

# Compatible Solutes in the Akinetes of the Terrestrial Cyanobacterium *Nostoc* sp. HK-01 Contribute to Its Heat Tolerance

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## Abstract

The detailed mechanisms that facilitate the heat tolerance of terrestrial cyanobacteria have not been completely elucidated, although several reports have revealed aspects of the heat tolerance mechanisms of several other organisms. The dormant cells, called akinetes, of the terrestrial cyanobacterium *Nostoc* sp. HK-01 can revive after dry heat exposure at 100°C for more than 10 h. We investigated the compatible solutes that protect the biomolecules in *Nostoc* sp. HK-01 akinetes using colonies containing various proportions of akinetes. We extracted the intracellular substances from each colony with 80% ethanol, which we purified with a series of analytical columns and analyzed by high-performance liquid chromatography and liquid chromatography-electrospray ionization-mass spectrometry. The compatible solutes were screened for their ability to prevent protein aggregation upon heating using the model enzyme lactate dehydrogenase. We detected an accumulation of glucosylglycerol, betaine, and glycine in akinetes. In addition, we confirmed that betaine, glycine, sucrose, and trehalose contributed to the prevention of the protein aggregation. The levels of sucrose and glycine in the colonies were approximately 1000× higher than those of glucosylglycerol, betaine, or trehalose. Our results indicated that sucrose and glycine are the main compatible solutes in the hydrophilic fractions of the cell extracts of *Nostoc* sp. HK-01 akinetes.

## Keywords

Akinete, Compatible Solute, Cyanobacteria, Dry Heat Tolerance, *Nostoc* sp. HK-01

## 1. Introduction

All levels of biological organization are affected by temperature [1]. Many organisms cannot live in high temperature environments as these environments cause damage to the essential macromolecules in their cells [1] [2] [3] [4]. However, some species exhibit either wet or dry heat tolerance.

Wet heat tolerance is the capacity to actively grow in high temperature environments. Hyperthermophile and thermophilic cyanobacteria are well-known wet heat-tolerant species. The archaeal species *Pyrolobus fumarii* can grow in temperatures up to 113°C [5]. The methanogen *Methanopyrus kandleri* strain 116 can grow at 122°C [6]. These organisms have hyperthermophilic mechanisms that stabilize their proteins [7]-[12], DNA [13] [14], and lipid membranes [15] [16]. Several species of thermophilic cyanobacteria are known to grow in temperatures over 50°C. *Synechococcus* cf. *lividus*, *Cyanothece* cf. *minervae*, and *Thermosynechococcus elongatus* BP-1 are cyanobacteria that can grow in geothermal habitats at 73°C - 74°C, 62°C, and 64°C, respectively [17]. In a previous study of *Thermosynechococcus elongatus* BP-1, thermal stability of the proteins made it possible to analyze the 3-dimensional structures of photosystems I and II using crystallization methods [18] [19] [20]. Thermophilic cyanobacteria have heat shock proteins (Hsp) [21]. A mutant cyanobacterium that overexpresses a Hsp, *Synechococcus* sp. PCC 7942, has a high survival rate, photosynthetic electron transport activity, and phycocyanin thermal stability at typically lethal temperatures [22].

Dry heat tolerance is the capacity to survive in high temperature environments, and later revive upon rehydration. *Bacillus subtilis*, *Tardigrades*, *Polypedilum vanderplanki*, and *Nostoc* are dry heat-tolerant species. Dry *B. subtilis* colonies, which are in a spore state with low water content, tolerate temperatures from 90°C - 120°C [3] [23]. The dipicolinic acid and small acid-soluble spore proteins that accumulate in the spores may contribute to the protection of the biomolecules in *B. subtilis* [3]. The cryptobiotic form of tardigrades, called tun, can revive after exposure to dry heat at temperatures of 90°C - 110°C [24]. Trehalose, which accumulates as cells adopt a dormant state, contributes to biomolecular protection in tardigrades [25] [26]. Larval *Polypedilum vanderplanki* in a cryptobiotic state were able to revive after exposure to dry heat at temperatures of 100°C [27]. Trehalose and glycerol, which accumulate during the transition of *Polypedilum vanderplanki* to a cryptobiotic state, may contribute to its biomolecular protection [28]. Dried colonies of the terrestrial cyanobacteria *Nostoc flagelliforme* and *Nostoc commune* were able to revive after exposure to dry heat at 85°C for 24 h [29] [30] or 1 h [31], respectively. We previously reported that dried colonies of the terrestrial cyanobacterium *Nostoc* sp. HK-01 were able to revive after exposure to dry heat at 100°C for more than 10 h [32] [33]. However, the detailed mechanisms of heat tolerance among terrestrial cyanobacteria have not been completely elucidated; several reports have partially revealed the

mechanisms of heat tolerance in other organisms.

