The Heat Tolerance of Dry Colonies of a Terrestrial Cyanobacterium, \textit{Nostoc} sp. HK-01

Shunta Kimura\textsuperscript{1}, Kaori Tomita-Yokotani\textsuperscript{1*}, Yuichi Igarashi\textsuperscript{1}, Seigo Sato\textsuperscript{1}, Hiroshi Katoh\textsuperscript{2}, Tomoko Abe\textsuperscript{3}, Kintake Sonoike\textsuperscript{4} and Masayuki Ohmori\textsuperscript{5}

\begin{itemize}
  \item \textsuperscript{1} Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan
  \item \textsuperscript{2} Division of Plant Functional Genomics, Life Science Research Center, Mie University, Tsu, Mie 514-8507, Japan
  \item \textsuperscript{3} School of Science and Engineering, Tokyo Denki University, Hiki-gun, Saitama 350-0394, Japan
  \item \textsuperscript{4} Faculty of Education and Integrated Arts and Sciences, Waseda University, Shinjuku-ku, Tokyo 162-8480, Japan
  \item \textsuperscript{5} Faculty of Science and Engineering, Chuo University, Bunkyo-ku, Tokyo, 112-8551, Japan
\end{itemize}

Abstract

The heat tolerance of dry colonies of a terrestrial cyanobacterium, \textit{Nostoc} sp. HK-01 by exposure to temperatures at 100 °C, the boiling point of liquid water, was examined. The cells of \textit{Nostoc} sp. HK-01 can live under these conditions for 10 hours. All of the viable cells were akinetes which are dormant cells. Akinetes have a high tolerance to high temperatures. The photosynthetic ability of cells of HK-01 was normal even after the heat exposure. The percentage of decreased water content in the dry colony was 5 percent during the heat exposure for 24 hours. The reasons for the decrease in the percentage of survival rates of colonies under the high heat condition are related to the remaining water in the dry colony, and/or, DNA damage of individual cells. Furthermore, there is a possibility that there are highly tolerant cells in the dry colony since there are cells that survived over 24 hours after the heat exposure. ©2015 Jpn. Soc. Biol. Sci. Space; doi: 10.2187/bss.29.12

Keywords; Akinete, Cyanobacteria, Heat tolerance, \textit{Nostoc} sp. HK-01

Introduction

There have been preparations for the establishment of a manned Mars mission (Badescu \textit{et al}., 2009; Casaregola \textit{et al}., 2009; Ehrenfreund \textit{et al}., 2012; International Space Exploration Coordination Group, 2013; Salotti and Heidmann, 2014). As one of the aspects of a manned Mars mission, there has been a study of space agriculture which is focused on the circulation of materials by bio-ecosystem function, for human habitation on Mars (Yamashita \textit{et al}., 2006, 2009; Wada, 2007; Kanazawa \textit{et al}., 2008). Terrestrial cyanobacteria can be utilized for material circulation as a bio-fertilizer for a manned Mars mission, because of their photosynthesis and nitrogen fixation abilities and drought tolerance (Ohmori \textit{et al}., 2006; Obana \textit{et al}., 2007; Yamashita \textit{et al}., 2009; Garcia-Pichel, 2010; Hu \textit{et al}., 2012; Katoh \textit{et al}., 2012).

A terrestrial cyanobacterium \textit{Nostoc} sp. HK-01 was isolated and characterized from \textit{Nostoc commune} crusts (Katoh \textit{et al}., 2003). \textit{Nostoc} sp. HK-01 is one of the candidate species of terrestrial organisms for the introduction to Mars. Arai \textit{et al}., (2008) reported that \textit{Nostoc} sp. HK-01 could grow for a period of 140 days on Martian regolith simulant which does not include a carbon source or a nitrogen source. \textit{Nostoc} sp. HK-01 may be able to create organic soil from Martian regolith (Arai \textit{et al}., 2008; Arai, 2009; Oarga, 2009). There are several different type of cells of \textit{Nostoc} sp. HK-01, vegetative cells, which have photosynthetic abilities, heterocysts, which have nitrogen fixation abilities, hormogonia, which are motile cells, and akinetes, which are dormant cells (Adams and Duggan 1999; Katoh \textit{et al}., 2003; Kaplan-Levy \textit{et al}., 2010). Generally, akinetes are known for having a high tolerance to desiccation, but their tolerance to high heat has not been yet investigated (Adams and Duggan 1999; Garcia-Pichel, 2010; Kaplan-Levy \textit{et al}., 2010). Hori \textit{et al}., (2003) have been reported about the heat tolerance of akinetes to 60 °C for 50 h.

