Growth of terrestrial cyanobacterium, Nostoc sp., on Martian Regolith Simulant and its vacuum tolerance

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Abstract The growth of terrestrial cyanobacterium, Nostoc sp., on the Martian Regolith Simulant (MRS) and its vacuum tolerance were studied as one of our challenges in this century to inhabit Mars. The viability of the tested cyanobacterium was evaluated by microscopic observation after staining by fluorescein diacetate (FDA). The general terrestrial cyanobacterial lump collected from the ground showed a significantly high tolerance to a high vacuum environment (10⁻⁵ Pa) for over one year. To scientifically elucidate its high tolerance function, Nostoc sp. HK-01 was used as another suitable scientific material for this study. After exposure to the high vacuum environment (10⁻⁵ Pa) for two weeks, Nostoc sp. HK-01 began to grow again. Some of it was also re-incubated again with a liquid culture medium. The A'MED (Arai’s Mars Ecosystem Dome) is designed to be installed on Mars for agricultural production. AMED was useful to conduct our study. We performed the fundamental experiment using the MRS. Nostoc sp. HK-01 was found to grow for over 140 days along with having the normal function of chlorophyll synthesis on the MRS. These results show the possibility that cyanobacteria could adapt to the MRS, and grow under the low pressure environment expected on Mars.

Key words: Mars, cyanobacteria, Nostoc, vacuum tolerance, A'MED

Introduction

The territory of humans has spread from the exploration of the moon surface in 1969 to the International Space Station construction, and recent attention to the moon has spread again for scientific research projects. The National Aeronautics and Space Administration (NASA) plans manned Mars missions after 2030 and has begun the study of the risk that space radiation has on the human body. There is a high possibility that humans might land on the Martian surface in the near future based on these plans (Horneck et al., 2001; Cucinotta et al., 2005). In Japan, the “Space Agriculture Task Force” consisting of 130 domestic and foreign researchers has a plan that habitation of 100 people can be accomplished on Mars for more than 20 years. They are doing significant research such as material circulation or food security (Yamashita et al., 2006, 2007).

There are several components of extreme environment, such as low pressure, high pressure, low temperature, high temperature, strong acidity, strong basicity and so on. The environment on Mars is a harsh environment for terrestrial life in terms of low temperature and low pressure. The comparisons of Mars and earth are as follows: the surface atmospheric pressure is about one-10⁶ (on average 7 × 10² Pa), the atmosphere consists of 95.32 % carbon dioxide. Based on the observations by the Mars space probes, Viking Landers 1 & 2 and the Mars Exploration Rovers, it is well known that the Martian average temperature is as low as −63 °C, and the major chemical components of the regolith covering the Martian surface are SiO₂, Al₂O₃, and Fe₂O₃ (Averner and MacElroy, 1976; Allen et al., 1979; Gross et al., 2003; Williams, 2004).

According to a numerical simulation modeled by Arai and Kimura (2008), the inside of the A'MED (Arai’s Mars Eco-systems Dome) warms by the greenhouse effect. A’MED is a closed dome, which will be installed on Mars. Temperature in the dome can be raised by the natural energy available on Mars by adjusting the number of layers on its top and its height after the introduction of inside air. If a man-made “closed dome” like the A’MED is installed on Mars, it is possible that a meteorologically mild environmental condition can be created that is suitable for the survival of terrestrial living creatures. Ultraviolet solar light and cosmic rays hitting Mars would be shielded, and the internal pressure, temperature, humidity, and light quantity would be controlled by the presence of a roof on the greenhouse. The crop production on Mars would occur by making the environment in the A’MED as close as possible to the terrestrial environment, and even humans could live in it. In addition, it would help us to reduce the materials supplied from the earth. However, the
process of introducing some living creatures on Mars or the selection of the most suitable creature has not yet been determined. Furthermore, in the early stage of the environmental condition in the A'MED installed on Mars, detailed experiments have not yet been performed to examine whether there is a problem with the photosynthetic functions of a plant becoming a crop and whether growth is possible on the oligotrophic Martian regolith. The A'MED would be useful to examine any of the biological experiments on earth as a simulated Mars environment.

