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A proposal for the unification of five species of the cyanobacterial genus *Microcystis* Kützing *ex* Lemmermann 1907 under the Rules of the Bacteriological Code

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Genomic DNA homologies were examined from six *Microcystis* (cyanobacteria) strains, including five different species, *Microcystis aeruginosa*, *Microcystis ichthyoblabe*, *Microcystis novacekii*, *Microcystis viridis* and *Microcystis wesenbergii*. All DNA–DNA reassociation values between two strains of *M. aeruginosa* and the other four species exceeded 70%, which is considered high enough for them to be classified within the same bacterial species. It is proposed to unify these five species into *M. aeruginosa* under the Rules of the Bacteriological Code and NIES843^T (= IAM M-247^T) is proposed as the type strain. Two other species, *Microcystis flos-aquae* and *Microcystis pseudofilamentosa*, should be regarded as morphological variations of this unified *M. aeruginosa*. The current taxonomy of cyanobacteria depends too much upon morphological characteristics and must be reviewed by means of bacteriological methods as well as traditional botanical methods.

Keywords: cyanobacteria, DNA–DNA hybridization, *Microcystis*, taxonomy, unification

INTRODUCTION

The blue-green algae, or cyanobacteria, have traditionally been classified on the basis of morphological and physiological characteristics and described under the rules of the Botanical Code. However, 16S and 5S rDNA sequence data placed this phylum within the *Bacteria* (Woese, 1987) which was supported by many other authors (e.g. Gupta, 1998). Describing this group in the same manner as most other bacteria is accompanied by the problem that existing genera and species have been described based solely on morphological characteristics observed in field samples (Castenholz & Waterbury, 1989). This problem has to date hindered the classification of cyanobacteria under the Bacteriological Code.

The colonial cyanobacterium *Microcystis* is a planktonic and toxigenic organism that usually occurs in eutrophic lakes, ponds and reservoirs and often forms water blooms from summer to late autumn (Carmichael, 1996). Delimitation and validity of *Microcystis* species have been discussed in a number of papers. Twenty-three *Microcystis* species were described by Geitler (1932), which included species both with and without gas vesicles. Stanier *et al.* (1971) suggested that only cells containing gas vesicles should be considered as *Microcystis*. According to the most recent edition of *Bergey's Manual of Determinative Bacteriology* (Holt *et al.*, 1994), *Microcystis* is characterized as having gas vesicles, a coccoid cell shape, a tendency to form aggregates or colonies, and an amorphous mucilage or a sheath. It must be noted, however, that gas vesicles are sometimes lost during subculture. Incidentally, some authors describe gas vacuoles (an assemblage of numerous gas vesicles) as aerotopes (Komárek & Anagnostidis, 1998). As species which meet these criteria, Watanabe (1996) recently distinguished five species in Japanese waters: *Microcystis aeruginosa* (Kützing) Kützing 1846, *Microcystis ichthyoblabe* Kützing 1843, *Microcystis novacekii*

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The DDBJ/EMBL/GenBank accession numbers for the 16S rDNA sequences of *Microcystis* strains used in this study are listed in Fig. 1.

(Komárek) Compère 1974, *Microcystis viridis* (A. Braun) Lemmermann 1903 emend. Kondrateva 1975 and *Microcystis wesenbergii* (Komárek) Komárek in Kondrateva 1968. In addition to these five species, Komárek (1991) distinguished *Microcystis flos-aquae* (Wittrock) Kirchner 1898, while Watanabe (1996) regarded this as one type of *M. ichthyoblabe*. In some cases, *M. ichthyoblabe* (including *M. flos-aquae*) and *M. novacekii* are not distinguished from *M. aeruginosa* (e.g. Kato *et al.*, 1991). It must be noted that although '*M. aeruginosa* (Kützing) Lemmermann 1907' is the correct type species name of *Microcystis* according to the last edition of *Nomina Conservanda*, the name *M. aeruginosa* (Kützing) Kützing 1846 is used by many authors (e.g. Watanabe, 1996; Komárek & Anagnostidis, 1998). The interpretation by Komárek (1957) is helpful to comprehend this nomenclatural problem. Although the genus name *Microcystis* Kützing 1833 was invalid according to the first diagnoses, Kützing himself used the name *M. aeruginosa* for the present *M. aeruginosa* in his publications in 1846 and later (Komárek, 1957). This is the reason why the name *M. aeruginosa* (Kützing) Kützing 1846 is accepted by many authors.