The heat tolerance of \textit{Nostoc} sp. HK-01 has not been investigated, although their tolerances to desiccation for 8 months, to 200 mM NaCl stress, and to vacuum (10\textsuperscript{5} Pa) for 2 weeks, have been investigated (Yoshimura \textit{et al}., 2006, 2012; Arai \textit{et al}., 2008; Arai, 2009). In low Earth orbit, the temperature of a body in space can become extremely high due to solar radiation (1,366 W m\textsuperscript{-2}) and no convection. The limit of tolerable temperatures for the ISS has been determined to be 120 °C (Nicholson \textit{et al}., 2000; Baglioni \textit{et al}., 2007; Horneck \textit{et al}., 2010). Heat tolerance is an important element of a candidate species for the introduction to Mars.

We will study the heat tolerance of dry colonies of \textit{Nostoc} sp. HK-01 by exposure to 100 °C, which is the boiling point of liquid water. The survival ability of the cells of \textit{Nostoc} sp. HK-01 is elucidated under the complete water loss condition, although under ordinary pressure conditions. This study was focused on the cell types, especially akinetes in \textit{Nostoc} sp. HK-01. To elucidate
the mechanism of high tolerance will contribute to the methods of production of highly tolerant colonies.

**Material and Methods**

**Biological materials**

Pure cultures of a terrestrial cyanobacterium, *Nostoc* sp. HK-01, and aquatic cyanobacterium, *Anabaena* sp. PCC 7120 stocked at the Ohmori Laboratory (present affiliation; Chuo University), were used as material.

**Preparation of dry colony**

Each cell was incubated in the liquid medium, BG-11 (Rippka *et al.*, 1979), at 26 °C, under the light condition, 74.3 ± 24.3 μmol m⁻² s⁻¹, with shaking at 120 rpm (BW101, Yamato, Japan). After the incubation, the wet colonies were dried at 23 °C with relative humidity 30 % in the dark. The colonies were dried under those conditions until the weight was stable.

**Preparation of heated colonies**

The dry colonies, ca. 100 μg dry weight, were placed in an oven (WFO-450, Eyela, Japan), and exposed to 100 °C, which is the boiling point of liquid water for 24 h.

**Pre incubation for each survival experiment**

Dry and heated colonies were rehydrated by 500 μl of BG-11 liquid medium. Each colony was kept in the dark at 30 ± 2 °C for 2 days and stirred for 1 minute by a mixer (TM-251, Iwaki, Japan) used for the viability assay. Each colony was incubated for 2 weeks, as described above, and was used for the photosynthetic activity assay.

**The viability assay**

Fluorescein diacetate (FDA) staining method (Jones and Senft, 1985; Mori *et al.*, 2002; Arai *et al.*, 2008) was used as the viability assay. One mg of dissolved FDA in 0.5 ml dimethylsulfoxide and 0.2 ml acetone, which was kept in a refrigerator as an FDA stock solution. At each stage of observation, 0.04 ml of the FDA stock solution were mixed with the 10 ml of DPBS (Dulbecco’s Phosphate Buffered Saline), and resulting the solution was used as the FDA staining solution (final FDA concentration; 0.002 %).

The 100 μl FDA staining solution was added to the 200 μl of suspended cells with water containing cyanobacteria, and mixed by shaking at 500 rpm, 37 °C for 10 minutes under dark conditions (M-BR-022UP, Taitec, Japan). After 5 minutes on ice, the cells were observed using a fluorescent microscope (BX50, Olympus, Japan), with a fluorescence NIBA filter (excitation wavelength, 470-490 nm). Viable cells emit green fluorescence during fluorescence observation.

**The survival rate and the akinete content rate**

The numbers of viable trichome cells (a), all trichome cells (b), viable akinetes (c) and all akinetes (d) were counted from the photomicrographs, at least 250 cells per examination. Trichome means vegetative cells, heterocyst and hormogonia. The survival rate of trichome cells was calculated as (a) / (b) × 100. The survival rate of akinetes (%) was calculated as (c) / (d) × 100. The total survival rate was calculated as ( (a) + (c) ) / ( (b) + (d) ) × 100. The akinete content rate was calculated as (d) / { (b) + (d) } × 100. Pearson correlations were used to examine the association between the akinete content rate and the total survival rate.

**The photosynthetic activity assay**

The photosynthetic activity of survival cells was assayed by flash-induced fluorescence yield decay. The rate of Qa⁻ reoxidation was measured with the double modulated fluorometer with a control unit (FL-200/PS, Photon Systems Instruments, Czech Republic). Approximately 1 ml suspension of cyanobacteria was placed in a cuvette. Short pulses (4 μs) from an orange LED were used as the measuring light. Qa⁻ was reduced by the saturating 30 μs flash from orange/red LED. Qa⁻ reoxidation kinetics were monitored as a decrease of fluorescence.

