Rubidibacter lacunae gen. nov., sp. nov., a unicellular, phycoerythrin-containing cyanobacterium isolated from seawater of Chuuk lagoon, Micronesia

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A unicellular cyanobacterium, designated KORDI 51-2^T, was isolated from surface seawater of Chuuk lagoon, Micronesia. The cells were wine-coloured rods and emitted red fluorescence under green excitation of an epifluorescence microscope. Thus, morphologically, the strain resembled *Synechococcus* species. However, based on 16S rRNA gene sequence similarities between strain KORDI 51-2^T and related strains belonging to cyanobacteria, the novel strain was distantly related to members of the '*Halotheca*' cluster. However, sequence similarities between strain KORDI 51-2^T and members of the '*Halotheca*' cluster were very low, ranging from 90.7 to 92.1 %, and phylogenetic analyses showed that the strain formed a distinct branch. Therefore, a polyphasic characterization including morphology, physiology and pigment composition was conducted to elucidate the taxonomic position of strain KORDI 51-2^T. The strain grew within a temperature range of 25–35 °C and a salinity range of 2–7 ‰. The optimal temperature and salinity were about 30 °C and 5 ‰, respectively. Strain KORDI 51-2^T contained phycoerythrin, and the dominant carotenoid pigments were zeaxanthin, β -carotene and echinenone. The DNA G + C content was 60.5 mol%. Based on phylogenetic analysis of the 16S rRNA gene sequence, and the physiological data and pigment compositions, strain KORDI 51-2^T is considered to represent a new genus and novel species of cyanobacteria for which the name *Rubidibacter lacunae* gen. nov., sp. nov. is proposed. The type strain is KORDI 51-2^T (=KCTC 40015^T=UTEX L2944^T).

Cyanobacteria have been found in various aquatic habitats including marine, freshwater, hot spring, hypersaline environments and ice (Castenholz, 2001), and play an ecological role as major contributors to photosynthesis in most environments (Whitton & Potts, 2000). However, the taxonomic classification of cyanobacteria is confusing due to dual nomenclature systems: bacteriological and botanical (Oren, 2004). Under the *Bacteriological Code* (1990 Revision) (Lapage *et al.*, 1992), the names of only five genera (*Halospirulina*, *Plankothricoides*, *Prochlorococcus*, *Prochloron* and *Prochlorothrix*) are validly published at present (Oren, 2004). However, according to the classification of the latest edition of *Bergey's Manual* (Castenholz, 2001), the phylum

Cyanobacteria is divided into five subsections. Among them, unicellular cyanobacteria with cylindrical, ellipsoidal or rod-shaped cells that reproduce by equal binary transverse fission or budding are categorized in Subsection I, which contains 14 form-genera (*Prochlorococcus*, *Prochloron*, '*Gloeobacter*', '*Chamaesiphon*', '*Cyanobacterium*', '*Cyanobium*', '*Cyanothece*', '*Synechococcus*', '*Dactylococcopsis*', '*Gloeotheca*', '*Microcystis*', '*Synechocystis*', '*Chroococcus*' and '*Gloeocapsa*') (Herdman *et al.*, 2001). In this study, a strain with unicellular and rod-shaped cells, designated KORDI 51-2^T, was isolated from seawater of Chuuk lagoon, Federated States of Micronesia. Phylogenetic analysis based on the 16S rRNA gene showed that the novel strain could be differentiated from previously described cyanobacterial strains. Thus, its taxonomic position was determined by using a polyphasic approach.

To isolate cyanobacteria, seawater samples were filtered through 3.0 μ m polycarbonate filters by gravity. For cultivation, about 4 ml of modified f/2 medium [NaNO₃, 75 mg; NH₄Cl, 5.3 mg; NaH₂PO₄·H₂O, 5 mg; 1 ml auto-

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KORDI 51-2^T is EF126149.

