Spirulina subsalsa var. salina var. nov.: Thermo-Halotolerant Cyanobacteria Accumulating Two Kinds of Compatible Solute, Originated from the Sultanate of Oman

Yoichi Kuroiwa1*, Rashid S. Al-Maamari2, Masaharu Tasaki1, Kazuo Okamura1, Mark Sueyoshi1, Ayaka Nakashima1, Eriko Yoshida3 and Kengo Suzuki3

1 Shimizu Corporation, Institute of Technology, 3-4-17 Etchu-jima, Koto-ku, Tokyo, 135-8530, Japan
2 Sultan Qaboos University, P. O. Box 33, Al-Khoud, PC 123, Sultanate of Oman
3 Euglena Co., Ltd., Yokohama Leading Venture Plaza 1, 75-1 Ono-cho, Tsurumi-ku, Yokohama-shi, Kanagawa 230-0046, Japan

*E-mail: kuroiwa@shimz.co.jp

(Received; 7 January, 2014/Accepted; 22 July, 2014)

Microalgal samples were collected from natural hypersaline sources in Oman. This resulted in the screening of thermo-halotolerant cyanobacteria, strain BAH8-10-45, having closed helically coiled trichomes and accumulating glycine betaine and O-α-D-glucopyranosyl-(1→2)-glycerole as compatible solutes. Morphological observation of BAH8-10-45 suggests that this strain belongs to the genus Supirulina Turpin ex Gomont and the species of Spirulina. subsalsa Oersted ex Gomont. Halospirulina has been proposed as a genus for cyanobacteria which has similar morphology to that of S. subsalsa but has halotolerance in range of 3–13 wt/vol % or higher. For BAH8-10-45, no growth was recorded in the salinity levels of fresh-water and seawater, while growth was confirmed in the range of 5–20 wt/vol %, indicating halotolerance different from even cyanobacteria belonging to Halospirulina. Descriptions for Halospirulina as well as the euryhaline character of Spirulina, were reviewed to examine classification for BAH8-10-45. Based on phycological taxonomy, mainly featuring morphological and physiological characteristics, Spirulina subsalsa var. salina var. nov. is proposed.

Key words: Spirulina subsalsa var. salina var. nov., Halospirulina, halotolerant, glycine betaine, O-α-D-glucopyranosyl-(1→2)-glycerole

1. Introduction

The increase in concentration of atmospheric carbon dioxide (CO2) is considered to be one of the main causes of global warming 63). Microalgae, which are photosynthetic microorganism with oxygen evolution 11), transform solar energy to chemical energy through CO2 fixation by photosynthesis and contribute to the reduction of atmospheric CO2 36,60). The transformed energy and carbon are mostly stored in lipids or carbohydrates in the algae-based biomass which are utilized as feedstock for bio-fuel 24,26) and fine chemical industries 60). Microalgae have the potential not only to reduce atmospheric CO2 but also to produce biomass on an industrial scale 24,26).

Much of the Middle East is located in areas geographically suitable for CO2 fixation and algae-based biomass production, due to high annual solar irradiance 57,59), favorable coastal climate (moderate temperature and low rain fall) 49), and availability of unutilized land (e.g. desert, sabkha). Additionally CO2 emissions from oil refineries 3) and produced water from oil wells 12) could be used for such biomass production. This study was carried out with a focus on utilizing the natural resources of the Sultanate of Oman to reduce atmospheric CO2 and produce algae-based biomass of commercial importance.

Recently, different compatible solutes have been identified in the cells of halotolerant microorganisms 57). Such compounds may serve as an energy reservoir, a protectant from a variety of physical and chemical stresses 30), and a protein stabilizer in their living cells 34,37,46). Compatible solutes have great potential for further commercial application. For example, their water-holding capacity may allow them to be used in additives, stabilizers, and sweeteners for agrichemical 10,40), food 40), cosmetic 34,37), and pharmaceutical industries 34,46).

During this study, thermo-halotolerant cyanobacteria (strain BAH8-10-45) accumulating glycine betaine and O-α-D-glucopyranosyl-(1→2)-glycerole (an isomer of glucosylglycerol) were successfully isolated, as the first steps towards fine-chemicals production from algae-based biomass in the Sultanate of Oman. From the morphological point of view, the strain BAH8-10-45 is closely related to genus Spirulina.
Spirulina Turpin ex Gomont characterized by closed helically coiled trichomes, gliding motility, invisible cross-walls, and no sheaths.

In 1827, genus Spirulina was proposed by Turpin as Spirulina oscillarioides. Gomont then proposed the genus Spirulina and genus Arthrospila in 1892, following their initial consideration by Stizenberger in 1852, resulting in the official listing of the genus Spirulina as Spirulina Turpin ex Gomont. Subsequently in 1932, as a result of a proposal by Geitler, the genus Spirulina and genus Arthrospila were combined into the single genus of Spirulina (strains of previous genus Spirulina were placed into the section Euspirulina in the re-established genus Spirulina). The single genus Spirulina continued for a while based on this “Geitlerian system” but a new proposal was made to redivide it into two genera of Spirulina and Arthrospila. In the mid-1990’s, phylogenetic analysis using the 16S rRNA gene sequence confirmed that there is a clear distinction between the genus Spirulina and genus Arthrospila in terms of the phylogenetic lineage. Evaluation results based on the phylogenetic analysis using the 16S rRNA gene sequence suggested that there would be further diversity in terms of the phylogenetic lineage within the genus Spirulina in that Spirulina major and Spirulina subsalsa would be classified as being from separate phylogenetic lineages. In the latest Bergey’s manual of systematic bacteriology published in 2001, Spirulina Turpin ex Gomont and Arthrospila Stizenberger were listed separately. It was also described that some strains in genus Spirulina characterized as halotolerant, formed a tight monophyletic cluster by phylogenetic analysis using the 16S rRNA gene sequence.

Prior to the publication of Bergey’s manual, Nübel et al. officially proposed in 2000, the new genus of Halospirulina characterized by halotolerance and forming a tight monophyletic cluster in phylogenetic analysis. Halospirulina is characterized by the use of its physiological specificity concerning salinity, described as, “Halotolerant, able to grow at salinity between 3–13% or above” as description of the genus Halospirulina or “growth range including fresh water of which the salinity is 3 wt/vol % or below” described by Margheri et al. in 2003.

The present study aimed to screen microalgae which were producing compatible solutes, and to also identify the classification of the glycine betaine and O-α-D-glucopyranosyl-(1→2)-glycerol producing strain BAH8-10-45 which was discovered during this search. During the work, the existing diagnosis of the genus Halospirulina were re-examined and the euryhaline described in the diagnosis on Spirulina Turpin ex Gomont was reviewed. The classification of BAH8-10-45 has been clarified based on the phylogenetic taxonomy mainly featuring the morphological and physiological characteristics of the strain.

