Possible involvement of extracellular polymeric substrates of Antarctic cyanobacterium Nostoc sp. strain SO-36 in adaptation to harsh environments

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Abstract (300 words)

Cyanobacteria are some of the primary producers in extremely cold biospheres such as the Arctic, Antarctic, and vast ice sheets. Many genera of cyanobacteria are identified from these harsh environments, but their specific mechanisms for cold adaptation are not fully understood. *Nostoc* sp. strain SO-36 is a cyanobacterium isolated in Antarctica more than 30 years ago and regarded as a psychrotolelant species. To determine whether the strain is psychrotolelant or psychrophilic, it was first grown at 30 °C and 10 °C. The cells grew exponentially at 30 °C, but their growth stopped at 10 °C, indicating that the strain is only psychrotolerant. Microscopic analysis revealed that the morphology of the cells grown at 30 °C was filamentous and differentiated heterocysts, which are specialized cells for gaseous nitrogen fixation under nitrogen-deprived conditions, indicating that the strain can grow diazotrophically. The cells grown at 10 °C have a smaller size, shortened filament length and decreased chlorophyll content per cell. At 10 °C, the cells are aggregated with extracellular polymeric substrates (EPSs), which is a common mechanism to protect cells from ultraviolet light. These results imply that segmentation into short filaments was induced by photodamage at low temperatures. To fully understand the adaptation mechanisms of *Nostoc* sp. strain SO-36 for low-temperature conditions, next-generation sequencing analyses were conducted. Complete genome sequence of the strain revealed that it has one main chromosome of approximately 6.8 Mbp with 4 plasmids, including 6855 coding sequences, 48 tRNA genes, 4 copies of rRNA operons, and 5 CRISPR regions. Putative genes for EPS biosynthesis were found to be conserved in Nostocaceae regardless of their habitat. These results provide basic information to understand the adaptation mechanisms at low temperatures, and the strain can be a model organism to analyze adaptation to extreme environments.

Key Words (4 to 6, alphabetical order)

Antarctica; Cyanobacteria; Extracellular polymeric substrate; Genome sequence; *Nostoc* sp. strain SO-36

Introduction

Cyanobacterium is a globally distributed microorganism that performs oxygenic photosynthesis. Their habitat is mainly in the hydrosphere of both fresh water and sea water, but they are also found in soil. Cyanobacteria can adapt to various temperatures. In hot springs, thermophilic cyanobacteria, such as *Thermosynechoccocus elongatus*, can grow at temperatures higher than 50 °C (Dyer and Gafford 1961), whereas some cyanobacteria can also survive at low temperatures, including glaciers, frozen soil, and polar areas, such as Antarctica, which has one of the most extreme conditions on earth with the coldest and driest climates. Antarctica has continuous darkness during winter and continuous sunlight during summer. Cyanobacterial species classified into several genera (Leptolyngbya, Oscillatoria, Phormidium, and Nostoc) were also isolated in exposed soils, ice-covered lakes, and ice surface in Antarctic regions (Cavacini 2001; Ohtani 1986; Ohtani et al. 1991). Most Antarctic cyanobacteria are psychrotolerant or psychrotrophic because of their viability near 0 °C, and their optimum temperature for growth is usually higher than 15 °C (Singh and Elster 2007).

Since the first exploration to Antarctica in 1895, many countries have dispatched Antarctic research expeditions to investigate its ecosystems, ice core structure, aurora generation mechanisms, etc. A permanent Japanese research station, Syowa Station, was established on the East Ongul Island in Antarctica in 1957, where research teams have isolated several cyanobacterial species from cyanobacteria-moss association and surface soils on the sunny places of exposed rocks. *Nostoc* sp. strain SO-36 (hereafter, *Nostoc* SO-36) was isolated from a cyanobacteria-moss community on Padda Island, which is located 80 km southwest of the Syowa Station, in 1988. Since most polar cyanobacteria are psychrotolerant and not psychrophile (Tang et al. 1997), *Nostoc* SO-36 was also regarded as a psychrotolerant strain. *Nostoc* SO-36 has been used in several studies on the cloning of acyl-lipid desaturase genes and lipid composition analysis (Chintalapati et al. 2006; Chintalapati et al. 2007). However, it remains to be elucidated whether *Nostoc* SO-36 is a truly psychrotolerant strain, and if so, its difference from strains sensitive to low temperatures has not yet been identified.

The mechanisms of cold shock responses of cyanobacteria have been investigated using mesophilic species such as *Synechocystis* sp. PCC 6803 (Los and Murata 1999; Morgan-Kiss et al. 2006). The mechanisms include membrane fluidity maintenance and secondary RNA structure unwinding. It was also reported that the expression of some genes was induced via cold shock treatment (Suzuki et al. 2001). However, how cyanobacteria adapt to extreme conditions remains unclear. This lack of knowledge could be a result of the inaccessibility to cyanobacterial samples that thrive in polar regions, their slow growth, and bacterial contamination. Further, the cyanobacteria that thrive in Antarctica are subjected not only to cold stress but also to desiccation, irradiation, and chronic nutrient depletion. Ecological relationships with other bacteria, fungi, and plants are also important to understand stress resistance. For example, the production of extracellular polymeric substrates (EPSs) has been reported to play a protective role for various bacteria and algae in extreme cold and dry environments (Bhagat et al. 2017; Krembs et al., 2002; Tamaru et al. 2005). In general, EPSs act as a water and nutrient reservoir of bacteria in the surrounding microenvironment, and they may act as gelatinous barriers to reduce water loss in cells (Roberson and Firestone 1992). EPSs also contribute to the formation of a stable biofilm (Rossi and De Philippis 2015; Zippel and Neu 2011) and endure freeze–thaw treatment (Tamaru et al. 2005) of cyanobacteria. However, genomic sequences and information of EPS biosynthesis–related genes of Antarctic cyanobacteria remain

86 insufficient.

87 Here, we report that *Nostoc* SO-36 can survive but cannot proliferate in temperatures less than 10 °C,
88 indicating that the strain is not psychrophilic but psychrotolerant. We also deciphered the complete genome
89 sequence of this legacy strain that was isolated more than 30 years ago by the Japanese Antarctic Research
90 Expedition and identified its phylogenetic position and EPS biosynthesis–related genes. This genomic information
91 contributes to the research on the genetic backgrounds of psychrotolerant mechanisms of Antarctic cyanobacteria.

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Materials and Methods

Cyanobacterial strains and growth conditions

Nostoc sp. strain SO-36 (NIES-3992) and *Anabaena* sp. PCC 7120 were grown in a liquid medium of $BG11_0$ ($BG11$ without nitrate) (Stanier et al. 1971) at 30 °C in light (30–50 μmol m^{-2} s^{-1}) on a rotary shaker (120 rpm) (Awai et al. 2007). For low-temperature condition experiments, the cells were first grown in a $BG11_0$ medium at 30 °C to an optical density at 730 nm (OD_{730}) of approximately 1.0 and were inoculated into fresh $BG11_0$ to OD_{730} of approximately 0.1. Then, the new diluted culture was incubated at 10 °C under illumination, as described above.

Measurements of growth curve and pigment contents

The growth curves of *Nostoc* sp. strain SO-36 and *Anabaena* sp. PCC 7120 were constructed by diluting the cells in a fresh liquid $BG11_0$ medium. The initial OD of 0.1 was measured at a wavelength of 730 nm. The cell density was measured every 24 h for 1 week using UV-2600 (Shimadzu, Japan). The chlorophyll and total carotenoid contents of the cells were measured by extraction with 100% methanol (Arnon et al. 1974; Wellburn 1994).

Alcian blue staining and sugar content measurement

Nostoc sp. strain SO-36 and *Anabaena* sp. PCC 7120 cells were observed under an upright microscope (BX53, Olympus, Tokyo, Japan) at 1000× magnification and a stereo microscope (SZX7, Olympus). Heterocyst and/or carboxylic polysaccharides in the culture were stained with 1% Alcian blue in 3% acetic acid (pH 3.0) solution and were incubated for 10 min before observation (Di Pippo et al. 2013).