There have been some studies related to the life of creatures under such a severe environment. As a creature with a tremendous drought tolerance, the dry larvae of the sleeping chironomid, *Polypedilum vanderplanki*, breeds in small pools in shallow hollows on unshaded rocks in dry Africa and the anhydrobiosis “tun stage” in water bears, *Milnesium tardigradum*, of Phylum Tardigrada have been investigated in resistant studies such as drying, temperature, pressure and chemicals. The revival of the dry larvae from the dormant chironomid was confirmed even after exposure to the high temperature of 103 °C and cryogenic temperature of −269 °C. The longest record was 17 years under the dry condition and also 168 hours of storage in 100 % ethanol or 30 minutes under a high vacuum (Hinton, 1960; Adams, 1985; Watanabe et al., 2005). There is a project in which “tun” is going into space to be exposed to the radioactive rays environment, because water bears have a high tolerance to the cryogenic temperature of −250 °C, and extreme pressure of 600 MPa (Saigusa et al., 2006). There are experiments related to the tolerance in extreme environments of plant functions about their seed germination after high vacuum exposure of about 46 species of seeds (Hashimoto, 2003). The study to confirm the revitalization of the photosynthetic function after a vacuum exposure of cyanobacteria as a prokaryotic organism has not yet been performed, although there such studies of plants (Hashimoto, 2003). Some terrestrial creatures have such a high tolerance to such extreme environments. During the evolution process of the earth’s atmosphere, the function of cyanobacterium, as one of the fundamental creatures, played extremely important roles. The cyanobacteria have a high nitrogen fixation ability and carbon assimilation by photosynthesis, and it is thought that they contribute to the formation of the earth’s atmosphere (Miyakawa, 2004). Cyanobacterium is a creature which has such features, so we suggest that it is possible that the cyanobacteria will significantly contribute to the changing composition of the primitive Martian atmosphere in the A'MED. The tolerance to extreme environments on earth involving 144 strains of cyanobacteria has already been studied. Especially, as for *Nostoc* sp., all the strains of *Nostoc* were revived after freezing to −196 °C by liquid nitrogen (Mori et al., 2002). Furthermore, Jones (1989) reported that when cyanobacterium, *Nostoc commune*, was re-wetted in the dark from the desiccated colonies, the ability of the nitrogen fixation was more than twice compared to nondesiccated colonies taken from the light and placed in the dark. These results indicate that *Nostoc* has a superior ability for nitrogen fixation (Jones, 1989). Recently, the possible application of *Nostoc* to increase soil organic matter and to reclaim degraded soil ecosystems was proposed(Obana et al., 2007). Above all, Kotoh et al. (2003) found that *Nostoc* sp. HK-01 has a superior drought tolerance, a high photosynthesis recovery ability, alkali tolerance and nitrogen fixing ability. The aquatic cyanobacterium, “Anabaena sp. Strain, PCC 7120”, has already had all its genomes decoded, while *Nostoc* sp. HK-01 has not yet been completed (Ehira et al., 2003). However, it has been elucidated that genes expressed more intensively during dehydration were screened in the terrestrial cyanobacterium, *Nostoc* sp. HK-01, which is phylogenetically very similar to the aquatic *Anabaena* sp., PCC7120. The gene expression during the hydration was expressed for a short time in *Anabaena*, but in the case of *Nostoc*, the expression increased until the wet mass decreased to 10 % of its original mass. It was shown that the higher desiccation tolerance of *Nostoc* was more intensely supported by the gene expression than in *Anabaena* (Yoshimura et al., 2006). These studies strongly indicated that *Nostoc* sp. HK-01 is a superior species for introduction into the dry land environment like Mars (Ohmori et al., 2006). However, there is no definite reference studies that utilized the Mars environment.

In this study, we assume that humans will perform agriculture using the lifeless Martian regolith in the previously-installed A’MED on Mars, and our final objective is to create the soil, in which plants can start to grow within a short time. When we perform agriculture on Mars, we suggest that *Nostoc* sp., especially the HK-01 species, will effectively create a medium for the stabilization of terrestrial creatures on Mars. It is particularly worth noting that information on the mechanism related to the tolerance of cyanobacteria to the high vacuum environment is required, since they will be exposed during transportation in addition to the growth adaptation ability of cyanobacteria on Mars under low ambient pressure. Therefore, the high vacuum tolerance and the revitalization capability of cyanobacterium were examined in this study.

