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NOTE

DNA–DNA reassociation among a bloom-forming cyanobacterial genus, *Microcystis*

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DNA base composition and DNA–DNA hybridization among the cyanobacterial genus *Microcystis* were determined using nine axenic *Microcystis* strains, including the three 'morphological' species of *Microcystis aeruginosa*, *Microcystis viridis* and *Microcystis wesenbergii*. These *Microcystis* species showed a similar DNA base composition (42·1–42·8 mol% G+C) and demonstrated more than 70% DNA relatedness, confirming their synonymy based on bacterial criteria.

Keywords: DNA hybridization, DNA G+C content, cyanobacteria, *Microcystis*

The genus *Microcystis*, which is known to be toxic and/or bloom-forming, is described in classical botanical literature as a cocoid unicellular cyanobacterium that forms spherical or lens-shaped colonies of irregularly or net-like arranged cells resulting from divisions in three planes (Geitler, 1932; Holt *et al*., 1994; Komárek & Anagnostidis, 1986). Further classification of *Microcystis* species is based on morphological features observed microscopically, such as cell size, cell arrangement in colonies, the existence of gas vesicles and characteristics of the mucilage of colonies (Geitler, 1932; Komárek, 1991). According to Geitler (1932), who established the current systematics of cyanobacteria, there are 32 species in the genus *Microcystis*, including eight species which have yet to be adequately described because there has been no clear delimitation of the form of the colonies and the cell diameter. The following six species of *Microcystis* were distinguished in eutrophic freshwaters in Japan; *Microcystis aeruginosa* with a cell diameter of 3·5–6·5 μm and diffluent mucilage, *Microcystis flos-aquae* with a cell diameter of 3·5–4·8 μm and very narrow and diffluent mucilage, *Microcystis ichthyoblabe* with a cell diameter of 2–3·2 μm and wide, fine and diffluent mucilage, *Microcystis novacekii* with a cell diameter of 3–5·5 μm and wide, limited but not refractive mucilage, *Microcystis viridis* with a cell diameter of 3·5–7 μm and limited, more or less refractive mucilage, and *Microcystis wesenbergii* with a cell diameter of 4–7 μm and limited, distinctly refractive mucilage (Komárek, 1991). The morphology of this cyanobacterium under selective culturing conditions, especially in unialgal or axenic cultures, however, is often markedly altered from the morphology in natural environments (Hagiwara *et al*., 1984; Komárek, 1991). Thus, we can not discriminate between species which are single cells and/or from the dissolution of colonies.

Genetic analyses of rRNA gene sequences (Neilan *et al*., 1994a, b, 1997; Kondo *et al*., 1998a, b; Otsuka *et al*., 1998), randomly amplified polymorphic DNA (RAPD) (Neilan, 1995; Nishihara *et al*., 1997) and RFLPs of phycocyanin intergenic spacer (Neilan *et al*., 1995) have been performed to create an alternative molecular genetics-based taxonomy for the cyanobacterial genus *Microcystis*. Nishihara *et al*. (1997) showed that three species, *M. novacekii*, *M. viridis* and *M. wesenbergii*, that possessed a single allozyme type (Kato *et al*., 1991) were genetically homogeneous by RAPD analysis. However, most of these genetic analyses indicated that no relationship exists between morphological characteristics and molecular analysis within the genus *Microcystis*. Moreover, the marked similarities in the complete 16S rDNA sequence and in phylogenetic analysis showed that the *Microcystis* species may be integrated into a single species (Otsuka *et al*., 1998). There was general agreement that the bacterial species was the only taxonomic unit that can be defined in phylogenetic terms since, in practice, DNA reassociation approaches the sequence standard and represents the most applicable procedure. The phylogentic definition of a species generally would

Abbreviation: RAPD, randomly amplified polymorphic DNA.

Table 1. *Microcystis* strains used in this study

Strain	Strain designation*	Isolation site in Japan
Microcystis aeruginosa (Küzing) Lemmermann f. *aeruginosa*	NIES87	Lake Kasumigaura, Ibaraki
Microcystis aeruginosa f. *aeruginosa*	NIES89	Lake Kawaguchi, Yamanashi
Microcystis aeruginosa (Küzing) Lemmermann f. *flos-aquae* (Wittrock) Elenkin	NIES98	Lake Kasumigaura, Ibaraki
Microcystis aeruginosa f. *aeruginosa*	NIES298	Lake Kasumigaura, Ibaraki
Microcystis viridis (A. Brown) Lemmermann	NIES102	Lake Kasumigaura, Ibaraki
Microcystis wesenbergii Komárek	NIES104	Chiyoda-ku, Tokyo
Microcystis wesenbergii	NIES111	Lake Kasumigaura, Ibaraki
Microcystis wesenbergii	NIES112	Lake Suwa, Nagano
Microcystis wesenbergii	NIES604	Lake Kasumigaura, Ibaraki

* NIES, National Institute for Environmental Studies, Environment Agency, Japan.