Cyanobacteria, which can perform oxygenic photosynthesis, are distributed worldwide in sea water, in fresh water, and on land. The terrestrial cyanobacterium *Nostoc* sp. HK-01, isolated from *Nostoc commune* crusts based on its drought tolerance, is capable of photosynthesis, nitrogen fixation, and growth under salted conditions, in environments with poor nutrition, and on Martian regolith simulant; it is also useful as a food [34]-[40]. Furthermore, dried colonies of *Nostoc* sp. HK-01 have high tolerances to vacuum, ultraviolet (UV), gamma rays, heavy particle beams, and dry heat at a temperature of 100°C [32] [33] [37] [40] [41]. Due to its capabilities, *Nostoc* sp. HK-01 is expected to be the best candidate organism in the future for initial soil formation on bare land and in closed ecosystems in human habitats on Mars [37] [39] [42] [43]. Dried colonies of *Nostoc* sp. HK-01 are able to grow after exposure to a temperature of 100°C, although the important pigment chlorophyll degrades above 75°C [44]. Dry heat tolerance of temperatures to 100°C is remarkable, especially in photosynthetic organisms. Thus, *Nostoc* sp. HK-01 may have a special mechanism that accounts for its tolerance to dry heat.

The tolerances of terrestrial cyanobacteria to desiccation, NaCl, and UV are closely linked to extracellular polysaccharides (EPS) [31] [36] [45] [46] [47] [48] [49]. However, the survival rates of *Nostoc* sp. HK-01 with EPS and without EPS were not markedly different after exposure to 100°C of dry heat [33]. This result indicates that the contribution of EPS to the dry heat tolerance of *Nostoc* sp. HK-01 is likely minimal. Several different types of cells comprise *Nostoc* sp. HK-01: vegetative cells with photosynthetic abilities, heterocysts with nitrogen fixation abilities, motile hormogonia, and dormant akinetes [32] [33] [34]. Kimura *et al.* [32] found that only the akinetes in the dry colonies of *Nostoc* sp. HK-01 are dry heat-tolerant. Those results indicated that the substances that contribute to dry heat tolerance are likely present in akinetes [33]. Akinetes of cyanobacteria are generally larger than other cell types, have a thick cell wall, and accumulate glycogen and granules of cyanophycin [50] [51] [52]. However, the substances that contribute to the environmental tolerances of akinetes have not been elucidated.

The roles of compatible solutes have been thoroughly investigated in the context of several tolerances. Compatible solutes can enhance the stability of a biomolecule during dehydration by forming hydrogen bonds with the polar residues of the biomolecule [53] [54] [55] [56]. They are known to accumulate in cyanobacteria, yeast, bacterial spores, tardigrades, *Polypedilum vanderplanki*, *Artemia*, and higher and lower plants [26] [28] [53] [54] [55] [57]-[65]. Various substances act as compatible solutes in different species. In response to dryness, trehalose and sucrose accumulate in cyanobacteria, *Phormidium autumnale*, *Chroococcidiopsis* spp., and *Anabaena* sp. PCC 7120 [57] [63]. However, the types of cells in which those compounds accumulate have not yet been elucidated. In *Nostoc* sp. HK-01, we previously confirmed the accumulation of sucrose in

akinetes by quantitative analysis. We used different ratios of akinetes in the colonies in order to verify that selected substances specifically accumulated in akinetes [33]. We also detected trehalose in colonies of *Nostoc* sp. HK-01, although not specifically in akinetes [33]. It is possible that sucrose and/or trehalose function in akinetes as compatible solutes, but there is no evidence that these compounds contribute to the dry heat tolerance of akinetes. However, there are methods available to identify compatible solutes that prevent protein aggregation and protect enzymatic activity [66] [67] [68] [69].

In this study, we isolated and identified substances that accumulated specifically in *Nostoc* sp. HK-01 akinetes. We isolated the substances from the hydrophilic layers of cell extracts because compatible solutes, which form hydrogen bonds with biomolecules instead of water, can be categorized as hydrophilic. We tested the biomolecular protective activities of the substances, based on the level of protein aggregation and enzymatic activity in their presence. We used lactate dehydrogenase (LDH), a model enzyme for the measurement of the activity of compatible solutes, for the screening assay [70]. In this report, we describe the mechanism of biomolecular protective activity and the intracellular concentrations of compatible solutes in *Nostoc* sp. HK-01 akinetes.