The total sugar content was analyzed using the phenol–sulfate method (DuBois et al. 1956). In brief, 200 μL of the samples was diluted with 200 μL of 5% (w/w) phenol and vortexed for 30 s. Then, 1 mL of sulfuric acid was added, and the tube was immediately vortexed for another 30 s and kept in a water bath at room temperature for 10 min. The total sugar contents were measured colorimetrically by measuring the absorption at 490 nm using UV-2600. Standard curves were determined via glucose dilution at various concentrations (0, 0.2, 1, 2, 10, 20, 100, and 200 nmol mL^{-1}).

Isolation of genomic DNA from *Nostoc* SO-36

The cells from 100 mL of 7–14-day cultures in BG11 (OD_{730} approximately 1.0–1.5) were collected via centrifugation, and the cell pellet was transferred to a mortar. Then, the cell pellet was frozen by liquid nitrogen and macerated into fine powder using a pestle. The powder was suspended in a lysis buffer (10 mM Tris-HCl pH 9.5, 10 mM EDTA, 100 mM KCl, 500 mM sucrose, 4 mM spermidine, 0.1% 2-mercaptoethanol, and 0.5% Triton X-100) (Nishi et al. 2019). Then, the suspension was centrifuged, and the supernatant was discarded. To remove polysaccharides, the precipitate was resuspended with sorbitol buffer (Souza et al. 2012) with slight modification (100 mM Tris-HCl, pH 8.0, 350 mM sorbitol, 5 mM EDTA, 1% polyvinylpyrrolidone-10, and 1% 2-mercaptoethanol) and centrifuged again to obtain a precipitate. Genomic DNA was extracted from this precipitate using Qiagen Genomic-tip 20/G (Qiagen, Venlo, The Netherlands) (Nishi et al. 2019). The concentrations of the samples were determined using NanoDrop (Thermo Fisher Scientific, Tokyo, Japan).

Whole-genome sequencing strategy

A sequencing library was synthesized using a PacBio Sequel Microbial Library Construction Kit (Pacific Biosciences of California, Inc., Menlo Park, CA) following the manufacturer's protocol. Whole-genome sequencing was performed using a massive parallel sequencer Sequel (Pacific Biosciences of California, Inc.) via the sequencing service of Macrogen Inc. (Macrogen Japan, Tokyo, Japan). A total length of 366 Mb of sequencing reads was obtained. The number of subreads, subread N50 values, and average read length were 46,289, 10,409 bases, and 7,914 bases, respectively. Sequencing reads were assembled *de novo* using Flye v.2.4.2 (Kolmogorov et al. 2019) with the following settings: selected minimum overlap, 2000 and predicted genome size, 8 Mbp. Assembly results were checked with QUAST v.5.0.2 (Gurevich et al. 2013) and Bandage v.0.8.1 (Wick et al. 2015), both with default settings and via a BLASTn search of the sequences. The single chromosome and four plasmid assemblies were circularized by removing the terminal overhanging region. No contamination of SMRTbell adaptor sequences in these super-contigs was confirmed by BLASTn search. The nucleotide sequence of the constructed circular genome was corrected by mapping the reads generated by the other massively parallel sequencer MiSeq (Illumina, Inc.). MiSeq reads were prepared with a read length of 302 base pairs (bp) and an average insert size of 700 bp. The reads were filtered for quality with a Phred score of 30 using a read-through adaptor trimming option in CLC Genomics Workbench v.11.0. (Qiagen) with the following parameters: mismatch cost, 2; indel cost, 3; length fraction, 0.95; similarity fraction, 0.95; and allow to read mapping to multi-loci, ignored. Gene prediction and functional annotation of the complete genome sequence were performed using the DFAST pipeline v.1.1.4 (Tanizawa et al. 2018) with an option using CyanoBase (Fujisawa et al. 2017) as the main annotation resource. The start codon of the *dnaA* gene was defined as the +1 position of the chromosome.

The nucleotide sequence similarity between the *Nostoc* SO-36 chromosome and that of closely related species was compared by constructing a diagonal dot plot using the Genome Traveler v.3.0.25 with default settings (In Silico Biology, Inc., Yokohama, Japan). The dot plot patterns were also confirmed using D-Genies v1.3.0 (Cabanettes and Klopp 2018).

Phylogenetic analysis

Phylogenetic analyses were performed by maximum-likelihood (ML) algorithms in a MEGA-X software ver. 10.1.7 (Kumar et al. 2018) with the following parameters: number of bootstrap replication, 1000; substitution model, Tamura-Nei model (Tamura and Nei 1993); rates among sites, uniform rates; gaps/missing data treatment, complete deletion; ML heuristic methods, NNI; initial tree for ML, default (NJ/BioN); and branch swap filter, moderate. The tree topology was also checked by comparing it with that obtained by neighbor-joining algorithms. A list of accession numbers of the 16S ribosomal RNA sequences used in this study is provided in Table S1.

Data availability

Original sequencing reads were deposited in the DRA/SRA/ENA database with accession numbers DRR337996 and DRR337997. The accession numbers of complete genome sequences were as follows: chromosome, AP025732; pANSO36A, AP025733; pANSO36B, AP025734; pANSO36C, AP025735; and pANSO36D, AP025736.

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Results

Nostoc SO-36 is a psychrotolerant cyanobacterium

Because *Nostoc* SO-36 was isolated in Antarctica, it was expected that the strain can grow (psychrotrophic) or at least survive (psychrotolerant) at low temperatures. To test this, this strain was grown at 10 °C and compared with a representative filamentous cyanobacterium, *Anabaena* sp. PCC 7120 (hereafter, *Anabaena* 7120) (Fig. 1). At 10 °C, *Nostoc* SO-36 could not grow but maintained its OD for at least 7 days. In contrast, after 7 days of culture, the OD of *Anabaena* 7120 decreased constantly and became ten times lower than that at the beginning. To confirm the viability of *Nostoc* SO-36 cells after incubation at 10 °C, the cold-treated cells were shifted to 30 °C. Since the cellular growth and color of *Nostoc* SO-36 were recovered within a day, we concluded the *Nostoc* SO-36 has ability to endure at 10 °C conditions (Figs. S1, S2). On the other hand, cellular growth of the *Anabaena* 7120 was not recovered in the same conditions (Fig. S2). Because *Nostoc* SO-36 could survive but not grow at low temperatures, its growth rate at a higher temperature (i.e., 30 °C) was examined. At 30 °C, *Nostoc* SO-36 and *Anabaena* 7120 grew exponentially. These results suggest that *Nostoc* SO-36 is psychrotolerant but not psychrotrophic.

Nostoc SO-36 grew in a filamentous shape very similar to that of *Anabaena* 7120 at 30 °C (Fig. 2a, c). The formation of heterocysts, which are cells specialized for gaseous nitrogen fixation, was observed in a medium without a fixed nitrogen source ($BG11_0$ medium), indicating that *Nostoc* SO-36 can grow diazotrophically. As for pigment contents, the low-temperature treatment decreased the chlorophyll content but not the total carotenoid content (Fig. 2i, j). At 10 °C, the filaments became much shorter compared with the cells grown at 30 °C (Fig. 2e). Moreover, the cells aggregated to make a small particle (Fig. 2g, h, about 100–500 μm in diameter) with some components, which are possibly EPSs.

To determine whether the EPS content increased at low temperatures, *Nostoc* SO-36 and *Anabaena* 7120 cells were stained with Alcian blue after 7 days of culture at 30 °C. Fig. 2b and d show that even at 30 °C, *Nostoc* SO-36 accumulated more oligosaccharides outside the cells compared with *Anabaena* 7120. The shorter filaments of *Nostoc* SO-36 grown at 10 °C showed EPS accumulation. Total sugar amount normalized by the value of OD_{730} which includes both EPS and intracellular sugars was higher in the cells grown at 10 °C compared with that of the cells grown at 30 °C (Fig. 2j). Although the increase of total sugar amount might indicate the accumulation not only EPS but also compatible solutes in the cells (Fig. 2j), the cells were aggregated at 10 °C and formed a large mass of surrounding EPS (Fig. 2g, h). We also tried to confirm that the total sugar accumulation and the aggregated cells with EPS barrier are effective to freeze-thaw tolerance using *Nostoc* SO-36 (Fig. S3). The cells of *Nostoc* SO-36 clearly showed tolerance to the freeze-thaw stress. Furthermore, pre-freezing temperature of the cells also important for the stress tolerance. Since *Nostoc* SO-36 survived in the extreme environments of Antarctica, such as freezing, desiccation, high light in summer, and low light in winter, it must have certain mechanisms to adapt to low temperatures. Genomic sequences contain fundamental information to understand these mechanisms (Chrismas et al. 2018); thus, a method was first developed to isolate the genomic DNA of *Nostoc* SO-36 at a grade high enough for next-generation sequencing (NGS) analysis.