Table 2. DNA relatedness among *Microcystis* species

'Morphospecies'	Strain	G+C content (mol%)	Reassociation (%) with biotinylated DNA from:				
			NIES87	NIES98	NIES102	NIES104	NIES111
M. aeruginosa f. *aeruginosa*	NIES87	42·8	100·0	100·4	90·5	96·1	99·2
M. aeruginosa f. *aeruginosa*	NIES89	42·1	83·6	99·0	91·2	94·1	99·8
M. aeruginosa f. *flos-aquae*	NIES98	42·3	84·8	100·0	81·7	89·2	74·3
M. aeruginosa f. *aeruginosa*	NIES298	42·5	74·0	71·1	79·7	82·6	79·5
M. viridis	NIES102	42·3	78·5	92·2	100·0	93·0	85·3
M. wesenbergii	NIES104	42·5	89·7	72·9	71·2	100·0	88·2
M. wesenbergii	NIES111	42·8	81·8	82·7	85·0	86·6	100·0
M. wesenbergii	NIES112	42·7	74·0	74·4	73·4	93·1	88·8
M. wesenbergii	NIES604	42·2	71·8	71·9	77·4	85·7	88·6

include that strains have approximately 70% or greater DNA–DNA relatedness (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987). Although *Microcystis* is a prokaryote, no data are available on DNA–DNA reassociation among *Microcystis* species concerning its taxonomy to date. We determined here, the DNA base composition and DNA–DNA reassociation in the cyanobacterial genus *Microcystis* to demonstrate genetic relatedness according to bacterial criteria.

Axenic strains of *Microcystis*, isolated from Japanese freshwater systems, were obtained from the National Institution for Environmental Science (NIES), Environmental Agency, Japan. The strains used in this study were all axenic strains preserved in the NIES collection (Table 1). They were cultured in MA medium (Ichimura & Itoh, 1977) at a temperature of 25 °C under illumination of approximately 20 μE m^{-2} s^{-1} with a 12:12 h light–dark cycle.

A 2 l culture at the late-exponential growth phase was sonicated for 5 min (47 kHz, 300 W) to remove the gas vesicles. Then, cell pellets harvested by centrifugation at 14400 ***g*** for 15 min were suspended in 5 ml NET buffer (50 mM Tris/HCl, 150 mM NaCl, 100 mM Na_2-EDTA, pH 8·0) containing 50 mg lysozyme and incubated at 37 °C for 1 h. After the addition of 0·25 ml 10% (w/v) SDS and 0·1 ml proteinase K solution (10 mg ml^{-1}), incubation was carried out at 56 °C for 30 min. Solutions were extracted with phenol/chloroform/isoamylalcohol (25/24/1, by vol.) two or three times. The genomic DNA was precipitated by the addition of a one-sixth volume of 2-propanol. After rinsing with 80% (v/v) ethanol, the DNA was dissolved in 1 ml TE buffer (10 mM Tris/HCl, 1 mM Na_2-EDTA, pH 8·0) containing DNase-free RNase A (100 μg ml^{-1}) and incubated at 37 °C for 1 h. Another purification was performed with the addition of 0·1 ml CTAB solution (10%, w/v, cetyltrimethylammonium bromide in 0·7 M NaCl) and incubation at 65 °C for

30 min. A final purification was carried out by phenol and chloroform/isoamylalcohol (24/1) extractions and ethanol precipitation. The G+C content of the purified DNA was determined by directly analysing the deoxyribonucleosides by reversed-phase HPLC (Tamaoka & Komagata, 1984) with some modifications. The DNA-GC kit (Yamasa Shoyu, Japan) was used as the standard. DNA–DNA hybridization experiments were performed in microdilution wells using a fluorometric binding method (Ezaki *et al*., 1989).

The DNA base compositions of the *Microcystis* species studied are shown in Table 2. The DNA G+C contents of *M. aeruginosa* and *M. wesenbergii* were 42·1–42·8 mol% and 42·2–42·8 mol%, respectively, and the DNA G+C content of *M. viridis* was 42·3 mol%, indicating that there was overlap. The range for members of the genus *Microcystis* was 41·6–42·5 mol% (Rippka & Herdman, 1992). There was no relationship between the DNA G+C content and morphological features of the species such as cell diameter or the characteristics of mucilage.

The complete 16S rDNA sequence similarities among *Microcystis* species including *M. aeruginosa*, *M. viridis* and *M. wesenbergii* were 91% or greater, sometimes identical (Neilan *et al*., 1997; Otsuka *et al*., 1998), indicating that DNA relatedness may be higher than 70% within a bacterial species (Stackebrandt & Goebel, 1994). The similarities of partial 16S rDNA sequences from *Microcystis* strains used in this study were also more than 97% except for *M. aeruginosa* NIES98 (Kondo *et al*., 1998b).

Levels of DNA relatedness were determined by the microplate hybridization method of Ezaki *et al*. (1989) and the results are shown in Table 2. The DNAs from *M. aeruginosa* NIES87, *M. aeruginosa* NIES98, *M. viridis* NIES102, *M. wesenbergii* NIES104 and *M. wesenbergii* NIES111 were labelled with photobiotin to determine their levels of relatedness to other DNAs. *M. aeruginosa* NIES87 shared high levels of DNA relatedness to the other strains exceeding 71·8%. *M. aeruginosa* NIES98, *M. viridis* NIES102, *M. wesenbergii* NIES104 and *M. wesenbergii* NIES111, whose DNA was biotinylated, also showed high levels (71·1–100·4%) of DNA relatedness. *Microcystis* species have been morphologically discriminated by microscopic observations of cell diameter, the cell arrangement in colonies, and characteristics of the mucilage of colonies. However, DNA–DNA reassociation values reached 70% among the genus *Microcystis* despite the morphological diversity of cell and colonial features in the *Microcystis* species.

Based on the results described above, we concluded that the genus *Microcystis* used in this study does not differ significantly in DNA base composition or DNA relatedness. The members of this genus seem to be integrated into one species according to bacterial criteria, in which a DNA reassociation value of about 70% plays a dominant role.

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