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Full Length Research Paper

A study of the morphological distinctions between the *Microcystis* morphospecies isolated from the same reservoir

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The toxic potential, morphological characteristics and sequences of the 16S rRNA gene, and restriction fragment length polymorphisms (RFLPs) of the amplified 16S-23S rDNA internal transcribed spacer (ITS) regions were investigated in three *Microcystis* morphospecies (MCYS-LB01, -LB02 and -LB03) isolated from Lebna dam in Tunisia, and compared to the reference strain *Microcystis aeruginosa* PCC 7806 and different known *Microcystis* species described in the literature. Based on their morphology, morphospecies MCYS-LB01 and -LB02 were assigned to *Microcystis aeruginosa* and morphospecies MCYS -LB03 to *Microcystis wesenbergii*. The phylogenic tree based on 16S rDNA sequences showed that these three morphospecies are indistinguishable from each other, from the reference strain PCC 7806 and from many other known *Microcystis* species and, therefore, this tree did not necessarily correlate to the distinctions between morphospecies. Furthermore, when using PCR amplification and restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA spacer region of these three morphospecies of *Microcystis*, a similar pattern type was obtained, even though they had been assigned to two distinct morphospecies on the basis of their morphological characteristics. This implies that some of the morphological distinctions between the *Microcystis* morphospecies isolated from the same reservoir must be viewed as intraspecific or phenotypic variations.

Key words: Cyanobacteria, *Microcystis*, 16S rRNA, ITS, RFLP, Microcystins.

INTRODUCTION

Mass 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 and 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), and neigh-boring 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 dominant group of toxin produced by *Microcystis* is the microcystin hepatotoxin, a cyclic heptapeptide that is formed non-ribosomally by peptide and polyketide synthetases (Dittmann et al., 1997; Tillett et al., 2001). These hepato-toxins 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 diffi-

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cult 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 and Anagnostidis, 1999). *Microcystis* colonies differ in shape and size, but also in the appearance of their mucilage (Via-Ordorika et al., 2004) . 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, for the different *Microcystis* species. Fahrenkrug et al. (1992) and Otsuka et al. (1999) examined the DNA base composition of four *Microcystis* strains, and they concluded that these strains had fairly similar base compositions.

Krüger et al. (1995) and Otsuka et al. (1999) investigated the fatty acid com-position of *Microcystis species* as a possible taxonomic criterion, and they reported that the fatty acid composition was irrelevant to species distinction. Neilan et al. (1997) and Otsuka et al. (1998) presented phylogenetic trees of the strains in the genus *Microcystis* based on 16S rDNA sequence comparisons, and they concluded that these trees did not match the classification based on mor- phological characteristics. In view of the frequent debates about the validity of morphological features for identifying *Microcystis* species, and the fact that previous molecular studies did not support correlations between phenotypes and genotypes (Otsuka et al., 1998, 1999, 2000), in this paper we intend to use the term “morphospecies” instead of 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 polymer-phism (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, mor-phological characteristics and phylogenetic analysis based on PCR-RFLPs of the internal transcribed spacer (ITS) and the 16S rDNA sequences of three non-axenic *Microcystis* morphospecies collected from a freshwater reservoir in Tunisia (Lebna dam). For the purposes of comparison, the axenic strain *M. aeruginosa* PCC 7806 was also included in this study as a reference strain.

MATERIALS AND METHODS

Study sites

The Lebna dam is located in the Cap Bon region of northeastern Tunisia, and has a surface area of 650 ha and a maximum depth of 10 m. This reservoir provides irrigation water for market gardening (lettuce, tomatoes, peppers, etc.) and arboriculture, and drinking water for domestic animals.

Sampling and morphological characterization of *Microcystis* morphospecies

Sampling in the reservoir for colony isolation and morphological characterization was carried in the autumn of 2005 using plankton nets (20 µm mesh size) and the dominant genus identified was *Microcystis*. Aliquots of the concentrated net samples were fixed with formalin (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 various *Microcystis* morphospecies. The cell diameter was determined for 50 cells (10 cells from 5 different colonies of each morphospecies) . The remaining fresh phytoplankton sample was used for colony isolation as described below.