Isolation of genomic DNA from *Nostoc* SO-36 and initial trial for NGS analysis

Nostoc SO-36 was isolated from a moss community on a southwest slope in Padda Island; thus, the sample could have a mixture of other microorganisms. Before this research was started, the strain was purified as mono cyanobacterial isolates using the agar plate method (Sakamoto et al. 2019). The obtained strain had no visible contamination under a microscope at this stage. We applied the DNA purification method with sorbitol (Nishi et al. 2019; Souza et al. 2012) to extract genomic DNA, because the removal of EPS of this strain was quite difficult by ordinary protocols with additional CsCl purification. Then, the isolated genome was subjected to NGS analysis using TruSeq Nano DNA Library Prep kits (Illumina, San Diego, CA) with eight cycles of polymerase chain reaction (PCR) amplification. A library with approximately 800 bp of averaged insert DNA size was sequenced using MiSeq following the manufacturer's protocol. Because this protocol and Illumina sequencing procedure include PCR for fragment amplification for analyses, it was found that almost half of the reads were sequences of other microorganisms. This was probably caused by GC-bias (more suitable GC content tends to have more Illumina reads) between the contaminant microorganisms and *Nostoc* SO-36. Then, the PacBio system was used because this method does not include many cycles of PCR steps.

Genomic features of *Nostoc* SO-36

The complete genome sequence of *Nostoc* SO-36 consists of 7,408,262 bp with a single circular chromosome and four plasmids named pANSO36A, pANSO36B, pANSO36C, and pANSO36D (Fig. 3, Table 1). The total length of the circular chromosome was 6,802,717 bp, with an average G + C content of 41.41%. The lengths and G + C contents of the four plasmids were as follows: pANSO36A, 271,158 bp, 40.81%; pANSO36B, 228,652 bp, 40.47%; pANSO36C, 69,903 bp, 41.25%; and pANSO36D, 35,832 bp, 39.98%. Functional annotation revealed a total of 6855 CDSs, 48 tRNA genes, 4 copies of rRNA operons, and 5 CRISPR regions. The genome was composed of CDSs with a coding ratio of 78.0%. Possibly due to a quality problem in the NGS reads, a small number of indels or frameshifts remained in open reading frames in the current version of the genome sequence. The completeness score and strain heterogeneity score of the genome calculated via the CheckM program v.1.0.18 (Parks et al. 2015) were 97.96% and 0.00%, respectively.

A BLASTn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with default settings using the 16S rRNA gene sequence of *Nostoc* SO-36 revealed a 100%, 98.99%, and 98.79% similarity with that of the *Nostoc* sp. strain SO-42 isolated from Antarctica (GenBank accession number: AB098071.1) by Dr. S. Ohtani (Arima et al. 2012), *Nostoc* sp. GT138 (accession number: KF494240.1) isolated from a symbiotic gland tissue of *Gunnera tinctoria*, and *Nostoc* sp. TCL240-02 (accession number: CP040094.1) isolated from the hornwort *Leiosporoceros dussii*, respectively. Some symbiotic or host-associated species were listed in BLASTn results with high similarity scores (both e-value and identity scores). To determine the phylogenetic position of *Nostoc* SO-36, a phylogenetic tree was constructed via the ML method using 16S rRNA sequences of various *Nostoc* species and some outgroup species (Fig. 4). The tree shape and division of clades were similar to those in previous studies with phylogenetic analyses of the genus *Nostoc* (Rajaniemi et al. 2005; Řeháková et al. 2007). Based on the phylogenetic analysis of 16S rRNA genes, *Nostoc* SO-36 was found to belong to the typical clade of *Nostoc sensu stricto* (Bagchi et al. 2017; Řeháková et al. 2007), which also includes *Nostoc punctiforme* PCC 73102, *Nostoc commune* NIES-4072, and many symbiotic *Nostoc* species. The *Nostoc* strains isolated as symbionts or from a host-associated environment are marked with black squares in Fig. 4. As several researchers have reported, symbiotic strains

belonging to the genus *Nostoc* were identified as a polyphyletic group (Bell-Doyon et al. 2020; Gagunashvili and Andresson 2018; Kanesaki et al. 2018; Ran et al. 2010; Yamada et al. 2012). As shown in Fig. 4, some free-living strains are also included in the clade of *Nostoc sensu stricto*.

Among these closely related strains, whole-genome sequences of five species, including *Nostoc* sp. TCL240-02, *Nostoc* sp. ATCC 53789, *Nostoc* sp. '*Peltigera_membranacea* cyanobiont' N6, *Nostoc* sp. C57, and *Nostoc punctiforme* PCC 73102, were available. These strains showed higher than 98.5% identity in 16S rRNA sequences relative to those of *Nostoc* SO-36. The nucleotide sequences of *Nostoc* SO-36 chromosomes were compared with those of other closely related strains via dot plot matrix with a window length of 1 kb and blast e-value < 10. However, no large genomic islands with a high similarity between *Nostoc* SO-36 chromosomes and those of the other five species were observed (Fig. 5a-f). In contrast, dot plot analysis revealed that the chromosome of *Nostoc punctiforme* PCC 73102 showed several highly homologous genomic islands with other closely related species, except the strain N6 (Fig. 5g-j). These results indicate that the chromosomal structure and sequence organization of *Nostoc* SO-36 were not similar to those of closely related species.

Genes for extracellular polymeric substrate synthesis conserved in Nostocaceae

Because the low-temperature treatment enhanced EPS production and cell aggregation, conserved genes for the EPS synthesis pathway were surveyed among *Nostoc* SO-36 and closely related species with complete or high-quality draft genome sequences (Table 2). The EPS synthesis pathways of gram-negative bacteria include the Wzx/Wzy pathway, ABC transporter–dependent Kps pathway, synthase-dependent pathway, and extracellular sucrase pathways (Pereira et al. 2013; Schmid et al. 2015). Whether the synthase-dependent and extracellular sucrase pathways are functional in cyanobacteria remains unknown.

Because heterocyst-forming cyanobacteria have a relatively larger genome and multicopy genes for EPS biosynthesis in general, Wzx/Wzy pathway genes were found in all strains. In *Nostroc azollae* 0708, *wzx* was missing. In *Nostoc* SO-36, *wzx* and *wzb* were found as a single-copy gene. A relatively small copy number of genes in the Wzx/Wzy pathways is a characteristic feature of *Nostoc* SO-36. The EPS-biosynthetic mechanisms in this strain are likely simpler than those of other related species. Recently, an EPS synthesis gene cluster of the Wzx/Wzy pathway encoded by the plasmid pSYSM of a unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis* 6803) was found to be involved in Synechan synthesis (Maeda et al. 2021), but an ortholog gene of *wzy* in the pSYSM cluster was not conserved in these strains. However, an ortholog gene of other putative *wzy* conserved in the *Synechocystis* 6803 chromosome was found in all strains. In contrast, an ortholog gene of *wzz* (PCP-2a) conserved in the pSYSM of *Synechocystis* 6803 was found among all strains examined (Table 2). Multicopy features of these genes indicated the complexity of the regulatory system for EPS biosynthesis in various Nostocaceae species.