Images of cells of strain KORDI 51-2^T from exponential and stationary growth phases and graphs showing the growth rate of strain KORDI 51-2^T at various temperatures and salinities are available as supplementary material with the online version of this paper.

claved *f/2* trace metal solution (1^{-1} deionized water: $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 3.15 g; $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, 4.36 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 9.8 mg; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 6.3 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 22.0 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 10.0 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 180 mg); and 0.5 ml filter-sterilized *f/2* vitamin solution (1^{-1} deionized water: vitamin B_{12} , 1 mg; biotin, 1 mg; thiamine.HCl, 200 mg) in 1 l 0.2 μm -filtered seawater; autoclaved], which has the same constituents as *f/2* medium (Guillard, 1975) except that ammonium chloride was added and sodium silicate was omitted, was added to a cell culture flask filled with 40 ml of the filtered seawater. The culture flask was incubated at 25 °C at about 20 $\mu\text{E m}^{-2} \text{s}^{-1}$ (light:dark=14:10). The culture was maintained routinely by transferring small amounts of the culture to modified *f/2* medium about every 4 weeks for 2 years. After successive culturing for about 2 years, cycloheximide (final conc. 20 $\mu\text{g ml}^{-1}$) was added to prohibit the growth of picoeukaryotes. Then, to obtain an axenic culture, an aliquot of 1 ml, diluted to give 0.2 cell ml^{-1} with fresh medium, was dispensed into each well of a sterile 96 deep-well plate. The plate was incubated at 25 °C under about 20 $\mu\text{E m}^{-2} \text{s}^{-1}$. After 1.5 months, cell growth was observed in 10 of the wells. One hundred microlitres of culture from wells that contained growing cells were transferred to fresh medium and incubated again. This dilution procedure was repeated using the isolate obtained after the first dilution. To confirm the axenic state of the culture, the culture was examined by using DAPI (4',6-diamidino-2-phenylindole) staining and epifluorescence microscopy and heterotrophic bacteria were not observed.

PCR amplification of the target DNA was carried out using DNA that had been extracted by using a bead-beating protocol, involving zirconium beads, chloroform and 2-propanol (McBain *et al.*, 2003). The 16S rRNA gene segments were amplified using five primers (27F, Oxy359F, Syn1017R, Oxy1313R and 1522R) according to the protocol described by Fuller *et al.* (2003). After purification of the PCR products using an AccuPrep PCR purification kit (Bioneer), the amplified DNA was sequenced with an Applied Biosystems automatic sequencer (ABI3730XL) at Macrogen Corp., Seoul, Korea. An almost-complete 16S rRNA gene sequence of strain KORDI 51-2^T (1359 bp) was obtained. The sequence of strain KORDI 51-2^T was compared with 16S rRNA gene sequences available in GenBank/EMBL/DDBJ using BLASTN searches (Altschul *et al.*, 1990). The 16S rRNA gene sequences were aligned manually with those of other related cyanobacterial strains obtained from the GenBank database, based on known 16S rRNA gene secondary structure information using the program jPHYDIT (Jeon *et al.*, 2005). Phylogenetic trees were obtained by using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. An evolutionary distance matrix for the neighbour-joining method was generated according to the model of Jukes & Cantor (1969). The robustness of the tree topologies was assessed by bootstrap analyses based on 1000 replications for the

neighbour-joining and the maximum-parsimony methods and 100 replications for the maximum-likelihood method. Phylogenetic analyses were carried out using MEGA 3 (Kumar *et al.*, 2004) and PAUP* 4.0 (Swofford, 1998). Likelihood parameters were estimated by using the hierarchical ratio tests in MODELTEST version 3.04 (Posada & Crandall, 1998). Carotenoid pigments were determined by using an HPLC method with a reversed-phase C_8 column (Zapata *et al.*, 2000). Ten millilitres of culture at the exponential phase were harvested by being filtered with a GF/F filter and the pigments were extracted with 95% methanol.

The temperature range for growth was determined using the modified *f/2* medium. Culture flasks inoculated with cells in the exponential growth phase were incubated at 15–45 °C, at intervals of 5 °C. Tolerance to sea salts was determined using a medium that had the same constituents as the modified *f/2* medium, but was made with autoclaved MilliQ water with various concentrations (0, 1, 2, 3, 5, 7 and 10%) of sea salts (Sigma), instead of seawater. Utilization of ammonium, nitrate and urea was examined using media that were prepared using artificial seawater (Lyman & Fleming, 1940), each nitrogen source as a sole nitrogen (final concentrations of 900, 100 and 50 μM sodium nitrate, ammonium chloride and urea, respectively) and other constituents of the *f/2* medium except nitrate. Growth was measured by monitoring the increase of chlorophyll *a* fluorescence with time using a fluorometer (TD700; Turner designs). All experiments were conducted at 30 °C (except temperature experiments) under light intensity of about 70 $\mu\text{mol E m}^{-2} \text{s}^{-1}$.