2. Materials and Methods

2.1. Sampling and algal screening

Environmental samples (soil, water, microbial mat and salt crystals) were collected from the shoreline at Shannah (20°44′N/58°41′E) and the surface of salt pan in Barr Al-Hikman (20°34′N/58°20′E).

These samples were collected in sterile plastic bottles and stored in a dark environment at ambient temperature. For screening of thermo-halotolerant microalgae, aliquots of environmental samples were suspended in 20 ml sterile IMK medium (Wako Pure Chemical Industries, Ltd., Japan) containing NaCl at 10–30 wt/vol %, and incubated under cool white light at 45°C. After the growth of microalgae was confirmed visibly (within 1–5 days), liquid culture was repeated for faster growth and screening of microalgae. Pure cultures were obtained after several rounds of serial dilution in liquid media to eliminate contamination, and inoculated onto solid media. Solid media were prepared with IMK medium containing NaCl at 10–20 wt/vol % with 1 wt/vol % Difco agar (Becton, Dickinson and Company) on sterilized petri dishes (Becton, Dickinson and Company). After 1–2 weeks, the isolates formed colonies on solid media.
2.2. Halotolerance and thermotolerance assays

For halotolerance assays, the isolated strain was inoculated into 100 ml flasks containing 20 ml of IMK medium at ten different salinity concentrations (0, 0.5, 1, 3, 5, 10, 15, 20, 25 and 30 wt/vol %). For thermotolerance assays, the isolated strain was inoculated into 100 ml flasks containing 20 ml of IMK medium at nine different temperatures (20, 25, 30, 35, 40, 45, 50, 55, and 60 °C) under the optimum growth salinity of each of the isolates. Triplicate cultures were grown under cool white light. Biomass was measured every two days using the dry weight method to determine the maximum growth density.

Evaluation of halotolerance of *Halospirulina tapetica* CCC Baja-95. Cl. 2 T which is type strain of the genus *Halospirulina* followed the method used by Nübel et al. 45. The modified 1/2 Provasoli’s enriched seawater culture medium 51 was adjusted by NaCl to different total salinities, was used for halotolerance assays. Algal cultures took place at 40 °C and the dry weight was measured every other day.

2.3. Light microscopic observation

Photomicrographs of Fig. 1 were taken using a digital camera (Nikon DS-L2) with a microscope (Nikon BX-51) equipped with Nomarski interference contrast (DIC) illumination.

For comparison of the cell size among strains of *S. subsalsa* and relates under the microscopic observation. *Spirulina subsalsa* NIES-27 was cultured in MA medium 27 at 25 °C, and *H. tapetica* CCC Baja-95. Cl. 2 T was cultured in modified 1/2 Provasoli’s enriched seawater medium 51 was adjusted to 7 wt/vol % in total salinities with NaCl 45.

2.4. Extraction of compatible solutes and measurement of 13C-NMR

Compatible solute was extracted from BAH8-10-45 cells, grown in IMK medium at 15 wt/vol % NaCl, essentially as described by Borowitzka et al. 61. The solute was freeze-dried and dissolved in D2O for further NMR analyses. Natural-abundance 13C NMR spectra of the solute were recorded at 100 MHz on a JEOL JNM-400A spectrometer. The chemical shifts were recorded in ppm using 3-(trimethylsilyl) propionate-2,2,3,3-d4 (TSP-d4) as an internal standard. The recorded chemical shifts were then compared with those of literature values for O-α-D-glucopyranosyl-(1→2)-glycerol and glycine betaine to determine the components 44.

2.5. PCR amplification

To determine a partial 16S rRNA gene sequence of isolates, a small number of cells were collected from a microbial colony on solid media using a needle and suspended in a PCR solution. This PCR solution (50 µl) contained 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.5 µl 10×PCR buffer, 0.2 mM of each dNTP, and 50 pmol of each primer (primers 27F and 1492R 61). Amplification conditions were as follows: 2 min activation of the polymerase at 95 °C, followed by 35 cycles consisting of 30 s at 94 °C, 30 s at 50 °C, and 2 min at 72 °C, and finally 7 min of extension at 72 °C. The PCR product was electrophoresed through a 0.8 wt/vol % agarose gel with TBE buffer, and then purified with a QIAquick gel extraction kit (QIAGEN). A nucleotide sequence of a PCR product was determined using a Bigdye terminator v3.1 cycle sequencing kit (Applied Biosystems) and a model 3730 DNA sequencer (Applied Biosystems).

2.6. Phylogenetic analysis

The 16s rRNA nucleotide sequences determined were aligned with reference sequences obtained from the GenBank database using Clustal W version 1.83 and edited manually using BioEdit Sequence Alignment Editor ver. 7.0.9. 23 A phylogenetic tree was constructed using the neighbor-joining method with K2 (Kimura-2-parameter 29) +I+G model in MEGA ver. 5.0 46. Bootstrap values were calculated based on 1000 replicates.

3. Results and Discussion

3.1. Selection of thermo-halotolerant microalgae

From the results of thermo-halotolerant screening, four algal mixed cultures (SHN3, BAH12, BAH13 and BAH8) were screened, and algal strains (SHN3-30-45, BAH12-20-45, BAH13-20-45 and BAH8-10-45) were isolated from each mixed culture (Table 1). Photomicrographs of the four isolates are shown in Fig. 1a-d. All isolates are simply identified using the “Key to the form-genera of Subsection I or III” in Bergey’s Manual of Systematic Bacteriology 8.

BAH12-20-45 and BAH13-20-45 are unicellular, ovoid,
Table 1. Characteristics of isolated thermo-halotolerant cyanobacteria originated from the Sultanate of Oman.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SHN 3-30-45</th>
<th>BAH 8-10-45</th>
<th>BAH12-20-45</th>
<th>BAH13-20-45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth temp. range (optimum temp.); °C</td>
<td>30–50 (35)</td>
<td>25–45 (30)</td>
<td>30–45 (40–45)</td>
<td>25–45 (35)</td>
</tr>
<tr>
<td>Growth salinity range (optimum salinity); %</td>
<td>3–30 (10)</td>
<td>5–20 (10)</td>
<td>10–30 (15)</td>
<td>10–30 (20)</td>
</tr>
<tr>
<td>Width (Mean &lt;±SE&gt;); μm</td>
<td>3.6 &lt;±0.5&gt;</td>
<td>4.1 &lt;±0.2&gt;</td>
<td>3.3 &lt;±0.4&gt;</td>
<td>3.9 &lt;±0.8&gt;</td>
</tr>
<tr>
<td>Length (Mean &lt;±SE&gt;); μm</td>
<td>970 &lt;±420&gt;</td>
<td>530 &lt;±210&gt;</td>
<td>5.4 &lt;±0.9&gt;</td>
<td>7.6 &lt;±1.7&gt;</td>
</tr>
<tr>
<td>Shape</td>
<td>Filamentous, straight cylindrical</td>
<td>Filamentous, closed helically</td>
<td>Unicellular, ovoid without mucilaginous envelope</td>
<td>Unicellular, ovoid without mucilaginous envelope</td>
</tr>
<tr>
<td>Morphological identification</td>
<td>Geitlerinema sp.</td>
<td>Spirulina sp.</td>
<td>Halothece sp.</td>
<td>Halothece sp.</td>
</tr>
<tr>
<td>Isolation source</td>
<td>Stagnant hyper saline pond (salinity 19%)</td>
<td>Microbial mat from heliothermal shallow saline shore (salinity 6%)</td>
<td>Microbial mat from surface soil in salt pan</td>
<td>Microbial mat from surface soil in salt pan</td>
</tr>
</tbody>
</table>