## 2. Material and Methods

### 2.1. Cyanobacterium and Chemicals

For all experiments in this study, we used a pure culture of the terrestrial cyanobacterium *Nostoc* sp. HK-01, stocked at the Ohmori Laboratory (present affiliation; Chuo University) [32] [34].

Betaine, glycine, and sucrose were purchased from Wako (Osaka, Japan). Trehalose was purchased from Tokyo Chemical Industry (Tokyo, Japan). Glucosylglycerol was purchased from Toronto Research Chemicals (Toronto, Canada). Each chemical was dissolved in the buffer for the protein aggregation assay kit, PROTEOSTAT® (Enzo Life Sciences, Farmingdale, New York, USA), to the experimental concentrations.

### 2.2. Incubation and Preparation of Colonies

We prepared dried colonies as described previously [32]. Each group of cells was incubated in the liquid medium BG-11 [71], at 26°C with light exposure at  $74.3 \pm 24.3 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  with a 3-cm amplitude for  $120 \text{ cycles}\cdot\text{min}^{-1}$  on an SR-1 reciprocating shaker (As One, Osaka, Japan). After incubation, we obtained several groups of wet colonies after counting the cells. The number of total cells and the number of akinetes were counted in photomicrographs taken under a microscope (BX50; Olympus, Tokyo, Japan), using a Thoma counting chamber (Nippon Rinsho Kikai Kogyo, Tokyo, Japan). We calculated the percentage of akinetes in the sample as  $\text{percentage of akinetes} = \frac{\text{the number of akinete}}{\text{the number of all cells}} \times 100$ .

### 2.3. Analysis of Intracellular Substances by Liquid Chromatography-Electrospray Ionization-Mass Spectrometry

We extracted the contents of the colonies with different ratios of akinetes with 80% ethanol at 65°C. The solutions were concentrated to dryness in vacuo at 38°C [63]. The extracts were further purified using a Sep-Pak C<sub>18</sub> cartridge (Waters, Milford, Massachusetts, USA) with a water-methanol solvent system, by increasing the methanol concentration in the series from 0% to 50% to 100% (10 mL per step). The 0% methanol fraction was purified through a 0.45- $\mu$ m filter (Tosoh, Tokyo, Japan), and each extract was analyzed by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). The positive mode of the ESI-MS spectra and several fragments were analyzed with a Waters Micromass ZQ Mass Spectrometer (Waters, Milford, Massachusetts, USA), consisting of an LC 2690 Separation Module with a 996 Photodiode Array and an HPLC/PD detector. The analysis system was used under the following operating conditions: capillary voltage, 3.5 kV; cone voltage, 30 V; extractor voltage, 5 V; lens voltage, 0.1 V RF; source temperature, 120°C; desolvation gas flow, 300 L·hr<sup>-1</sup>; cone gas flow, 50.0 L·hr<sup>-1</sup>; column, TSKgel® Amide-80 (particle size, 3  $\mu$ m; and dimensions, 2.0 mm in diameter  $\times$  15 cm) (Tosoh, Tokyo, Japan); column temperature, 30°C; solvent, CH<sub>3</sub>CN (80/20); and flow rate, 0.2 mL·min<sup>-1</sup>.

### 2.4. Analysis of Intracellular Amino Acids by High-Performance Liquid Chromatography

We used the AccQ-Tag Amino Acid Analysis Method (Waters, Milford, Massachusetts, USA). We extracted the colonies with the varying ratios of akinetes with 80% methanol. The solutions were concentrated to dryness in vacuo at 38°C, as described above. The extracted solutions were acid-hydrolyzed with 6N HCl at 110°C for 6 h. The solutions were evaporated to dryness in vacuo at 38°C to remove the acid. The extracted water solution that resulted from the acid hydrolysis was further purified through a 0.45- $\mu$ m filter (Tosoh, Tokyo, Japan). The solutions were again evaporated to dryness in vacuo at 38°C. We added 20  $\mu$ L of HCl (20 mM), 60  $\mu$ L of AccQ Fluor Borate Buffer, and 20  $\mu$ L of AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) to the solutions. We performed a derivatization of the amino acids in the solutions by heating at 55°C for 10 min. The AccQ Fluor Reagent reacts rapidly with primary and secondary amino acids. Each extract was analyzed by high-performance liquid chromatography (HPLC) with a 2695 Separation Module with a 2996 Photodiode Array (Waters, Milford, Massachusetts, USA). The analytical conditions were as follows: column, AccQ-Tag Amino Acid Analysis Column (particle size, 4  $\mu$ m; and dimensions, 3.9 mm in diameter  $\times$  15 cm) (Waters, Milford, Massachusetts, USA); eluted with Eluent A (aqueous buffer), Eluent B (100% acetonitrile), and Eluent C (MilliQ water); flow rate, 1.0 mL·min<sup>-1</sup>; and detection at 254 nm. The gradient conditions were in accordance with the gradient table in the instruction manual for the AccQ-Tag Amino Acid Analysis Method, starting