**Materials and Methods**

**Biological materials**

Two species of terrestrial cyanobacteria, *Nostoc* sp. collected from the ground in Tsukuba City as an ordinary species, and *Nostoc commune* HK-01 as a purified one for the laboratory experiment to examine the tolerance in a low pressure environment and adaptation to the MRS, were used as the biological materials in this study. The collected species from the ground in Tsukuba City is a
popular species and the lump of cyanobacteria was not pure. Pure cyanobacterium was also used to determine their scientific function. Kotoh et al. (2003) isolated Nostoc sp. HK-01 from the soil in Hyogo Prefecture, Japan, and purified it using the method of Rippka (1988). Nostoc sp. HK-01 has been investigated in some studies at the molecular level. It has been stored at the Ohmori Laboratory of Saitama University.

Preparation of experiments for Nostoc sp.

For each experiment, a liquid culture of cyanobacteria was used as previously described (Rippka et al., 1979; Watanabe, 1960). A small amount of stored Nostoc sp. suspended in 4 ml of distilled water was incubated with shaking at 120 min⁻¹ in 40 ml of the liquid medium, BG-11, consisting of 0.5 M HEPES(C₈H₁₈N₂O₄S), ethanesulfonic acid, contained in a 100 ml Sakaguchi flask for 4 days under a fluorescent light (74.3 ± 24.3 μmol m⁻² s⁻¹) at 26 °C and atmospheric pressure, 10³ Pa (Rippka et al., 1979; Hughes et al., 1958; Allen and Stanier, 1968).

After the incubation, a 50 ml volume of the incubated suspension culture containing Nostoc sp. was aseptically separated from the liquid medium by centrifugation at 2000 rpm for five minutes (H-18 series desk centrifuge, Kokusan, Japan). After the centrifugation, the obtained pellet, which was almost the aggregated Nostoc sp., was moved to a 9-cm sterilized Petri dish. The Petri dish was stored in a desiccator (Nikko, Japan) that had been initially sterilized with ethanol and ultraviolet light before this experiment in the dark at 26 °C for further concentration to dryness. The dried Nostoc sp. was then subdivided into microtubes, and each mass was measured every day. The confirmation of sufficient dried materials was judged at the time when a change in the mass was within an error equal to or less than 1 mg. The dried materials were used for a high vacuum experiment.

Exposure of Nostoc sp. to a high vacuum

A pressure of 10⁻¹ Pa was used as the high vacuum pressure in this study. The high vacuum environment, 10⁻¹ Pa, in the chamber for Nostoc sp. was maintained by continuous exhaustion made by a turbo molecular pump. It took about 24 hours to reach the vacuum of 10⁻¹ Pa from the normal atmospheric pressure of 10⁵ Pa. The tubes had ten small holes made by a needle in their cap, and then the tubes with the dried Nostoc sp. were placed in the chamber for the high vacuum exposure for two weeks. As the control condition, an atmospheric pressure of 10⁵ Pa was prepared in the same room and run for two weeks. In the case of the ordinary Nostoc sp., a lump of them was used and exposed for over one year under the high vacuum condition.

Treatment of Nostoc sp. after exposure to high vacuum environment

After the high vacuum experiment, a 1000 µl of sterilized water was added to the tube containing the dried Nostoc sp. for swelling and kept in the dark at 25 °C for one day. The swelled Nostoc, which absorbed sufficient water, was placed in a 100 ml Erlenmeyer flask containing 40 ml of a BG-11 medium. They were incubated under continuous light (74.3 ± 24.3 μmol m⁻² s⁻¹) at 26 °C and 10³ Pa with 120 min⁻¹ of shaking for a certain period. After the incubation, we took pictures to record the cell proliferation, removed some cellular mass from the liquid medium every few days and confirmed the revitalization of the cell by fluorescent microscopic observations after cell staining.

Viability assay of Nostoc sp.

Fluorescein diacetate, FDA, was used for the staining of Nostoc sp. in this study, because the solution has already been shown as the most suitable and easy staining solution for cyanobacteria (Suzuki, 1999; Ishizuka and Sato, 2002). One mg of FDA pre-dissolved in 0.5 ml dimethylsulfoxide was re-dissolved in 0.2 ml acetone, and it was kept in a refrigerator as a stock solution of FDA. At each observation, 10 ml of Dulbecco’s Phosphate Buffered Saline, DPBS, was added to 0.04 ml of the FDA stock solution (final FDA concentration; 0.002 %). This solution was used as the FDA staining.