The morphology and motility of cells of strain KORDI 51-2^T were examined by using epifluorescence and phase-contrast microscopes in each growth phase. Thylakoid arrangement and internal inclusions of strain KORDI 51-2^T at the exponential growth phase were observed using transmission electron microscopy after thin-sectioning and negative staining at MicroID (Korea). An *in vivo* adsorption spectrum from 400–800 nm was obtained using a spectrophotometer (Lambda 19; Perkin Elmer) after collection of 5 ml of exponential phase culture using a GF/F filter. To test whether strain KORDI 51-2^T was able to show chromatic adaptation, four sets of culture in culture flasks were incubated in blue, red, green and yellow bottles (Nalgene), respectively, under about 20 $\mu\text{E m}^{-2} \text{s}^{-1}$ (light:dark=14:10). An *in vivo* adsorption spectrum for each sample was measured after incubation for 0, 10 and 20 days.

The DNA G+C content was determined by using HPLC analysis of deoxyribonucleosides as described by Mesbah *et al.* (1989) after DNA purification using the method of Marmur (1961), at KCCM (Seoul, Korea).

Cells were non-motile, unicellular rods of 0.9–1.5 μm in diameter and 2.5–4.7 μm in length in the exponential growth phase (see Supplementary Fig. S1a, available in IJSEM Online). However, elongated cells of 10 μm or more appeared at the stationary phase (Supplementary Fig. S1b, in IJSEM Online), due probably to stress as observed for

Fig. 1. *In vivo* absorption spectrum for strain KORDI 51-2^T.

other unicellular cyanobacteria (Koblížek *et al.*, 2000). No sheath or aggregation was observed during growth. Cells divided by transverse binary fission in a single plane. *In vivo* absorption spectra showed peaks at 548, 628 and 679 nm, corresponding to peaks of phycoerythrobilin, phycocyanobilin and chlorophyll *a* (Fig. 1). Chromatic adaptation was not observed for strain KORDI 51-2^T. The culture was wine coloured because of the presence of phycoerythrin. The strain contained peripheral thylakoids and dark and white inclusions were observed inside the cells after thin-sectioning and transmission electron microscopy (Fig. 2). Growth of strain KORDI 51-2^T was observed at temperatures of 25–35 °C, with optimal growth at about 30 °C (see Supplementary Fig. S2a, in IJSEM Online). Strain KORDI 51-2^T grew at concentrations of sea salts of 2–7 % (optimal growth occurred at about 5 %) (Supplementary Fig. S2b, in IJSEM Online). The strain was able to grow with ammonia, nitrate and urea as a sole nitrogen source. The dominant carotenoid pigments were zeaxanthin, β -carotene and echinenone (relative molar ratio of 1.4:1:0.1); myxoxanthophyll was also found in trace amounts (Table 1).

The morphological characteristics of strain KORDI 51-2^T (i.e. shape and presence of phycoerythrin and peripheral thylakoids) are common to *Synechococcus* strains belonging to Marine cluster 5.1 (Herdman *et al.*, 2001). However, strain KORDI 51-2^T formed a clade that was separate from

Synechococcus strains in phylogenetic trees based on 16S rRNA genes constructed by using three different tree-building methods (Fig. 3); sequence similarities of the 16S rRNA genes were less than 89 %. Thus, strain KORDI 51-2^T was genetically distant from the genus *Synechococcus*. In addition, strain KORDI 51-2^T resembled a botanically described taxon, *Synechococcus rhodobaktron* (Komárek & Anagnostidis, 1995), which is devoid of mucilage, has rod-shaped cells (3–11 μ m long and 0.8–2.3 μ m wide) and is a phycoerythrin-containing freshwater strain. However, strain KORDI 51-2^T could be differentiated from *S. rhodobaktron* with respect to the obligate requirement of salts for growth. Instead, strain KORDI 51-2^T was distantly related to members of the extremely halotolerant ‘*Halothece*’ cluster (90.7–92.1 % sequence similarity) and formed a sister branch with a clade including ‘*Halothece*’ sp. MPI 96P605 and ‘*Halothece*’ sp. PCyano42 (Fig. 3), based on 16S rRNA gene sequence similarity and phylogeny. The robustness of the clade including strain KORDI 51-2^T and the ‘*Halothece*’ cluster was confirmed in the maximum-likelihood and neighbour-joining trees. In the maximum-parsimony tree, however, they were not clustered in a clade (Fig. 3). Thus, the low level of 16S rRNA gene sequence similarity and the unstable tree topology suggest that strain KORDI 51-2^T is genetically distant from members of the ‘*Halothece*’ cluster and could be assigned as representing a novel genus and species. In addition, physiological characteristics, such as temperature range for growth, sea salts tolerance range, absence/presence of phycoerythrin and composition of carotenoid pigments strongly support that strain KORDI 51-2^T represents a novel species (Table 1). Common features of strains pertaining to the ‘*Halothece*’ cluster are growth at 45 °C (moderate thermophiles), extreme halotolerance (optimal growth at salt concentrations of between 5 and 20 %) and the absence of phycoerythrin as light-harvesting phycobiliproteins (Garcia-Pichel *et al.*, 1998). In contrast, strain KORDI 51-2^T had upper temperature and salinity limits of 35 °C and 7 %, respectively, and contains phycoerythrin (Table 1).