without mucilaginous envelope, and with binary fission in one plane in subsequent generations. BAH12-20-45 has the cell width of 2.5–4.2 μm and the cell length of 4.1–7.1 μm. BAH13-20-45 has the cell width of 2.9–5.5 μm and the cell length of 5.5–10.6 μm. These morphological features indicated that BAH12-20-45 and BAH13-20-45 are closely related to genus *Cyanothece*. According to the description of the Bergey’s manual, genus *Cyanothece* are divided into three clusters on the phylogenetic tree of cyanobacteria based on 16S rRNA gene sequences. Cluster 3 contains strains PCC 7418, PCC 9718, MPI95AH11, MPI95AH13, MPI96AL06, MP96P408, MPI96AL03, and Syn C1 P22, differing from cluster 1 and cluster 2 in growing well at salinity greater than that of seawater (3 wt/vol % NaCl) and tolerating salinities as high as 20 wt/vol % NaCl, reflecting their original habitat, in a solar evaporation pond. According to the description by Garcia Pichel et al., the strain MPI95AH13 has diverse at the cell shape, the fusiform (*Dactylococcopsis*-like shape) is sometimes observed as one of its cell shape. The fusiform also observed at the strain BAH12-20-45 showing by the arrow in Fig. 1a. After the publication of latest Bergey’s manual (2001), Margheri et al. proposed the new genus *Halothece* in 2008, as the new classification for the strain MPI96P605 in the genus *Cyanothece*. The genus *Halothece* is considered to consist of a group of strains, ranging from those similar to the genus *Cyanothece* to those of which the similarity of the 16S rRNA gene sequence is above 91% with *Halothece californica* MPI96P605 as the type strain.

Morphological characteristic of BAH12-20-45 and BAH13-20-45 is similar to those of the *Cyanothece* mentioned above, and similarity with *H. californica* MPI96P605 was higher than 91% (data not shown). According to some morphological features, BAH12-20-45 and BAH13-20-45 were identified *Halothece sp.*

BAH8-10-45 is characterized by filamentous, closed helically coiled trichomes, with invisible cross-walls, no sheaths, gliding motility, helix widths between 3.7 and 4.5 μm, and trichome widths between 1.2–1.6 μm. These morphological features indicated that BAH8-10-45 is closely related to genus *Spirulina* Turpin ex Gomont. According to above mentions, BAH8-10-45 was identified as *Spirulina sp.*

SHN3-30-45 is characterized by filamentous, straight cylindrical trichomes, with gliding motility, and absence of constrictions between adjacent cells, and trichome widths between 2.9–4.0 μm. These morphological features indicated that SHN3-30-45 is closely related to genus *Geitlerinema* (Anagnostidis & Komárek) Anagnostidis. SHN3-30-45 was identified as *Geitlerinema sp.* from the mentions above.

Growth characteristics of the four isolates are shown in Table 1. Temperature tolerance for BAH12-20-45 ranged from 30–45°C, with an optimum at 40–45°C. Salinity tolerance ranged from 10–30 wt/vol % total salinity, with an optimum of 15 wt/vol %. Temperature tolerance for BAH13-20-45 ranged from 25–45°C, with an optimum at 35°C. Salinity tolerance ranged from 10–30 wt/vol % total salinity, with an optimum of 20 wt/vol %. Temperature tolerance for SHN3-30-45 ranged from 30–50°C, with an optimum at 35°C; no growth was observed at 55°C. Salinity tolerance ranged from 3–30 wt/vol % total salinity, with an optimum of 10 wt/vol %. Temperature tolerance for BAH8-10-45 ranged from 25–45°C, with an optimum at 30°C; no growth was observed at 50°C. Salinity tolerance ranged from 5–20 wt/vol % total salinity, with an optimum of 10 wt/vol %; no growth was observed at <5 wt/vol %.

According to the description of Borowitzka & Borowitzka, *Dunaliella* species are known as halophilic chlorophytes, and their commercial production was started in Australia, Israel and USA during the 1970s and 1980s. The optimum temperature for *Dunaliella salina* has been reported to be in the range 20–40°C, and growth salinity at <approx. 32 wt/ vol % saturation point. *D. salina* is the first micro alga to be used commercially to produce fine chemicals, because its extreme salinity tolerance simplifies maintenance of a unialgal culture, relatively free of competitors, pathogens and predators. All our isolates are also characterized as thermo-halotolerant similar to *Dunaliella* species. This indicates that these isolates are also suitable for simplifying maintenance of a unialgal culture for the commercial mass production. In addition, commercial production from *D.*
Spirulina subsalsa var. salina var. nov. also requires a suitable, low-cost harvesting method 46. Shelef et al. 49 also pointed out that cell size is an important factor since low-cost filtration procedures are presently applicable only for harvesting fairly large cell size (e.g. Coelastrum, Spirulina).

Among our four thermo-halotolerant isolates (SHN3-30-45, BAH12-20-45, BAH13-20-45, and BAH8-10-45), BAH8-10-45 was found to have fairly large cell size (Fig. 1d and Table 2) suitable for low-cost filtration procedures for mass production. In addition, cells of BAH8-10-45 accumulated glycine betaine and O-\(\alpha\)-D-glucopyranosyl-(1→2)-glycerole (Fig. 2) as compatible solutes, indicating potential for production of fine chemicals of commercial importance.

Gabby-Azaria et al. pointed out glycine betaine accumulating under hypersaline growth conditions in S. subsalsa 16 and Mackay et al. also described glycine betaine in halotolerant species of Spirulina 38. There had been reported glycine betaine accumulation in other species of Spirulina, but BAH8-10-45 accumulated glycine betaine and O-\(\alpha\)-D-glucopyranosyl-(1→2)-glycerole as compatible solutes. It was suggested that BAH8-10-45 have characteristic compatible solute accumulation different from other halotolerant species of Spirulina.

Glycine betaine and O-\(\alpha\)-D-glucopyranosyl-(1→2)-glycerole have great potential for further commercial application. Glycine betaine have been known to function as osmoprotectant, for reduction of plant stress from salt 30 and drought 10,48, as skin protection from ultraviolet induced cell damage 34, and as inhibitor of \(\beta\)-amyloid formation in Alzheimer’s disease 34, used in agricultural, cosmetic, and pharmaceutical industries. O-\(\alpha\)-D-glucopyranosyl-(1→2)-glycerole is also expected to have potential in cosmetic, food, and pharmaceutical industries 37,46.