In all of these studies, it was primarily cell size, colonial form and sheath characteristics that were used as the taxonomic criteria, and the term 'morphospecies' was given to this classification of *Microcystis* species. Colonies of *Microcystis*, however, can easily change in form or disaggregate during serial subculture and it becomes difficult to re-identify them. Several attempts have been made to define taxonomic criteria, other than morphological ones, for cultured and disaggregated *Microcystis* cells. Fahrenkrug *et al.* (1992) examined DNA base composition of a number of cyanobacteria, including four *Microcystis* strains. The *Microcystis* strains used in this study had fairly similar base compositions, 38–43 mol% G+C, which was confirmed (39–43 mol% G+C) by Otsuka *et al.* (1999b). Krüger *et al.* (1995) investigated the fatty acid composition of *Microcystis* species as a taxonomic criterion and concluded that this attribute may be particularly helpful in cyanobacterial taxonomy, including the genus *Microcystis*. However, their investigation was not performed in relation to species delimitation and Otsuka *et al.* (1999b) reported that the fatty acid composition of *Microcystis* morphospecies was irrelevant to species distinction. Neilan *et al.* (1997) and Otsuka *et al.* (1998) presented phylogenetic trees of strains in the genus *Microcystis* based on 16S rDNA sequence comparisons. These trees did not reflect the classification based on morphological characteristics and Otsuka *et al.* (1998) concluded that the five *Microcystis* morphospecies investigated may possibly be unified into one species. This idea was partially supported by Kondo *et al.* (2000) with the result of DNA–DNA hybridization tests using three *Microcystis* morphospecies. Cell size, growth temperature range, salinity tolerance, restriction fragment length polymorphism on PCR amplification products (PCR-RFLP) of the *cpcBA* intergenic spacer and flanking region (IGS), and a phylogenetic analysis based on 16S–23S internal transcribed spacer (ITS) sequence comparisons also failed to support the validity of the current taxonomy of *Microcystis* (Neilan *et al.*, 1995; Bolch *et al.*, 1996; Otsuka *et al.*, 1999a, b).

In this paper we report the genomic DNA homologies among six strains, including five *Microcystis* morphospecies, and reconsider the current classification of *Microcystis* species according to bacteriological taxonomic criteria.

Methods

Strains. Six strains, including five morphospecies used in the hybridization study are listed in Table 1. Throughout our study, *M. flos-aquae* is not distinguished from *M. ichthyoblabe*, according to Watanabe (1996). The five strains other than TAC86 were isolated from 1996 to 1997 and their morphospecies-characteristic colonial forms have been recorded photographically to certify the correct identification of these strains (Otsuka *et al.*, 2000). *M. aeruginosa* strain NIES843^T (= IAM M-247^T) was previously designated *M. aeruginosa* strain NC7 (Otsuka *et al.*, 2000). The strains were purified and cloned by the micropipette-washing method. Purity of the strains was checked by bacterial check media (Watanabe & Hiroki, 1997) and fluorescent microscopy with DAPI staining. Cultures were maintained in MA medium (Ichimura, 1979) at 20 °C under a 12:12 h light/dark cycle with a photon flux density of about 30 μmol m^{-2} s^{-1} provided by daylight fluorescent lamps.

Sequencing and phylogenetic analysis. The 16S rDNA sequence of *M. aeruginosa* TAC86 had been determined previously (Otsuka *et al.*, 1998) and the sequences of other five strains were determined in this study. DNA for use in PCR was extracted according to Palinska *et al.* (1996) with minor modifications. PCR amplification and sequencing were also carried out as described previously (Otsuka *et al.*, 1998). The sequence alignment, including the six strains under examination here as well as 10 other strains of *Microcystis*, other cyanobacteria and related organisms, was obtained using the multiple sequence alignment tools in CLUSTAL X (Thompson *et al.*, 1997), then converted to a distance matrix. The distance matrix was used to reconstruct a phylogenetic tree using the neighbour-joining algorithm of CLUSTAL X, with multiple substitutions corrected and positions with gaps excluded, and the seed number for random number generation and numbers of bootstrap trials were set to 111 and 1000, respectively.

