better than that in the DTN media (Fig. 5A). The optimum pH for growth was 8.0 (Fig. 5B), which agreed well with that for cyanobacterial growth. Thus, the pH of the WK medium was adjusted to 8.0 with 5 mM TES-NaOH.

DNA sequencing and phylogenetic analysis

To determine the genus of strain HK-01, the 16S rDNA region was sequenced. The 16S rDNA sequence of this strain was homologous to that of *Anabaena* and *Nostoc*. No significant difference was found between the distance tree for the NJ method and that for the MP method. The distance tree for 16S rDNA shows that HK-01 belongs to a cluster of heterocystous cyanobacteria, particularly *Anabaena* and *Nostoc* (Fig. 6). The identity of the sequence of strain HK-01 with *Anabaena* sp. PCC 7120, *Anabaena variabilis* IAM M-3 (same as PCC7118), *Anabaena variabilis* NIES23 and *Nostoc punctiforme* was 97.4%, 97.3%, 97.3% and 93.7%, respectively. To further identify the phylogenetic position of strain HK-01, the *trnL*(UAA)-intron region, which has been used to classify species within the family *Nostoc*11,21, was determined. The conserved region of the *trnL*(UAA)-intron shows that HK-01 belongs to the cluster of *Anabaena* and *Nostoc*. The alignment of DNA sequences of the so-called hypervariable region21 shows that the sequence of HK-01 is highly homologous to that of *Nostoc* (Fig. 7). It was also shown that the hypervariable region of another drought-tolerant cyanobacterium, *Nostoc commune*, was 15 bp shorter than that of HK-01. On the other hand, the sequence of variable region I in HK-01 was somewhat different from that in other *Nostoc* species (Fig. 7).

Fig. 6. Distance tree of cyanobacteria constructed on the basis of almost complete 16S rDNA sequences (more than 1300 nucleotides). The phylogenetic tree was constructed using the NJ algorithm as implemented within CLUSTAL W. The root of the tree was determined using the 16S rDNA of *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 6301 as the outgroup. Bootstrap values calculated by the NJ method (above the branches) and MP method (under the branches) are indicated in more than 50% of 1000 bootstrap replicates.
Isolation of Drought-Tolerant Nostoc

Discussion

The isolation and purification of cyanobacteria are difficult and time-consuming procedures. Methods should be deliberately chosen taking into account the physiological characteristics of the isolated cyanobacterium. In the present study, much attention was paid to drought-tolerance and starvation-tolerance. The cyanobacteria taken from the ground were first dried for more than one week before the isolation process began and then grown on a nutrient-poor medium. Such a procedure helped the Nostoc sp. HK-01 to predominate over other bacteria and fungi. Also, the motile hormogonia of this species made the isolation easier, i.e., some hormogonia on the plate moved far from their original colonies and settled where no other bacteria or fungi existed. Based on these methods, we could isolate and purify Nostoc sp. HK-01.

In the course of cultivation it was found that dark and poor-nutrient conditions induced the cells to differentiate into hormogonia and that irradiation inhibited the differentiation. This dark-dependent formation of hormogonia is not observed in Nostoc muscorum A and Calothrix sp. PCC 7601, where red light is required for cells to differentiate into hormogonia and green light reverses the effect. The differentiation into hormogonia under dark and poor-nutrient conditions seems to be a strategy to spread widely on nutrient-poor soils. In the daytime, the colonies photosynthesize and increase in cell number, while during the night they produce motile hormogonia and expand their territories.

Although strain HK-01 could grow on agar plates, whether the DTN medium was diluted or not, it could not grow in the liquid DTN medium. However, this strain could grow well in liquid WK medium which was originally developed for symbiotic bacteria. The major difference in the composition of the media was the presence of EDTA. The DTN medium contains EDTA but the WK medium does not, suggesting that EDTA inhibits the growth of strain HK-01. EDTA is an effective chelator for divalent cations such as Ca$^{2+}$ and Mg$^{2+}$, and thus the observed growth inhibition in the medium with EDTA may due to depletion of divalent cations. Another difference between the DTN and WK medium...
media was the concentration of nitrate. The concentration of nitrate needed for good growth was between 0.46 mM (1/20 DTN plate) and 0.99 mM (WK medium), although strain HK-01 could grow without a nitrogen source (data not shown). A high concentration of nitrate (9.23 mM, DTN plate) was inhibitory to growth (data not shown).

The 16S rDNA and trnL(UAA) intron sequence analyses indicate that strain HK-01 is part of the Nostoc cluster. The profile of the phylogenetic tree based on the 16S rDNA sequence was very similar to that based on DNA-DNA hybridization (4). The phylogenetic tree of 16S rDNA implies that HK-01 belongs to the Cluster 3 (4), which include Anabaena sp. PCC 7120. The bootstrap values of 99.4% (NJ method) and 74% (MP method) imply that the clade including Nostoc sp. HK-01 is the same as the clade of Anabaena sp. PCC7120 and Anabaena variabilis. On the other hand, strain HK-01 is distant from Nostoc punctiforme PCC 73102, a Cluster 1 (4). In spite of its sequence similarity to a symbiotic Nostoc, HK-01 is non-symbiotic and makes extracellular polysaccharides like non-symbiotic Nostoc commune. Taking these matters into account, it is concluded that Nostoc sp. HK-01 is a new species that can be classified as a Cluster 3 Nostoc very similar to Anabaena sp. PCC 7120.

Acknowledgments

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References