Isolation and Characterization of a Drought-Tolerant Cyanobacterium, *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 *Nostoc* species. This *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 *Nostoc*. The maximum growth rate was attained at pH 8. The DNA sequences of the 16S rRNA region and *trnL*(UAA)-intron region were determined to classify this *Nostoc* species. The *trnL*(UAA)-intron sequence was homologous to a symbiotic *Nostoc*, and also to some extent to *Anabaena* sp. PCC 7120. This cyanobacterium, named *Nostoc* sp. HK-01, is promising as an improver of arid and nutrient-poor soils.

Key words: cyanobacterium, drought-tolerance, hormogonia, *Nostoc*

Cyanobacteria of the genus *Nostoc* are found on every continent on earth in a wide range of terrestrial and aquatic ecosystems. Although numerous strains of *Nostoc* have been identified, only a few *Nostoc* species have been characterized in detail⁴. *Nostoc commune* is a well-known, terrestrial and highly drought-tolerant cyanobacterium¹², and desiccated *N. commune* has been shown to survive for almost 100 years¹. Desiccated *N. commune* exhibits a rapid recovery of photosynthetic activity, attaining almost half of the maximum level within 1 h of rewetting¹⁶. The *Nostoc* species are thought to be very useful for agricultural applications, their N₂-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 cyanobacteria²⁰. The sequence of the *trnL*(UAA) intron (group I intron) is also well used for identifying cyanobacterial taxonomic levels^{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 *Nostoc* sp. and cultivated it axenically. The phylogenetic position of the isolated *Nostoc* was determined from the sequences of the 16S rDNA and *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^{2,9}. The DTN medium contains in one liter; 0.19 g Na₂EDTA, 52.5 mg MgSO₄, 0.1 g KNO₃, 0.7 g NaNO₃, 0.122 g NaH₂PO₄·2H₂O, 42 mg CaCl₂, 1 mg FeCl₃·6H₂O, 5 mg NH₄Cl, 0.1 g Na₂S₂O₃·5H₂O, 83 mg NaHSO₃, 0.42 g NaHCO₃, and 0.5 ml trace elements (0.5 ml H₂SO₄, 0.5 g H₃BO₄, 2.28 g MnSO₄·5H₂O, 0.5 g ZnSO₄·7H₂O, 25 mg CuSO₄·5H₂O, 25 mg Na₂MoO₄·2H₂O, 45 mg CoCl₂·6H₂O, 19 mg NiSO₄(NH₄)SO₄·6H₂O and 4 mg NaSeO₄ in one liter²) and 8 mM Tricine-NaOH (pH 8.0). A medium developed by Watanabe and Kiyohara¹⁹ was used for the liquid culture. The medium contains in one liter; 0.125 g K₂HPO₄,

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0.1 g KNO₃, 0.275 g MgSO₄·7H₂O, 50 mg CaCl₂, 50 mg NaCl, 4 mg FeSO₄·7H₂O, and 1 ml of trace elements A₅ (2.86 g H₃BO₃, 1.81 g MnCl₂·4H₂O, 0.22 g ZnSO₄·7H₂O, 80 mg CuSO₄·5H₂O, 21 mg Na₂MoO₄ and 20 µl concentrated sulfuric acid in one liter). This medium is referred to herein as WK medium (Watanabe and Kiyohara¹⁹).

Isolation and purification of Nostoc sp.

Crusts of cyanobacteria were collected at the Harima Science Garden City Campus of Himeji Institute of Technology, Hyogo Prefecture, Japan¹⁶) (134.5°E, 35°). The crusts were dried for a week at 25°C in dark and stored in darkness at room temperature. These crusts were soaked with sterile MQ-water for 1 day and washed twice with sterile water. Wetted crusts were ground with a mortar and pestle and then spread on a 1/20 DTN agar plate. They were grown under a 16 h light (50 to 200 µE m⁻² s⁻¹)-8 h dark cycle from 25 to 30°C for about 1 month. The colonies of *Nostoc* sp. excreting polysaccharide were cultured on another 1/20 DTN plate. When a layer of extracellular polysaccharide 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 Rippka¹³). 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-hydroxyethylpiperazine-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 $A_{665} \times 13.42 = \mu\text{g Chl/ml}^{17}$.