Isolation and toxic potential of *Microcystis* morphospecies

For isolation of colony of *Microcystis*, 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 cyano-bacteria and algae stuck in the mucilage of *Microcystis* spp., but the absence of other cyanobacteria was checked by visual inspection under the microscope. Each series of ten isolated colonies from each morphospecies of *Microcystis* were pooled separately in a sterilized Eppendorf tube (1.5 ml) and then lyophilized. Aliquots from each species were then used to determine the toxic potential by the protein phosphatase type 2A (PP2A) inhibition assay and the microcystin biosynthesis gene cluster (*mcy*) amplification. For the PP2A inhibition assay, lyophilized morphospecies of *Microcystis* colonies were extracted with 100 µl aqueous methanol (75 % [v/v]), and then centrifuged at 5 000 g for 10 min. An aliquot from each supernatant was then analyzed by the PP2A inhibition assay as described in Bouaïcha et al. (2001). PCR amplifications of *mcy*A, *mcy*B and *mcy*C, which are indicative of the presence of the microcystin biosynthesis gene cluster (Dittmann and Börnerb, 2005), were performed by isolating the DNA from each morphospecies of *Microcystis* 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 *N*-methyl transferase (NMT) domain of the microcystin synthetase genes *mcy*A, *mcy*B, and *mcy*C. The PCR mixture contained 2.5 µl of 10 x PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 5 pmol of each primer, 10 µl lysate cells, 2.5 Units of 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

Table 1. Primers used in amplifying of the 16S rRNA and the 16S-23S rRNA ITS regions and detecting *mcyA*, *-B*, and *–C* genes of the different *Microcystis* morphospecies (MCYS-LB01, -LB02 and -LB03) isolated from Lebna dam (Tunisia) and the reference strain *M. aeruginosa* PCC 7806.

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

[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*).

for 7 min. The reaction mixtures were stored at 4°C. A negative control without template was included in some experiments. The PCR products were then analyzed by electrophoresis on 1.5% agarose gel in 1 x TBE (Tris-borate-EDTA) buffer, stained with SYBR Safe™ 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).

PCR amplification and sequencing of the 16S rDNA regions

PCR amplifications of the 16S rDNA regions were performed directly with cell lysates obtained as described above. A set of two primers, 27F1 and 1494Rc (Table 1), was used for the PCR amplification of 16S rRNA genes from the axenic reference strain PCC 7806 isolated from the Braakman reservoir (Netherlands), and from the three non-axenic *Microcystis* morphospecies (MCYS-LB01, -LB02 and -LB03). The PCR mixture contained 2.5 µl of 10 x PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 5 pmol of each primer, 5 µl lysate cells, 2.5 Units of Taq DNA polymerase, and water to give a final volume of 25 µl. All PCR reagents were purchased from Invitrogen, France. 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 reaction mixtures were stored at 4°C. A negative control without template was included in some experiments. The PCR products were then analyzed by electrophoresis as indicated above.

The PCR products of three independent reactions were mixed 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). The 16S rDNA sequences of each *Microcystis* morphospecies 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. 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. Relationships between the strains were inferred using the maximum likelihood method (Olsen et al., 1994). The phylogenetic tree was midpoint rooted, using the strain *Synechoccus elongates* PCC 7942 (accession number AF132930) as the out group. The statistical significance of the branches was estimated by boostrap analysis of the tree programs, involving the generation of 1000 trees.

PCR amplification of the ITS regions

PCR amplifications of the ITS regions were performed directly using cell lysates obtained 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. The PCR mixture contained 2.5 µl of 10 x PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 5 pmol of each primer, 5 µl lysate cells, 2.5 units of Taq DNA polymerase, and water to give a final volume of 25 µl. All PCR reagents were purchased from Invitrogen, France. 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 product was denatured as described in Jensen and Straus (1993) by combining 5 µl of amplification product with 10 µl of deionized water and 6 µl of loading buffer (TrackIt™ Cyan/Yellow containing 0.1% SDS; Invitrogen, France), and then heating to 95°C for 3 min. Samples were then immediately placed in dry ice/ethanol for storage, and thawed on wet ice just before electrophoresis. The direct PCR products and denatured PCR products were then analyzed by electrophoresis as indicated above.