Overall, the evidence from the polyphasic taxonomic study based on phylogenetic analysis of 16S rRNA gene sequences, physiological features and pigment compositions indicates that strain KORDI 51-2^T should be classified as representing a novel genus and species, for

Fig. 2. Transmission electron micrographs of thin sections of cells of strain KORDI 51-2^T. Bars, 1 μ m.

Table 1. Selected characteristics that differentiate strain KORDI 51-2^T and phylogenetically related strains

Strains: 1, strain KORDI 51-2^T (*Rubidibacter lacunae* gen. nov., sp. nov.); 2, '*Halotheca*' sp. MPI 96P605 (Garcia-Pichel *et al.*, 1998); 3, '*Euhalotheca*' cluster (Garcia-Pichel *et al.*, 1998). ND, No data available.

Characteristic	1	2	3
Isolation source	Seawater, Micronesia	Solar evaporation ponds, Spain	Solar lake, evaporation ponds, Benthic microbial mats
Cell morphology	Rods	Short rods	Cocci to rods
Length (µm)	2.5–4.7	9.9	4.0–82
Width (µm)	0.9–1.5	6.5	2.6–10.3
Temperature range (optimum) (°C)	25–35 (30)	20–45 (30–38)	20–45 (25–38)
Sea salt tolerance range (optimum) (%)	2–7 (5)	2.5–24.5 (5–20.5)	1.5–28 (2–25)
DNA G + C content (mol%)	60.5	ND	42.4*
Phycocyanin	Present	Absent	Absent
Carotenoid pigments†			
Zeaxanthin	1.40	0.34	0.04–0.33
Echinenone	0.10	0.13	0.37–1.19
Myxoxanthophyll	0.04	Traces	0.05–1.50

*Data for *Cyanotheca* sp. PCC 7418.

†Relative molar ratio of each carotenoid to β-carotene.

Fig. 3. Maximum-likelihood tree showing the relationships between strain KORDI 51-2^T and other related species belonging to the cyanobacteria. Bootstrap values above 60% are shown at branch nodes. Solid circles indicate that the corresponding nodes were also recovered in maximum-parsimony and neighbour-joining trees. *Escherichia coli* ATCC 25922^T (X80724) was used as an outgroup (not shown). Bar, 0.05 nucleotide substitutions per site.

which the name *Rubidibacter lacunae* gen. nov., sp. nov. is proposed.

Description of *Rubidibacter* gen. nov.

Rubidibacter (Ru.bi.di.bac'ter. L. adj. *rubidus* red, reddish; N.L. masc. n. *bacter* rod; N.L. masc. n. *Rubidibacter* red-coloured rod).

Non-motile, unicellular rods and oxygenic photoautotroph. Reproduction occurs by transverse binary fission in a single plane. Do not produce well-defined sheath layers. Contain phycoerythrin and photosynthetic thylakoids located peripherally. Require seawater for growth. Contain zeaxanthin, β -carotene and echinenone as major carotenoid pigments. The type species is *Rubidibacter lacunae* sp. nov.

Description of *Rubidibacter lacunae* sp. nov.

Rubidibacter lacunae (la.cu'nae. L. gen. n. *lacunae* a pool of water, of a pond).

Exhibits the following properties in addition to those given in the genus description. Cells are 0.9–1.5 μ m wide and 2.5–4.7 μ m long. Growth occurs at 25–35 °C (optimum, 30 °C) and in concentrations of sea salts of 2–7 % (w/v). Utilizes ammonia, nitrate and urea as a sole nitrogen source. *In vivo* absorption spectrum shows peaks at 548, 628 and 679 nm and a shoulder at 565 nm. Not capable of chromatic adaptation. The G+C content of the genomic DNA of the type strain is 60.5 mol%.

The type strain, KORDI 51-2^T (=KCTC 40015^T=UTEX L2944^T), was isolated from seawater of Chuuk lagoon, Micronesia.

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