It was first equally subdivided into a microtube shaded from the light, so that the FDA did not fade, and the cultured Nostoc sp. was crushed using a mixer (Test Tube Mixer TM-251, Iwaki, Asahi Techno Glass, Japan) for one minute. A 200 µl cell suspension of Nostoc sp. was then placed in by a new tube. A 100 µl accessible FDA solution was added to the tube and mixed under a concussion culture at 37 °C for 10 minutes, then the

Table 1 Composition of Martian Regolith Simulant (Weight%, oxides)

<table>
<thead>
<tr>
<th></th>
<th>Na</th>
<th>Mg</th>
<th>Al</th>
<th>Si</th>
<th>P</th>
<th>K</th>
<th>Ca</th>
<th>Ti</th>
<th>Mn</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Martian Regolith (observed value)²⁴</td>
<td>1.34</td>
<td>6.00</td>
<td>7.20</td>
<td>43.40</td>
<td>0.68</td>
<td>0.10</td>
<td>5.80</td>
<td>0.60</td>
<td>0.45</td>
<td>18.20</td>
</tr>
<tr>
<td>Martian Regolith Simulant (Total of three ingredients)</td>
<td>2.54</td>
<td>2.11</td>
<td>12.74</td>
<td>59.67</td>
<td>–</td>
<td>0.27</td>
<td>3.12</td>
<td>0.28</td>
<td>0.07</td>
<td>14.81</td>
</tr>
<tr>
<td>Three ingredients</td>
<td>Basaltic sand from Miyake Island (20%)²³</td>
<td>3.97</td>
<td>2.86</td>
<td>14.00</td>
<td>52.66</td>
<td>–</td>
<td>0.67</td>
<td>8.62</td>
<td>1.42</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>Kunigel (70%)²⁵</td>
<td>2.50</td>
<td>2.20</td>
<td>14.20</td>
<td>70.20</td>
<td>–</td>
<td>0.20</td>
<td>2.00</td>
<td>–</td>
<td>2.50</td>
</tr>
<tr>
<td></td>
<td>Ferric oxide powder (10%)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

²⁴Data based on direct soil analyses from Viking X-ray Fluorescence Spectrometer and SNC meteorite reported by Kieffer et al. (1992).
²³Data based on direct soil analyses from X-ray Fluorescence Spectrometer reported by Kato et al. (2005).
stained cells were agitated in order to have the FDA permeate the cell membrane (Deep Well Maximizer) (Bio Shaker, TAITEC, Japan). After 5 minutes on ice, an appropriate amount of the cell suspension was placed on a slide glass and observed under a fluorescent microscope (BX50 type, OLYMPUS, Japan). We used optics and a fluorescence NIBA filter (excitation wavelength, 470-490 nm) for observations at 100, 400 and 1000 magnifications (10×10, 10×40, 10×100). The observed images were recorded by a microscopic digital camera and the intensity of the fluorescence and the number of viable cells were compared. The rate, %, of the vital cells was counted within several viewing fields with an area of 22500 µm² each, and statistically calculated.

The growth of Nostoc sp. on the Martian regolith simulant (MRS)

The mixture of basaltic sand from Miyake Island, KUNIGEL-VI (Kunimine Industries Co., Ltd., Japan) and ferric oxide powder at the ratio of 2:7:1 simulated the Martian regolith analyzed by NASA’s Mars probes, Viking landers and Mars Exploration Rovers (Allen et al., 1979; Gross, 2003). The detailed composition of the regolith stimulant is shown in Table 1. In this study, we used this as the “Martian Regolith Simulant” (MRS). A glass cup (snap cup) filled with a certain volume of MRS was sterilized at 120 °C for 3 hours (Eyela Windy Oven, WFO-450SD, Tokyo, Japan) for each experiment.

For the regolith adaptation examination of Nostoc sp., the “regolith MDM medium”, MRS-MDM, was prepared by mixing the MRS and MDM medium in the volume ratio of 1:3. The MDM medium has been used as a medium for Nostoc sp., and its components are the same as described by Watanabe (1960). The agar (Nacalai Tesque, Inc., Kyoto, Japan), gelled at low temperature around 30 to 31 °C, was added to the MDM medium. The ratio of MDM to the MRS medium (the MDM-MRS medium) in each snap cup was adjusted to 0, 20, 40, 60, 80 and 100 % (MDM concentration). The 300 µl cell suspension of Nostoc sp. HK-01 was directly placed on top.