Preparation of DNA for DNA–DNA hybridizations. A 30 ml aliquot of culture was centrifuged at 5000 ***g*** for 10 min at 4 °C. The pellet was broken with a beadbeater (4600 r.p.m., 30 s × 2) and resuspended in 10 mM Tris/HCl buffer (pH 8·0) containing 1 mM EDTA. One-tenth vols 10% SDS solution was added to the suspension and mixed well. The lysate was extracted at least twice with Tris-buffered phenol and at least three times with chloroform. DNA was precipitated by addition of 0·1 vols 3 M sodium acetate and 2·5 vols ethanol. Enzyme hydrolysis of RNA was achieved by treatment with Ribonuclease T1 (Sigma-Aldrich) and ribonuclease A (Sigma-Aldrich).

DNA–DNA hybridizations. DNA–DNA hybridization experiments were performed with DNA from both *M. aeruginosa* strains as probes. Percentages of DNA reassociation were

Table 1. Strains used in DNA–DNA hybridization experiments

Morphospecies	Strain*	Geographic origin	16S rDNA sequence reference
M. aeruginosa	NIES843^T (= IAM M-247^T)	Japan	This study
M. aeruginosa	TAC86	Japan	Otsuka *et al.* (1998)
M. ichthyoblabe	TC24	Thailand	This study
M. novacekii	BC18	United Kingdom	This study
M. viridis	CC9	Peoples Republic of China	This study
M. wesenbergii	TC7	Thailand	This study

* IAM, Institute of Molecular and Cellular Biosciences Culture Collection, The University of Tokyo, Tokyo, Japan; NIES, National Institute for Environmental Studies Collection, Tsukuba, Japan; TAC, Tsukuba Algal Collection, National Science Museum, Tsukuba, Japan.

determined fluorometrically from the extent of hybridization by the method of Ezaki *et al.* (1989). The optimal renaturation temperature, calculated from the G+C content (Fahrenkrug *et al.*, 1992; Holt *et al.*, 1994; Otsuka *et al.*, 1999b), was approximately 31 °C, which is 55 °C lower than the T_m according to Meinkoth & Wahl (1984) and Ezaki *et al.* (1989), and a concentration of 100 μg DNA ml^{-1} was used. Each experiment was repeated at least five times.

RESULTS AND DISCUSSION

The taxonomic relationships between the *Microcystis* morphospecies are uncertain. Recently published data based on physiological and biochemical characteristics, PCR-RFLP of the *cpcBA* IGS, 16S rDNA and 16S–23S ITS sequences strongly indicated that the current morphological classification of the *Microcystis* species is invalid (Neilan *et al.*, 1997; Otsuka *et al.*, 1998, 1999a, b). The lengths of the 16S rDNAs of the present six strains were identical, 1457 bp, between positions 33 and 1542 of the 16S rDNA of *Escherichia coli*. Similar to the results of Otsuka *et al.* (1998), all six strains had high sequence similarity, exceeding 99·5% (data not shown). While *M. aeruginosa* NIES843^T had 100% sequence similarity with *M. viridis* CC9, it had 99·5% sequence similarity to *M. aeruginosa* TAC86, currently classified as the same morphospecies. Thus the six strains are closely related to one another, regardless of species distinction. In the *Microcystis* cluster of the neighbour-joining tree based on 16S rDNA sequences (Fig. 1), there is no clear division