with 100% Eluent A, followed by 99% Eluent A to 1% Eluent B after 0.5 min, 95% Eluent A to 5% Eluent B after 18 min, 91% Eluent A to 9% Eluent B after 19 min, 83% Eluent A to 17% Eluent B after 29.5 min, 60% Eluent B to 40% Eluent C after 33 min, and 100% Eluent A after 36 min (all ratios are volume per volume).

### 2.5. Measurement of Level of Protein Aggregation

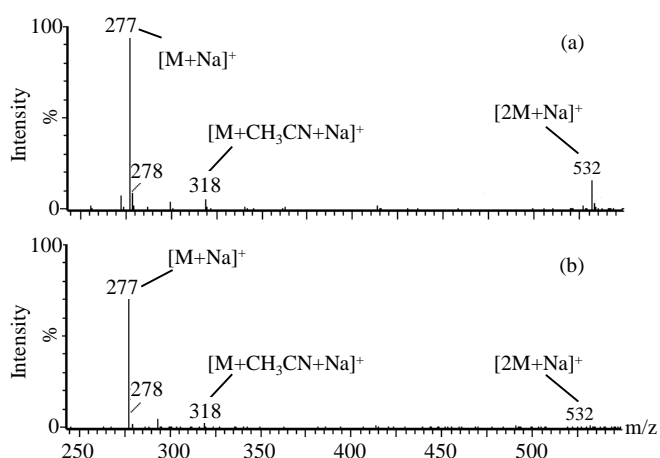
We use a protein aggregation assay kit, PROTEOSTAT® (Enzo Life Sciences, Farmingdale, New York, USA), to measure the extent of protein aggregation. Each sample was mixed with 200 µg/mL LDH (Roche, Berlin, Germany), then heated in an ND-M01 Thermoblock (Nissin, Tokyo, Japan) at 50°C for 90 min. We added the PROTEOSTAT® detection reagent (Enzo Life Sciences, Farmingdale, New York, USA), which emits fluorescence when bound to aggregated proteins, to the samples in a 96-well plate. After the samples were kept in the dark for 15 min at 26°C, we measured the fluorescence of the samples (excitation: 500 nm, emission: 603 nm) with a Varioskan™ microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

### 2.6. Measurement of Enzymatic Activity

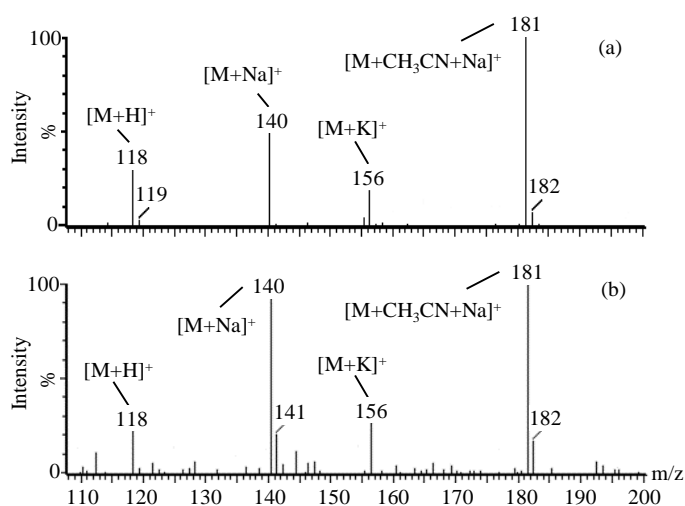
The Cytotoxicity LDH Assay Kit-WST (Dojindo, Kumamoto, Japan) was used to measure enzymatic activity. We heated 1 µg/mL LDH in an ND-M01 Thermoblock (Nissin, Japan) at 50°C - 70°C for 0 - 60 min. We then added 50 µL of working solution to 50 µL LDH. After the samples were kept in the dark for 30 min at 26°C, we added 25 µL of stop solution. We measured the absorbance ( $\lambda$ , 490 nm) with a Varioskan™ microplate reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