Four genes, KpsD (OPX family), KpsE (PCP family), KpsM, and KpsT, which are core components of the ABC transporter–dependent pathway, were found in all strains, suggesting that this pathway is functional in these cyanobacteria. However, other accessory protein groups, such as KpsC/KpsS and KpsU, were not conserved in *Nostoc* SO-36 (Table 2). The genes for synthase-dependent pathways were not found in the genome of *Nostoc*

275 SO-36.

Discussion

Phylogenetic position of *Nostoc* SO-36

In the BLASTn search of 16S rRNA of *Nostoc* SO-36, a number of symbiotic *Nostoc* species were closely related. So far, a number of symbiotic or host-associated cyanobacteria have been identified from various plants and fungi, such as lichen, azolla, cycad, and gunnera. Most of these symbiotic relationships with plants and fungi were established by nitrogen-fixing cyanobacterial species under the order Nostocales. Although the strains isolated as symbiotic species were polyphyletic among Nostoclaes, a number of symbiotic strains belonged to a phylogenetic clade of *Nostoc sensu stricto* (Bell-Doyon et al. 2020; Papaefthimiou et al. 2008). Many researchers have tried to identify common genomic features for the host-specificity of these strains, but persuasive explanations have not yet been found, except nitrogen fixation or hormogonium formation. Recently, Hashidoko et al. (2019) identified a *Nostoc* strain that can form a hormogonium in response to a diacylglycerol compound, which was extracted from leaf litters of cycad (Hashidoko et al. 2019). Because *Nostoc* SO-36 was isolated from Bryophyte communities on a sunny slope in Antarctica, this strain might also respond to some metabolites released from these plants to establish a host-associated habitat and endure the extremely cold and dry climate. Interestingly, the phylogenetic clade of *Nostoc sensu stricto* includes a lot of strains isolated from both temperate or subtropical climates and polar areas. Furthermore, the fact that a number of strains from various cyanobacterial genera were isolated from the Antarctic region indicates that psychrophilic or psychrotolerant phenotypes would be a polyphyletic feature in the evolutionary history of cyanobacteria.

Genes for EPS biosynthesis and export pathways in *Nostoc* SO-36

Generally, in bacteria, four pathways are known for EPS biosynthesis: 1) Wzx/Wzy-dependent pathway, 2) ABC transporter–dependent pathway, 3) synthase-dependent pathway, and 4) a minor pathway, extracellular synthesis by a single sucrase protein (Schmid et al. 2015). Because orthologous genes of alginate polymerase genes for pathway 3 and fructansucrase proteins for pathway 4 were not found in the complete genome sequence of *Nostoc* SO-36 and closely related species in Table 2, the EPS biosynthesis in these species would depend on pathways 1 and 2. Recently, Maeda et al. (2021) identified that proteins encoded by a cluster of Wzx/Wzy pathway genes on the plasmid pSYSM play a primary role in EPS synthesis in *Synechocystis* 6803, which has an additional Wzy-like gene *sll0923* in the main chromosome. The putative Wzy genes found in *Nostoc* SO-36 and closely related species were more similar to the chromosomal gene of *Synechocystis* 6803. In the Wzz (PCP-2a) gene, *Synechocystis* 6803 also has two homologous genes: *sll5052* on the plasmid and *sll0923* on the chromosome. *Nostoc* SO-36 and closely related species possess only a homologous gene of plasmid type (*sll5052*) in *Synechocystis* 6803. This information suggests that acquired, loss, or complex horizontal transfers of a group of Wzx/Wzy-dependent pathway genes have occurred and resulted in diverged EPSs in this phylum. In the ABC transporter–dependent pathway, only core complex genes are conserved in *Nostoc* SO-36 and its closely related species. Other accessory proteins or genes for polymer unit synthesis were not found, except the *kpsF* gene. It is still unclear whether the Wzx/Wzy-dependent pathway and ABC transporter–dependent pathway perform EPS export coordinately or in selective use under stress conditions. The disruption of single-copy genes in Table 2 might be an effective strategy to propose the function of these pathways. Further investigations on the temperature-

specific expression of EPS synthesis pathway genes can help understand their functional importance.

Putative factors for psychrotolerant phenotype of Antarctic cyanobacteria

Biondi et al. (2008) collected 51 Antarctic cyanobacterial strains that include six *Nostoc* strains from the benthic mat of a frozen lake. They reported that although photosensitivity varied among the strains, five *Nostoc* strains showed a slight or strong photosensitivity with changing yellowish color when the cells were cultivated under 30–40 μmol m^{-2} s^{-1} irradiation in laboratory conditions. Shorter filaments of *Nostoc* SO-36 were observed when grown at 10 °C (Fig. 2). This is probably due to the photoinhibitory effect induced by a low temperature. Filamentous cyanobacteria form short filaments, which most likely cleave at the site between heterocysts and vegetative cells. In line with this hypothesis, a decrease in chlorophyll content was observed, whereas no heterocysts were observed in cells grown at 10 °C. Biondi et al. (2008) also observed that a *Nostoc* strain produced high amounts of polysaccharides. It is possible that EPS under low temperatures form large aggregates of *Nostoc* SO-36 cells and contribute to a reduced light irradiance to the cells. To ascertain whether EPS is involved in protective mechanisms against excess light in *Nostoc* SO-36, it is crucial to analyze knock-out mutants of EPS synthetic genes. We tried to knock-out several genes in *Nostoc* SO-36 using the triparental method used for *Anabaena* 7120 (Elhai and Wolk 1988), but it was not successful. We could not isolate even a partial knock-out mutant of the multicopy of cyanobacterial genomes, indicating that the *Nostoc* SO-36 mechanism for the restriction–modification system is different from that of *Anabaena* 7120. Further studies on the photosensitivity of *Nostoc* SO-36 could provide clarity on the function of EPS under low temperatures.

It is also well-known that fatty acid desaturation is essential for low-temperature tolerance by maintaining membrane fluidity and photosynthesis recovery from low-temperature photoinhibition (Gombos et al. 1994; Wada et al. 1990). Chintalapati et al. (2007) reported that when the growth temperature shifted from 25 °C to 10 °C, *Nostoc* SO-36 membrane lipids showed an increase in tri-unsaturated fatty acid content [C18:3(9,12,15)]. It was also revealed that the transcript levels of genes for fatty acid desaturase, *desA*, *desB*, *desC*, and *desC2*, are constitutively expressed regardless of the growth temperature. These results suggest that the cold adaptation of *Nostoc* SO-36 is not simply regulated at the transcriptional level but also at the posttranscriptional or translation level. These types of regulations might be important for fatty acid desaturation and other mechanisms in *Nostoc* SO-36 to acquire phychrotolerance.

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Tables

Table 1. Genomic features and summary of genome analysis.

Features	Length (bp)	G + C content	Number of CDSs	Number of tRNA	Number of rRNA operon	Number of CRISPR
Chromosome	6,802,717	41.41	6252	48	4	5
pANSO36A	271,158	40.81	279	0	0	0
pANSO36B	228,652	40.47	207	0	0	0
pANSO36C	69,903	41.25	87	0	0	0
pANSO36D	35,832	39.98	30	0	0	0

CDSs, coding sequences

476 Table 2. Copy number of the putative genes involved in polymerization, chain length control, and EPS export in *Nostoc* sp. SO-36 and related species. Wzx/Wzy pathway
477 proteins, other EPS−related genes, and ABC-dependent Kps pathway proteins are listed below. (nf: not found or uncertain results)

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Gene Name	Product	*Nostoc* sp. SO-36	gene ID in SO-36	*Nostoc punctiforme* PCC 73102	*Nostoc* sp. ATCC 53789	*Nostoc* sp. TCL240-02	*Nostoc* sp. C057	*Nostoc sp. cyanobiont* N6	*Nostoc cycadae* WK-1	*Nostoc* sp. 7107	*Anabaena* sp. PCC 7120	*Nostoc piscinale* CENA21	*Nostroc azollae* 0708	*Synechocystis* sp. PCC 6803
wzx	polysaccharide flippase	1	ANSO36C_54270	4	3	2	3	3	2	3	5	2	nf	1
wzy	polysaccharide polymerase	2	ANSO36C_43250 ANSO36C_22980	5	4	4	4	4	4	3	5	1	2	4
wza/kpsD	outermembrane EPS exporter, OPX	2	ANSO36C_54160 ANSO36C_22930	2	2	2	2	2	2	2	3	2	2	1
wzb	low-molecular weight protein-tyrosine-phosphatase	1	ANSO36C_23020	2	2	1	1	2	2	2	4	2	2	1
wzz/wzc/kpsE	polysaccharide biosynthesis tyrosine autokinase, PCP	4	ANSO36C_03200 ANSO36C_05340 ANSO36C_54210 ANSO36C_22920	7	5	4	5	5	3	3	7	2	4	2
exoD	CPS/EPS-related protein	2	ANSO36C_02710 ANSO36C_57750	2	2	2	2	2	2	2	2	1	nf	1
kpsM	Capsule polysaccharide transport permease	3	ANSO36C_08310 ANSO36C_32170 ANSO36C_24590	5	3	5	5	2	4	2	8	3	3	3
kpsT	Capsule polysaccharide transport ATP binding component	1	ANSO36C_08300	1	1	1	1	1	1	1	1	1	1	1
kpsC/kpsS	Capsule polysaccharide export protein	nf	-	nf	nf	nf	nf	nf	nf	nf	nf	nf	nf	nf
kpsF	arabinose 5-phosphate isomerase	2	ANSO36C_45460 ANSO36C_38440-38450 *	2	2	2	2	2	2	2	2	2	2	2
kpsU	3-deoxy-manno-octulosonate cytidylyltransferase	nf	-	2	nf	1	1	nf	nf	nf	nf	nf	nf	1

*This gene was splited by farmeshift probably due to sequencing read error.