Table 2. Diagnostic and descriptive features of cyanobacteria having tightly coiled trichomes.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Trichome width ((\mu)m)</th>
<th>Helix width ((\mu)m)</th>
<th>Trichome length ((\mu)m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spirulina subsalsa NIES-27</td>
<td>1.5 ±0.1 &gt;</td>
<td>4.2 ±0.3 &gt;</td>
<td>390 ±160 &gt;</td>
</tr>
<tr>
<td>Halospirulina tapaticola CCC Baja-95 Cl. 2</td>
<td>1.5 ±0.1 &gt;</td>
<td>4.2 ±0.2 &gt;</td>
<td>530 ±250 &gt;</td>
</tr>
<tr>
<td>Spirulina subsalsa BAH8-10-45</td>
<td>1.4 ±0.1 &gt;</td>
<td>4.1 ±0.2 &gt;</td>
<td>540 ±210 &gt;</td>
</tr>
<tr>
<td>Spirulina subsalsa Oersted ex Gomont</td>
<td>0.8-1.4</td>
<td>2.3-5 (5.6)</td>
<td>150-500 (~700)</td>
</tr>
<tr>
<td>Spirulina labyrinthiformis Kützing ex Gomont</td>
<td>0.8-1.4</td>
<td>2.3-5 (5.6)</td>
<td>75-120 (~160)</td>
</tr>
<tr>
<td>Halospirulina Nübel, Garcia-Pichel et Muyzer</td>
<td>1.5</td>
<td>4</td>
<td>NA</td>
</tr>
<tr>
<td>Halospirulina tapaticola Nübel et al.</td>
<td>1.5–3</td>
<td>4–6</td>
<td>NA</td>
</tr>
</tbody>
</table>

Mean ±SE mean values of at least 50 samples. NA: information not available.

Fig. 2. Natural abundance 13C NMR spectrum of an aqueous extract from cells of BAH8-10-45. Each resonance is identified as to the particular solute molecule, B: glycine betaine; G: O-\(\alpha\)-D-glucopyranosyl-(1→2)-glycerole (details of the identification, see reference 5,44).
BAH8-10-45 was selected from isolates, to accumulate compatible solutes and to have fairly large cell size suitable for low-cost filtration procedures for mass production. Subsequently, the identification of BAH8-10-45 was performed.

3.2. Identification of BAH8-10-45

3.2.1. Morphology of BAH8-10-45

Morphology of BAH8-10-45 (Fig. 1d–f) is characterized as follows: filamentous closed helically coiled trichomes, with invisible cross-walls and no sheaths. Trichome width is between 1.2–1.6 μm, and helix width is between 3.7–4.5 μm (Table 2). While the helix width is almost constant up to the head, the body is straight or slightly bent (as indicated by the arrow in Fig. 1e). Trichomes coiled counter-clockwise attach to one another at certain intervals, forming a helix (Fig. 1d). These trichomes are viscous and the cells are either solitary or aggregated (Fig. 1d). Trichomes regularly turn around spirally with little forward motion. Morphological characteristics are similar to Spirulina Turpin ex Gomont. According to above mentions, BAH8-10-45 was defined genus Spirulina.

Further morphological comparison was conducted with the diagnosis of S. subsalsa in the original article by Gomont. Gomont described the morphological characteristics of S. subsalsa as “Trichomala pallide aeruginosa, in stratum saturate aerugineum aut aerugineo-lutescens agglomerase, vel inter varias Oscillatoriae sparsa, ambitu irregulariter tortuosa, rarissime recta, in spiram densam subirregularem, passim laxiunculam, aut rarius regularem, diametro 3 μ and 5 μ aequantem contorta, 1 μ and 2 μ crassa; anfractus contigui vel subcontigui (v.s.)". The morphological characteristics of BAH8-10-45 agreed with those of S. subsalsa described above. The trichome of BAH8-10-45 shown in Fig. 1 is either blue-green or greyish white (showing by the arrow in Fig. 1d) and inside of the trichome is homogenous (Fig. 1f). The helix is almost straight or slightly bent (showing by the arrow in Fig. 1e). Trichomes coiled regularly closed (Fig. 1f). The helix is almost straight or slightly bent (showing by the arrow in Fig. 1e). Trichomes coiled regularly closed (Fig. 1f). The helix is almost straight or slightly bent (showing by the arrow in Fig. 1e). Trichomes coiled regularly closed (Fig. 1f). The helix is almost straight or slightly bent (showing by the arrow in Fig. 1e).

3.2.2. Salinity response of BAH8-10-45

There are two groupings of Spirulina according to halotolerance (Fig. 3); a higher tolerance group (growth at >15 wt/vol %) and a lower tolerance group (no growth at >15 wt/vol %). The lower tolerance group consists of S. subsalsa, Spirulina labyrinthiformis and S. major. The higher tolerance group consisted of Halospirulina strains and S. subsalsa including BAH8-10-45. Among the higher tolerance group, all strains were able to grow at around seawater salinity (approximately 3–4 wt/vol %) except BAH8-10-45 (Fig. 3). Around freshwater salinity (approximately less than 0.5 wt/vol %), Halospirulina sp. IR21 and PE1 strains were able to grow as described by Margheri et al. The strain of Spirulina subsalsa BAH8-10-45 was unable to grow in freshwater, brackish-water and seawater salinities, and its halotolerance ranged from 5–20 wt/vol %.

By Gomont’s re-establishment of the genus Spirulina in 1892, S. major, S. labyrinthiformis and S. subsalsa were incorporated into the genus Spirulina, especially S. subsalsa, is characterized by its habitat in diverse environmental conditions around the world as suggested by its expression of “cosmopolitan” by many researchers. Gomont’s original diagnosis of the distribution and habitat of Spirulina Turpin ex Gomont also mentioned its euryhaline using the expression of “hydrophilae vel halophilae” after the description of the diagnosis composed of its morphological characteristics. Fig. 3 summarized halotolerance of these three Spirulina species, i.e. S. major, S. labyrinthiformis and S. subsalsa including BAH8-10-45. The halotolerance range of S. major and S. labyrinthiformis was generally between freshwater and salinity of 13 wt/vol %.

In contrast, S. subsalsa was found to generally grow between freshwater and salinity of 20 wt/vol %. These results indicate that the euryhaline of the genus Spirulina, its distribution and habitat, ranging from freshwater, brackish-water, and seawater includes a much higher salinity of at least around 20 wt/vol %, at least. In the diagnosis of Gomont, it was suggested that S. subsalsa has a typical euryhaline, meaning higher halotolerance than S. major or S. labyrinthiformis, the former described as “aquas salinas aut subsalsas” while the latter two were described simple as “aquas subsalsas”. Halotolerance of the S. subsalsa distinguished from others with an expression of “salsas”, is inferred to be between at least 13–15 wt/vol % and approximately 20 wt/vol % (Fig. 3).