## 3. Results

We previously reported that some substances that accumulate in *Nostoc* sp. HK-01 akinetes could contribute to its heat tolerance as compatible solutes, including sucrose [33]. We isolated and identified substances other than sucrose from the hydrophilic fractions of *Nostoc* sp. HK-01 cell extracts that accumulated specifically in akinetes. Three candidate compounds, in addition to sucrose, were confirmed in the extracts from the colonies of *Nostoc* sp. HK-01. We measured the molecular weight of the extracts via the positive mode of LC-ESI-MS. We detected the molecular ions  $[M + Na]^+$  277,  $[M + CH_3CN + Na]^+$  318, and  $[2M + Na]^+$  532. These ions were consistent with glucosylglycerol ( $C_9H_{18}O_8$ , 254.23), and were detected at a retention time of 10.5 min (**Figure 1**). The molecular ions  $[M + H]^+$  118,  $[M + Na]^+$  140,  $[M + K]^+$  156, and  $[M + CH_3CN + Na]^+$  181, which are specific to betaine ( $C_5H_{11}NO_2$ , 117.15), were detected at a retention time of 11.7 min (**Figure 2**). We analyzed the amino acids in the cell extracts of *Nostoc* sp. HK-01 by HPLC with the AccQ-Tag Amino Acid Analysis Method. The HPLC peak of glycine was detected at a retention



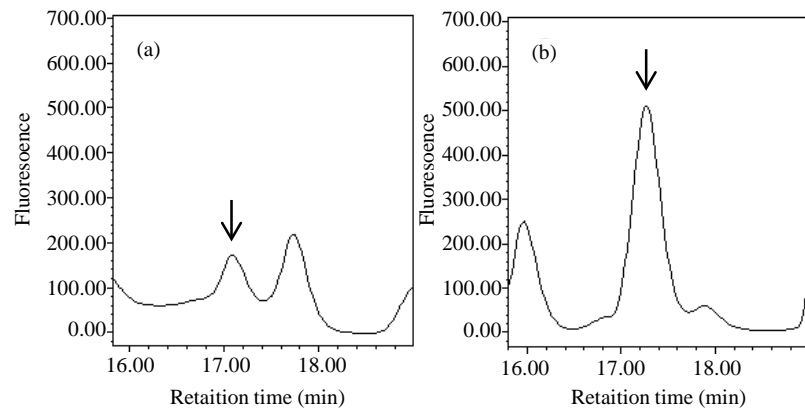
**Figure 1.** Mass spectra of glucosylglycerol ( $C_9H_{18}O_8$ , 254.23) and the cell extracts of *Nostoc* sp. HK-01 analyzed by positive-mode LC-ESI-MS. (a): Glucosylglycerol. (b): Cell extract of *Nostoc* sp. HK-01.



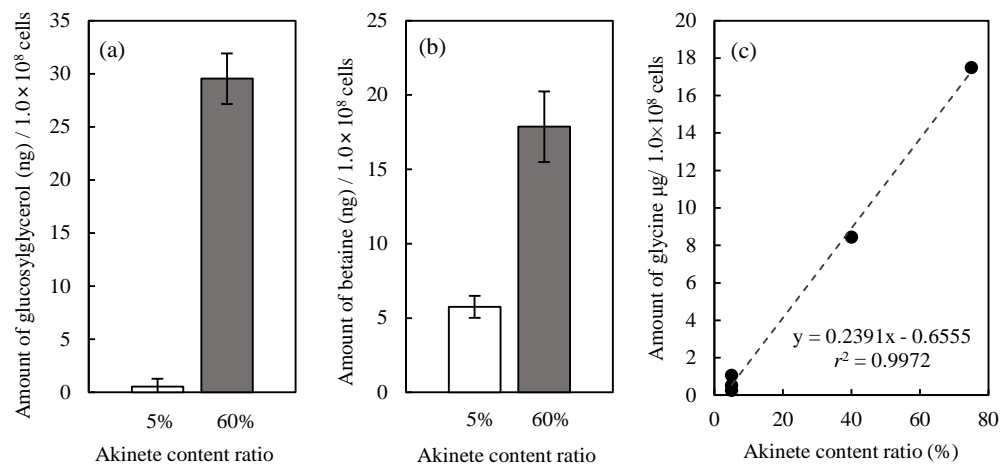
**Figure 2.** Mass spectra of betaine ( $C_5H_{11}NO_2$ , 117.15) and the cell extracts of *Nostoc* sp. HK-01 analyzed by positive-mode LC-ESI-MS. (a): Betaine. (b): Cell extract of *Nostoc* sp. HK-01.