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Figure legends

Fig. 1. Growth curve of *Nostoc* SO-36 and *Anabaena* 7120. Both were cultured in $BG11_0$ at 10 °C (dotted line) or 30°C (solid line). The black and gray lines represent *Nostoc* SO-36 and *Anabaena* 7120, respectively. The error bars indicate the standard deviation based on three independent experiments.

Fig. 2. Phenotypes of *Nostoc* SO-36 and *Anabaena* 7120 at low temperature. **a**–**f**. Cell shapes and accumulation of extracellular polymeric substrates of *Nostoc* SO-36 (**c**–**f**) and *Anabaena* (**a**, **b**). **a**, **c**, **e**: Cell shapes observed under a blight field microscope. **b**, **d**, **f**: Culture stained with Alcian blue. **a**–**d**: Grown at 30 °C, **e**, **f**: Grown at 10 °C. Size bar = 20 μm. **g**, **h**. Morphology of *Nostoc* SO-36 grown at 10 °C observed under a stereomicroscope. Size bar = 200 μm. **i**. Chlorophyll content normalized by the value of OD_{730}. **j**. Total carotenoid content normalized by the value of OD_{730}. Total sugar content normalized by the value of OD_{730}. The error bars indicate the standard deviation based on three independent experiments.

Fig. 3. Genomic structure of chromosome and four plasmids. The outer to inner circles represent strand information. The outer black circle shows the scale bars in Mbps. In the second outer circle, the blue bars indicate the genes transcribed in a clockwise direction. In the third outer ring, the blue bars indicate the genes transcribed in a counter-clockwise direction. The inner ring shows the GC skew, $(G - C)/(G + C)$, which was calculated with a 10-kb window at a step size of 100 bp. Similar to other *Nostoc* species, a significant change of the GC skew pattern of the chromosome was not clearly observed in this species.

Fig. 4. Maximum-likelihood phylogenetic tree based on the 16S rRNA gene sequences of *Nostoc* SO-36 and related species of Nostocacean cyanobacteria. The bootstrap values obtained are displayed at the nodes when they are higher than 50%. GenBank sequence accession numbers are listed in Table S1. *Nostoc* SO-36 is indicated by an arrow. The strains isolated as symbiotic or host-associated species are indicated with black squares.

Fig. 5. Dot plot matrix comparisons of the nucleotide sequence of chromosome of *Nostoc* SO-36 with that of closely related strains. The conserved regions in the dot plot are represented by red dots. The continuous red dots indicate highly conserved genomic regions. (**a**-**f**) Comparison of *Nostoc* SO-36 with other strains; **a**: *Nostoc* SO-36 itself, **b**: *Nostoc punctiforme* PCC 73102, **c**: *Nostoc* sp. ATCC 53789, **d**: *Nostoc* sp. TCL-240-02, **e**: *Nostoc* sp. *'Peltigera_membranacea* cyanobiont' *N6*, **f**: *Nostoc* sp. C057. (**g**-**j**) Comparison of *Nostoc punctiforme* PCC 73102 with other strains; **g**: *Nostoc* sp. ATCC 53789, **h**: *Nostoc* sp. TCL-240-02, **i**: *Nostoc sp. 'Peltigera_membranacea* cyanobiont*' N6*, **j**: *Nostoc* sp. C057.

512 Fig. 1

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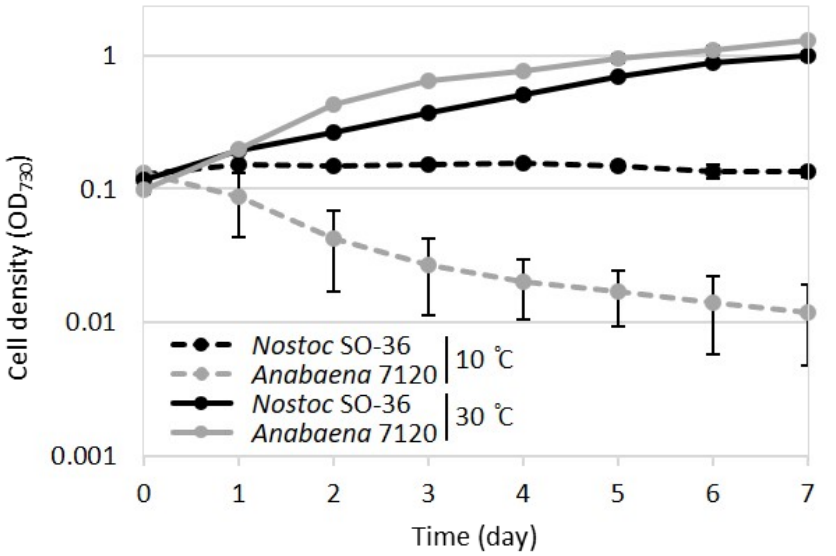

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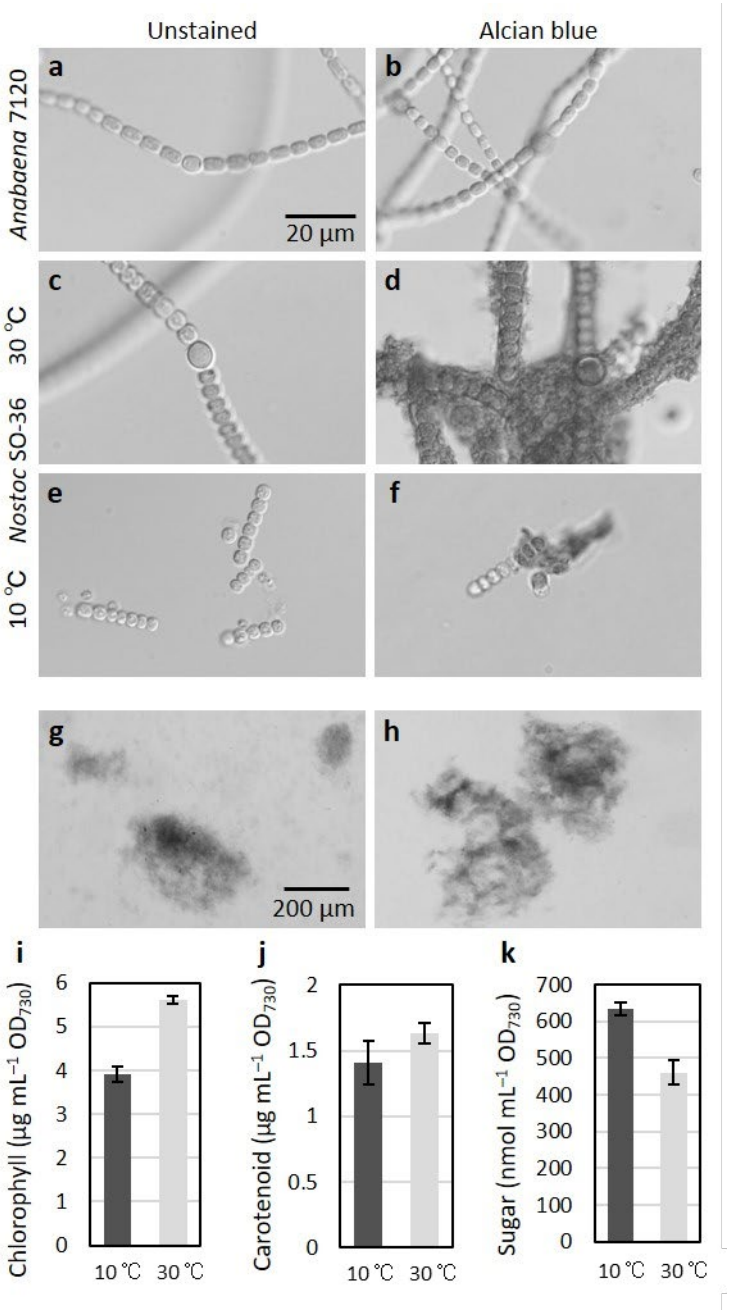
516 Fig. 2