DNA sequencing

DNA was extracted from *Nostoc* sp. HK-01 according to the method of Murray and Thompson¹⁰) using cetyltrimethylammonium 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'-AGAGTTTGATCCTGGCTC-3' and 5'-AAAGGAGGTGATCCAGCC-3'), tRNA^{-Leu} (UAA)(5'-TGTGGCGGAATGGTAGACGCTAC-3', and 5'-GGGTGGAGGGACTTGA-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'-GTCTCTCTAGAGTGCCCA-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 W¹⁸), 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. 4¹⁷) 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-

Table 1. Nucleotide sequences used in this study.

a) 16S rDNA sequence	
strain	accession no.
<i>Nostoc</i> sp. HK-01 ^a	AB085687
<i>Anabaena</i> (<i>Nostoc</i>) sp. PCC 7120	AP003598
<i>Anabaena variabilis</i> IAM M-3	AB016520
<i>Anabaena variabilis</i> NIES23	AF247593
<i>Cylindrospermum</i> sp. PCC 7417	CSP133163
<i>Nostoc punctiforme</i> PCC 73102	AF027655
<i>Nostoc</i> sp. 152	NSP133161
<i>Nostoc</i> sp. ATCC 53789	AF062638
<i>Nostoc</i> sp. AWT 203	AF317630
<i>Nostoc</i> sp. DM103	NDM344563
<i>Nostoc</i> sp. GSV224	AF062637
<i>Nostoc</i> sp. PCC 9709	AF027654
<i>Nostoc</i> sp. TDI#AR94	AF027653
<i>Synechococcus</i> sp. PCC 6301	X03538
<i>Synechocystis</i> sp. PCC 6803	D64000
^a Isolated <i>Nostoc</i> in this study	
b) <i>trnL</i> (UAA) intron sequence	
strain	accession no.
<i>Nostoc</i> sp. HK-01 ^a	AB085686
<i>Anabaena</i> (<i>Nostoc</i>) sp. PCC 7120	AP003597
<i>Nostoc commune</i> strain DRH1	AF204090
<i>Nostoc commune</i> UTEX584	AF204098
<i>Nostoc punctiforme</i> PCC 73102	NSU83254
<i>Nostoc</i> sp. 'Peltigera aphthosa' sample 1	AF055655
<i>Nostoc</i> sp. TCS-2001/2	NSP422008

pping. Bootstrap analysis³⁾ with 1,000 replications was performed.

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 AB085686 and AB085687, 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 iso-

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

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 μ m.

lated *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.

As shown in Fig. 2A, the isolated cyanobacterium is filamentous and the vegetative cells are 3 to 4 μ m in length and 2 to 3 μ m in width. This strain has differentiated cell types: heterocysts (Fig. 2A and 2C, arrows), motile hormogonia (Fig. 2B), and akinetes (Fig. 2C, arrowheads), and these features are characteristics of the genus *Nostoc* (4).

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

Fig. 3. Drought test for isolated *Nostoc* sp. strain HK-01. Wet cells of the isolated strain HK-01 (cultivated *Nostoc*), and desiccated cells of the same strain (dried *Nostoc*) and of *Synechocystis* sp. PCC 6803 (dried *S. 6803*) were grown on a 1/20 DTN plate.

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 condi-

Fig. 4. Effects of nutrients and light on hormogonia differentiation. Scale bar=100 μ m.

tions (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 previously^{4,12}. 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-

Fig. 5. Growth of the isolated *Nostoc* sp. HK-01 in liquid media. Cells were grown in various liquid media (panel A), and liquid WK media at various pH (panel B).

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.

oped for symbiotic *Nostoc*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 symbiotic *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).

