

Full Paper

Physiological properties and genetic analysis related to exopolysaccharide (EPS) production in the fresh-water unicellular cyanobacterium *Aphanothece sacrum* (Suizenji Nori)

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The clonal strains, phycoerythrin(PE)-rich- and PE-poor strains, of the unicellular, fresh water cyanobacterium *Aphanothece sacrum* (Suringar) Okada (Suizenji Nori, in Japanese) were isolated from traditional open-air aquafarms in Japan. *A. sacrum* appeared to be oligotrophic on the basis of its growth characteristics. The optimum temperature for growth was around 20°C. Maximum growth and biomass increase at 20°C was obtained under light intensities between 40 to 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (fluorescent lamps, 12 h light/12 h dark cycles) and between 40 to 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for PE-rich and PE-poor strains, respectively, of *A. sacrum*. Purified exopolysaccharide (EPS) of *A. sacrum* has a molecular weight of ca. 10⁴ kDa with five major monosaccharides (glucose, xylose, rhamnose, galactose and mannose; $\geq 85 \text{ mol}\%$). We also deciphered the whole genome sequence of the two strains of *A. sacrum*. The putative genes involved in the polymerization, chain length control, and export of EPS would contribute to understand the biosynthetic process of their extremely high molecular weight EPS. The putative genes encoding Wzx-Wzy-Wzz- and Wza-Wzb-Wzc were conserved in the *A. sacrum* strains FPU1 and FPU3. This result suggests that the Wzy-dependent pathway participates in the EPS production of *A. sacrum*.

Key Words: *Aphanothece sacrum*; Exopolysaccharide (EPS); unicellular cyanobacterium; Wzy-dependent pathway

Introduction

Cyanobacteria are regarded as good candidates for the conversion of light energy into other useful, renewable molecules, particularly in the food, pharmaceutical, and other, industries. Among cyanobacterial products, exopolysaccharide (EPS) is currently attracting attention because it is water soluble and easily recovered from liquid cultures (De Philippis and Vincenzini, 1998; De Philippis et al., 1993, 2001; Li et al., 2001; Pereira et al., 2009). According to the review by Pereira et al. (2015), more than one hundred cyanobacteria belonging to Sections I, III, IV, and V (Rippka et al., 1979) have been reported to synthesize a significant amount of EPS. The molecular weight of EPSs range from 0.2- to 2.0×10^4 kDa, and more than 75% of those so far characterized are heteropolysaccharides consisting of six or more different kinds of monosaccharides. Most EPSs of cyanobacteria are strongly anionic because of the presence of one or two different uronic acids and sulfate-containing sugars. Owing to the presence of a large number of negative charges, EPS-producing cyanobacteria and their EPSs have been considered as chelating agents for the removal of heavy metals from water (De Philippis et al., 2011; Okajima et al., 2010a, b; Tran et al., 2016).

The unicellular cyanobacterium *Aphanothece sacrum* (Suringar) Okada (Suizenji Nori, in Japanese) is the only microalga that has been cultured as a food source for more than 300 years so far recorded; it has been cultured since 1763 using cool (ca. 20°C), oligotrophic running waters obtained from a river and/or underground in a traditional aquafarm that still exists in Asakura City, Fukuoka Prefecture, Japan. Surrounded by a large amount of EPS, cells

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Table 1. Monosaccharide compositions of exopolysaccharides of *Aphanothece sacrum* FPU1 and FPU3.

Sample	Glc	Xyl	Rha	Gal (mol%)	Man	Fuc	Others
FPU1	29.6	18.6	18.6	11.1	11.0	4.4	6.7*
FPU3	27.1	20.1	19.5	11.4	11.2	4.4	6.3*
Aqua-farm cultured**	28.2	17.6	11.1	12.0	10.9	7.5	12.7

*Including ribose, arabinose, galacturonic acid, glucuronic acid and N-acetyl-mannosamine.

**EPS (Sacran®) obtained from *A. sacrum* cultured in the aqua-farm (Okajima et al., 2008).

Abbreviations: Glc, glucose; Xyl, xylose; Rha, rhamnose; Gal, galactose; Man, mannose; Fuc, fucose.

of *A. sacrum* form a stable thick sheet which can reach more than 20 cm in length and width, and float in water. Cell sheets of *A. sacrum* are preserved as salted or dried forms, and served in traditional Japanese cuisine.

Okajima-Kaneko et al. (2007) determined the partial molecular structure of the EPS using *A. sacrum* that was cultured in the open-air aquafarm. The EPS of *A. sacrum* has an extremely high molecular weight of over 1.6×10^4 kDa, and has carboxylate groups in 17 mol% and sulfate groups in about 12 mol% of the sugar residues (Okajima et al., 2007). The purified EPS of *A. sacrum* in hot pure water became physical hydrogels after cooling. The hydrogels showed an ultra-high absorption efficiency not only for water (6,100 mL g⁻¹), but also for saline (2,700 mL g⁻¹) (Okajima et al., 2008). It also adsorbs divalent, as well as trivalent, metal ions with high efficiency (Okajima et al., 2009, 2010a, 2010b). At present, EPS produced by *A. sacrum* is used as a pharmaceutical compound and sold as a registered trademark, “Sacran®”.

Fujishiro et al. (2004) reported the establishment of a pure culture of *A. sacrum*. The phylogenetical analysis using genes coding for ferredoxin and 16S-rRNA revealed that it was closely related to *Cyanotheca* sp. PCC8801. However, the physiological properties and molecular mechanisms relating to EPS production in *A. sacrum* have not been elucidated yet.

We have successfully isolated several clonal strains of *A. sacrum* from the cells cultured in traditional aquafarms in Kumamoto and Fukuoka Prefectures, Kyushu, Japan, because the strain of Fujishiro et al. (2004) mentioned above was not available. The aim of the present study was to characterize the physiological properties of *A. sacrum* strains related to EPS synthesis, the chemical properties of their purified EPS, and to identify the genes related to the final steps of assembly and export of EPS.

Materials and Methods

Isolation of clonal strains. Aquafarm-cultured *A. sacrum* was obtained from Kumamoto prefecture (Suizenji-Nori Honpo, Kashima Town) and Fukuoka prefecture (Endoh-kinkawadoh and Fresh Water Laboratory, Fukuoka Research Center, Fish and Marine Research Center, Asakura City), Kyushu, Japan. Single cells without an EPS matrix were obtained as follows: a small (<5 mm in diameter)

aliquot of cell aggregate was washed several times with sterilized water and then dispersed with a Teflon homogenizer. The homogenate was suspended in a liquid medium mentioned below) and sonicated (15 s \times 8 times, cooling with ice; Astrason Ultrasonic Processor XL2020 equipped with standard microchip No.4418, Misonix Inc. Farmingdale, NY, USA). Cell suspension after sonication was passed through a nylon-mesh (opening size of 10 μ m, NYTAL, SEFAR AG, Heiden, Swaziland) to remove aggregated cells and water-insoluble EPS, and then the cell density was adjusted to 10^7 – 10^8 cells L⁻¹. After addition of germanium (final concentration of 10 mg L⁻¹) to prevent the growth of diatoms that adhered to aquafarm-cultured *A. sacrum*, the cell suspension was mixed with an equal volume medium containing 1.6% w/v agarose (cooled to ca. 40°C after autoclaving; A0169, Sigma-Aldrich, St. Louis, MO, USA), and poured into plastic Petri dishes. Many colonies, including eukaryotic microalga, appeared after 1 to 2 months incubation in agarose plates. Colonies of unicellular cyanobacteria that produces EPS were picked up by a glass capillary under a microscope, and then inoculated into a small amount (~3 mL) of liquid medium. The medium used for isolation was modified AQUIL (Ohki et al., 2014) after altering the concentrations of salts and nutrients as follows: salt concentrations 0.6, 0.3, 0.15, 0.06 or 0.03‰; nutrient concentrations K₂HPO₄, 15, 3 or 1.5×10^{-7} M; KNO₃, 10 or 1×10^{-4} M; metals same as that of the original, or 1/2 or 1/5 of the original, concentrations (Table S1). After repeating the isolation procedure several times, clones of unicellular EPS-producing cyanobacteria were obtained. Isolation and maintenance of the isolated clones were carried out at 20°C under daylight-type fluorescent lamps (about 20–40 μ mol m⁻² s⁻¹, 14 h light–10 h dark cycles). All media and equipment were used after autoclaving or dry heat sterilization (150°C for 3 h).

EPS-producing colonies of three different appearances were recovered from the agar plates. The components of modified AQUIL are presented in Table 1: (1) a stable jelly-like colony colored brown that consists of cell aggregates with a common capsule-like thick EPS layer (Figs. S1A, S2A, and S2C; isolated from all aquafarms); (2) the same as (1) but the color of the colony was green (Figs. S1B, S2B, and S2D; isolated from Suizenji-Nori Honpo Aquafarm, Kumamoto); and (3) dark-green cells forming

a loose aggregate by slime-like EPS (isolated from Endoh-kinkawadoh Aquafarm, Fukuoka). A representative clone was chosen from the respective types. The selected clones were named FPU1, FPU3 (both were isolated from the aquafarm in Kumamoto) and FPU101 (isolated from the aquafarm in Fukuoka) for type (1), type (2) and type (3), respectively. Two of the strains, FPU1 and FPU3, were used in all experiments, and FPU101 for phylogenetical analysis only.

16S rRNA gene sequence. For the identification of isolated clones, DNA was extracted using a FastDNA Spin kit (MP Biomedicals, Solen, OH, USA). Most regions of the 16S rRNA gene and ITS between 16S and 23S rRNA genes were amplified using primers as follows: 8F (forward, Lane, 1991) and PISTE-Cyano-R (reverse, 5'-CTCTGTGCCAAGGTATC-3'; corresponding to positions 27 to 43 of 23S rRNA gene, Harvrkamp, unpublished). The conditions for the polymerase chain reaction (PCR) were the same as those used in Ohki et al. (2014). The PCR products were directly sequenced using a ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The DNA fragment sequenced was 1856 bp. The partial 16S rDNA sequences (from position 1 to 1422) from 26 unicellular cyanobacteria were aligned using ClustalW (Thompson et al., 1994). The phylogenetic tree was inferred using the neighbor-joining method (Saitou and Nei, 1987) and was visualized by MEGA, version 6.06 (Tamura et al., 2011). The partial sequence of the 16S rRNA gene of isolates were deposited in to DDBJ/ENL/GenBank with the following accession numbers, LC229080–LC229082.

Characterization of pigments. Absorption spectra of the cell suspensions of isolated clones were measured using a spectrophotometer (U3000, Hitachi Co. Ltd., Tokyo, Japan) equipped with a head-on type photomultiplier.

Growth and EPS production. When cell suspensions were aerated or shaken, cells formed clumps and attached to the wall of the culture vessels, dried-up, and then stopped growing. Therefore a batch culture (liquid culture without aeration or shaking) was used for the experiments. The growth rate was calculated on the basis of chlorophyll *a* concentrations, as explained below. Cell aggregates in an early linear growth phase were homogenized with a Teflon homogenizer and inoculated into a fresh medium at a chlorophyll *a* concentration of *ca.* 10^{-4} g L⁻¹ (*ca.* 1.5×10^{-2} g L⁻¹ dry weight). Cultures performed at different temperatures (15, 20, 25 or 30°C) and light intensities (20, 40, 80, or 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$) under a 14-h light/10-h dark cycle for 5 weeks. Cells were collected onto a polycarbonate filter (pore size 8 μm ; Advantec, Tokyo, Japan) every week, and kept at -80°C until the chlorophyll *a* measurements. The chlorophyll *a* content was measured by methanol extraction using the absorption coefficient of Mackinney (1941). The average growth rate, μ (d⁻¹), was determined from the increase of chlorophyll *a* concentration during 5 weeks. The increase of biomass was determined as an increase in dry weight. Cells cultured for 5 weeks were collected onto a nylon mesh (opening size of 10 μm), scooped into a plastic tube, lyophilized, and kept at room temperature until measurement. The EPS contents

were measured after purification from lyophilized samples with the same methods used in a previous paper (Ohki et al., 2014). The reported values were an average of three independent cultures with \pm SD and evaluated with a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using JSTAT version 12.8 (Vec-tor, Tokyo, Japan).

Chemical analysis of EPS. Two clones, FPU1 and FPU3, which were cultured at 20°C under a light intensity of around 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$, were used. The methods used for the purification of EPS, the determination of the absolute molecular weight, the detection of carboxyl and sulfate groups, and the estimation of the element CHLN and uronic acid contents, were the same as those used in our previous paper (Ohki et al., 2014).

The monosaccharide composition of EPS was determined by Protenova Co., Ltd. (Takamatsu, Kagawa, Japan). Acid hydrolyzed and N-acetylated EPS was derivatized with *p*-aminobenzoic ethyl ester (ABEE). The ABEE-derivatized monosaccharides were detected with a fluorescence detector (excitation at 306 nm, emission at 360 nm) after separation by an HPLC system (BioAssist ez, Tosoh Co., Ltd., Tokyo, Japan) equipped with PN-PAK C18 columns (3.0 \times 75 mm). The elution buffer was acetonitrile containing boron buffer (0.2 M boron buffer pH8.9:acetonitrile = 93:7) at a flow rate of 0.5 ml/min.

Genome analysis. Genomic DNA was extracted from the frozen cells of the strains FPU1 and FPU3 using Multi-Beads Shocker (MB601NIHS, Yasui Kikai Co., Osaka, Japan) with the manufacturer's equipment with the following settings; 3000 rpm, 5 times an on-time 60-s and off-time 60-s cycle, followed by phenol/chloroform extraction, RNase treatment and ethanol precipitation (Hihara and Ikeuchi, 1997). Whole genome sequencing by PacBio RSII (Pacific Biosciences of California, Inc., Menlo Park, CA, USA) with the P5-C3 sequencing reagents with two SMRT cells was performed by outsourcing (TaKaRa Bio, Dragon Genomics, Yokkaichi, Japan). After quality evaluation and filtering, 420,523 and 450,535 subreads for FPU1 and FPU3, respectively, were assembled using Celera Assembler version 7.0 (Berlin et al., 2015) with default parameters. Identification and annotation of genes in the assembled contigs were performed by MiGAP (Sugawara et al., 2009) and manual curation using the information in CyanoBase (Fujisawa et al., 2017). Suspicious contigs derived from contaminated microbes were removed using the results of MiGAP and additional BLAST searches. Finally, we obtained 20 and 95 cyanobacterial contigs with high similarity to the genera *Cyanothece*/*Aphanothece* for FPU1 and FPU3, respectively. The lengths of the longest contig were 1,186,619 bp and 207,764 bp for FPU1 and FPU3, respectively. Draft genome sequences of the strains FPU-1 and FPU-3 have been deposited into DDBJ/ENL/GenBank with the following accession numbers: BDQK01000001-BDQK01000020 and BDQL01000001-BDQL01000095, respectively. The identification of genes related to the EPS synthesis in these two strains was performed by the similarity search programs BLASTN and BLASTP.

Results and Discussion

Identification of unialgal clones of *A. sacrum*

Phylogenetic analysis using a partial sequence of the 16S rRNA gene revealed that FPU1 and FPU3 formed a single clade with *A. sacrum* of which sequences were reported previously as follows: *A. sacrum* cultured in Endoh-Kinkawadoh Aquafarm, Fukuoka (AB094350), clone derived from the same aquafarm (AB116658; Fujishiro et al., 2004); and *A. sacrum* strain SAG2412 that is maintained at the Experimental Phycology and Culture Collection of Algae at the University of Goettingen (KF417640) (Fig. 1). This clade was a sister to the clade consisting of *Aphanocapsa*, *Cyanothece*, *Gloeotheca* and *Gloeocapsa* species. The sequences of 1855 bp of our clones were >99% identical to those of *A. sacrum* previously reported. Results indicate that FPU1 and FPU3 are *A. sacrum*. The absorption spectra of cell suspensions of FPU1 and FPU3 revealed that the content of phycoerythrin of FPU3 was lower than that of FPU1 (Fig. 2). As sequences of 16S rRNA gene and ITS between 16S and 23S rRNA genes are identical, FPU1 and FPU3 are the same species (Stackebrandt and Goebel, 1994). FPU3 is suggested to be a mutant defect in phycoerythrin syntheses that occurred spontaneously at the aquafarm, because FPU1 and FPU3 were isolated from the same aquafarm. FPU101 was not included in *A. sacrum* nor *Aphanocapsa*-*Cyanothece*-*Gloeotheca*-*Gloeocapsa* clades. It belonged to the *Chroococcus* clade (*Chroococcus* sp. FPU101, Fig. 1).

Growth of isolated *A. sacrum*

First, we used the modified AQUIL medium that had been used previously to isolate EPS producing unicellular cyanobacteria (*Cyanothece* sp. Viet Num 01, Ohki et al., 2014) after slight modifications (Table S1). Cells proliferated well in a salinity range of 0.3 to 0.06‰. Concentrations of NO_3^- (as KNO_3) higher than 10^{-4} M and PO_4^{3-} (as K_2HPO_4) higher than 3×10^{-7} M produced marked inhibitory effects on the growth. Using NH_4^+ (as NH_4Cl) as a nitrogen source killed the cells within several days, even at very low concentrations (ca. 10^{-6} M). Better growth was obtained when the concentrations of Fe-EDTA and metals were reduced to 1/5 of that used in the previous studies (Ohki et al., 2014). These results indicate that *A. sacrum* is oligotrophic. The BG11 medium (Rippka et al., 1979), commonly used for cyanobacteria, and the AST medium of Fujishiro et al. (2004), that was developed for *A. sacrum* culture, did not support the active growth of our clones. As modified AQUIL medium produces precipitations after autoclaving, we have established a simple medium—FPU medium for *A. sacrum* (Table S2d)—that can be autoclaved without producing precipitations. Using the FPU medium, the optimum temperature and light intensity for the growth of *A. sacrum* strains FPU1 and FPU3 were studied.

Both strains were able to grow between 15 and 30°C under light intensities between 20 and $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ with the only exception being under the lowest light intensity ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 30°C (Figs. 3 and 4). Under this condition, the cells gradually stopped proliferating,

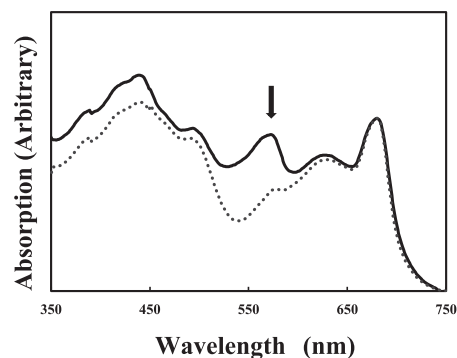


Fig. 1. Absorption spectra of cell suspensions of strains, FPU1 (solid line) and, FPU3 (dotted line).

A distinct peak at ca. 570 nm corresponding to phycoerythrin (indicates by an arrow) was observed in FPU1, while only a small absorption shoulder was detected in FPU3.

and then changed to pale yellow as they lost the phycobilin pigments and a part of chlorophyll *a*. This inhibition is considered to occur because the consumption by respiration during the dark period exceeded the production by photosynthesis, because growth was observed when the temperature of the dark period was lowered to 20°C. The maximum growth rate of FPU1 measured on the basis of chlorophyll *a* was obtained at 20°C at a light intensity of $80 \mu\text{mol m}^{-2} \text{s}^{-1}$. Growth was markedly inhibited at 15°C. The increase in biomass of FPU1 followed the same trend at the evolution of the chlorophyll *a* (Fig. 3). The growth rate of FPU3 was also high at 20°C, but was not affected by light intensities (Fig. 4). In contrast to the growth rate, the increase in biomass of FPU3 at 20°C was light-dependent and was maximal at $120 \mu\text{mol m}^{-2} \text{s}^{-1}$. The increase in biomass of FPU3 was also markedly reduced at 15°C. However, the growth inhibition at 15°C was less important than in the case of FPU1. This temperature did not provide such large inhibitory effects on the growth rate as was observed with FPU1. Results indicated that the optimum temperature for the growth of *A. sacrum* was around 20°C. This temperature is similar to the water temperature used in traditional aquafarms. The light intensities to obtain the highest biomass production at this temperature were lower for FPU1 ($80 \mu\text{mol m}^{-2} \text{s}^{-1}$) than for FPU3 ($\geq 120 \mu\text{mol m}^{-2} \text{s}^{-1}$). This difference may occur because the fluorescent lamps used for culturing are rich in green wavelengths that are more efficiently absorbed by phycoerythrin than phycocyanin. Thus, we hypothesize that the strain FPU3, which produces less phycoerythrin, can photosynthesize without inhibition under higher intensities of light rich in green wavelengths. Preliminary analysis showed that the EPS content per dry weight was around 55% at 20°C under light intensities of 40, 80 and $120 \mu\text{mol m}^{-2} \text{s}^{-1}$.

The increase in biomass of *A. sacrum* measured in the traditional aquafarm at Fukuoka ranged from 0.05 to 0.17 d^{-1} (the deviation was thought to be dependent on the seasons) though it was estimated on the basis of wet-weight (Asakura Management and Construction Office, Japan Water Agency, unpublished data). The maximum increase in biomass, 0.38, on the basis of dry-weight in this study was more than 2 times higher than that of aquafarm-cul-

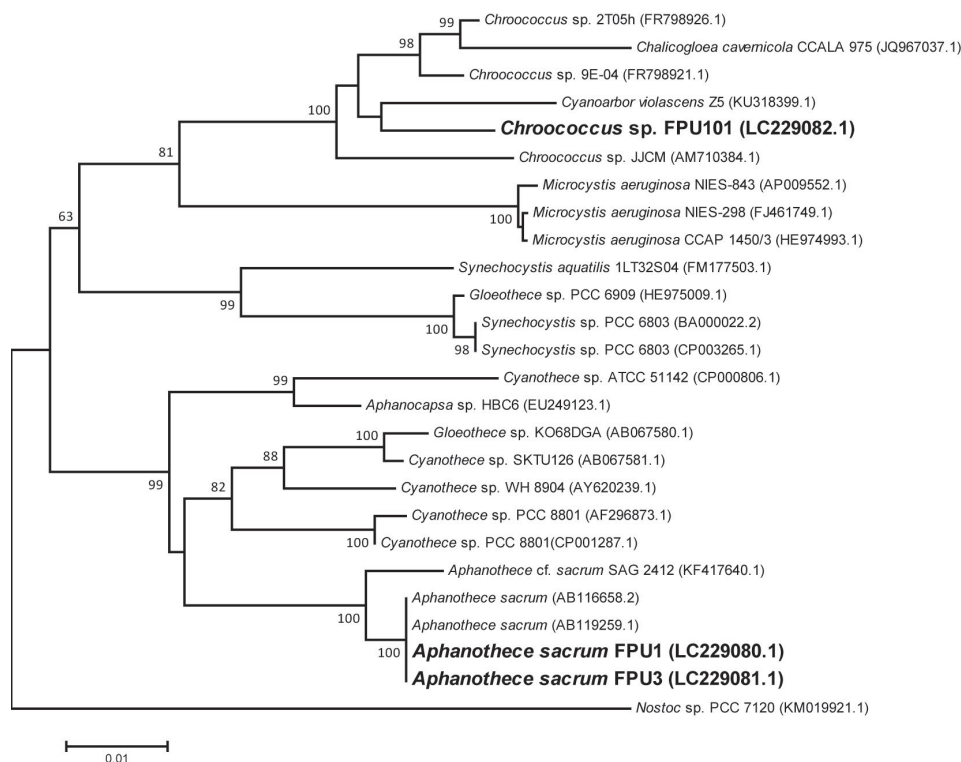


Fig. 2. Neighbor-joining tree based on the 16S rRNA sequences (1422 bases from the position 1) of exopolysaccharide-synthesizing cyanobacteria isolated from traditional open-air aquafarms, including *Aphanothece* clones: FPU1, the type (1) clone; FPU3, the type (2) clone and FPU101, the type (3) clone.

All major nodes are labeled with bootstrap values ($n = 1000$). Branch lengths are drawn to scale, in units of distance as calculated in the distance matrix. The tree was rooted with a single outgroup, *Nostoc* sp. PCC7120 (filamentous cyanobacterium).

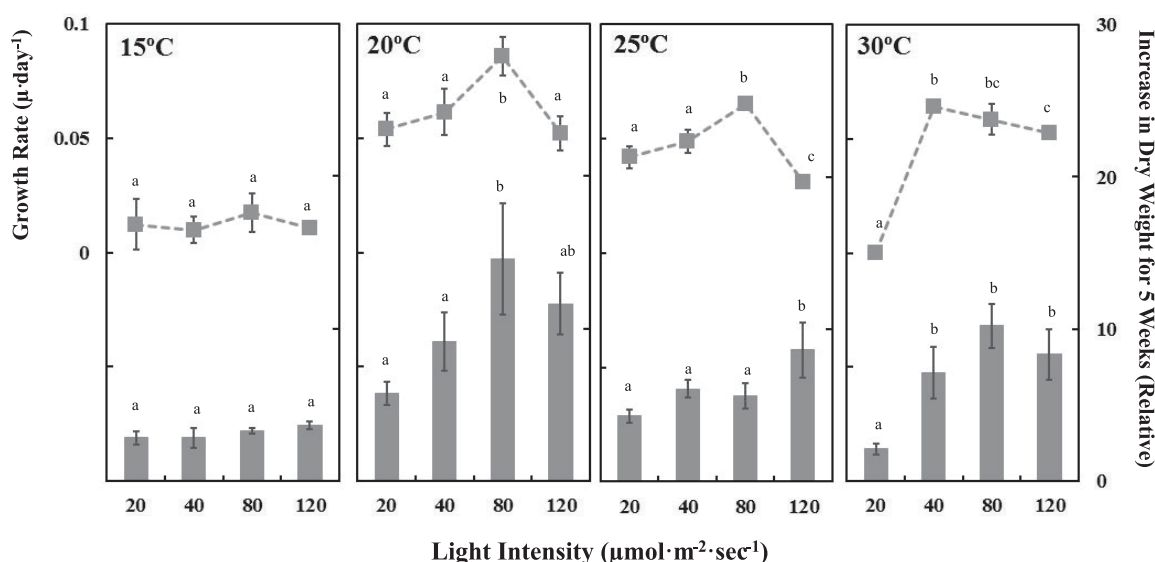


Fig. 3. Growth of *Aphanothece sacrum* strain FPU1 under different temperatures and light intensities.

Cells were inoculated into fresh medium, and cultured at combinations of 15, 20, 25, 30°C and 20, 40, 80, and 120 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Average growth rate, μ (d^{-1}) (line graph) determined from the increase in chlorophyll *a* concentrations and biomass increase determined from the relative increase of dry weight (bar graph) for 5 weeks. Results are the mean \pm SD of three independent cultures. Means followed by the same letter are not significantly different ($P < 0.001$).

tured *A. sacrum*. The EPS contents obtained in this study was also comparable with, or slightly higher than, those of aquafarm-cultured *A. sacrum* (Okajima and Kaneko, unpublished data).

Chemical properties of EPS

The absolute molecular weight of the EPS was estimated to be 2.0×10^4 and 0.7×10^4 kDa for FPU1 and FPU3, respectively. The results indicate that *A. sacrum* produces

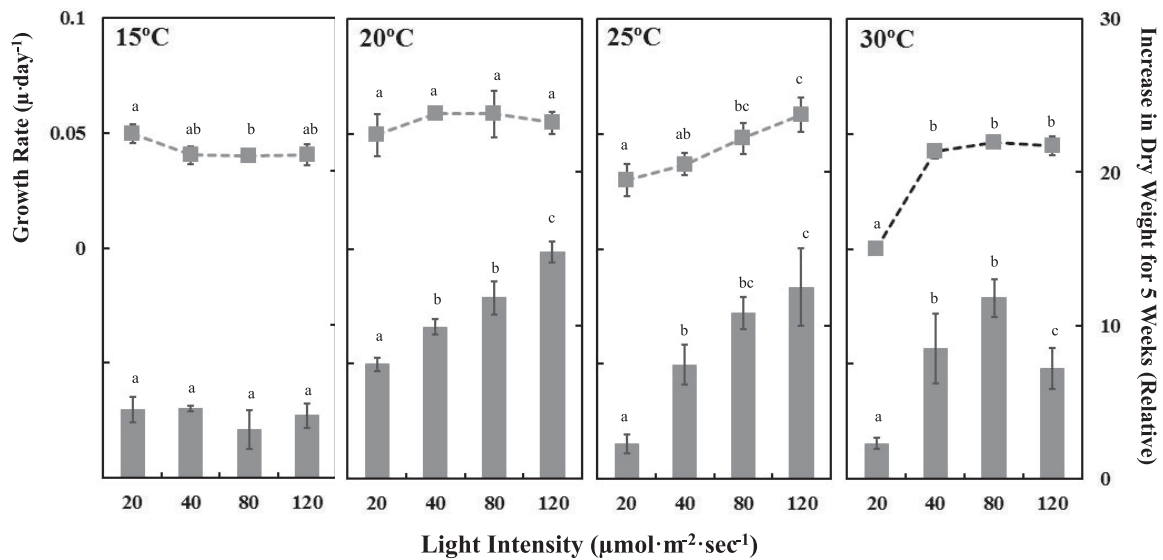


Fig. 4. Growth of *Aphanothece sacrum* strain FPU3 under different temperatures and light intensities. The other experimental details are the same as Fig. 3.

Table 2. Copy number of genes involved in the polymerization, chain length control and export of EPS in *Aphanothece sacrum*.

Wzx-dependent	Strains		ABC-dependent	Strains	
	FPU1	FPU3		FPU1	FPU3
wzx	1	1	kpsC	nf	nf
wzy	2	2	kpsD	1	1
wzz	2	2	kpsE	nf	nf
wza	1	1	kpsF	1	1
wzb	1	1	kpsM	4	4
wzc	2	2	kpsS	nf	nf
waaL	1	1	kpsT	3	3
			kpsU	1	1

nf: not found yet.

one of the highest molecular weight EPSs among cyanobacteria (cf. De Philippis et al., 1998; Ohki et al., 2014; Pereira et al., 2009). The molecular weight of EPS produced by FPU3 was about 1/3 of that produced by FPU1. The molecular weights of the EPSs produced by aquafarm-cultured *A. sacrum* deviated between 2.9×10^4 and 1.6×10^4 kDa (Okajima and Kaneko, unpublished data). However, the EPS produced by FPU3 was too small to regard as a deviation. The EPS of strain FPU3 may differ from that of FPU1. The EPS primary-structure analysis of FPU3 is required to elucidate this question. Their FT-IR spectra indicated that the EPS had carboxylate and sulfate groups, as well as the typical functional groups of sugars. The results of the elemental analysis showed that the ratio of S was 14 and 22 mol% of total monosaccharide for FPU1 and FPU 3, respectively. The monosaccharide composition of EPS are summarized in Table 1. Though the presence of uronic acid of ca. 16 mol% was confirmed by the carbazole-sulfuric acid method, only galacturonic and glucuronic acids with less than 2 mol% were detected. Incomplete acid hydrolysis, degradation and/or lactonization of uronic acids tends to occur upon EPS hydrolysis (De Philippis et al., 1998; Radhakrishnamurthy and Berenson, 1963). Alternatively,

a unique uronic acid(s) such as nosturonic acid (Helm et al., 2000) might be contained in the sugar chains. The chemical properties of EPS were almost the same as that of *A. sacrum* cultured in a traditional aquafarm (Okajima-Kaneko et al., 2007).

Genome analysis

We obtained 857 Mb and 925 Mb of sequencing reads for FPU1 and FPU3, respectively, by PacBio RSII (Pacific Bioscience) (see Table 2 and also Section “Materials and Methods”). These read amounts correspond to more than 190-fold or 205-fold depth of coverages. Sequence reads were assembled using Celera Assembler version 7.0 and, finally, 20 and 95 contigs were obtained as draft genome sequences for FPU1 and FPU3, respectively. The total lengths of contigs were 4.493 Mb and 4.485 Mb, and GC contents were 36.94% and 37.02%, for FPU1 and FPU3, respectively. The total numbers of genes were 4399, including 16 rRNA and 42 tRNA for FPU1, whereas they were 4371, including 16 rRNA and 39 tRNA, for FPU3. Draft genome sequences of FPU1 and FPU3 are almost identical, with the following exceptions. The terminal regions of several contigs of FPU3 were truncated in the middle of glycosyltransferase genes. It might mean copy

numbers of some glycosyltransferases are different in these two species. The total number of transposase genes also seemed to be different and a small number of local rearrangements were found in these two strains. To identify the related genes for the phenotypic differences, such as cellular colors between FPU1 and FPU3, is the next subject for our studies. We also tried genomic rearrangement analysis using nucleotide sequence between the draft genome of FPU1 and related cyanobacterial species, *Cyanothece* sp. PCC 8801, *Cyanothece* sp. PCC 7822, and *Gloeocapsa* sp. PCC 7428, using the genome rearrangement map function of GenomeTraveler (In silico biology Inc., Yokohama, Japan) with default parameters. However, we could not find any large syntenic portions (Data not shown). This suggests that the physical structure of *A. sacrum* genome is not similar to those of related cyanobacterial species.

According to the recent reviews of Pereira et al. (2009, 2015), the final steps of assembly and export of EPS in cyanobacteria are thought to be performed by Wzy- and/or ABC-dependent pathways. The protein complexes, Wzx-Wzy, Wza-Wzc, and probably Wzz and Wzb, are involved in the Wzy-dependent pathway, and the complexes of KpsC-KpsU-KpsS-KpsF, KpsM-KpsT and KpsD-KpsE are involved in the ABC-dependent pathway. The putative genes involved in the polymerization, chain length control, and export of EPS, and their copy numbers, found in the draft genomes of *A. sacrum* are listed in Table 2. The Wzx-Wzy-Wzz and Wza-Wzb-Wzc encoding genes were detected in the genome of *A. sacrum*. On the other hand, *kpsC* and *kpsS*, which encode β -Kdo-transferases that are involved in the capsular polysaccharides assembly in *Escherichia coli* (Willis and Whitfield, 2013), were not found in FPU1 and neither in FPU3. These genes usually consist of large gene clusters with a number of EPS-related genes as found in a related species *Gloeocapsa* sp. PCC 7428 (Shih et al., 2013) or in *Nostoc punctiforme* (Pereira et al., 2009). However, in other related species *Cyanothece* sp. PCC 7822, *Cyanothece* sp. PCC 8801, and in *A. sacrum*, most of these genes were located in individual genomic loci. The copy number of each EPS-related gene and similarities of Wzx-Wzy-Wzz proteins were also varied, even among the related species. These results suggest that the mechanisms for polymerization of the EPS units in *A. sacrum* are unique. Further functional analyses are necessary to understand the details of EPS production in *A. sacrum*.

Conclusion

We have established an *A. sacrum* batch culture system using a newly developed simple medium. The productivity of our system was the same or slightly higher than that of traditional aquafarms, and the chemical properties of purified EPS was also the same as that of “Sacran[®]” (Okajima-Kaneko et al., 2007, cf. Table1). The ability to synthesize EPS did not change after isolation and maintenance with indoor culture systems.

A. sacrum has been distributed widely in oligotrophic waters of the Kyushu area, Japan. However, it is listed as an endangered species (Ministry of the Environment of

Japan, Red List, 2017) because its habitat is vanishing due to changes in the water environments. It is only maintained in several aquafarms in the Kyushu area in Japan. However, the productivity of *A. sacrum* in two major aquafarms of the Fukuoka prefecture has been reduced to ca. 1/5 during the last decade, mainly because of water eutrophication. The aquafarm in Kumamoto prefecture was destroyed by an earthquake that occurred in the Kumamoto area on April, 2016. Therefore, our strains will be useful for preserving the species *A. sacrum* that is extinct in its natural habitat, in addition to their usefulness to the study of the molecular mechanisms of EPS production in unicellular cyanobacteria.

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Supplementary Materials

Supplementary figures and tables are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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