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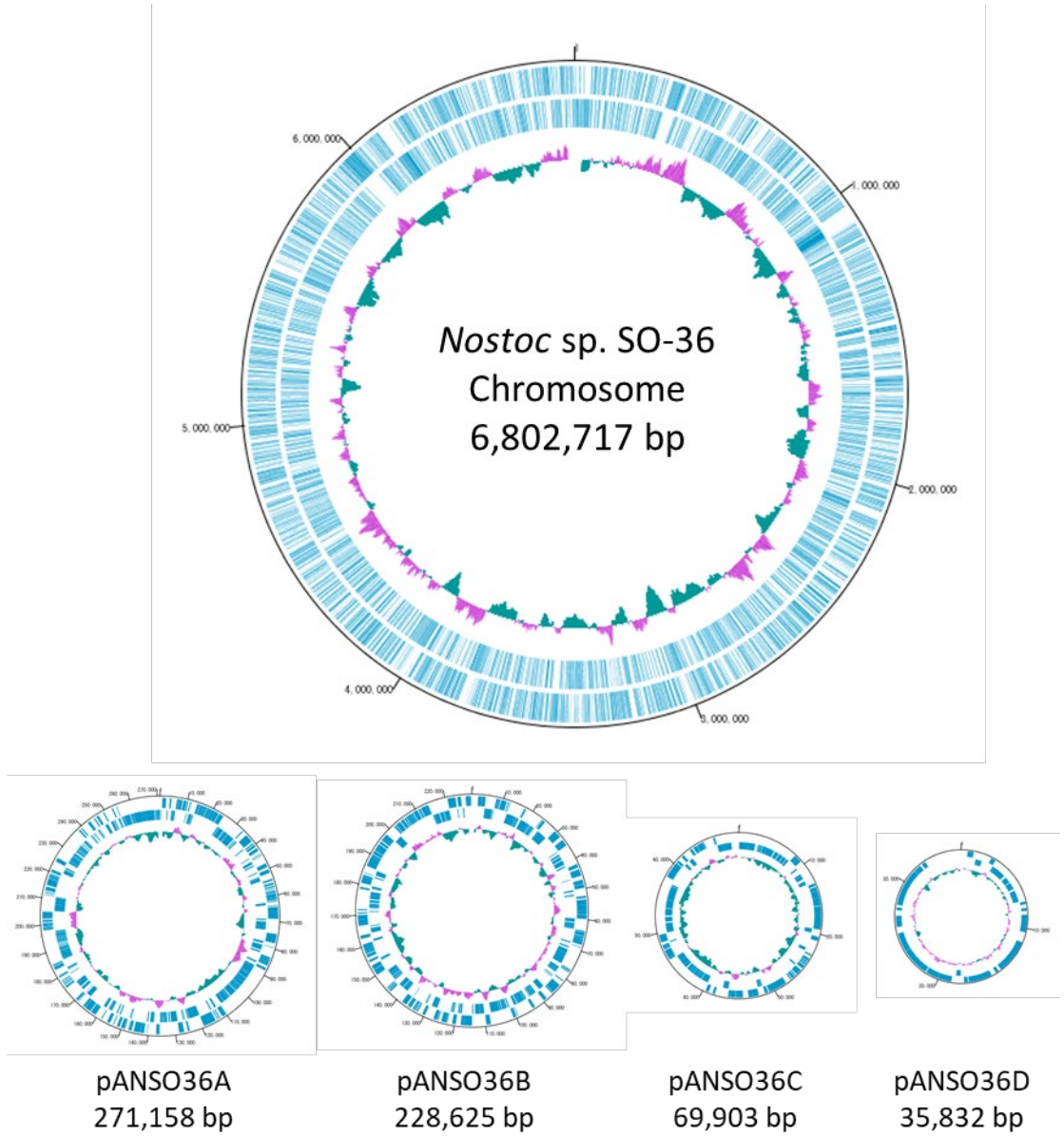

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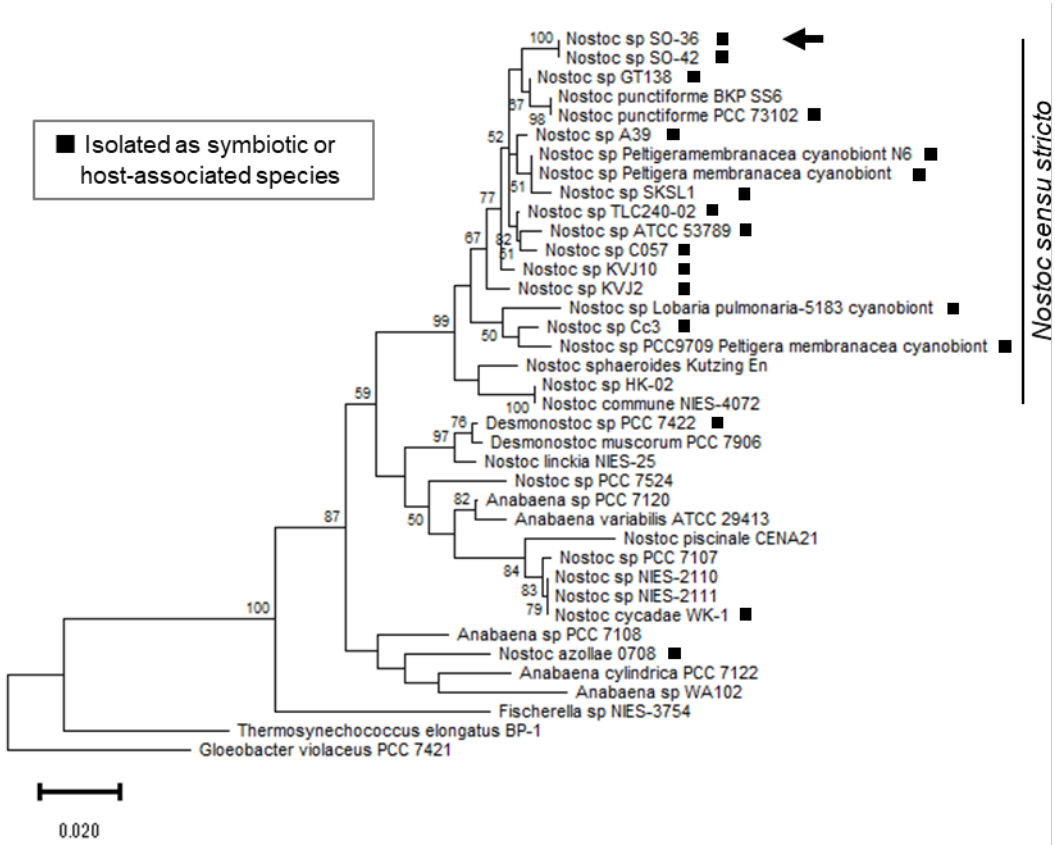
522 Fig. 4