time of 17.1 - 17.3 min (**Figure 3**). We performed a quantitative analysis of glucosylglycerol, betaine, and glycine in several colonies with differing akinete content ratios. We detected 0.5 ng glucosylglycerol per  $1.0 \times 10^8$  cells in a colony with 5% akinetes and 29.5 ng per  $1.0 \times 10^8$  cells in a colony with 60% akinetes (**Figure 4(a)**). We also detected 5.8 ng betaine per  $1.0 \times 10^8$  cells in a colony with 5% akinetes and 17.9 ng per  $1.0 \times 10^8$  cells in a colony with 60% akinetes (**Figure 4(b)**). Glycine was present at 0.3  $\mu$ g per  $1.0 \times 10^8$  cells in a colony with 5% akinetes, 8.4  $\mu$ g per  $1.0 \times 10^8$  cells in a colony with 40% akinetes, and 8.4  $\mu$ g per  $1.0 \times 10^8$  cells in a colony with 75% akinetes (**Figure 4(c)**).

The accumulation of sucrose specifically in the akinetes of *Nostoc* sp. HK-01 has already been reported by Kimura *et al.* [33]. The presence of trehalose in the colonies has also already been reported, although not specifically in the akinetes



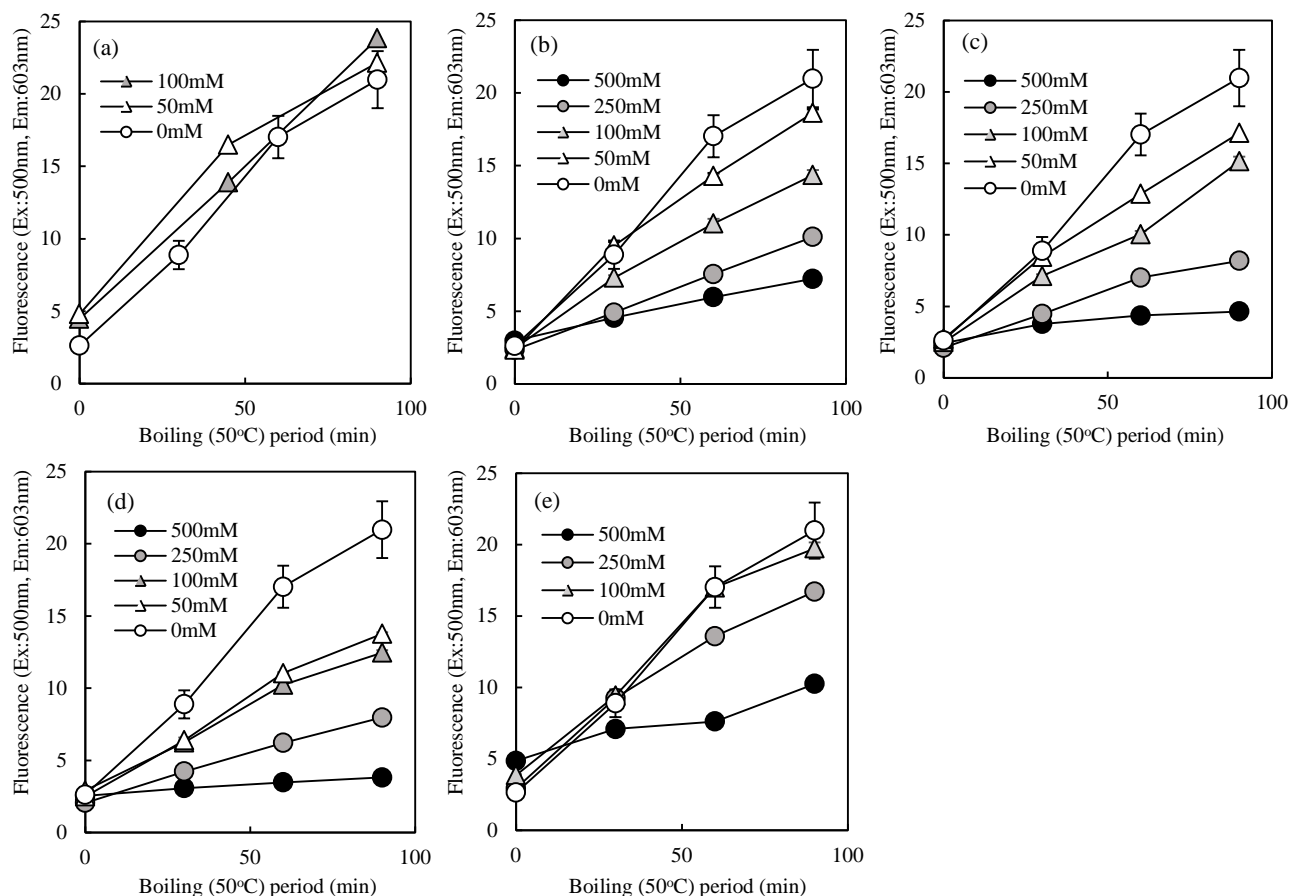
**Figure 3.** HPLC chromatograms of glycine ( $C_2H_5NO_2$ , 75.07). (a): Standard amino acids. (b): Cell extracts of *Nostoc* sp. HK-01. Arrows show peaks for glycine.