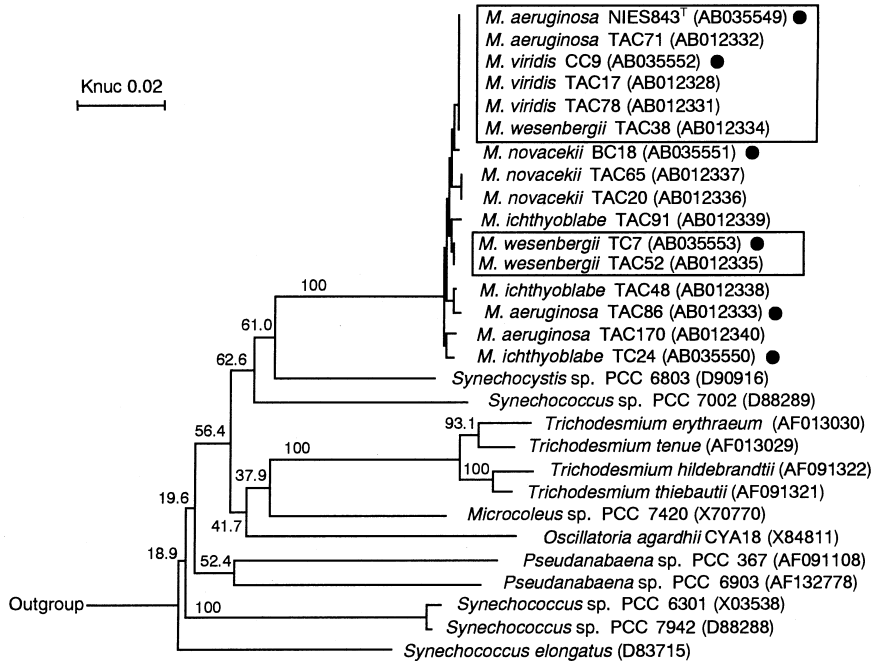

Fig. 1. Neighbour-joining tree of the *Microcystis* strains and related organisms. An alignment of 1347 nt after excluding positions with gaps was used. Local bootstrap probabilities (for branches except those within the *Microcystis* cluster) are indicated at nodes. Strains marked with a black circle were used for DNA–DNA hybridization and strains in each rectangle have an identical sequence. Accession numbers in the DDBJ/EMBL/GenBank databases are indicated in parentheses. *E. coli* (accession no. E05133), used as an outgroup, is not shown.

Table 2. Genomic DNA homologies (%) between each *Microcystis* strain and two strains of *M. aeruginosa* used in DNA–DNA hybridization experiments

Probe	*M. aeruginosa* NIES843^T	*M. aeruginosa* TAC86	*M. ichthyoblabe* TC24	*M. novacekii* BC18	*M. viridis* CC9	*M. wesenbergii* TC7
M. aeruginosa NIES843^T	100	74·1	80·7	74·0	91·7	89·7
M. aeruginosa TAC86	75·0	100	86·4	85·2	75·9	74·6

which agrees with the current species classification. Compared with the cluster of *Trichodesmium* spp. or *Pseudanabaena* spp., 16S rDNA differentiation within the genus *Microcystis* is low. DNA–DNA binding percentages were all greater than 70 % (Table 2). While the percentage hybridization between the two strains of *M. aeruginosa*, NIES843^T and TAC86, was 74·1–75·0 %, that between *M. aeruginosa* NIES843^T and *M. viridis* CC9 was 91·7 %, despite the fact that these two strains have been classified as different morphospecies. As with the 16S rDNA similarities, the genomic DNA homologies did not correspond with the current species classification. It is considered that the relative binding percentage should be higher than 70 % within a bacterial species (Wayne *et al.*, 1987). According to this criterion the present six strains can be classified as members of the same species.

Microcystis spp. classification has traditionally been chiefly based on morphological characteristics. There is a problem, however, in the application of morphological criteria to cyanobacterial classification because of the variability of characteristics in response to changes in the environment (Dor & Hornhoff, 1985). Palinska *et al.* (1996) showed that the great morphological diversity observed in nature and (partially) in culture does not necessarily reflect genetic diversity and mentioned the likelihood that more 'ecophenic' and/or phenotypic forms have been described rather than genotypic species. Otsuka *et al.* (2000) showed that *Microcystis* strains can change their colonial forms in culture and, as a result, the morphological characteristics of many morphospecies overlap. *Microcystis* spp. possibly change colony forms in the field as well as in culture, which is thought to have caused uncertainty in the present classification of *Microcystis* species. It has been suggested that genetic data must be taken into consideration for classification of *Microcystis* species. The difference in colonial form must be regarded as intraspecific or phenotypic variation. Kato *et al.* (1991) concluded that *M. viridis* and *M. wesenbergii* are both well-established species based on allozyme tests. This, however, was not supported by Otsuka *et al.* (1999a, b). Additionally, Kato *et al.* (1991) did not illustrate the validity of using genotypes derived from allozyme tests for species-level classification. The differences in the genotype can also be regarded as intraspecific variation.

In this study, we propose the unification of the five (or more) morphospecies of the genus *Microcystis*. This unification is necessary since even a single strain sometimes has characteristics corresponding to more than two morphospecies or an intermediate form between two or more morphospecies (Otsuka *et al.* 2000). For valid publication of cyanobacterial names and type strains under the Rules of Bacteriological Code, the following points are important.

1. New names or combinations must be published in the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM) and if published elsewhere, the new names or combinations are not validly published until they are published in IJSEM.

2. The Botanical Code specifies that names of Cyanophyta validly published under the Bacteriological Code as cyanobacteria are valid according to the Botanical Code and the International Committee on Systematic Bacteriology (ICSB) in 1986 approved that names of cyanobacteria described and validly published as blue-green algae under the International Code of Botanical Nomenclature (ICBN) are recognized as having been validly published under the International Code of Nomenclature of Bacteria (ICNB) (see Castenholz & Waterbury, 1989).