3.2.3. Taxonomic consequences

Taxonomic account

Spirulina subsalsa var. salina Kuroiwa et Tasaki var. nov.

Spirulina subsalsa var. salina (sa.li’na. L. adj. salina, salted, saline). Fig. 1d.

Description: halophilic, no growth below 5 wt/vol % and
above 20 wt/vol % able to grow at least 13–15 wt/vol % as total salinity. No growth at freshwater, brackish-water, and seawater. Morphological characteristics are the same as *Spirulina subsalsa* Oersted ex Gomont. The optimum growth temperature is observed at 30 °C while no growth is observed at 20 °C or 50 °C. It accumulates glycine betaine and O-α-D-glucopyranosyl-(1→2)-glycerol as compatible solutes at 15 wt/vol % in total salinity.

Habitat: heliothermal hypersaline environment.

Type Locality: sere algal mat on the surface of marine sabkha (salt pan) in Barr Al-Hikman, the Sultanate of Oman (20°35′24.72″N/58°16′8.69″E).

Type: preserved specimen number NIES-3373, deposited in the Microbial Culture Collection at the National Institute for Environmental Studies (NIES), Ibaraki, Japan.

Type Strain: BAH8-10-45, isolated by Yoichi Kuroiwa.

Castenholz *et al.* described some species belonging to genus *Spirulina* having worldwide distribution in freshwater, brackish-water, and seawater. *Spirulina* species are also common in inland saline lakes and in some hot springs at temperatures as high as 50 °C (7). The halotolerant morphospecies of *Spirulina* have been observed in North America (8), South America (9), Europe (7), Australia (30), Africa (31), and Asia (18,42). Many ecological studies clearly indicate that the distribution of morphospecies of *Spirulina* is widespread in hypersaline environments throughout the world.

In a hypersaline endoevaporitic microbial community in Eilat (Israel), morphospecies of *Spirulina*, both Halothecelike and Phormidium-like cyanobacteria, were identified by microscopic observation (49). Nübel *et al.* also described that Halospirulina (characterized morphospecies of *Spirulina*), Oscillatoria limnetica-like, and Euhalothece group species were detected by DGGE analysis, in the microbial mat in evaporation ponds of a saltern in Guerrero Negro, Baja California, Mexico (No. 6 pond: ca. 14% wt/vol total salinity) (45). Ecological studies report the distribution of *Spirulina* morphospecies in hypersaline environments at Solar Lake, Eilat in Israel (49), Yallahs Salt Ponds in south Jamaica (20), salterns of Alicante in Spain (47), coastal salt lakes in Western Australia (28), salt pans of southeastern coast in India (20), hypersaline microbial mats in Sultanate of Oman (1), and solar salterns of Petchaburi in Thailand (6). These ecological studies clearly show that phylogenetic identification based on ‘genus or species’ phenotypes has not changed since establishment of genus *Spirulina* Turpin ex Gomont and related species (21).

The proposal for the new genus of Halospirulina by Nübel *et al.* (45) meant that at least two genera and two species (*H. tapeticola* and *S. subsalsa*) exist in the grouping described as “Spirulina morphospecies in hypersaline environments” as pointed out by Komárek and Anagnostidis (33). It is difficult to separately identify them based only on morphological

### Growth of the Spirulina/Halospirulina strains in response to salinity

<table>
<thead>
<tr>
<th>Strain</th>
<th>Possible Classification</th>
<th>Total salinity (wt/vol %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBMM Hi-45&lt;sup&gt;(5)&lt;/sup&gt;</td>
<td><em>Spirulina major</em></td>
<td>0, 5, 10, 15, 20</td>
</tr>
<tr>
<td>PCC6313&lt;sup&gt;(6)&lt;/sup&gt;</td>
<td><em>Spirulina major</em></td>
<td></td>
</tr>
<tr>
<td>MPI S1&lt;sup&gt;(7)&lt;/sup&gt;</td>
<td><em>Spirulina labyrinthiformis</em></td>
<td>0, 5, 10, 15, 20</td>
</tr>
<tr>
<td>CCC Snake P. Y-85&lt;sup&gt;(8)&lt;/sup&gt;</td>
<td><em>Spirulina subsalsa</em></td>
<td></td>
</tr>
<tr>
<td>MPI S2&lt;sup&gt;(9)&lt;/sup&gt;</td>
<td><em>Spirulina subsalsa</em></td>
<td></td>
</tr>
<tr>
<td>MPI S4&lt;sup&gt;(9)&lt;/sup&gt;</td>
<td><em>Spirulina subsalsa</em></td>
<td></td>
</tr>
<tr>
<td>P 7&lt;sup&gt;(40)&lt;/sup&gt;</td>
<td><em>Spirulina subsalsa</em></td>
<td></td>
</tr>
<tr>
<td>UBMM Bo 89&lt;sup&gt;(9)&lt;/sup&gt;</td>
<td><em>Spirulina subsalsa</em></td>
<td></td>
</tr>
<tr>
<td>BAH 8-10-45&lt;sup&gt;(17)&lt;/sup&gt;</td>
<td><em>Spirulina subsalsa</em></td>
<td></td>
</tr>
<tr>
<td>IR 21&lt;sup&gt;(10)&lt;/sup&gt;</td>
<td>Halospirolea sp.</td>
<td>0, 5, 10, 15, 20</td>
</tr>
<tr>
<td>PE 1&lt;sup&gt;(10)&lt;/sup&gt;</td>
<td>Halospirolea sp.</td>
<td></td>
</tr>
<tr>
<td>J. Som 6&lt;sup&gt;(39)&lt;/sup&gt;</td>
<td>Halospirolea sp.</td>
<td></td>
</tr>
<tr>
<td>ML 3&lt;sup&gt;(30)&lt;/sup&gt;</td>
<td>Halospirolea sp.</td>
<td></td>
</tr>
<tr>
<td>MPI S3&lt;sup&gt;(17)&lt;/sup&gt;</td>
<td>Halospirolea sp.</td>
<td></td>
</tr>
<tr>
<td>CCC Baja-95 Cl 3&lt;sup&gt;(30)&lt;/sup&gt;</td>
<td><em>Halospirolea tapeticola</em></td>
<td></td>
</tr>
<tr>
<td>CCC Baja-95 Cl 2&lt;sup&gt;(30)&lt;/sup&gt;</td>
<td><em>Halospirolea tapeticola</em></td>
<td></td>
</tr>
<tr>
<td>CCC Baja-95 Cl 2&lt;sup&gt;(30)&lt;/sup&gt;</td>
<td><em>Halospirolea tapeticola</em></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Growth yields of the Spirulina/Halospirulina strains in response to salinity.