**Figure 4.** The quantity of the low molecular weight substances per  $1.0 \times 10^8$  cells in colonies of *Nostoc* sp. HK-01. (a): glucosylglycerol (ng). (b): betaine (ng). (c): glycine ( $\mu$ g).

[33]. We next tested the activities of glucosylglycerol, betaine, glycine, sucrose, and trehalose for the capacity to prevent protein aggregation in the context of heating. The fluorescence intensity of the control, LDH, with no additives after exposure to heat at  $50^\circ\text{C}$  for 90 min was 21.0. The fluorescence intensities of LDH in the presence of 50 mM and 100 mM glucosylglycerol were 22.2 and 23.8, respectively (Figure 5(a)). The fluorescence intensities of LDH with 50 mM and 100 mM betaine were 18.6 and 14.4, respectively (Figure 5(b)). The fluorescence intensities of LDH with 50 mM and 100 mM glycine were 17.3 and 15.1, respectively (Figure 5(c)). The fluorescence intensity of LDH with 50 mM and 100 mM sucrose were 13.8 and 12.5, respectively (Figure 5(d)). The fluorescence intensities of LDH with 100 mM and 250 mM of trehalose were 19.7 and 16.7, respectively (Figure 5(e)). Betaine, glycine, sucrose, and trehalose demonstrated activity against protein aggregation (Figures 5(b)-(e)), but glucosylglycerol did not (Figure 5(a)). We tested the relationship between the level of protein aggregation and the enzymatic activity of LDH. The enzymatic activities were 79.7%, 56.8%, and 5.3% when the fluorescence intensities were 5.7, 7.0, and 23.5,





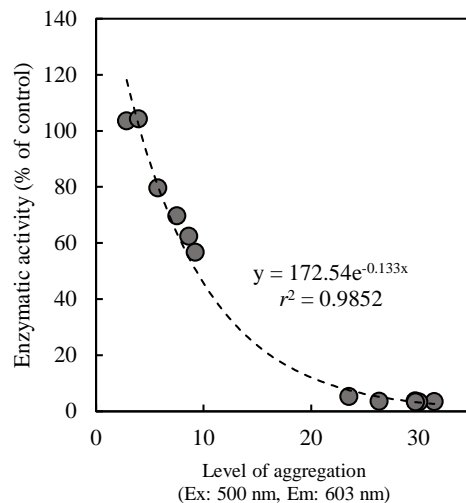
**Figure 5.** Effects of the substances from the colonies of *Nostoc* sp. HK-01 on the aggregation of 200 µg/mL LDH boiled at 50°C. (a): glucosylglycerol. (b): betaine. (c): glycine. (d): sucrose. (e): trehalose.

respectively, indicating a correlation (Figure 6;  $y = 172.54e^{-0.133x}$ ,  $r^2 = 0.9852$ ).

#### 4. Discussion

Our summarized results are shown in Table 1. The quantities of glucosylglycerol, betaine, and trehalose in the colonies containing a high proportion of akinetes were 30 ng, 18 ng, and 30 ng per  $1.0 \times 10^8$  cells, respectively. We did not detect protein protective activity by glucosylglycerol. In addition, the accumulation of trehalose was not specific to akinetes [33]. The quantities of sucrose and glycine in the colonies with a high proportion of akinete were approximately 1000× higher than those of glucosylglycerol, betaine and trehalose, at 17 µg and 18 µg per  $1.0 \times 10^8$  cells, respectively. Thus, our results indicate that sucrose and glycine may be the main compatible solutes among the low molecular weight compounds in the hydrophilic fractions of the *Nostoc* sp. HK-01 akinetes. We confirmed that sucrose is not only an energy source, but that it also contributes to biomolecular protection in akinetes [33].

The dry state of an *Anabaena variabilis* akinete contains  $2.06 \times 10^{-10}$  g of solids and comprises 63% water [72] [73]. On average, the biomass of an *Aphanizomenon ovalisporum* (Nostocales) akinete was 0.4 ng (wet weight) [52] [74].