We examined the revitalization from the dormant HK-01 after high vacuum exposure

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Revitalization and recovery of the growth of Nostoc sp. HK-01 after high vacuum exposure

A lump of cyanobacteria was used in this study to determine the general cyanobacterial tolerance ability to a high vacuum condition. Their revitalizing ability has been maintained over 20 % even after a long exposure such as over one year at 10⁻⁵ Pa (Fig. 1). It showed a high tolerance to the vacuum condition. The lump was quite contaminated and many species of organisms were recognized by the microscopic observation (data not shown). This result indicates that terrestrial cyanobacteria have an extremely high tolerance to a high vacuum environment, although they are not a pure lump. During the long exposure at 10⁻⁵ Pa, there would be the possibility of an expression related to some functions by their interaction. To scientifically study the functions in detail during their tolerance, a pure species is should to be examined.

Results and Discussion

Revitalizing ability of general cyanobacterial lump(GCL) after extremely long exposure to high vacuum

A lump of cyanobacteria was used in this study to determine the general cyanobacterial tolerance ability to a high vacuum condition. Their revitalizing ability has been maintained over 20 % even after a long exposure such as over one year at 10⁻⁵ Pa (Fig. 1). It showed a high tolerance to the vacuum condition. The lump was quite contaminated and many species of organisms were recognized by the microscopic observation (data not shown). This result indicates that terrestrial cyanobacteria have an extremely high tolerance to a high vacuum environment, although they are not a pure lump. During the long exposure at 10⁻⁵ Pa, there would be the possibility of an expression related to some functions by their interaction. To scientifically study the functions in detail during their tolerance, a pure species is should to be examined.

Revitalization and recovery of the growth of Nostoc sp. HK-01 after high vacuum exposure

We examined the revitalization from the dormant
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Table 2 The fresh mass of Nostoc after 8 day incubation with liquid medium

<table>
<thead>
<tr>
<th>Ambient pressure in prior to incubation (Pa)</th>
<th>Fresh mass (mg)</th>
<th>growth rate (b/a)</th>
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</thead>
<tbody>
<tr>
<td>exp. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^5$</td>
<td>1.49</td>
<td>31.9</td>
</tr>
<tr>
<td>$10^3$</td>
<td>1.23</td>
<td>14.0</td>
</tr>
<tr>
<td>exp. 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^5$</td>
<td>0.84</td>
<td>29.0</td>
</tr>
<tr>
<td>$10^3$</td>
<td>0.66</td>
<td>16.0</td>
</tr>
</tbody>
</table>

state, i.e., the state without metabolic activity, of the cyanobacterium, *Nostoc* sp. HK-01, which was naturally dried, after being exposed for two weeks in the high vacuum environment at $10^{-5}$ Pa, then incubated in the liquid BG-11 nutrient medium. We confirmed the revitalization of the cells in *Nostoc* sp. by the increase in the population of the cells and fluorescence after the FDA cell staining. The mass reduction rate of exposed *Nostoc* sp. was calculated using the following equation: $((A-B)/A)\times100$, where $A$ indicates the sample mass before its exposure, and $B$ indicates the sample mass after its exposure. The reduction of the mass of the exposed *Nostoc* sp. was 7.5% - 14.2% which was 10.6% on average, while that of the control was 0% - 5.7% or 2.2% on average. Some past reports showed that there was a correlation between the drought tolerance and freeze tolerance, and there was non frozen water in the cyanobacterium cells. However, there has been no reports about the relationship between the desiccation process of the cell and its period as to whether the short-term desiccation of the cell by the high vacuum leads to extinction of the cyanobacteria (Cameron, 1962; Katoh et al., 2003; Lin et al., 2004; Yoshimura et al., 2006).