The growth was defined as optimal: 75–100%, sub-optimal: 50–74%, weak: up to 49%. Originated from a: Nübel *et al.* (Salinity tolerance tested at total salinity 0–25 wt/vol %) (45); b: Margheri *et al.* (Salinity tolerance tested at total salinity 0–18 wt/vol %) (39) and c: This study (Salinity tolerance tested at total salinity 0–30 wt/vol %). Classification based on originated articles or trichome morphology, generic and species names given are sensu Castenholz (8), Komárek & Anagnostidis (33) and Gomont (21).
observation, possibly creating a problem that multi-step identification process, such as evaluation using the 16s rRNA gene sequence (phylogenetic analysis etc.) or evaluation of the halotolerance after the isolation of a strain, may be required. In this study, the results of the morphological observation of *H. tapetica* CCC Baja-95 Cl. 2 have re-confirmed that this strain is morphologically identified as *S. subsalsa* 30, and the same as *S. subsalsa* NIES-27 or *S. subsalsa* var. *salina* BAH8-10-45.

Nübel *et al.* commented on the conventional classification of cyanobacteria that, “morphological classification may provide insufficient taxonomic resolution and cyanobacteria with similar or identical morphology may have slightly different physiology”. They proposed a new genus of *Halospirulina* based on the extreme halotolerance as a physiological characteristic can be used to define a phylogenetically coherent group 45.

Many phylogenical researchers have attempted over a period of some 100 years to clarify the diversity of the halotolerance of *S. subsalsa* Oersted ex Gomont, starting in 1892 by Gomont who used the words “aquas salsas aut subsalsas” to describe its distribution and habitat 21. In 1932, Geitler wrote, “In stehenden salzhaltigen Gewässern, in Meer, in Brackwasser, in Thermen, auch in Hochmooren; Kosmopolitisch” 19. In 2005, Komárek and Anagnostidis wrote, “In marine biotopes and in inland salty and brackish stagnant waters, frequently also in mineral and thermal springs, sometimes (?) in freshwaters, bogs ...; possibly distributed worldwide, cosmopolitan” 13. As a result of their work, it is now clearly established that diversity in terms of halotolerance as a morphological characteristic of *S. subsalsa*. If it is necessary to present such character as a classification, the diversity of halotolerance of the genus *Spirulina* must be taxonomically evaluated and classified with variety (or subspecies) or even lower taxonomic groups than the genus level where the morphological classification has been established. The findings of the present study suggest that as far as the halotolerance of those classified in the genus *Spirulina* is concerned, any effort to improve the classification accuracy should be based on a more detailed classification of the variety or subspecies, etc. mainly below *S. subsalsa*.

After the description of a new genus by Nübel *et al.* 45, Margheri *et al.* in 2003 verified the growth of *Halospirulina* strains (Strains IR21 and PE1) in freshwater and a salinity of 3 wt/vol % or lower (Fig. 3) 39. It is indicated that there are some strains, having the defining halotolerance of the genus *Halospirulina*, in this *Halospirulina* cluster. In this study, it was confirmed that BAH8-10-45 was also included in the *Halospirulina* cluster as described later. In addition, it is worth noting that our isolate BAH8-10-45 showed no growth below 5 wt/vol % total salinity (Fig. 3), although growth salinity response of genus *Halospirulina*, described as one of the character of the genus, ranged from 3–13 wt/vol % total salinity or above 45. This strongly suggests that there are at least three genotypes, based on growth salinity response, in the *Halospirulina* cluster/genus.

Based on the above, it is evident that taxonomical status of genus *Halospirulina* and the taxonomical positions of strains in the *Halospirulina* cluster/genus are unclear. In addition, the taxonomical status of genus *Halospirulina* should be re-examined by further studies without making exceptions on the phylogenological taxonomy. From the diagnosis of the euryhaline characteristics of *S. subsalsa* in terms of its distribution and habitat by Gomont in 1892 21, to the more recent description by Komárek and Anagnostidis in 2005 33, it was steadily established within the framework of conventional physiological taxonomy that there was a diversity of *S. subsalsa* in terms of a physiological characteristic, namely its halotolerance. Based on the results this study’s original descriptions, we propose that the halotolerant BAH8-10-45 having phenotype of *S. subsalsa*, should be placed under the species *S. subsalsa*, and we also propose *Spirulina subsalsa* var. *salina* for this strain. The description of this new taxonomical status is given above.

3.2.4. Molecular phylogenetic relationship of BAH8-10-45 among traditional morphospecies of *Spirulina*

The following evaluation using the 16S rRNA gene sequence has two purposes. One is to clarify the phylogenetic position of BAH8-10-45 which has been identified as *Spirulina subsalsa* var. *salina*. The other is to clarify the phylogenetic status of the “Highly halotolerant/Halospirulina cluster” (sensu Nübel *et al.* 45) as a new cluster that should be included in the genus *Spirulina*.

Phylogenetic analysis of the 16S rRNA gene sequence also included comparable long sequences (from 101 to 1450 corresponding to Escherichia coli str. K-12 substr. MG1655 [U00096] numbering) of particularly well defined strains belonging to cyanobacteria available in GenBank 64 (Fig. 4a). The strains identified as *Spirulina* and available in GenBank were divided into two clusters (tentatively named *Spirulina* cluster A and *Spirulina* cluster B) in the phylogenetic tree of cyanobacteria (Fig. 4a). The branching of these clusters was supported by high bootstrap values (96 or 100%) in Neighbor-Joining analysis (Fig. 4a).

Fig. 3b shows the phylogenetic analysis of the 16S rRNA gene sequences having a sequential similarity to *Spirulina* (from 78 to 1469 corresponding to E. coli numbering). Five *Spirulina* clusters (*Spirulina* cluster 1.1, 1.2, 1.3, 2 and 3) were formed in the phylogenetic tree among *Spirulina* and related cyanobacteria. Cluster A was subdivided into four clusters (*Spirulina* cluster 1.1, 1.2, 1.3 and cluster 3). Cluster 1.1 contained traditional morphospecies of *S. subsalsa*. *S. subsalsa* CCAP 1475/1 [HF678502], *Spirulina* sp. MPI 54 [Y18792], *Spirulina* sp. P7 [AF091109], uncultured bacterium clone GBI-65 [GQ441246], GBI-66 [GQ441247], and GBIII-78 [GQ441342] were isolated from marine and brackish water 48. Cluster 1.2 contained traditional morphospecies of *S. subsalsa*. *S. subsalsa* NIES-27 [AB003166], *S. subsalsa* [AF329394], *S. subsalsa* FACHB351 [FJ862662], and uncultured bacterium clone SA 82 [JQ738968] were isolated from coastal and sediment on surface rocks 4. *S. subsalsa*
strains in Cluster 1.2 exhibit drought resistance⁴. Cluster 1.3 was “Highly halotolerant/Halospirulina cluster” (sensu Nübel et al.⁴⁵) containing traditional morphospecies of S. subsalsa. Spirulina sp. EEW1 [HQ008224], Halospirulina sp. CCC Baja-95 Cl. 3 [Y18790], Halospirulina sp. MPI S3 [Y18789], H. tapetica CCC Baja-95 Cl. 2T [Y18791], and strain S. subsalsa var. salina BAH8-10-45 [AB873003] were isolated from hypersaline environments⁴,⁴⁵ and exhibit a hyper salt tolerance⁴,⁴⁵. Cluster 2 mainly contained traditional morphospecies of S. major strains. Spirulina sp. PCC 6313 [AM709631], S. major OBB36S18 [AJ639890], S. major 1LT27S0 [FM177505], S. major OBB22S09 [AJ635436], Spirulina sp. GLS010 [FJ546714], Spirulina sp. X75045, and S. subsalisa CCAP 1475/2 [HF678507] were isolated from a wide salinity range of habitats (freshwater, blackish and marine)³¹,⁴⁵. Cluster 3 contained traditional