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526 Fig. 5

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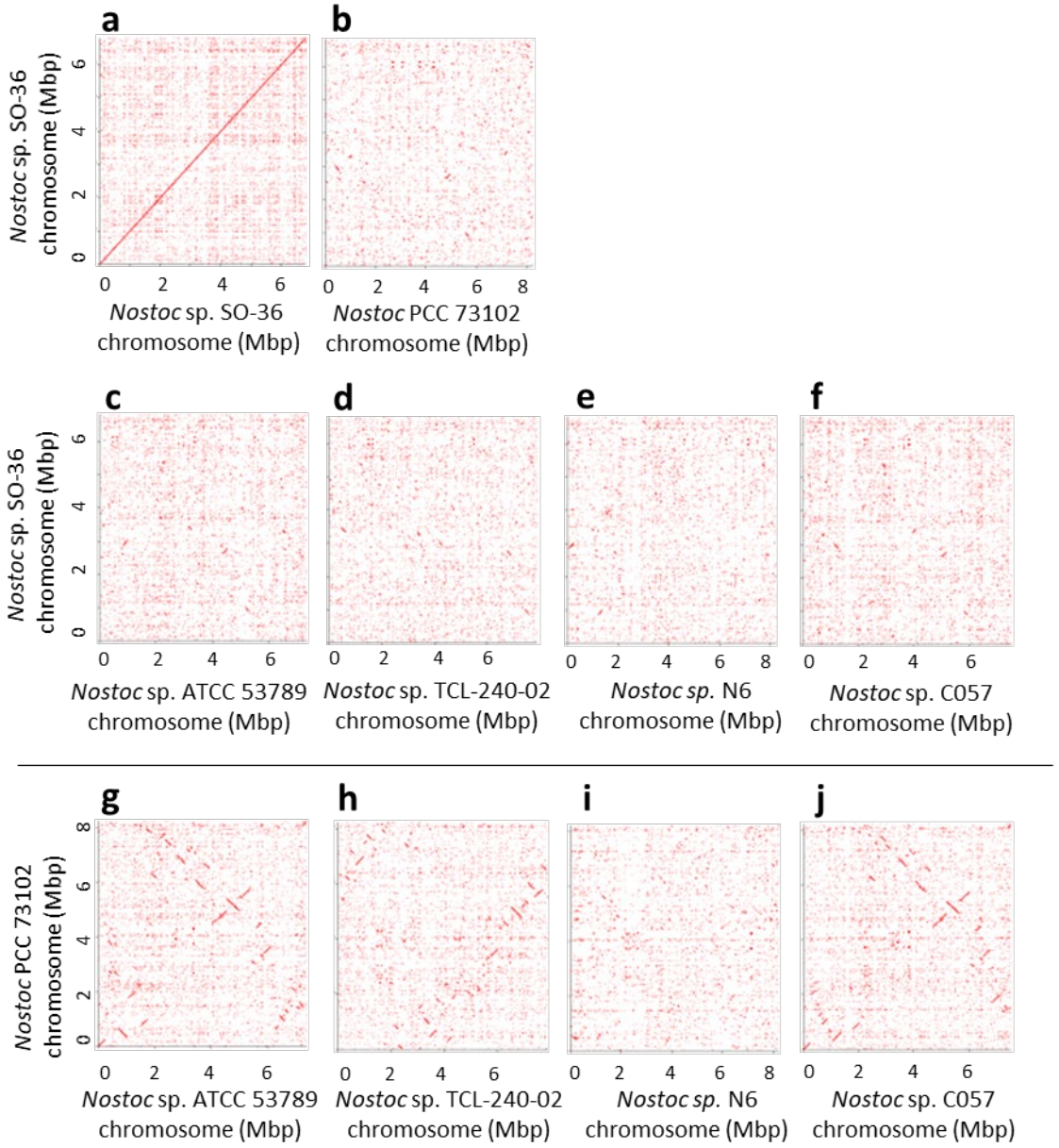

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Electronic supplementary materials

Title: Possible involvement of extracellular polymeric substrates of Antarctic cyanobacterium *Nostoc* sp. strain SO-36 in adaptation to harsh environments.

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Content: **Table S1, Figs. S1-S3**

549 Table S1. 16S rRNA gene list with accession numbers used in phylogenetic tree.

Strain name	Accession no.	Types of habitat Free-living or Symbiotic (Host information)
Gloeobacter violaceus PCC 7421	NC_005125	Free-living
Thermosynechococcus elongatus BP-1	NC_004113	Free-living
Fischerella sp. NIES-3754	NZ_AP017305	Free-living
Anabaena sp. PCC 7120	NC_003272	Free-living
Nostoc punctiforme PCC 73102	NC_010628	*Macrozamia* sp., Various hosts
Nostoc sp. SO-36	in this work	Bryophyte
Nostoc sp. SO-42	AB098071	Algal mat
Nostoc sp. TLC240-02	CP040094	*Leiosporoceros dussii*
Nostoc linckia NIES-25	AP018223	Free-living
Nostoc cycadae WK-1	NZ_DF978488	*Cycas revoluta*
Nostoc sp. PCC 7107	NC_019676	Free-living
Nostoc sp. HK-01	AP018318	Free-living
Nostoc sp. HK-02	AP018326	Free-living
Nostoc azollae 0708	CP002059	*Azolla filiculoides*
Desmonostoc sp. PCC 7422	HG004586	*Cycas* sp.
Nostoc sp. Cc3	HG004580	*Cycas circinalis*
Nostoc sp. KVJ2	EU022712	*Blasia pusilla*
Nostoc sp. KVJ10	EU022708	*Blasia pusilla*
Anabaena sp. PCC 7108	AJ133162	Free-living
Nostoc sp. GT138	KF494240	*Gunnera tinctoria*
Nostoc sp. '*Peltigera membranacea* cyanobiont'	JX181775	*Peltigera membranacea*
Nostoc sp. ATCC 53789	CP046703	Lichen
Nostoc piscinale CENA21	CP012036	Free-living
Desmonostoc muscorum PCC 7906	AB325908	Free-living
Nostoc commune NIES-4072	GCA_003113895	Free-living
Nostoc sphaeroides Kutzing En	GCA_003443655	Free-living
Nostoc sp. NIES-2111	LC322125	Free-living
Nostoc sp. NIES-2110	LC228974	Free-living
Nostoc sp. '*Lobaria pulmonaria*-5183 cyanobiont'	CP026692	*Lobaria pulmonaria*
Nostoc sp. ATCC53789	AF062638	Lichen thallus
Nostoc sp. PCC 7524	CP003552	Free-living
Anabaena cylindrica PCC 7122	NR_102457	Free-living
Anabaena sp. WA102	CP011456	Free-living
Nostoc sp. PCC9709 '*Peltigera membranacea* cyanobi	AF027654	*Peltigera membranacea*
Nostoc sp. A39	KF494247	*Anthoceros punctatus*
Nostoc sp. '*Peltigera membranacea* cyanobiont N6'	JX975209	*Peltigera membranacea*
Nostoc sp. SKSL1	EU022726	*Peltigera canina*
Nostoc sp. C057	NZ_CP040281	*Phaeoceros* sp.
Nostoc punctiforme BKP_SS6	MW383844	Free-living
Anabaena variabilis ATCC 29413	CP000117	Free-living

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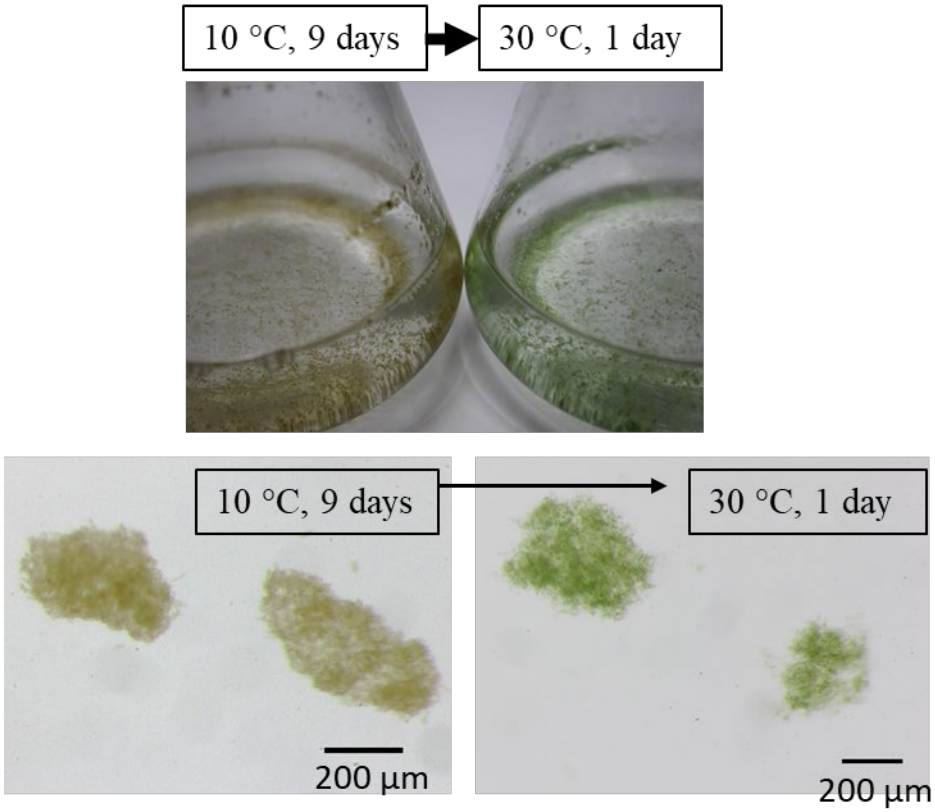