**Figure 6.** Correlation between enzymatic activity (% of control) and the level of LDH aggregation (n = 12).

**Table 1.** Compatible solutes in akinetes of *Nostoc* sp. HK-01.

|                   | Chemical formula                                | Molecular weight | Amount /10 <sup>8</sup> cells** | Accumulation in akinetes specifically | Protein protect activity to heat*** | Structure |
|-------------------|-------------------------------------------------|------------------|---------------------------------|---------------------------------------|-------------------------------------|-----------|
| Glucosyl-glycerol | C <sub>9</sub> H <sub>18</sub> O <sub>8</sub>   | 254.23           | 30 ng                           | +                                     | -                                   |           |
| Betaine           | C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub>  | 117.15           | 18 ng                           | +                                     | +                                   |           |
| Glycine           | C <sub>2</sub> H <sub>5</sub> NO <sub>2</sub>   | 75.07            | 18 µg                           | +                                     | +                                   |           |
| Sucrose           | C <sub>12</sub> H <sub>22</sub> O <sub>11</sub> | 342.30           | 17 µg*                          | +                                     | +                                   |           |
| Trehalose         | C <sub>12</sub> H <sub>22</sub> O <sub>11</sub> | 342.30           | 30 ng*                          | -*                                    | +                                   |           |

\*Data from Kimura *et al.* 2017 [33]. \*\*Values from colonies with high akinete content. \*\*\*Protein protective activity tested by LDH aggregation after heating (50°C).

Our data indicated that the dry weight of a *Nostoc* sp. HK-01 akinete is 0.25 - 0.4 ng (Ong *et al.*, unpublished data). We previously found 0.17 pg sucrose per cell in colonies with 60% akinetes [33]. In this study, we found 0.18 pg glycine per cell in colonies with 75% akinetes. Thus, the concentration of sucrose in akinetes would be 2 - 3.3 mM and the concentration of glycine 9.6 - 15.8 mM, assuming that the water content of a dried akinete is 63% [73]. The triggers for the differentiation of *Nostoc* sp. HK-01 vegetative cells to akinetes have not yet been

identified, although triggers including light intensity, temperature, and the concentration of inorganic nutrients have been reported for other species [50] [51] [74]. We were unable to prepare colonies with 100% akinetes in this study as the cultures contain many types of cells in various life cell cycle states [33]. Thus, our suggestion that akinetes accumulate higher concentrations of sucrose and glycine is based on the use of colonies with different ratios of akinetes. There is a possibility that the sucrose and glycine concentrations in akinetes were sufficient to protect essential biomolecules.

Several reports have described mechanisms for the prevention of protein aggregation by compatible solutes. Compatible solutes affect the surface tension of proteins [73] [75]. Arginine can prevent protein aggregation through the destabilization of intermediate proteins [75]. Sugars can decelerate the degradation of proteins in the solid state by reducing their mobility via hydrogen bonding [56]. Proteins are denatured when the free energy of the denatured state is lower than the free energy of the native state [76]. Several reports have suggested that protein aggregation can be prevented by the addition of low molecular weight compounds [66] [67] [68] [69] [70] [77]. These compounds might reduce the free energy of the native state, or increase the free energy of the denatured state [76].

We found that the levels of betaine, glycine, sucrose, and trehalose, which are related to heat tolerance, vary between colonies with low akinete contents and high akinete contents (Figures 4(a)-(c); [33]). The accumulation of these compounds could account for the ability of akinetes to tolerate dry heat. The protein protective activities of betaine, glycine, sucrose, and trehalose would have occurred at physiologically optimum concentrations, because each substance could function at different concentrations (Figures 5(a)-(e)). In this study, we demonstrated that the compounds of interest specifically accumulated in akinetes. We identified compatible solutes in *Nostoc* sp. HK-01 for the first time. The detailed mechanisms of the substances identified will be further investigated in our future studies.

## 5. Conclusion

In this study, we investigated the compounds that were responsible for the dry heat tolerance of *Nostoc* sp. HK-01. Our findings indicated that sucrose and glycine are the main compatible solutes in the hydrophilic fractions of cell extracts from *Nostoc* sp. HK-01 akinetes. This exceptional tolerance of *Nostoc* sp. HK-01 makes it a candidate organism for promoting soil formation on bare land and in closed ecosystems. We believe that our study makes a significant contribution to the literature, because it provides an insight into previously unknown compatible solutes that enable *Nostoc* sp. HK-01 to survive at temperatures to 100°C.

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