The amount of dry weight of *Nostoc* sp. exposed to a high vacuum for two weeks was reduced faster than that of the control. After the high vacuum exposure, we performed their incubation using a liquid culture in a BG-11 medium under the condition of light irradiation $(74.3 \pm 24.3 \mu\text{mol} \text{m}^{-2}\text{s}^{-1})$, $10^3$ Pa, 120 shaking min$^{-1}$, 26 °C for eight days. After the incubation and filtration, the fresh mass of *Nostoc* sp. was measured. The increase in mass of *Nostoc* sp. exposed to the high vacuum was either equal to or surpassed that of the control (Table 2).

FDA stained cells were observed by a fluorescent microscope with excitation at the wavelength of 470-490 nm (Fig.2). The viable cells showed a green fluorescence. Dittmer and Weltzien (1990) reported the staining mechanism of FDA as follows. Since FDA is a non-polar molecule, it is carried into a cell by active transport. It is then hydrolyzed by esterase, and turns into a polar molecule, fluorescein. Because of its polarity, fluorescein cannot be eliminated from the cell, and is accumulated there. Fluorescein trapped in the cell emits its green fluorescence when activated by a blue light. The FDA staining indicates the esterase activity by the multiple esterase enzymes in the viable cell. In this context, the fluorescence of FDA staining does not verify the metabolism activity of the cell (Steward et al., 1999a, 1999b). We should limit our conclusion to the esterase activity of the *Nostoc* sp. incubated with the liquid medium after being exposed to a high vacuum environment for two weeks.

Figure 2 shows the optical and fluorescence microphotographs (150 µm × 150 µm) of *Nostoc* sp. HK-01 incubated in liquid medium for 0 to 8 days. They were stained by FDA after the exposure to a high vacuum; (b), (f) and (j)). Viable cells stained by the FDA emitted a green fluorescence with an excitation wavelength at 470-490 nm. The *Nostoc* after being exposed to a high vacuum increased as well as the *Nostoc* that was not exposed. The proliferation rate of the vacuum exposed *Nostoc* was similar to that of the control, and a green fluorescence was observed for the long chained *Nostoc*.

Figure 3 shows the occurrence of viable cells stained by the FDA solution. They were observed as a green fluorescence made by the esterase activity at a wavelength of 470-490 nm. After the high vacuum exposure, a small amount of *Nostoc* sp. was stained by the FDA solution during the 0- to 8-day incubation, and was compared to the *Nostoc* sample incubated under the control pressure of $10^3$ Pa. The vertical axis indicates the ratio of the green fluorescent viable cells observed several times in a specific area (150 µm × 150 µm). The occurrence of living cells during the early incubation period of the vacuum at $10^{-5}$ Pa was higher than that of the control at $10^3$ Pa (Fig. 3). The occurrence of living cells during the 8-day incubation in the vacuum at $10^{-5}$ Pa reached 100% as did the control. These results show clear evidence that there was almost no difference in the proliferation ability between the exposed high vacuum and control conditions (Fig. 3). The esterase activity of the cells would be recovered within 8 days after the high vacuum exposure.

Based on these results, it was found that the cyanobacterium *Nostoc* sp. has not only a dry tolerance, but also a high vacuum tolerance function. The high vacuum tolerance of the cyanobacteria along with the cell observations and re-incubated investigation has never been documented until this present report. Because the atmospheric pressure on Mars is about $10^3$ Pa, much lower than that on earth, $10^5$ Pa, it is thought that the cyanobacteria including *Nostoc* sp. would be suitable as a
photosynthetic organism having a low pressure tolerance for uses in the Mars environment.