<i>Nostoc</i> sp. HK-01	AGA AA CTGAGCCTTGA SA AGAAAT TC TTCAAGTGAAGCTCTCAA ACT CAGGAAACCTAAATCTG TT C CAGACA AG GCAATCCTGA	90
<i>Nostoc</i> sp. TCS-2001/2	AGATA AA CTGAGCCTTGAAGGAGAAATCCCTCAAGTGAAGCTCTCAA ACT CAGGAAACCTAAATCTGGTGACAGACATGGCAATCCTGA	90
<i>Nostoc</i> 'P. aphthosa'-1	AGATA AA CTGAGCCTTGAAGGAGAAATCCCTCAAGTGAAGCTCTCAA ACT CAGGAAACCTAAATCTGGTGACAGACATGGCAATCCTGA	90
<i>N. punctiforme</i>	AGATA AA CTGAGCCTTGAAGGAGAAATCCCTCAAGTGAAGCTCTCAA ACT CAGGAAACCTAAATCTGGTGACAGACATGGCAATCCTGA	90
<i>N. commune</i> DRH1	AGATA AA CTGAGCCTTGAAGGAGAAATCCCTCAAGTGAAGCTCTCAA ACT CAGGAAACCTAAATCTGGTGACAGACATGGCAATCCTGA	90
<i>N. commune</i> UTEX584	AAATAATTGAGCCTTGAAGGAGAAATCCCTCAAGTGAAGCTCTCAA ACT CAGGAAACCTAAATCTGGTGACAGACATGGCAATCCTGA	90
<i>Anabaena</i> 7120	AAATAATTGAGCCTTAGAGA AA AGAAAT TC TTAAGTGGATGCTCTCAA ACT CAGGAAACCTAAATCTAG CT TATAGACA AG GCAATCCTGA	90
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<i>Nostoc</i> sp. HK-01	GCCAAGCCCAAAAATTTTAGATT TAA ATTTTAGATT TAG ATT GCA CTTCAGTCCAAAATTCAAAATCCAAAATCCAAAATTA AG GGA	180
<i>Nostoc</i> sp. TCS-2001/2	GCCAAGCCCAAAAATTTTAGATT TGT GATT TTAG ATT TAC GATTAGTCTTCAATCCAAAATTCAAAATCCAAAATTA T -GGGA	179
<i>Nostoc</i> 'P. aphthosa'-1	GCCAAGCCCAAAAATTTTAGATT TGT GATT TTAG ATT TAT GATTAGTCTTCAATCCAAAATTCAAAATCCAAAATTA TGC -GGGA	179
<i>N. punctiforme</i>	GCCAAGCCCAAAAATTTTAGATT TGCG ATT TTAG ATT TGG GATTAGT TTT CAATCT-----AAAATCCAAAATCCAAAATTA TGA -GGGA	172
<i>N. commune</i> DRH1	GCCAAGCCCAAAAATTTTAGATT TGCG ATT TTAG ATT TACC ATTAGTCTTCAATCC-----AAAATCCAAAATTA TGA -GGGA	165
<i>N. commune</i> UTEX584	GCCAAGCCCGAAAATTTTAGATT TGCG ATT TTAG ATT TAC GATTAGTCTTCAATCC-----AAAATCCAAAATTA TGA -GGGA	165
<i>Anabaena</i> 7120	GCCAAGCCGAAGTA-----GTAATTAGTAAGTTAACAACAGATAACTTACAGCT-AATCGGA	146
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<i>Nostoc</i> sp. HK-01	AGGTGCAGAGAC TC GACGGGAGCTACCTTAACGT CA AGGCGAGGGTAAAGGGAGAGTCCAATTCTCAA ACC GA AGGTAGTAGT GA AG	270
<i>Nostoc</i> sp. TCS-2001/2	AGGTGCAGAGACCCGACGGGAGCTACCTTAACGT TA AGTCGAGGGTAAAGGGAGAGTCCAATTCTCAA ACC TGATTGGCTATTGTCAT	269
<i>Nostoc</i> 'P. aphthosa'-1	AGGTGCAGAGACCCGACGGGAGCTACCTTAACGT TA AGTCGAGGGTAAAGGGAGAGTCCAATTCTCAA ACC TGCGCTTGTAAAGCCAT	269
<i>N. punctiforme</i>	AGGTGCAGAGACCCGACGGGAGCTACCTTAACGT TA AGTCGAGGGTAAAGGGAGAGTCCAATTCTCAA ACC TGATTGGCTATTGTCAT	262
<i>N. commune</i> DRH1	AGGTGCAGAGACCCGACGGGAGCTACCTTAACGT TA AGCCGAGGGTAAAGGGAGAGTCCAATTCTTAA ACC TGATTGGCTAAAGTCAT	255
<i>N. commune</i> UTEX584	AGGTGCAGAGACCCGACGGGAGCTACCTTAACGT TA AGCCGAGGGTAAAGGGAGAGTCCAATTCTTAA ACC TGACTTTGCTAAAGCCAT	255
<i>Anabaena</i> 7120	AGGTGCAGAGAC TC GACGGGAGCTACCTTAACGT CA AGACGAGGGTAAAGAGAGAGTCCAATTCTCAA AGC CA TAG GCAGTAGC GA AG	236
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<i>Nostoc</i> sp. HK-01	CTGC AA GAGAAATGAAAA TC CGTTGACCTTA	300
<i>Nostoc</i> sp. TCS-2001/2	CAGGTAGCAG-TGAA AA CTGCGGGAGGATG	298
<i>Nostoc</i> 'P. aphthosa'-1	CAGGTAGCAG-TGAA AA CTGCGGGAGGATG	298
<i>N. punctiforme</i>	CAGGTAGCAG-TGAA AA CTGCGGGAGGATG	291
<i>N. commune</i> DRH1	CAGGTAGCAG-TGAA AA CTGCGGAAGAATG	284
<i>N. commune</i> UTEX584	CAGGTAGCAG-TGAA AA CTGCGGAAGAATG	284
<i>Anabaena</i> 7120	CTC GGG AGAA TGAAAA TC CGTTGACCTTA	266
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Fig. 7. Alignment of *trnL*(UAA) intron sequences. Black boxes: the nucleotides of *Nostoc* sp. HK-01 different from others. Double upper line: hypervariable region. Upper line: variable region I. Bold letters: the nucleotides that agree with those of *Anabaena* sp. PCC 7120. Asterisks: conserved nucleotides for all species.

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^{5,8)} and green light reverses the effect⁶⁾. 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 produced 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 Ca²⁺ and Mg²⁺, 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

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 *Nostoc* (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 *Nostoc* (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.

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