3. A proposal of neotype strains for consideration by the Judicial Commission of the ICSB must be published in the IJSEM.

Although none of the *Microcystis* morphospecies meet the criteria in point 1 above, each name must be regarded as valid under the ICNB, according to point 2. Since *M. aeruginosa* is the type species of this genus under the ICBN, this is the most suitable species name for the unified species. *Microcystis* morphospecies have to date had no valid type strain. In the strain catalogue of Pasteur Culture Collection (Rippka & Herdman, 1992), *M. aeruginosa* PCC 7941 was proposed as the type strain, but this proposal was not made under the Rules of the Bacteriological Code (see point 3). The origin of strain PCC 7941 was strain NRC-1, which was isolated in the study of Hughes *et al.* (1958). However, the authors failed to describe morphological characteristics from which they used to identify the strain as *M. aeruginosa* and for this reason strain PCC 7941 is not a suitable type strain. We propose *M. aeruginosa* NIES843^T (= IAM M-247^T) as the type strain of the unified *M. aeruginosa*. Since the correct identification of *Microcystis* morphospecies is some-

times in doubt, we selected strains whose characteristics are well documented (cf. Otsuka *et al*., 2000) and did not include strain PCC 7941 in the present study. Incidentally, the 16S rDNA sequence of strain PCC 7941 (accession no. AJ133171) has 99·4 % similarity to that of *M. aeruginosa* NIES843^T (data not shown).

M. flos-aquae and *Microcystis pseudofilamentosa* Crow 1923 should be included in the unified *M. aeruginosa*, since the colonial characteristics of these two species were shown to be morphological variations of one or more of the five morphospecies (Otsuka *et al*., 2000). In addition, Kondo *et al*. (2000) reported that DNA–DNA relative binding percentages between a strain of *M. flos-aquae* [*M. aeruginosa* (Kützing) Lemmermann f. *flos-aquae* (Wittrock) Elenkin 1938 in their study] and strains of *M. aeruginosa*, *M. viridis* and *M. wesenbergii* were greater than 70 %. An intermediate colonial form between *Microcystis botrys* Teiling 1942 and *M. novacekii* was observed during our study (data not shown) and partial sequences of 16S rDNA (478 bp) of *M. botrys* strains NIVA-CYA 161/1 and NIVA-CYA 264 (accession nos Y12608 and Y12609, respectively) have 100 % similarity with that of *M. aeruginosa* NIES843^T. These facts indicate the possibility that *M. botrys* could also be included in the unified *M. aeruginosa*. *Microcystis elabens* Kützing 1846, *Microcystis holsatica* Lemmermann 1907 and *Microcystis incerta* (Lemmermann) Lemmermann 1907 have sometimes been described in recent papers (e.g. Neilan *et al*., 1997). However, *M. holsatica* and *M. incerta* always lack gas vesicles. *M. elabens* NIES42 and *M. holsatica* NIES43 were shown to be phylogenetically distant from the major *Microcystis* cluster, but clustered with *Synechococcus* sp. PCC 6301 (Neilan *et al*., 1997). In addition, *M. elabens* has rod-shaped cells. These facts indicate that these morphospecies should not be included in *Microcystis*. Komárek & Anagnostidis (1998) described a number of *Microcystis* morphospecies in their recent revision of *Chroococcales* cyanobacteria (including 10 as species in Europe, 11 as species outside Europe and some other 'unrevised taxa'). They suspected that some of the morphospecies were synonyms of other *Microcystis* or should be moved to other genera. Besides these 'suspected' morphospecies and those proposed to be unified in this study, there are still eight to ten other morphospecies in their revision. Among these morphospecies, diagnostic characteristics of *Microcystis firma* (Kützing) Schmidle 1902, *Microcystis natans* Lemmermann ex Skuja 1934 and *Microcystis smithii* Komárek et Anagnostidis 1995 were well documented and seem not to fit within the variations of the unified *M. aeruginosa* summarized in the description below (cf. Otsuka *et al*., 2000). The cell sizes of *M. natans* and *M. firma* are much smaller than the unified *M. aeruginosa* and *M. smithii* usually has a solitary aerotope (Komárek & Anagnostidis, 1998).