---

**Fig. 4a.** Neighbor-Joining phylogenetic tree of cyanobacteria based on 16S rRNA gene sequences. Numbers at nodes indicate bootstrap percentages from 1000 replicas obtained with distance and parsimony analyses, respectively; values were reported only at nodes where both methods gave bootstrap percentages as 100%. Accession codes of sequences retrieved from GenBank⁴. The position of the 16S rRNA gene sequence of cyanobacteria with helical, tightly coiled trichomes were marked in bold. The scale bar indicates 1% estimated sequence divergence. The analysis involved 46 nucleotide sequences of 16S rRNA gene. There were a total of 1284 positions in the final dataset.
morphospecies of *S. labyrinthiformis* strain exhibiting a thermotolerance. *S. tapetica* sp. CCC Baja-95 Cl. 2 (97.8%), *S. subsalsa* NES-27: 92.1% and *S. major* 45) : 90.9% and *Spirulina* sp. CCC Snake P. Y85 as *S. labyrinthiformis* 45) : 90.8% showing in Table 3). The percentage of similarity among BAH8-10-45 and related strains clearly justifies the definition of a new species according to common bacteriological practice 50) . However, when the similarity within each cluster is evaluated regarding the current species belonging to the genus *Spirulina*, the percentage of similarity within each species is
91.0–99.4% for *S. subsalsa* (Cluster 1.1; 97.5–99.4% (n = 3), Cluster 1.2: 94.7–99.2% (n = 2) and Cluster 1.3: 97.3–98.7% (n = 4)), 98.8–100% for *S. major* (Cluster 2, n = 5), and 100% for *S. labyrinthiformis* (Cluster 3, n = 1). It is clear that the similarity threshold of the species belonging to the genus *Spirulina* is equal to or slightly lower than threshold range of 98.7–99% for new species of common bacteriological practice. In other words, there appears to be a situation where clusters including species of the genus *Spirulina* in the phylogenetic analysis based on the 16S rRNA gene sequence correspond to the genus (or higher) which is a broad concept bundling species of most common bacteria. The similarity within *S. subsalsa* having three sub-clusters is low (91.0–99.4%). The range of similarity within each of the sub-clusters forming *S. subsalsa* is generally similar to that of *S. major* (Cluster 2), suggesting a possibility that the future progress of research may divide *S. subsalsa* into several species. Of the three sub-clusters forming *S. subsalsa*, Cluster 1.3 appears to show a different character in terms of halotolerance of its strains from strains forming other clusters of *S. subsalsa*. It must be noted that this difference was used by Nübel et al. to propose the genus *Halospirulina* 43. If halotolerance described above are re-arranged (for example, condition pointed out by Margheri et al. in 2003 that a strain must be resistant to a salt concentration of 15 wt/vol % or higher 39) with related strains for the further advancement of research, there is a possibility of new developments in the near future. Such developments may include a change of the diagnosis of *S. subsalsa* in the genus *Spirulina* Turpin ex Gomont based on plant taxonomical evaluation and the establishment of a new species as a morpho/ecospecies. Another possibility is the introduction of a new genus with the condition that species grow with a salinity of approximately 13–15 wt/vol % or higher or re-definition of the genus *Halospirulina* based on classification and analysis with emphasis on phylogenetic analysis using the 16S rRNA gene sequence.

Accordingly, until a new definition of this new taxonomic group is given to Cluster 1.3, this cluster is considered to be a *S. subsalsa* cluster based on the classification of the genus *Spirulina* which is firmly established at present. If the strains in this cluster are to be further classified using significant characteristics, a taxonomic group below *S. subsalsa* should be established in respect of the classification conforming to phycological taxonomy as in the case of BAH8-10-45 so that its status is clear. As far as evaluation of the phylogenetic analysis of the genus *Spirulina* is concerned, there appears to be a gap in the sense that the species and variety (or subspecies) in the classification based on phycological taxonomy generally correspond to such higher taxonomic groups as genus and species in the phylogenetic analysis and evaluation of the similarity of most common bacteria using the 16S rRNA gene sequence.

This gap between phylogenetic analysis using the 16S rRNA gene sequence and the conventional classification also exists within the taxonomy of most common bacteria. While