Fig. S1

Viability of *Nostoc* SO-36 after incubated in 10 °C

To analyze neither the *Nostoc* SO-36 are still viable even after incubated at 10 °C, two analysis was conducted. The cells of *Nostoc* SO-36 were shifted to 30°C for 1 day, after the incubation at 10 °C for 9 days. The chlorophyll content was rapidly recovered. Cells in the glass flask (Upper). Microscopic pictures of the same sample culture (Lower).

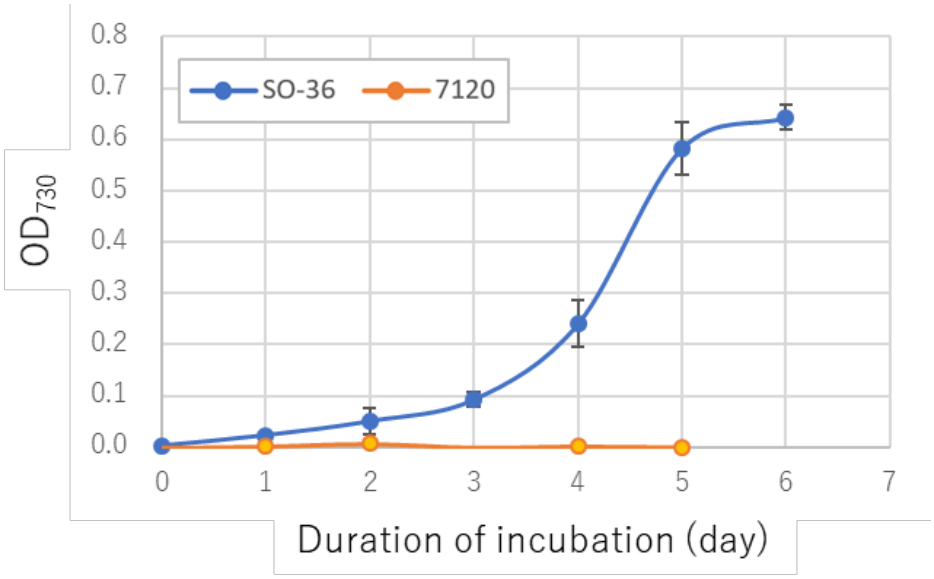

Fig. S2

Viability of the cells of *Nostoc* SO-36 and *Anabaena* 7120 after the incubation at 10 °C.
After 9 days of incubation at 10 °C, 3 mL aliquots of each culture were transferred to fresh BG-11_0 medium.
New cultures were incubated at 30 °C for 5 or 6 days. Cellular growth was analyzed at OD_{730} for each day.
The average values of three-independent experiments were plotted. Error bars indicated standard deviation.
Rapid recovery of cellular growth was only observed in case of *Nostoc* SO-36.

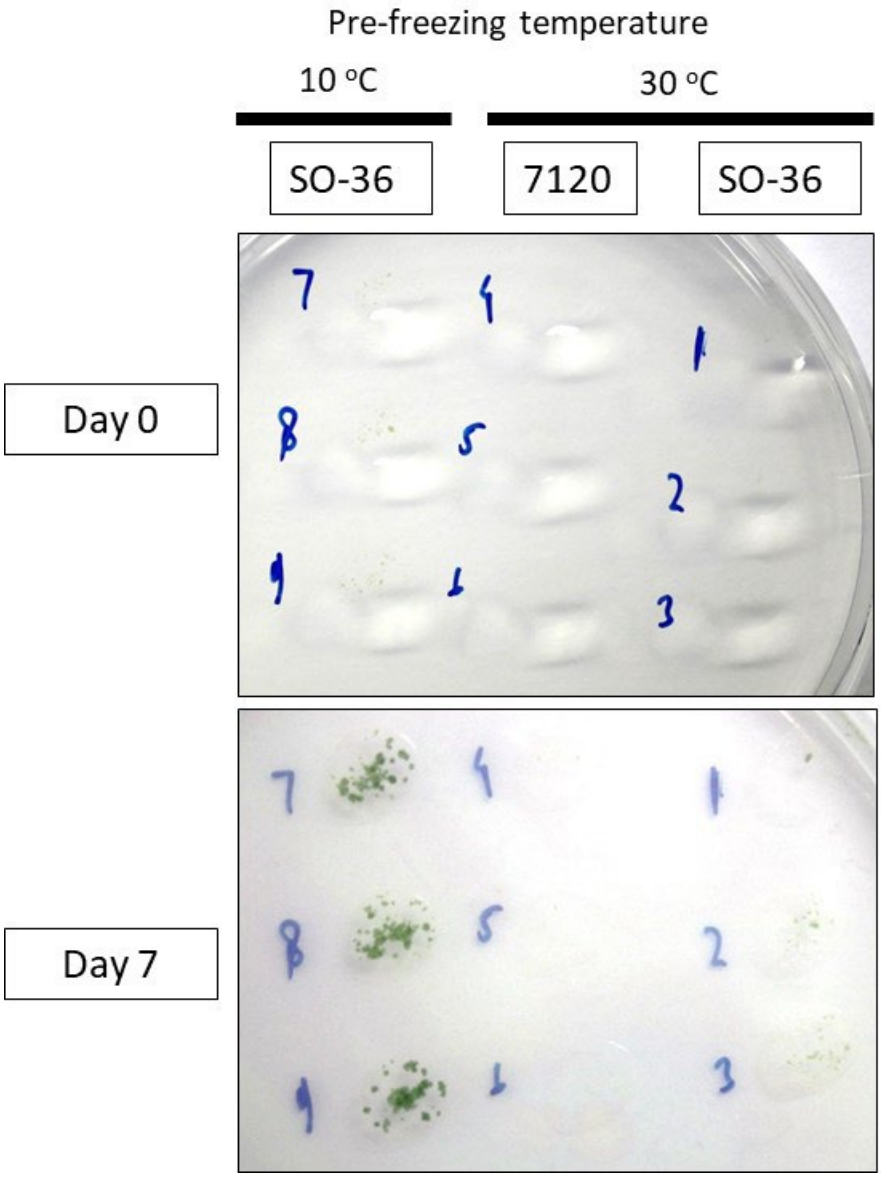

Fig. S3

Freezing test of the cells of *Nostoc* SO-36 and *Anabaena* 7120.

Cells were adjusted to OD_{730} = 1.0 and centrifuged. Cellular pellets were frozen by liquid nitrogen and thawed on ice for 1.5-2 hours until the last crystals of ice had melted. Cells were re-suspended with an aliquot of liquid BG-11_o medium and were spread on BG-11_o solid agar plate. The plates was incubated at 30 °C with illumination of 30-50 μmol m^{-2} s^{-1}. Growth recovery of the cells were checked after 7 days. Upper and lower panels showed the colony of the cells on the plate at day 0 and 7, respectively. *Nostoc* sp. SO-36 showed freeze-thaw tolerance, but *Anabaena* PCC 7120 did not. Pre-freezing temperature was also important to the freeze-thaw tolerance. Experiments were replicated for three times.