Among strains currently classified as any of the five morphospecies (or seven, including *M. flos-aquae* and *M. pseudofilamentosa*), there may or may not be some strains which cannot be included in the unified *M. aeruginosa*. These strains would need to be distinguished from the unified *M. aeruginosa* and classified, where appropriate, using genetic, biochemical, physiological and invariable morphological characteristics.

Morphological characteristics can give important information about the organism of interest; however, it is necessary to bear in mind that there are organisms which cannot be classified solely by morphological characteristics. The current taxonomy of cyanobacteria depends too much upon morphological characteristics and must be reviewed by means of bacteriological methods as well as traditional botanical methods. Molecular investigations, especially those based on 16S rDNA and DNA–DNA hybridization, would bring unification or division to some species, genera and even taxa of higher rank. There remain many cyanobacteria to be reconsidered regarding their taxonomy. The genus *Synechococcus* is an example; genetic distances among *Synechococcus* spp. in the neighbour-joining tree (Fig. 1) seem too large for them to be classified in a single genus and it has been shown that this is another example of an unnatural taxon (Honda *et al*., 1999).

Description of *Microcystis aeruginosa* comb. nov. Kützing 1833

Microcystis aeruginosa (ae.ru.gi.no′sa. L. fem. adj. *aeruginosa* full of copper rust or verdigris, hence green).

The description is based on the data from Geitler (1932), Waterbury & Rippka (1989), Komárek (1991), Fahrenkrug *et al*. (1992), Holt *et al*. (1994), Krüger *et al*. (1995), Watanabe (1996) and Otsuka *et al*. (1999a, b, 2000). Under the Botanical Code, the species has been described as *M. aeruginosa* (Kützing) Lemmermann 1907 [= *M. aeruginosa* (Kützing) Kützing 1846, formerly '*Micraloa aeruginosa*' Kützing 1833], *M. ichthyoblabe* Kützing 1843, *M. novacekii* (Komárek) Compère 1974 (formerly '*Diplocystis novacekii*' Komárek 1958), *M. viridis* (A. Braun) Lemmermann 1903 emend. Kondrateva 1975 [formerly '*Polycystis viridis*' A. Braun in Rabenhorst 1862, '*Diplocystis viridis*' (A. Braun in Rabenhorst 1862) Komárek 1958], *M. wesenbergii* (Komárek) Komárek in Kondrateva 1968 (formerly '*Diplocystis wesenbergii*' Komárek 1958), *M. flos-aquae* (Wittrock) Kirchner 1898 and *M. pseudofilamentosa* Crow 1923. The G+C content of the DNA is 38–45 mol % (type strain G+C content is 41 mol %). Fatty acid composition is tetradecanoic acid, hexadecanoic acid, Δ9-hexadecenoic acid, octadecanoic acid, Δ9-octadecenoic acid, Δ9,12- octadecadienoic acid, Δ9,12,15-octadecatrienoic acid, Δ6,9,12-octadecatrienoic acid and Δ6,9,12,15-octadecatetraenoic acid. Hexadecanoic acid is the main component, which ranges from about 40 to 70 %. Cells are coccoid, 2·5–7·6 μm in

diameter and tend to form aggregates or colonies, with amorphous mucilage or a sheath. Morphological characteristics of the colony vary as follows: (1) more or less firm, elongated or lobated usually with distinct holes, subspherical and reticulated, and the margin of the colonial envelope is invisible under a microscope without treatment (colony type 1); (2) soft, the distribution of cells in colonies is homogeneous, irregular or sponge-like, and the margin of the colony is irregular and invisible under a microscope without treatment (colony type 2); (3) small and firm, not lobated, composed of tightly aggregated cells and a thick surrounding gelatinous substance, sometimes elongate discontinuously (colony type 3); (4) firm, small and composed of cubic subcolonies that contain eight cells and the margin of colonies is usually visible, undulating in natural populations (colony type 4); (5) spherical, elongated, lobated, with visible margin, limited by smooth, continual, refractive line and filled with mucilage, with cells arranged in a single layer inside the margin or aggregated in the centre of the colony (colony type 5); (6) intermediate form between any two or more types; and (7) other forms corresponding to none of the above types 1–5 (cf. Otsuka *et al*., 2000). The colonies can easily change in form or disaggregate during serial subculture. The cell usually has gas vesicles which are sometimes lost. Some strains produce the cyclic heptapeptide hepatotoxin, microcystin (type strain is positive for this). Type strain is NIES843^{T} (= IAM M-247^{T}).

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