**Results**

The microscopic observation of wet cells, dry cells and heated colonies of *Nostoc* sp. HK-01 was performed with fluorescein diacetate (FDA) staining method. In the case of wet colonies, viable trichome cells were observed easily (Fig. 1. A-a-1, A-b-1, A-a-2, A-b-2). The viable akinetes larger than other cell types, were also observed, easily (Fig. 1. A-a-3, A-b-3). All of the cell types under the wet conditions were viable. In the case of dry colonies, a large number of viable cells were akinetes and trichome cells were rarely observed (Fig. 1B). The trichome cells were almost always not viable after the dry treatment. In the heated colony after the exposure to 100 °C for 2 h, trichome cells were not viable (Fig. 1. C-a-1, C-b-1), while the akinetes were still viable (Fig. 1. C-a-2, C-b-2, C-a-3, C-b-3). These results indicate that only akinetes were viable under heated conditions. The survival rate (%) after the exposure to 100 °C was calculated in wet, dry, and heated colonies. Regarding the other three types of cells, trichome which means vegetative cells, heterocysts and hormogonia, the survival rate (%) decreased remarkably during the dry treatment (Fig. 2). In heated colonies after the exposure to 100 °C for 2 h, viable trichrom cells were not detected (Fig. 2). The survival rates of akinetes under wet, dry, and heated condition were not remarkable different (Fig. 2). The correlation between the rate of existence of akinetes in the colony and their survival rate was examined. In wet colonies, no correlation was observed (Fig. 3A; r = -0.2). In dry and heated colonies, certain correlations were observed (Fig. 3B and C; r = 0.73, p < 0.001 in both). These results indicate that akinete is the cell type which has a tolerance to dry and heated conditions of *Nostoc* sp. HK-01.

The heat tolerance of dry colonies of *Nostoc* sp. HK-01 and *Anabaena* sp. PCC 7120 were tested by exposure to 100 °C. In a terrestrial cyanobacterium, *Nostoc* sp. HK-01, the survival rates were not changed compared to the rate of control, even after the exposure to 100 °C for 3, 4, 5, and 6 h (Fig. 4). A reduction in survival rates of cells in HK-01 was observed during exposure to 100 °C beyond 7 h. After 10 h exposure, 36.4 ± 9.9 % of HK-01
cells had survived. After 11–24 h of exposure, living cells of HK-01 were not detected. In dry colonies of an aquatic cyanobacterium, *Anabaena* sp. PCC 7120, living cells were not detected after exposure to 100 °C for 1–3 h (data not shown). This result indicates that dry colonies of *Nostoc* sp. HK-01 can survive after exposure to 100 °C for 10 h, and *Nostoc* sp. HK-01 has superior heat tolerance compared to *Anabaena* sp. PCC 7120.

The photosynthetic activity of cyanobacteria cells after exposure to 100 °C was investigated. The electron transfer from Q$_A$ to Q$_B$ shows a peak on the shoulder of the relaxation kinetics of flash-induced fluorescence yield. In the cells from the dried colonies of *Nostoc* sp. HK-01 after exposure to 100 °C for 10 h, the same level of photosynthetic activities as control was recognized (Fig. 5-A and B). In the cells from dry colonies of *Anabaena* sp.
PCC 7120 which cannot survive after exposure to 100 °C, no photosynthetic activity was observed, even after 1 h, although a photosynthetic activity was recognized in the fresh cells (Fig. 5-C and D).

The dry weight of dry colonies of *Nostoc* sp. HK-01 during exposure to 100 °C was measured. There was a 5% decrease in the weight of dry colonies after exposure to dry heat (Fig. 6). Their weight was re-increased under the laboratory condition, 23 °C and 30% of humidity during the following 24 h.

In the tested sample, living cells of *Nostoc* sp. HK-01 after the exposure to 100 °C for 24 h were undetectable in statistically (Fig. 4). However, a very small number of viable cells were detected (Fig. 7).
Discussion

Akinete is the cell type which has a tolerance to dryness and dry heat (100 °C) in a terrestrial cyanobacterium, Nostoc sp. HK-01 (Fig. 1-3). The heated condition can be regarded as the same as a highly desiccated condition. Trichome which did not have tolerance to dryness, could not survive after heat exposure. It was suggested that akinetes possess a tolerance to dryness and heat.

Metabolic activities of matured akinetes are very low or undetectable (Adams and Duggan, 1999; Kaplan-Levy et al., 2010). Leprince et al., (1999) and Hoekstra et al., (2001) suggested that reduction of metabolism was an important factor to avoid oxidative stress from reactive O₂ spicies (ROS) during dryness. In plants, the accumulation of ROS during desiccation occurs mainly from the decline in CO₂ fixation, leading to leakage of electrons from electron transport chains to O₂ (Leprince et al., 1999; Dat et al., 2000; Hoekstra et al., 2001). ROS causes extensive peroxidation and de-esterification of membrane lipids (Hoekstra et al., 2001). Depression of metabolic activities in akinetes may be related to the desiccation tolerance of Nostoc sp. HK-01 (Fig. 1-3).

Akinetes accumulate glycogen and granules of cyanophycin (Adams and Duggan 1999; Garcia-Pichel, 2010; Kaplan-Levy et al., 2010). Accumulation of a compatible solute such as sucrose and/or trehalose is well known as dry tolerance in cyanobacteria, yeast, fungal spores, artemia, tardigrade, Polypedilum vanderplanki and lower and higher plants (Hershkovitz et al., 1991; Hill et al., 1994; Crowe et al., 1998; Hoekstra et al., 2001; Potts, 2001; Elbein et al., 2003; Higo et al., 2006; Hengherr et al., 2008; Sakurai et al., 2008; Jönsson and Persson, 2010). It is thought that cellular membranes and proteins stabilized by hydrogen bonding between hydroxyl groups in these sugar and polar residues in macromolecules (Crowe et al., 1998; Hoekstra et al., 2001; Elbein et al., 2003). Hershkovitz et al., (1991) reported that drought resistant cyanobacteria, Phormiumidum autumnale, strain LPP4, and a Chroococcidiopsis sp. accumulate trehalose and sucrose in response to drought stress, although drought sensitive cyanobacteria, Plectonema boryanum and Synechococcus strain PCC 7942 did not accumulate. Higo et al., (2006) reported that a strain of cyanobacteria, Anabaena sp. PCC 7120, with greater accumulation of sucrose has higher dehydration tolerance. Although the accumulation of sucrose and trehalose in akinetes has not been elucidated, any compatible solute may play an important role in the dryness tolerance of akinetes of Nostoc sp. HK-01.

The viable cells of Nostoc sp. HK-01 were observed after exposure to 100 °C for 24 h (Fig. 4). If too much damage has been accumulated during dormancy, this damage overwhelms the capacity of DNA repair systems and leads to death (Setlow, 2006). It was conceivable that exposure to heat accumulates DNA damage in cells of Nostoc sp. HK-01 and lead to death such as in spores of Bacillus. The photosynthetic activity of Nostoc sp. HK-01 was detected after exposure to 100 °C for 10 h (Fig. 5). This result implies that DNA involving photosynthetic activity was intact or DNA repair systems related to photosynthetic activity could function after heat exposure.

Water is essential to maintaining cell membrane and proteins (Hoekstra et al., 2001; Potts, 2001). The ca. 5 % weight of dry colonies of Nostoc sp. HK-01 was decreased by exposure to 100 °C for 24 h (Fig. 6). This change in weight can be thought water loss from air-dry colonies. And the reason for the increased weight might be water absorption under room conditions (23 °C, relative humidity 30 %). This change in weight might be absorbed water from the atmosphere (Fig. 6). A small amount of water from the atmosphere may be essential for cell survival, and loss of this water may lead to cell death.

Viable cells of Nostoc sp. HK-01 were rarely found after exposure to 100 °C for 24 h, although the survival rate was undetectable in statistically (Fig. 4 and 7). These viable cells may have survived under complete water loss. There are two possibilities regarding these cells, (1) the dry process was optimum and (2) mutation of heat tolerant cells. In Polypedilum vanderplanki, the slowly dehydrated samples can recover and the content of trehalose in the samples was remarkably increased during the dehydration, although the quickly dehydrated sample can not recover (Sakurai et al., 2008). In this study, the survival limit of Nostoc sp. HK-01 was exposure to 100 °C for 10 h (Fig. 4). If the survival limit and survival rate was changed by dry process, possibility (1) can be supported. If survival limit and the survival rate were not changed by dry process, possibility (2) can be supported. Highly tolerant colonies can be obtained by the investigation of dry process or screening of heat tolerant cells.

The terrestrial cyanobacterium, Nostoc sp. HK-01 has tolerances to desiccation, NaCl and vaccum (see introduction). In addition, the heat tolerance of akinetes of Nostoc sp. HK-01 was revealed in this study. Nostoc sp. HK-01 would be a good candidate species as a bio-fertilizer for a manned Mars mission.

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