Digestion of amplified ITS regions

The PCR products of three independent reactions were mixed and purified using the ChargeSwitch® PCR Clean-Up kit (Invitrogen, France) to remove any remaining amplification reaction compo-

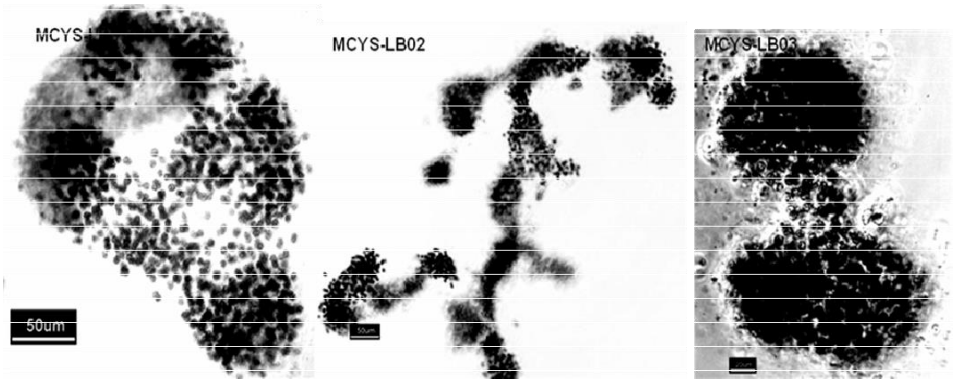

Figure 1. Micrographs showing morphological characteristics of *Microcystis* colonies of morphospecies MCYS-LB01, MCYS-LB02 and MCYS-LB03 isolated from Lebna dam (Tunisia) (El Herry et al., 2008).

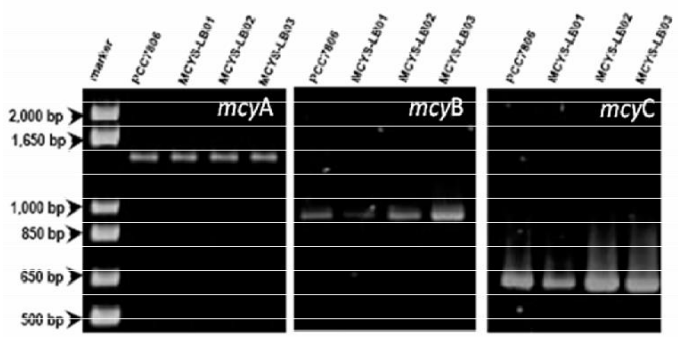

Figure 2. Gel electrophoresis of PCR products of the *mcyA*, *-B*, and *-C* genes using primer sets MSF-MSR, 2156F-3111R, and PSCF1-PSCR1, respectively, for the three morphospecies (MCYS-LB01, - LB02 and -LB03) isolated from Lebna dam (Tunisia) and the reference strain *M. aeruginosa* PCC 7806.

components, including unincorporated primers and nucleotides. The ITS amplification regions were then digested separately with six restriction endonucleases which recognize and cleave specific nucleotide motifs. Approximately 5 µl of PCR product for each morphospecies was combined with 1 µl of the corresponding enzyme buffer and 5 U of restriction enzyme in a 10 µl-digest. Reaction mixtures were incubated overnight at the temperature suggested by the enzyme supplier. The DNA restriction enzymes *Eco*RV, *Taq*I, *Bam*HI, *Alu*I, *Hin*dIII, and *Hae*III (Invitrogen, France) were used to generate specific RFLP patterns for each *Microcystis* morphospecies studied and for the reference strain, *Microcystis aeruginosa* PCC7806. The reaction mixture was then analyzed by electrophoresis as indicated above.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences reported in this paper was deposited in the GenBank database under the following accession numbers (the *Microcystis* morphospecies are shown in the parentheses): EU541970 (*M. aeruginosa* MCYS- LB01), EU541971 (*M. aeruginosa* MCYS-LB02) and EU541972 (*M. wesenbergii* MCYS-LB03).

RESULTS

Morphological characteristics and toxigenic potential of the different *Microcystis* morphospecies

Three *Microcystis* morphospecies were isolated (MCYS-LB01, -LB02 and -LB03) in Tunisia from the Lebna dam and their morphological characteristics were compared. As shown in Figure 1, these morphospecies had differing cell sizes, colony morphology and size, and mucilage characteristics. In the MCYS- LB01 morphospecies, the colonies were more or less spherical, and the cells usually 4 - 6 µm in diameter, sparsely to densely agglomerated, and contained gas vesicles. In contrast, the MCYS-LB02 colonies were elongated, the cells usually 4 - 6 µm in diameter, not very densely and irregularly agglomerated, and contained gas vesicles. The mucilage of the MCYS-LB01 and MCYS-LB02 morphospecies was colorless, structureless, and diffluent, and did not form a very wide margin around the cells.