<table>
<thead>
<tr>
<th>Strain</th>
<th>GenBank Accession Number</th>
<th>Cluster number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Spirulina subsalsa</strong> var. salina var. nov.</td>
<td><strong>Cluster 1.1</strong></td>
<td>1</td>
<td>100.0</td>
<td>97.8</td>
<td>97.5</td>
<td>92.7</td>
<td>92.8</td>
<td>91.7</td>
<td>91.6</td>
<td>91.6</td>
<td>91.0</td>
<td>92.7</td>
<td>92.3</td>
<td>92.4</td>
<td>91.5</td>
<td>91.0</td>
<td>91.5</td>
<td>91.0</td>
</tr>
<tr>
<td></td>
<td>Cluster 1.2</td>
<td>2</td>
<td>100.0</td>
<td>99.4</td>
<td>93.0</td>
<td>93.0</td>
<td>92.8</td>
<td>92.8</td>
<td>91.8</td>
<td>91.3</td>
<td>91.6</td>
<td>91.0</td>
<td>92.9</td>
<td>92.6</td>
<td>92.9</td>
<td>92.9</td>
<td>92.7</td>
<td>92.7</td>
</tr>
<tr>
<td></td>
<td>Cluster 1.3</td>
<td>3</td>
<td>100.0</td>
<td>99.4</td>
<td>92.9</td>
<td>92.6</td>
<td>92.4</td>
<td>94.7</td>
<td>91.6</td>
<td>91.6</td>
<td>91.2</td>
<td>91.0</td>
<td>92.6</td>
<td>92.6</td>
<td>92.6</td>
<td>92.6</td>
<td>92.3</td>
<td>92.1</td>
</tr>
<tr>
<td></td>
<td><strong>Cluster 2</strong></td>
<td>4</td>
<td>100.0</td>
<td>92.1</td>
<td>91.5</td>
<td>91.8</td>
<td>92.6</td>
<td>92.5</td>
<td>91.9</td>
<td>91.8</td>
<td>91.9</td>
<td>91.8</td>
<td>91.7</td>
<td>91.8</td>
<td>91.9</td>
<td>91.8</td>
<td>91.8</td>
<td>91.7</td>
</tr>
<tr>
<td></td>
<td><strong>Cluster 3</strong></td>
<td>5</td>
<td>100.0</td>
<td>97.8</td>
<td>97.3</td>
<td>91.3</td>
<td>91.3</td>
<td>91.7</td>
<td>91.7</td>
<td>91.8</td>
<td>91.7</td>
<td>91.5</td>
<td>91.4</td>
<td>91.2</td>
<td>91.1</td>
<td>91.3</td>
<td>91.4</td>
<td>91.7</td>
</tr>
<tr>
<td></td>
<td><strong>Cluster 4</strong></td>
<td>6</td>
<td>100.0</td>
<td>98.8</td>
<td>92.0</td>
<td>92.0</td>
<td>91.8</td>
<td>92.0</td>
<td>91.8</td>
<td>91.9</td>
<td>91.8</td>
<td>91.9</td>
<td>91.8</td>
<td>91.9</td>
<td>91.9</td>
<td>91.8</td>
<td>91.9</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td><strong>Cluster 5</strong></td>
<td>7</td>
<td>100.0</td>
<td>98.8</td>
<td>92.0</td>
<td>92.0</td>
<td>91.8</td>
<td>92.0</td>
<td>91.8</td>
<td>91.9</td>
<td>91.8</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td><strong>Cluster 6</strong></td>
<td>8</td>
<td>100.0</td>
<td>98.8</td>
<td>92.0</td>
<td>92.0</td>
<td>91.8</td>
<td>92.0</td>
<td>91.8</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td><strong>Cluster 7</strong></td>
<td>9</td>
<td>100.0</td>
<td>98.8</td>
<td>92.0</td>
<td>92.0</td>
<td>91.8</td>
<td>92.0</td>
<td>91.8</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td><strong>Cluster 8</strong></td>
<td>10</td>
<td>100.0</td>
<td>98.8</td>
<td>92.0</td>
<td>92.0</td>
<td>91.8</td>
<td>92.0</td>
<td>91.8</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td><strong>Cluster 9</strong></td>
<td>11</td>
<td>100.0</td>
<td>98.8</td>
<td>92.0</td>
<td>92.0</td>
<td>91.8</td>
<td>92.0</td>
<td>91.8</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td><strong>Cluster 10</strong></td>
<td>12</td>
<td>100.0</td>
<td>98.8</td>
<td>92.0</td>
<td>92.0</td>
<td>91.8</td>
<td>92.0</td>
<td>91.8</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td><strong>Cluster 11</strong></td>
<td>13</td>
<td>100.0</td>
<td>98.8</td>
<td>92.0</td>
<td>92.0</td>
<td>91.8</td>
<td>92.0</td>
<td>91.8</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td><strong>Cluster 12</strong></td>
<td>14</td>
<td>100.0</td>
<td>98.8</td>
<td>92.0</td>
<td>92.0</td>
<td>91.8</td>
<td>92.0</td>
<td>91.8</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td><strong>Cluster 13</strong></td>
<td>15</td>
<td>100.0</td>
<td>98.8</td>
<td>92.0</td>
<td>92.0</td>
<td>91.8</td>
<td>92.0</td>
<td>91.8</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td><strong>Cluster 14</strong></td>
<td>16</td>
<td>100.0</td>
<td>98.8</td>
<td>92.0</td>
<td>92.0</td>
<td>91.8</td>
<td>92.0</td>
<td>91.8</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
<td>91.9</td>
</tr>
</tbody>
</table>
the 16S rRNA gene is used as the most effective molecular marker for the taxonomical study of the bacteria, it has been pointed out that resolution regarding the taxonomy of species is limited for some existing taxonomic groups of bacteria of which the taxonomy has been finely established. In such cases, this limitation is sometimes compensated for by phylogenetic analysis using the sequence of a specific housekeeping gene, such as the gyrB gene, of which the evolutionary speed is faster than that of the 16S rRNA gene. Contrary to the situation described above, phylogenetic analysis using the 16S rRNA gene sequence with the genus *Spirulina* suggests a possibility of analysis to the variety (or subspecies) level beyond the species level dealt with the existing classification system because of the slow evolutionary speed of the 16S rRNA gene. As such, this evaluation approach is inferred to offer an effective resolution when the evolutionary relationship of species/strains belonging to the genus *Spirulina* is to be evaluated.

The taxonomic work this time was conducted primarily with cyanobacteria obtained in the screening process of halophilic microalgae which autotrophically produce highly value-added compatible solutes from the viewpoint of their commercial production for industrial use. When this screening was planned, it was difficult to find effective screening conditions based on the existing taxonomical knowledge of microalgae which was largely confirmed to morphological information.

For the identification of BAH8-10-45 this time, as the historical background and definition of the genus *Halospirulina* were somewhat problematic, the taxonomy of this strain was clarified.

From the viewpoint of industrialists, we are hoping to see the establishment of at least the species and preferably the variety (or subspecies) as well, within the current phylogenotypical classification system so that diverse physiological data can be accumulated and systematized through the classification work, as in the case of most common bacteria to facilitate the utilization of cyanobacteria in a number of different industrial fields. In the case of most common bacteria, industrialists themselves can identify and classify them without the involvement of highly specialized experts. It is hoped that a similar environment allowing the easy classification of cyanobacteria can be established in the near future. In other words, this environment will allow the establishment of a new phylogenotypical classification system in which new genus, species and variety (or subspecies) can be backed by simple identification results through readily available and convenient phylogenetic analysis using the 16S rRNA gene sequence. Although phylogenetic analysis using the 16S rRNA gene sequence as the starting point, has become a common practice today in the case of cyanobacteria; the present situation appears to be that the phylogenotypical classification system for higher classes than genus to form the background has not yet been fully established.

Acknowledgments

This study has been generously supported by the Japan Cooperation Center, Petroleum (JCCP) under the auspices of the Ministry of Economy, Trade and Industry, Japan. We are grateful to Dr. Y. Kasai, MBI Chair of Marine Biosciences, Kamaishi Research Laboratory, Kitasato University for his valuable suggestions and discussions on algal isolation and phylogenetic analysis. We would like to thank Dr. R. W. Castenholz, Culture Collection of Microorganisms from Extreme Environments, for his generous gift of the strain *H. tapetica* CCC Baja-95 Cl. 2’. Our thanks also go to Dr. M. Kawachi (National Institute for Environmental Studies) and President T. Hasegawa (Ecorenaissance, Co., Ltd.) for their many helpful suggestions regarding taxonomic research.

References

15) Felsenstein, J. 1981. Evolutionary trees from DNA sequences:


