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Morphological characteristics and phylogenetic analyses of unusual morphospecies of *Microcystis novacekii* forming bloom in the Cheffia Dam (Algeria)

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ABSTRACT

The toxicological potential and morphological characteristics and phylogenetic analysis based on the 16S rDNA sequence and the 16S-23S rDNA internal transcribed spacer (ITS) were investigated in unusual morphospecies of Microcystis *(MCYS-CH01) isolated from the Cheffia Dam in Algeria. The presence of microcystin synthetase genes (mcyA, -B, and -C) in isolated colonies of this morphospecies, and the fact that serine/threonine phosphatase (PP2A) was inhibited by its crude extract indicated that this morphospecies was microcystin-producer. The morphological features of this unusual morphospecies were very different from any of those described in the literature of all known species of* Microcystis*. The phylogenic tree based on 16S rDNA sequences shows that this morphospecies is indistinguishable from the reference strain* Microcystis aeruginosa *PCC 7806 and from many other known* Microcystis *species and, therefore, this tree did not necessarily correlate to the distinctions between morphospecies. However, phylogenetic analysis based on the 16S-23S rRNA spacer region could be an effective way to assign this unusual morphospecies MCYS-CH01 to the Asian species* Microcystis novacekii*. Comparison of the ITS sequence of this morphospecies with sequences available in the GenBank database showed that some highly conserved genotypes are found throughout the world.*

Key words: Cyanobacteria, 16S rRNA, ITS, Microcystins

1. INTRODUCTION

Blooms of toxic cyanobacteria constitute a threat to the safety and ecological quality of surface waters worldwide. The genus *Microcystis* constitutes one of the most widely distributed toxic bloom-forming genera of cyanobacteria (Sivonen & Jones 1999). Within the North-African basin, several studies in Morocco (Oudra *et al*. 2001; Sabour *et al*. 2002), Algeria (Nasri *et al.* 2004), and Tunisia (El Herry *et al*. 2008), neighboring countries with similar climatic conditions, have shown that natural cyanobacterial blooms containing microcystins are dominated by the genus *Microcystis*. In Egypt, however, microcystins have been isolated and characterized from both *Microcystis aeruginosa* (Abdel-Rahman *et al.* 1993; Mohamed *et al*. 2003) and *Oscillatoria tenuis* (Brittain *et al.* 2000). The group of toxin produced by *Microcystis* is the microcystin hepatotoxins, a cyclic heptapeptides which are formed non-ribosomally by peptide and polyketide synthetases (Dittmann *et al*. 1997; Tillett *et al*. 2001). They have been implicated in deaths due to microcystin-induced liver failure in domestic and wild animals (Codd *et al*. 2005), as well as in human illness (Kuiper-Goodman *et al.* 1999, Codd *et al*. 2005) and, as a result of exposure through hemodialysis, even in human death (Jochimsen *et al*. 1998; Pourria *et al*. 1998; Carmichael *et al*. 2001).

All species within the genus *Microcystis* have been reported to include microcystin-producing strains as well as strains that do not synthesize microcystin. Characterization of *Microcystis* species using conventional methods based on morphological features is very difficult, and only limited differentiation is possible below genus level. The genus *Microcystis* is clearly delimited at the genus level by molecular sequencing (Li *et al*. 1998) but in the field it occurs in the form of characteristic colonies that can be classified as different morphological types (morphotypes), each of which is equivalent to a species (morphospecies) (Komárek & Anagnostidis 1999). *Microcystis* colonies differ in shape and size, but also in the appearance of their mucilage (Watanabe 1996). However, the validity of the morphological taxonomy of these species has always been questioned. Several attempts have been made to define taxonomic criteria, other than morphological ones, based specially on 16S rDNA sequence comparisons (Neilan *et al*. 1997; Otsuka *et al*. 1998) for the different *Microcystis* species. Given that the taxonomic resolution offered by 16S rRNA genes is insufficient to distinguish between closely related organisms, research has increasingly focused on the rRNA 16S to 23S internal

transcribed spacer (rRNA-ITS). Restriction enzyme digestion of rRNA-ITS has been used to resolve closely-related cyanobacterial strains (Lu *et al*. 1997; Neilan *et al*. 1997; Laloui *et al*. 2002), and direct sequencing has been used to study subgeneric phylogenetic relationships in genera such as *Microcystis* (Otsuka *et al*. 1999). Furthermore, analysis of the length polymorphism and restriction fragment length polymorphism (RFLP) of the amplified rRNA-ITS region has generally made it possible to assign the cyanobacteria tested at genus and species level (Boyer *et al*. 2001). In fact, the high interspecific variability reported for this rRNA spacer makes it a promising candidate for RFLP. In this paper, we present the toxicological potential, morphological characteristics and phylogenetic analysis based on the 16S rDNA sequence of unusual non-axenic *Microcystis* morphospecies (MCYS-CH01) collected from the Cheffia Dam in Algeria. Furthermore, the 16S-23S rRNA ITS of this morphospecies was sequenced, and compared with some entire ITS sequences of different species of *Microcystis* available in GenBank. For the purposes of comparison, the axenic strain *M. aeruginosa* PCC 7806 was also included in this study as a reference strain.

2. MATERIALS AND METHODS

2.1. Study sites

The Cheffia Dam is located in the El Taref Wilaya in the north-eastern Algeria with the coordinates of 36°07'N and 8°03'E, it covers 1000 hectares and has a maximum depth of 30 m, and it provides drinking water for the Wilaya of Annaba and the surrounding area (population 1 million).

2.2. Sampling and morphological characterization of Microcystis *morphospecies*

Sampling for colony isolation and morphological characterization was carried in the autumn of 2005, and identified dominance of the genus *Microcystis*, in the Cheffia Dam by means of hauls with plankton nets (20-µm mesh size). Aliquots of the concentrated net samples were fixed with formaldehyde (5% f.c.) solution, and stored in the dark before being used for detailed determinations of the cell size, colony form, and sheath characteristics of the *Microcystis* morphospecies MCYS-CH01. The cell diameter was determined for 50 cells (10 cells from 5 different colonies). The remaining fresh phytoplankton sample was used for colony isolation as described below.

2.3. Isolation and toxic potential of the Microcystis *morphospecies MCYS-CH01*

For isolation of colony of the *Microcystis* morphospecies MCYS-CH01, fresh phytoplankton samples were diluted in sterilized Milli-Q water and individual colonies picked out by means of tiny Pasteur pipettes under binocular microscopes. Isolated colonies were then washed by transferring them into several drops of sterilized Milli-Q water until all other organisms had been removed. It was not possible to remove epiphytic cyanobacteria and algae stuck in the mucilage of *Microcystis* sp., but the absence of other cyanobacteria was checked by visual inspection under the microscope. Each series of ten isolated colonies were pooled separately in a sterilized Eppendorf tube (1.5 mL) and then lyophilized. Aliquots were then used to determine the toxic potential by the PP2A inhibition assay and mcy genes cluster amplification. For the PP2A inhibition assay, lyophilized materials of the morphospecies MCYS-CH01 and the reference strain *Microcystis aeruginosa* PCC 7806 were extracted with 100 µL aqueous methanol (75%, v/v), and then centrifuged at 5000 g for 10 min. An aliquot from each supernatant was then analyzed by the PP2A inhibition assay as described in Bouaïcha *et al*. (2001).

2.4. PCR amplification of the microcystin biosynthesis genes mcy*A,* mcy*B and* mcy*C*

PCR amplifications of *mcy*A, *mcy*B and *mcy*C, which are indicative of the presence of the microcystin biosynthesis genes cluster (Dittmann & Börner 2005), were performed by isolating the DNA directly from cell lysates obtained after five alternating cycles of freezing in liquid nitrogen and thawing at 55 °C (Iteman *et al*. 2000). The primers listed in table 1 were used to amplify the NMT domain of the microcystin synthetase genes *mcy*A, *mcy*B, and *mcy*C. The PCR mixture contained 2.5 µL of 10× PCR Buffer, 0.75 µL of 50 mM $MgCl_2$, 0.05 µL of a 100 mM concentration of each deoxynucleoside triphosphate, 0.5 µL of 10 pmol μL^{-1} of the NMT primers, 10 µL lysate cells, 0.5 µL of a 5 U μL^{-1} Taq DNA polymerase, and water to give a final volume of 25 µL. All PCR reagents were purchased from Invitrogen, France. The reaction mixtures were incubated in a Hybaid PCRExpress Thermal Cycler using the following program. After an initial cycle consisting of 5 min at 94 °C, and then 35 cycles of 95 °C for 60 s, 52 °C for 30 s and 72 °C for 60 s, the reaction was terminated by a cycle of 7 min at 72 °C for *mcy*B and *mcy*C. The *mcy*A gene PCR amplification involved an initial cycle of 7 min at 94 °C, followed by 30 cycles with 94 °C for 10 s, 60 °C for 20 s and 72 °C for 60 s, terminating with a cycle at 72 °C for 7 min. The reaction mixtures were stored at 4 °C. The PCR products were then analyzed by electrophoresis on 1.5% agarose gel in 1× TBE (Tris-borate-EDTA) buffer, stained with SYBR SafeTM DNA gel stain (Invitrogen, France), and photographed under UV light. The length of DNA fragments was estimated by comparison with a 1 Kb plus DNA ladder (Invitrogen, France).

Tab. 1. Primers used in amplifying and sequencing of the 16S rRNA and the 16S-23S rRNA ITS regions and detecting *mcy*A, -B, and -C genes of the *Microcystis* morphospecies (MCYS-CH01) isolated from the Cheffia dam (Algeria) and the reference strain *M. aeruginosa* PCC 7806.

[a]F designates forward primer, R designates reverse primer. *Primer 322 initiates amplification at a region near the end of the 16S rDNA on the RNA-like strand (positions 1338-1354 in *Synechocystis* PCC 6803; *Escherichia coli* numbering 1391-1407), and primer 340 is complementary to a region on the opposite strand at the beginning of the 23S rDNA (positions 26-45 in both *Synechocystis* PCC 6803 ; E. coli).

Genes	Primers	Sequences (5'-3')	Amplified fragments (bp)	References
*mcy*A	MSF[a] MSR[a]	ATCCAGCAGTTGAGCAAGC TGCAGATAACTCCGCAGTTG	1300	Tillett *et al.* 2001
*mcy*B	2156-F[a] 3111-R[a]	ATCACTTCAATCTAACGACT AGTTGCTGCTGTAAGAAA	955	Mikalsen *et al.* 2003
*mcy*C	PSCF1[a] PSCR1[a]	GCAACATCCCAAGAGCAAAG CCGACAACATCACAAAGGC	674	Ouahid *et al.* 2005
16S rDNA	27F1[a] 1494Rc[a]	AGAGTTTGATCCTGGCTCAG TACGGCTACCTTGTTACGAC	1467	Neilan *et al.* 1997
ITS	322Fa 340R[a]	TGTACACACCGCCCGTC CTCTGTGTGCCTAGGTATCC	about 560*	Iteman *et al*. 2000

2.5. PCR amplification and sequencing of the 16S rDNA regions

PCR amplifications of the 16S rDNA regions of the morphosepcies MCYS-CH01 and the reference strain PCC 7806 were performed directly with 5 µL lysate cells as described above. A set of primers (27F1 and 1494Rc) was used, and the sequences of each primer are indicated in table 1. Incubation of the reactions was performed in a Hybaid PCRExpress Thermal Cycler using the following program. After an initial cycle consisting of 5 min at 94 °C, 35 amplification cycles were started (30 s at 94 °C, 30 s at 50 °C and 1 min at 70 °C). The reaction was terminated by a cycle of 3 min at 72 °C. The PCR products were then analyzed by electrophoresis as explained before.

In order to obtain enough DNA quantity, the PCR products of three independent reactions were pooled and purified using the ChargeSwitch® PCR Clean-Up kit (Invitrogen, France) to remove amplification reaction components, including unincorporated primers and nucleotides, and then sequenced using the same set of primers as for amplification (27F1 and 1494Rc) by Plate-forme Génotypage des Pathogènes et Santé Publique (Institut Pasteur, Paris, France). DNA was sequenced with the big dye-terminator cycle sequencing kit using an ABI PRISM 3730XL DNA sequencer (Applied Biosystems). The 16S rDNA sequences of the morphospecies MCYS-CH01 and the reference strain PCC 7806 were aligned using Genedoc v2.6.0002 software (www.psc.edu/biomed/genedoc), with a representative data set of sequences of *Microcystis* species available in GenBank. Relationships between the strains were inferred using the maximum likelihood method (Olsen *et al.* 1994). The phylogenetic tree was midpoint rooted, using the strain Synechococcus elongatus PCC 7942 (accession number AF132930) as the out-group. The statistical significance of the branches was estimated by analysis of the tree programs, involving the generation of 1000 trees.

2.6. PCR amplification and sequencing of the ITS regions

PCR amplifications of the ITS regions were performed directly using 5 µL lysate cells as described above. A set of primers (322 and 340) was used to amplify specifically the part of the rRNA operon containing the ITS region. The sequences of each primer are indicated in table 1. After an initial cycle consisting of 5 min at 94 °C, 30 cycles of amplification were started (0.5 min at 94 °C, 0.5 min at 50 °C and 1 min at 70 °C). The termination cycle consisted of 3 min at 72 °C. The PCR products were then analyzed by electrophoresis as explained before.

In order to obtain enough DNA quantity, the PCR products from five independent reactions of each morphospecies were pooled and purified using the ChargeSwitch® PCR Clean-Up kit (Invitrogen, France), and then sequenced using the same set of primers as for amplification (322 and 340) by GenoScreen (Campus Pasteur, Lille, France). DNA was sequenced with the Applied Biosystems ready reaction kit using an ABI PRISM 3730XL DNA sequencer (Applied Biosystems). Methods used for DNA alignment and phylogenetic analyses of the 16S-23S ITS region were the same as those described above for the 16S rDNA sequence.

2.7. Nucleotide sequence accession numbers

The 16S and ITS sequences of the unusual morphospecies of *Microcystis* MCYS-CH01 reported in this paper were deposited in the GenBank database under the following accession numbers. The 16S sequence: EU541973, and the ITS sequence: EU541975.

3. RESULTS

3.1. Morphological characteristics and toxigenic potential of the morphospecies MCYS-CH01

The dominant morphospecies (MCYS-CH01) observed in the autumn in the samples from the Cheffia

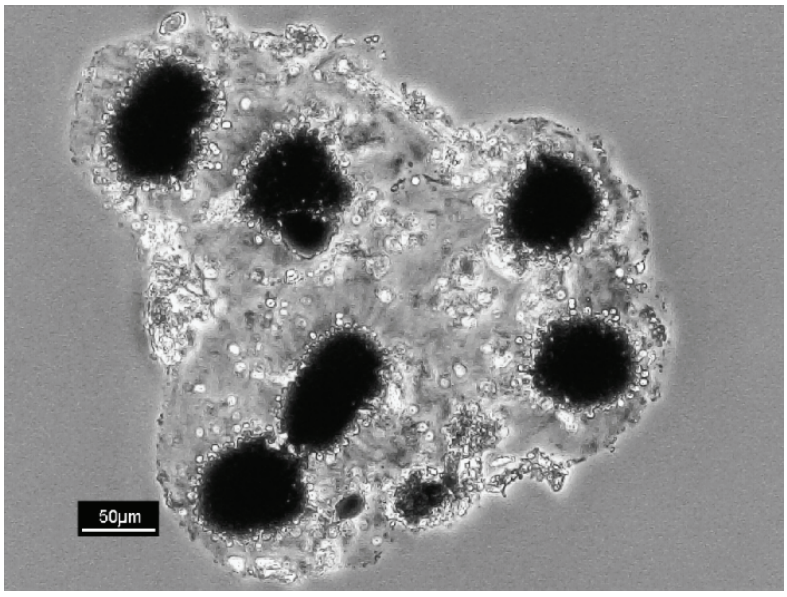

Fig. 1. Light micrograph showing morphological characteristics of colony of the morphospecies *Microcystis* sp. (MCYS-CH01) isolated from the Cheffia Dam (Algeria).

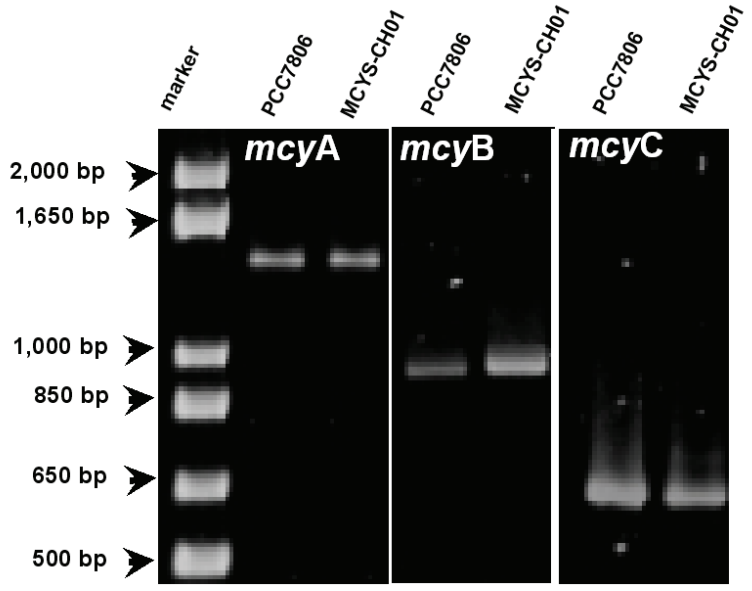

Fig. 2. Gel electrophoresis of PCR products of the morphospecies (MCYS- CH01) and the reference strain *M. aeruginosa* PCC 7806 of the genus *Microcystis* for *mcy*A, -B, and -C genes using primer sets MSF-MSR, 2156F-3111R, and PSCF1-PSCR1, respectively.

Dam (Algeria) was isolated, and its morphological characteristics were described. As shown in figure 1 this morphospecies displayed large colonies, up to the macroscopic level. These colonies were irregular in outline, and were composed of small subcolonies each containing densely packed cells. The mucilage was colorless, very thick, and clearly extended more than 50 µm beyond the outline of the cell cluster. The cells were spherical and small (diameter 3-4 µm), and contained only a few gas vesicles. The morphological features of this unusual morphospecies were different from any of those described in the literature of all known species of *Microcystis*, and may constitute a new species. The PP2A inhibition assay showed that crude methanol extracts of the morphospecies MCYS-CH01 and of the reference strain *M. aeruginosa* PCC 7806 all inhibited the activity of the PP2A enzyme. Furthermore, as shown in figure 2 the presence of microcystin synthetase genes *mcy*A, -B, and –C indicated that these two morphospecies are microcystin-producers.

3.2. Comparison of 16S rRNA gene sequences for different Microcystis *morphospecies*

A set of 2 primers 27F1 and 1494Rc, was used for the PCR amplification of 16S rRNA genes from the axenic reference strain PCC 7806 isolated from the Bra-

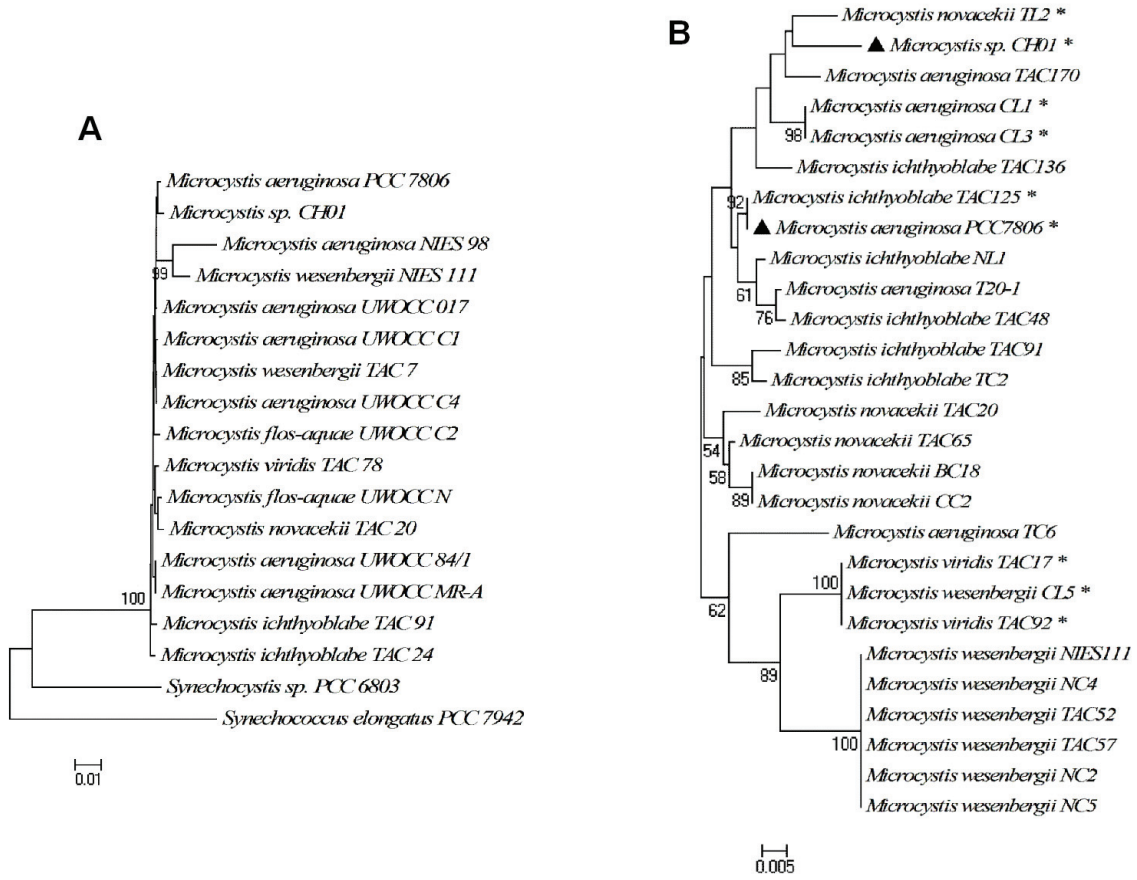

Fig. 3. Phylogenetic trees based on (A) 16S rDNA sequences showing the relationships between cyanobacteria strains of *Microcystis*. Outgroup *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803. An alignment of 1400 nucleotides after excluding positions with gaps was used. Scale bar =1 base substitution per 100 nucleotide positions. Local bootstrap probabilities (for branches except those within the *Microcystis* cluster) are indicated at nodes. Accession numbers in the GenBank databases are *Microcystis aeruginosa* D89032, AF139316, AF139315, AF139294, AF139301, AF139320, AF139299, *M. novacekii* AB012336, *M. wesenbergii* D89034, AB035553, *M. ichthyoblabe* AB035550, AB012339, and *M. viridis* AB012331, and *Microcystis* sp. (MCYS-CH01) EU541973 and on (B) 16S-23S rRNA sequences showing the relationships between the morphospecies *Microcystis* sp. (MCYS-CH01) isolated from Cheffia Dam (Algeria) and the toxic reference strain *M. aeruginosa* PCC 7806 and some Asian *Microcystis* strains sequenced by Otsuka *et al*. (1999). Bootstrap probabilities (>50%) are indicated at the nodes. Strains asterisked are microcystin-producers. Black triangle indicates strains sequenced in this study. Accession numbers in the GenBank databases of the morphoespecies *Microcystis* sp. (MCYS-CH01) EU541975 and of the different Asian strain sequenced by Otsuka *et al.* (1999) are given in table 2.

akman reservoir (Netherlands), and from the non-axenic *Microcystis* morphospecies (MCYS-CH01) isolated from the Cheffia Dam (Algeria). The specifically designed primers (27F1 and 1494Rc) enabled us to sequence both strands of 16S rDNA with overlaps. Complete sequences for both strands of the 16S rDNA were generated for the region extending from position 27 to position 1494 (*E. coli* numbering) for the two morphospecies tested. The assembled sequences were analyzed using the NCBI BLASTN 2.1.3 (http:///www.ncbi.nlm.nih.gov/blast/) program to align them with database sequences, and to check that the sequences generated were cyanobacterial in origin. The 16S rDNA sequences identified were compared to each other and to those of previously published almost-complete 16S rDNA sequences for *Microcystis* species and related organisms available in GenBank. After ambiguous characteristics had been removed from the alignment, 1,400 nucleotide positions were used for successive phylogenetic analyses. The morphospecies MCYS-CH01 and the reference strain tested, and some previously published complete 16S rDNA sequences for some *Microcystis* species, showed high DNA sequence similarity exceeding 99%. Constructed phylogenetic neighbor-joining trees (Fig. 3A) revealed that all these *Microcystis* strains formed a clearly-defined cluster with no clear divisions between them.

3.3. rRNA ITS gene sequence and phylogenetic analysis of the morphospecies MCYS-CH01

To elucidate phylogenetic difference between morphospecies MCYS-CH01, reference strain PCC 7806, and various *Microcystis* morphospecies described in the literature, DNA fragments covering the ITS of the refer-

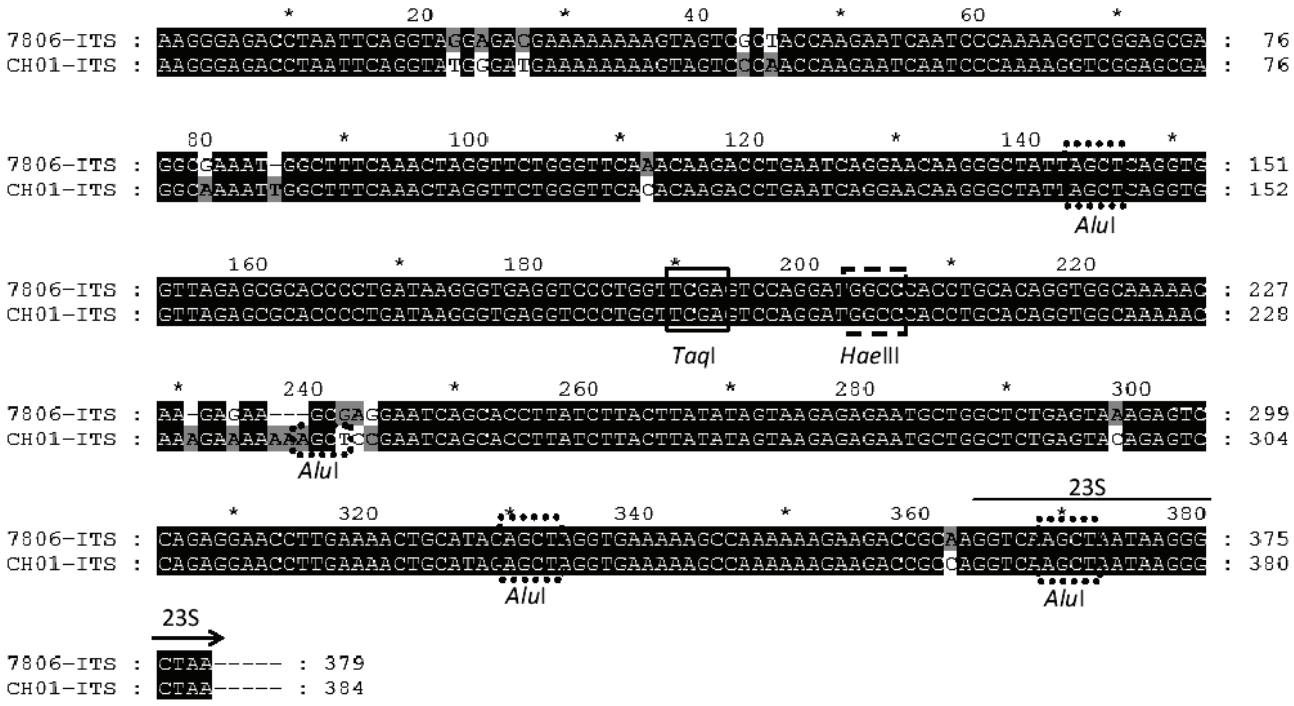

Fig. 4. Alignment of the nucleotide sequences of the ITS regions of the morphospecies *Microcystis* sp. (MCYS-CH01) isolated from Cheffia Dam (Algeria) and the reference strain *M. aeruginosa* PCC 7806. Rectangles indicate the cleavage site of the restriction enzymes (dotted line) AluI, (continuous line) TaqI, and (broken line) HaeIII. The arrow above the sequence of the ITS of the reference strain PCC 7806 indicates the 5' beginning of the 23S rRNA gene.

ence strain and of morphospecies MCYS-CH01 were sequenced and compared to 26 Asian Microcystis species sequenced by Otsuka *et al.* (1999). The length of the entire ITS sequences of the reference strain PCC 7806 and of morphospecies MCYST-CH01 are 358 and 363 bp, respectively. This length range is in overall agreement with the band (about 560 bp) observed by gel electrophoresis that, for the set of primers used, should have been 200 bp longer. The conserved domains (D1, D1', D2, D3, D4, D5 and box 5) and one tRNA gene, tRNAIle described by Iteman *et al.* (2000), were found in both these sequences. Recognition sites for each of the restriction enzymes TaqI and HaeIII were observed at positions 190 and 202, respectively, in the sequences (Fig. 4). However, three cleavage sites for the restriction enzyme AluI were observed in the ITS sequence of morphospecies MCYS-CH01, but only two in that of reference strain PCC 7806 (Fig. 4). This was confirmed by the point mutation (position 238) at the cleavage site of restriction enzyme AluI in the gene sequence of reference strain PCC 7806 (Fig. 4). In addition, one cleavage site of AluI was also observed at the beginning of 23S rDNA, and at the same position for both the PCC 7806 and MCYS-CH01 morphospecies (Fig. 4).