**Incubation of Nostoc sp. HK-01 on the regolith nutrient medium (MRS-MDM)**

The growth of *Nostoc* on the MRS containing the nutrition of the MDM adjusted to 0, 20, 40, 60, 80, 100 %, for the long term of 75 days, was observed and a remarkable growth was seen even in the oligotrophic medium, MDM 0 % (Fig. 4-a). These results confirmed that *Nostoc* sp. could grow on the Martian surface for a long time without nutritional compounds in the simulated small scale of the A'MED, φ 30 cm and 30 cm high, with the capacity of about 20 liters in the laboratory. The water content in the “Regolith medium” in the 75 ml snap cup is 37.7 %, for the 40 ml regolith volume. As the MRS was losing water, the cracks in the MRS were expanding day by day, and the capacity to retain water was observed to decrease. Because the water absorption coefficient of the KUNIGEL is 800 ml water / 100 g dry KUNIGEL for the regolith medium,
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Water is in the unsaturated state. Under this condition, the KUNIGEL does not release free water (Kunimine Industries Co., Ltd., URL). Water would mainly be discharged into the air by the aspiration of *Nostoc* sp. After 75 days from the initiation, a large quantity of dew drops of water was observed on the wall surface of the container of the A’MED, and the dropped water stuck to the transparent cover which covered the snap cup. Some cracks and cavities developed in the MRS during drying inside the snap cup (Fig. 4b). In the MRS - MDM 80 % ratio, over ten new colonies of *Nostoc* sp., each having a diameter of approximately 2 mm, grew in part of the regolith gap inside the cup (Fig. 4c). In the other MRS - MDM ratios, we could not observe the formation of a new colony. *Nostoc* sp. mainly formed a colony on the cracked part of the regolith and grew (Fig. 4d). In all the MRS - MDM ratios, the surfaces of the *Nostoc* sp. were moist, and dozens of air bubbles of 2 mm size were confirmed. Especially, in the MRS without MDM, MRS - MDM 0 %, big bubbles growing over 5 mm were observed on the surface of *Nostoc* sp. (Fig. 4a). The bubbles seem to have grown without being broken in the presence of highly viscous compounds excreted from the cell of *Nostoc*. In the MRS - MDM 60 % ratio, *Nostoc* was seen growing in the 5 mm cracks and grew in the form of a bridge, which was sustained due to the highly viscous substance (Fig. 4b). It is suggested that this high viscosity is associated with polysaccharides, since Yoshimura *et al.* (2006) reported that *Nostoc* sp. HK-01 produces extracellular polysaccharides. On the other hand, in all the MDM ratios (0, 20, 40, 60, 80, 100 %) without MRS, regardless of the MDM contents, all the *Nostoc* sp. whitened after the 75-day incubation (data not shown). From these observations and findings, *Nostoc* sp. has a suppressive effect on the crack expansion by the desiccation of the regolith. Furthermore, it also released extracellular polysaccharides inside the regolith layer during their invasion into it. It is regarded that *Nostoc* sp. is an extremely effective organism, which convert regolith to organic soil (Fig. 4).

**Fig. 5.** Ecopoisis in Martian Regolith by cyanobacterium, *Nostoc* sp. HK-01, in A’MED (Arai’s Mars Eco-systems Dome, A’MED).

![Fig. 5. Ecopoisis in Martian Regolith by cyanobacterium, Nostoc sp. HK-01, in A’MED.](image)

**Fig. 6.** The 140 - day incubation of *Nostoc* sp.HK-01 on the oligotrophic MRS medium (MDM = 0 %, 20 %) in a mini-scale A’MED. a; Regolith medium of MDM 0 %, b; Regolith medium of MDM 20 %, c; MDM 0 % medium, d; MDM 20 % medium.
the growth of the *Nostoc* and pH of the MRS in a future study.

The recovery of the chlorophyll synthesis in cyanobacteria after exposure to a high vacuum was an important subject to be examined. We examined this function using the materials incubated for a longer period, 140 days, with the filter placed on the MRS. In this study, we assumed the initial quantity of nourishment and water would be given to start growth of the plant on the Martian regolith, when humans introduced them to the Martian environment. Based on this assumption, we tested the growth of *Nostoc* sp. on the MRS in the oligotrophic state for a longer term and examined it using a laboratory scale model of the A'MED (Fig. 5). In the small scale A'MED (φ = 4 cm, 6 cm in height, snap cup of about 75 ml capacity), *Nostoc* sp. was incubated over 140 days with the oligotrophic MRS medium including the 0 % and 20 % MDM nutrients. It was shown that they could be grown for the 140 days (Figs. 6 a and b). Furthermore, a different growth of *Nostoc* was remarkably observed on day 140, between the MRS - MDM (0 % and 20 %) and only the MDM. The growth of *Nostoc* on the MRS - MDM was higher than that on the MDM based on the observation and the analysis of the chlorophyll synthesis (Fig. 6). The amount of chlorophyll a made by the *Nostoc* sp. grown on the MRS 100 % was 6.4 μg ml⁻¹ and that on the MDM 100 % was 2.5 μg ml⁻¹, thus a remarkable difference had developed.