A phylogenetic tree was constructed based on the alignment of the rDNA ITS sequences of morphospecies MCYS-CH01, reference strain PCC 7806 and 26 Asian *Microcystis* species sequenced by Otsuka *et al.* (1999). The similarity of the ITS sequence of morphospecies MCYS-CH01 was still high (92-98% sequence identity) compared to the other strains (Tab. 2). The distribution of the 28 sequences in a phylogenetic tree (Fig. 3B) did not reveal any obvious segregation between morphospecies MCYS-CH01, isolated from the Cheffia Dam (Algeria), reference strain PCC 7806 isolated from the Braakman reservoir (Netherlands), and the 26 sequences obtained by Otsuka *et al.* (1999) from *Microcystis* species isolated from Asian lakes and added to our analysis. Consequently, morphospecies MCYS-CH01 and reference strain PCC 7806 sequenced in this study are included in cluster I described by Otsuka *et al.* (2000) for some Asian strains belonging to all *M. novacekii* and *M. ichthyoblabe* strains, and most *M. aeruginosa* strains.

4. DISCUSSION

The genus *Microcystis* is usually linked to hepatotoxic blooms world-wide (Sivonen & Jones 1999). According to Komárek & Anagnostidis (1999), *Microcystis* is characterized by having gas vesicles, a coccoid cell shape, a tendency to form aggregates or colonies, and an amorphous mucilage or a sheath. Based on these criteria, ten species have been distinguished in Europe: *Microcystis aeruginosa* (Kützing) Kützing, *M. viridis* (A. Braun in Rabenhorst) Lemmermann, *M. wesenbergii* (Komárek) Komárek in Kondratieva, *M. novacekii*

Tab. 2. Similarities between the 16S-23S rRNA ITS sequence of the morphospecies *Microcystis* sp. (MCYS-CH01) isolated from Cheffia dam and the ITS sequences of the reference strain *M. aeruginosa* PCC 7806 and some Asian *Microcystis* strains sequenced by Otsuka *et al*. (1999). *Strain marked with "+" produces microcystin(s) and one with "-" does not.

Morphospecies	Reference strain	Accession number	Source of isolate	Microcystin*	% of sequence identity
Microcystis novacekii	TL2	AB015380	Thaïland	+	98
Microcystis ichthyoblabe	TC2	AB015372	Thaïland	-	98
Microcystis aeruginosa	TAC170	AB015365	Japan	Unknown	98
Microcystis ichthyoblabe	NL1	AB015371	Japan	-	97.6
Microcystis ichthyoblabe	TAC125	AB015368	Japan	+	97.6
Microcystis aeruginosa	PCC 7806	-	The Netherlands	+	97.3
Microcystis novacekii	TAC65	AB015375	Japan	-	97.3
Microcystis aeruginosa	T20-1	AB015384	Thaïland	-	97
Microcystis ichthyoblabe	TAC48	AB015366	Japan	-	97
Microcystis novacekii	CC2	AB015378	China	-	97
Microcystis novacekii	BC18	AB015377	United Kingdom	-	97
Microcystis novacekii	TAC20	AB015374	Japan	-	96
Microcystis aeruginosa	CL1	AB015381	China	+	96
Microcystis aeruginosa	CL3	AB015382	China	+	96
Microcystis wesenbergii	CL5	AB015392	China	+	95
Microcystis viridis	TAC92	AB015402	Japan	+	95
Microcystis ichthyoblabe	TAC91	AB015367	Japan	-	96
Microcystis viridis	TAC17	AB015398	Japan	+	95
Microcystis ichthyoblabe	TAC136	AB015369	Japan	-	95
Microcystis wesenbergii	NIES111	AB015388	Japan	-	94
Microcystis wesenbergii	TAC52	AB015390	Japan	-	94
Microcystis wesenbergii	TAC57	AB015391	Japan	-	94
Microcystis wesenbergii	NC4	AB015396	Japan	-	93
Microcystis wesenbergii	NC2	AB015394	Japan	-	93
Microcystis wesenbergii	NC5	AB015397	Japan	-	93
Microcystis aeruginosa	TC6	AB015385	Thaïland	-	92

(Komárek) Compère, *M. ichthyoblabe* (Kützing), *M. flos-aquae* (Wittrock) Kirchner. *M. natans* (Lemmermann) ex Skuja, *M. firma* (Kützing) Schmidle, *M. smithii* (Kützing et Anagnostidis), and *M. botrys* (Teiling). Many other species have been also characterized outside Europe (Komárek & Anagnostidis 1999). In this study, unusual morphospecies, *Microcystis* sp. (MCYS-CH01), has been identified in Algeria in the Cheffia Dam (Fig. 1). Colonies of this morphospecies are lenticular or almost spherical, irregularly spherical or slightly elongate or with wavy outline, compact, with packed cells, without holes and not lobate, in old stages composed of several clustered subcolonies, with a large number of densely aggregated cells. Mucilage was colorless, very thick, delimited at the margin, not diffluent; the wide margins around central cell clusters reach to 50 µm width. The cells were spherical and small (diameter 3-4 µm), and contained only a few gas vesicles.

Three species, *Microcystis aeruginosa*, *M. wesenbergii* and *M. ichthyoblabe* are often found in North African freshwater bodies (Oudra *et al.* 2001, Oudra *et al.* 2002, Nasri *et al.* 2004), however, this unusual *Microcystis* morphospecies was reported for the first time by Nasri *et al.* (2007) as the dominant *Microcystis* sp. forming bloom in the Cheffia Dam (Algeria). The presence of microcystin synthetase genes *mcy*A, -B, and –C in colonies of this morphospecies, and the fact that serine/threonine phosphatase (PP2A) was inhibited by its crude methanol extract indicated that it was microcystin-producer.

In contrast to its morphological classification, analysis of the 16S rDNA sequence of this non-axenic morphospecies revealed a high degree of similarity (>99% sequence identity) between them and the reference strain PCC 7806, and previously published almost complete 16S rDNA sequences for known *Microcystis* species (Fig. 3A). Several previous studies based on 16S rDNA have shown that different species of *Microcystis* can be clustered together (Neilan *et al*. 1997; Lyra *et al*. 2001). Otsuka *et al*. (1998) found that five *Microcystis* species: *Microcystis aeruginosa*, *M. ichthyoblabe*, *M. wesenbergii*, *M. viridis*, and *M. novacekii*, were so closely related in terms of 16S rDNA sequence that they can be grouped as a single species, and concluded that the 16S rDNA sequence is insufficiently variable to be used for phylogenetic analysis of these organisms at species level. Moreover, Neilan *et al*. (1997) have reported that minor and variable morphometric parameters may have led to the identification of *M. wesenbergi* and *M. viridis*, although it is difficult to justify their separation from *M. aeruginosa* on the basis of the results of 16S rRNA gene analyses. The difference in resolution from 16S rRNA in *Microcystis* matches the reported average sequence diversity of less than 1% in this gene (Otsuka *et al.* 1998, Boyer *et al*. 2001). Knowing that the internal transcribed spacer (ITS)

region between 16S and 23S rRNA genes is less conserved than the 16S rRNA gene in cyanobacteria (Neilan *et al*. 1997, Otsuka *et al*. 1999), we investigated the possible use of this domain for genotyping the unusual non-axenic morphospecies MCYS-CH01. To provide a comparison, the axenic strain *Microcystis aeruginosa* PCC 7806 was also included in this study as a reference strain. We found that reference strain PCC 7806 and morphospecies MCYS-CH01 displayed similar ribotypes, with an ITS size of about 360 bp (PCR product of about 560 bp). This is consistent with results reported in several studies (Lu *et al*. 1997; Otsuka *et al.* 1999; Janse *et al*. 2004; Humbert *et al*. 2005), where the size of ITS for some *Microcystis* species ranged from 320 to 365 bp.

In order to compare the genetic diversity of the unusual morphospecies MCYS-CH01, isolated from the Cheffia Dam (Algeria), to that of morphospecies isolated from other water-bodies separated by geographic distance as well as having different physical and chemical parameters, 26 of the 47 sequences obtained by Otsuka *et al.* (1999) from different *Microcystis* morphospecies isolated from Asian lakes were included in the analysis. Although the variation in the sequence of 16S-23S ITS regions of different *Microcystis* morphospecies was found to be more variable (92-98% sequence identity) than the corresponding 16S rDNA sequences (>99% sequence identity), the ITS sequences of the different *Microcystis* morphospecies were homogenous regardless of their geographical origin. Here we show that morphospecies MCYS-CH01 had a very similar 16S-23S ITS sequence (98% sequence identity) to that of the toxic strains *M. novacekii* T20-3 and TL2 described by Otsuka *et al.* (1999), and it was therefore assigned to *Microcystis novacekii.* The high degree of relatedness between these strains is entirely consistent with their common phenotypic characters: colonies are small and firm, not lobular, composed of tightly aggregated cells, and are surrounded by a thick gelatinous substance (Otsuka *et al*. 2000). In contrast, the non-toxic strains *M. novacekii* TAC65, CC2, BC18, and TAC20, share only 96-97.3% sequence identity (Tab. 2). Otsuka *et al*. (1999) reported that cluster I, which includes all the *M. novacekii* and *M. ichthyoblabe* strains and most *M. aeruginosa* strains, included both toxic and non-toxic strains. Watanabe (1996) used the term '*M. aeruginosa* complex' for these last three morphospecies, since their properties are obscure. It has previously been demonstrated that there is no clear relationship between rRNA gene phylogeny and microcystin production in the genus *Microcystis* (Tillett *et al*. 2001). Furthermore, since microcystins can also be synthesized by other cyanobacteria genera (e.g., *Anabaena* spp., *Nostoc* spp., *Oscillatoria* spp., and *Planktothrix* spp.), the production of these toxins must have originated in a common ancestral cyanobacterium, and the observed heterogeneous distribution of toxic and non-toxic strains may result from gene deletions occurring a number of times during evolution.

5. CONCLUSION

In conclusion, the presented morphological characteristics such as the presence of very thick and clearly extended mucilage and specially the molecular results based on the analysis of the 16S-23S rRNA spacer region, suggested that the unusual *Microcystis* morphospecies MCYS-CH01 isolated from the Cheffia Dam (Algeria) should be assigned to *Microcystis novacekii*. The comparison of the ITS sequence of this morphospecies with those of different species of *Microcystis* available in the GenBank database showed that some highly conserved genotypes are found throughout the world.

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