On the other hand, on a ceiling part of the small scale A'MED, many water drops were always observed. These drops of water dropped onto the regolith surface like rain. Therefore, *Nostoc* sp. was always kept in the wet state. On the surface of the cyanobacterium mat on the MDM regolith medium, many air bubbles were always observed. In the small scale A'MED, the cracking of the MDM - MRS medium and the capacity decrease of the medium was due to the direct falling of the water drops. Based on these results, if, as for *Nostoc* sp., there is a small amount of water, carbon dioxide and light in the alkaline oligotrophic MRS of pH 8 - 9, *Nostoc* can generate oxygen by photosynthesis inside the A'MED, and it was shown that it could grow for more than 140 days. Furthermore, *Nostoc* could directly grow on the MRS. It could be interpreted that they can live without liquid fertilizer. It also strongly suggests that it could produce a good promoting material for the organification of the regolith, and it also has a humidity adjustment function in the A'MED.

The composition of the regolith of Mars is estimated on the basis of the observations from the Mars space probe Viking Lander 1 in 1976 and the survey results of the surface material in the Gusev crater by the Mars Exploration Rover *Spirit*-1 in 2004. Based on these observed results, as for a Martian regolith simulant called JSC Mars-1, which NASA and ORBITEC jointly developed, the particle size consists of palagonite tephra equal to or less than 1 mm. The materials of this Martian regolith simulant was gathered on a slope of the Pu 'n Nene cinder located between the volcano of Mauna Kea and Mauna Loa in the Hawaii Islands (ORBITEC URL, Allen et al., 1979; Gross et al., 2003; Williams, 2004). The Martian regolith simulant of this study simulates the Martian regolith, but that the pH and dust particle size distribution of the Martian regolith are unconfirmed variables. In addition, the grain size of the Martian regolith simulant in this study is dependent on the microscopic KUNIGEL-VI (grain size of 63 μm mesh passing more than 90 %) holding volume of 70 % (Kunimine Industries Co., Ltd., URL). KUNIGEL-VI is a clay material, which is based on montmorillonite gathered from the bentonite mine of the Tsukinuno mineral deposit in Yamagata. Generally, the bentonite is a weathered mineral of volcanic ashes and the lava, which accumulated on the bottom of either a sea or lake. The bentonite used in this study is the volcanic vitric tuff, which has experienced a diagenetic alteration (Yokoyama et al., 2004). The main component of both JSC Mars-1 and the MRS used in this study is the volcanic ashes of basalt. In addition, the Mars exploration rover *Opportunity* discovered many characteristics of the volcanic action such as black basalt on the Meridiani Planum of abundant Fe₂O₃ (hematite). A globe of hematite of several centimeters in diameter was discovered from the bright Slickrock, and it was estimated that it was formed by subsurface water based on evidence from Earth. A vitriolic hydrated mineral, Jarosite (KFe(SO₄)₂(OH)₆), was also found in a sedimentary rock on the Martian surface as a hydrate. *Opportunity* discovered a density accumulation of chlorine and bromine on the rock surface inside the Endurance Crater. However, its concentration decreased when the rock was polished. It was estimated that the explored site became a temporary lake after the crater formation. Thus, this is mineralogic evidence that a volcano and a lake once existed on Mars in the past (Squyres et al., 2004a, 2004b, 2006; Klingelhöfer et al., 2004). If we consider this information, there could be a rock of alkaline volcanic ash origin that underwent anamorphism on the Martian surface that became the present regolith. Based on these findings, we definitely verified that *Nostoc* might grow in the Martian Regolith Simulant, MRS, which did not contain any organic matter. It will be necessary to perform a study that simulated more of Mars in the future by considering changes in the pH or the quantity of oxygen.

On the other hand, the fact that *Nostoc* sp. HK-01 can be revitalized after its exposure to a high vacuum environment confirmed that *Nostoc* as the best species to be introduced in the Mega-scale A'MED and the later colonization of Mars. It is also convenient as a fuss-free organism. A'MED will become an effective model for performing ecology experiments on Mars in the future.

This study showed that *Nostoc* sp. HK-01 could adapt on the MRS and sufficiently grow even if exposed
to a low pressure environment on Mars. The results of the significant tolerance to a high vacuum environment shown by the cyanobacterial lump would also provide useful information including some clues to elucidate the possible spreading of life from earth to extraterrestrial bodies.

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