However, colonies of the MCYS-LB03 morphospecies were composed of two small subcolonies, each with densely packed cells. The MCYS-LB03 cells were spherical, 5 - 7 µm in diameter, and contained gas vesi-cles. The mucilage of this morphospecies was colorless, structureless, although distinctly delimited, and firm, it was not diffluent, and displayed a refractive outline. According to Komárek and Anagnostidis (1999), morphospecies MCYS-LB01 and MCYS-LB02 can both be assigned to *Microcystis aeruginosa*, and MCYS-LB03 to *M. wesenbergii*.

The PP2A inhibition assay showed that crude methanol extracts of all these cyanobacterial morphospecies and of the reference strain *M. aeruginosa* PCC 7806 all inhibited the activity of the PP2A enzyme. The results in Figure 2 also show that all three morphospecies of *Microcystis* and the reference strain PCC 7806 expressed the *mcy*A, -B, and -C genes, indicating that they are all microcystin-producers.

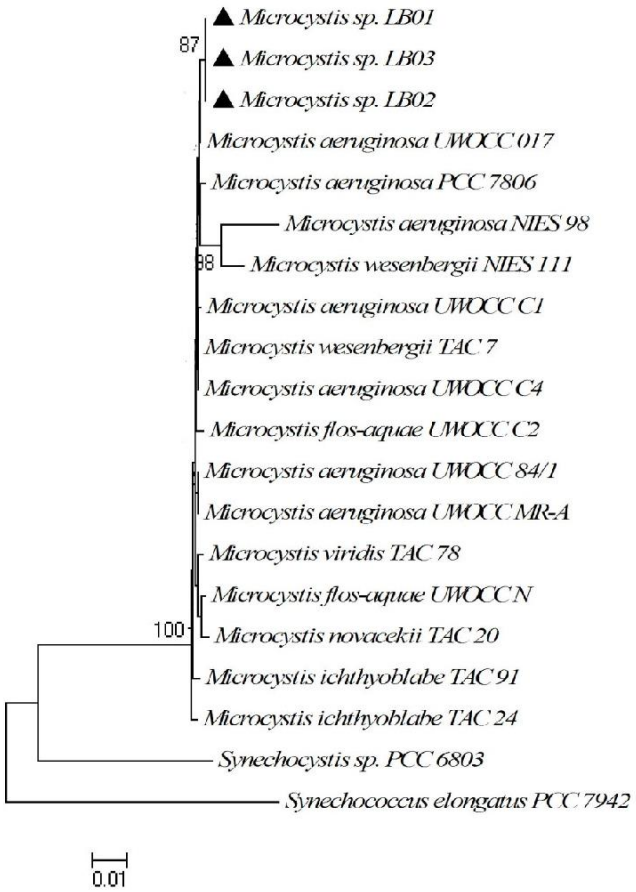

Figure 3. Phylogenetic tree based on 16S rDNA sequences showing the relationships between cyanobacteria strains of *Microcystis*. Outgroups were *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC6803. Black triangle indicated morphospecies sequenced in this study. An alignment of 1 400 nucleotides 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 *M. aeruginosa* D89032, AF139316, AF139315, AF139294, AF139301, AF139320, AF139299, *M. novacekii* AB012336, *M. wesenbergii* D89034, AB035553, *M. ichthyoblabe* AB035550, AB012339, *Microcystis* viridis AB012331.

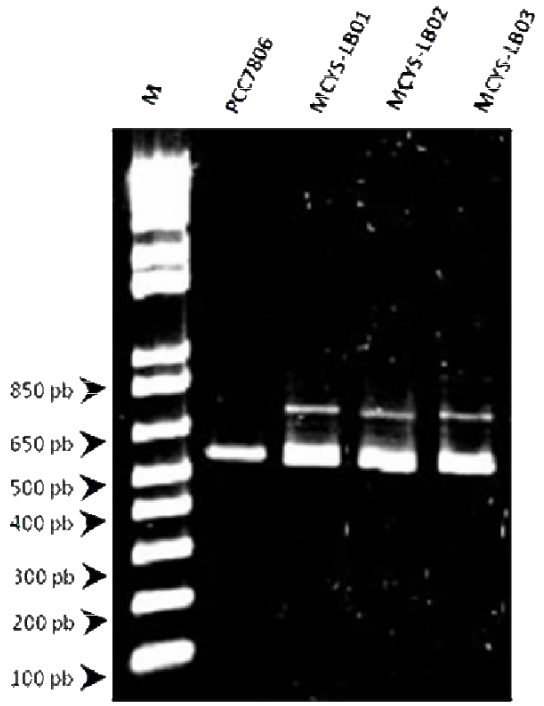

Figure 4. ITS amplicons of *Microcystis* morphospecies (MCYS-LB01, -LB02 and -LB03) isolated from Lebna dam (Tunisia). *M. aeruginosa* PCC 7806 is included as reference strain.

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

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 all morphospecies tested. After ambiguous characteristics had been removed from the alignment, 1 400 nucleotide positions were used for successive phylogenetic analyses. The three *Microcystis* morphospecies (MCYS-LB01, -LB02 and -LB03) shared 100% similarity. Constructed phylogenetic neighbor-joining trees (Figure 3) revealed that these *Microcystis* strains formed a clearly-defined cluster with no clear divisions between them.

Differentiation of *Microcystis* morphospecies based on PCR-RFLP of the 16S-23S rRNA ITS

To enable us to distinguish between the *Microcystis* morphospecies, the 16S- 23S rRNA ITS regions were amplified and analyzed by RFLP. With primers 322 and 340, a single PCR amplicon (about 560 bp) was observed for the axenic reference strain PCC 7806 (Figure 4) . After subtracting a total of 200 bp contributed by the 3' end of the 16S rRNA (150 bp) gene and the beginning of the 23S rRNA (50 bp) gene, the size of the ITS region amounted to about 360 bp for reference strain PCC 7806. However, amplification with these primers performed on the non-axenic morphospecies MCYS-LB01, -LB02 and -LB03 isolated in Tunisia from the Lebna dam yielded two major bands (about 560 bp = ITS of about 360 bp similar to that of the axenic reference strain PCC 7806) with equal intensity, and an additional faint band of higher molecular size (about 710 bp) (Figure 4). Since PCR products of 16S rRNA-23S rRNA spacer amplifications may contain artificial bands corresponding to heteroduplexes that result from associations of single strands of the different ITS species (Carmichael et al., 2001) , we also separated the PCR amplicons in a denaturing gel. Under these conditions, we still obtained the

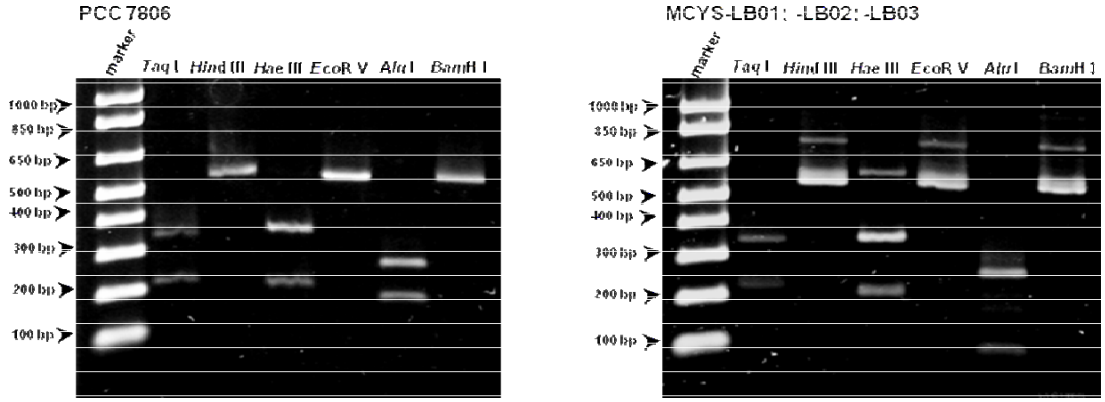

Figure 5. RFLP patterns of amplified 16S- 23S rRNA Intergenic Spacer Regions (ITS) from the axenic reference strain *M. aeruginosa* PCC7806 and the non-axenic morphospecies of the genus *Microcystis* (MCYS-LB01, -LB02 and -LB03) isolated from Lebna dam (Tunisia). The DNA restriction enzymes *Taq*I, *Hin*dIII, *Hae*III, *Eco*RV, *Alu*I and *Bam*HI were used to generate RFLP patterns specific of each *Microcystis* morphospecies.

the same two band profile (560 and 710 bp) (data not shown), indicating that the longer fragment of 710 bp was not a heteroduplex. Therefore, if we suppose that the band at 560 bp was the ITS amplicon, and the second band (about 710 bp) was an artifactual product, we can conclude that these morphospecies showed similar ribotypes.

To discriminate these morphospecies further, the PCR products obtained with primer set 322 and 340 were digested separately using six restriction enzymes (*Eco*RV, *Taq*I, *Bam*HI, *Alu*I, *Hin* dIII and *Hae*III) in order to reveal sequence polymorphism within the spacer regions. Representative gels of amplicons restricted using these enzymes are shown in Figure 5. Three of the enzymes, *Hin*dIII, *Eco*RV, and *Bam*HI, did not cut the DNA of the reference strain PCC 7806 or of the three morphospecies MCYS-LB01, -LB02 and -LB03. However, *Taq*I, *Hae*III and *Alu*I provided potentially useful discrimination between the different morphospecies and the reference strain (Figure 5). Reference strain PCC 7806 and the three morphospecies from the Lebna dam displayed the same RFLP pattern with *Taq*I (generally consisting of two bands), however, they differed by having different *Alu*I and *Hae*III RFLP patterns (Figure 5). As indicated in Figure 5, with the 16S-23S rDNA spacer of the three morphospecies from the Lebna dam, the restriction enzyme *Alu*I generated two intense and two weak bands, however, only two intense bands were observed with the reference strain PCC 7806 (Figure 5). The restriction enzyme *Hae*III generated two fragments with the ITS amplicons of the reference strain, however, with the morphospecies MCYS-LB01, -LB02 and - LB03, another band (about 560 bp) was also observed in addition to these two bands (Figure 5). This meant that RFLP pattern types could be distinguished for the reference
strain PCC 7806 tested, with the three morphospecies (MCYS-LB01, -LB02, and -LB03) isolated from the Lebna dam (Tunisia) displaying a similar RFLP pattern type (Table 2).

DISCUSSION

The genus *Microcystis* is usually linked to hepatotoxic blooms world-wide (Sivonen and Jones, 1999). According to Komárek and Anagnostidis (1999), *Microcystis* is characterized by having gas vesicles, a coccoid cell shape, a tendency to form aggregates or colonies, and amorphous mucilage or a sheath. Based on these criteria, ten species have been distinguished in Europe: *Microcystis aeruginosa* (Kützing) Kützing, *Microcystis viridis* (A. Braun in Rabenhorst) Lemmermann, *Microcystis wesenbergii* (Komárek) Komárek in Kondratieva, *Microcystis novacekii* (Komárek) Compère, *Microcystis ichthyoblabe* (Kützing), *Microcystis flos-aquae* (Wittrock) Kirchner. *Microcystis natans* (Lemmermann) ex Skuja, *Microcystis firma* (Kützing) Schmidle, *Microcystis smithii* (Kützing et Anagnostidis), and *Microcystis botrys* (Teiling). Many other species have been also characterized outside Europe (Komárek and Anagnostidis, 1999). In this study, two morphospecies, *M. aeruginosa* (MCYS-LB01, -LB02) and *M*. *wesenbergii* (MCYS-LB03) have been identified in one freshwater reservoir from Tunisia (Figure 1). The species *M. aeruginosa* and *M*. *wesenbergii* are often found in North African freshwater bodies (Oudra et al., 2001, 2002; Nasri et al., 2004). The presence of microcystin synthetase genes *mcy*A, -B, and -C in colonies of the three morphospecies isolated in this study, and the fact that serine/threonine phosphatase (PP2A) was inhibited by their crude methanol extracts indicated that they were all microcystin-producers.

In contrast to their morphological classification, analysis of the 16S rDNA sequences of the three non-axenic morphospecies revealed a high degree of similarity (>99% sequence identity) between them and the reference strain PCC 7806, and previously published almost

Table 2. Toxigenic potential and RFLP patterns and pattern types obtained for the different *Microcystis* morphospecies (MCYS-LB01, -LB02 and -LB03) isolated from Lebna dam (Tunisia) and the reference strain *Microcystis aeruginosa* PCC 7806. Pattern type derives from the pattern combination with the three restriction enzymes (*Taq*I, *Hae*III and *Alu*I)

Morphospecies	Geographical origin	Toxigenic Potential**	Size (bp) of PCR amplicon or ITS (in parenthesis)	RFLP patterns obtained			Pattern type
				***Taq*I**	***Hae*III**	***Alu*I**	
PCC 7806	Reservoir Braakman, Netherlands	+	560 (360)	T1	H1	A1	A
MCYS-LB01	Dam Lebna, Tunisia	+	560 (360), 710*	T1	H2	A2	B
MCYS-LB02	Dam Lebna, Tunisia	+	560 (360), 710*	T1	H2	A2	B
MCYS-LB03	Dam Lebna, Tunisia	+	560 (360), 710*	T1	H2	A2	B

*Band was likely generated by bacterial contamination using primers set 322 and 340.
**Toxicity of each morphospecies has been determined by PP2A assays and by the detection of microcystin synthetase genes (*mcyA*, -*B*, and -*C*).

complete 16S rDNA sequences for known *Microcystis* species (Figure 3). Our findings therefore did not confirm the taxonomic validity of the existing *Microcystis* species. Several previous studies based on 16S rDNA have shown that different *Microcystis* species can be clustered together (Neilan et al., 1997; Lyra et al., 2001). Otsuka et al. (1998) found that five *Microcystis* species: *M. 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 these three non-axenic morphospecies by PCR amplification and RFLP of their ITS regions. To provide a comparison, the axenic strain *M. aeruginosa* PCC 7806 was also included in this study as a reference strain. We found that reference strain PCC 7806 displayed 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. However, an additional band of about 710 bp was generated by the same primer set, and this was faint for morphospecies MCYS-LB01, -LB02 and -LB03 (Figure 4). This length PCR product band (about 710 bp) was repeatedly observed with this primer set in these morphospecies, and could be the result of bacterial contamination, as suggested by Laloui et al. (2002). In fact, primer 340 is specific for cyanobacteria (Wilmotte et al., 1993; Iteman et al., 2000; Laloui et al., 2002), but primer 322 described by Wilmotte et al. (1993) targets highly conserved bacterial sequences, and is therefore only suitable for studies of axenic cultures of cyanobacteria (Iteman et al., 2002; Laloui et al., 2002). Nevertheless, we considered it unlikely that this additional band originated from just a few contaminating bacterial cells, since it was of similar intensity in the profiles of the three morphospecies MCYS-LB01, -LB02 and -LB03 isolated at the same sample site (Figure 4). A possible alternative explanation for multiple bands is contamination by aggregated colonies of other cyanobacteria genera that had not been separated despite being washed thoroughly during isolation. On the other hand, the amplification of two equal band intensities (about 560 bp) with the three morphospecies MCYS-LB01, -LB02 and -LB03 would be consistent with the presence of two different rRNA operons in one *Microcystis* strain. Iteman et al. (2000) reported that it is also possible that, at least in some unicellular cyanobacteria, more than one copy of the *rrn* operon may be present in their genome, but that the corresponding ITS regions are the same or very similar in size, as is the case for strains of the unicellular cyanoabacterium *Synechococcus* PCC 6301 and *Synechocystis* PCC 6803. However, the unambiguous sequences retrieved from all 47 Asian strains (Otsuka et al., 1999) argue against the presence of two different operons in *Microcystis* strains. It may be necessary to purify and sequence these two similar bands to confirm the presence of two slightly different operons in these morphospecies MCYS-LB01, -LB02 and -LB03 in the Lebna dam.

From Table 2, it can be seen that *Taq*I digests reference strain PCC 7806 and the three morphospecies (MCYS-LB01, -LB02 and -LB03) from the Lebna dam as a single profile type T1. *Hae*III distinguished reference strain PCC 7806 and the three morphospecies as a H2-type MCYS-LB01, -LB02 and -LB03. The continued presence of a band of about 560 bp after the digestion of the ITS amplicons of morphospecies MCYS-LB01, -LB02 and LB03 (Figure 5) with *Hae*III indicated that only one

operon of the ITS spacer was digested by this enzyme. This could be well explained by a base pair substitution or an insertion/deletion in the cleavage site of the restriction enzyme *Hae*III in one operon and, therefore, suggests the presence of two operons as indicated above. Conversely, *Alu*I grouped the morphospecies MCYS-LB01, -LB02 and -LB03 as a single, type-A2 profile, but differentiated between type A1, reference strain PCC 7806. Therefore, two RFLP pattern types A and B can be assigned to the *Microcystis* morphospecies tested (Table 2). We can conclude a similar type B pattern obtained for morphospecies MCYS-LB01, -LB02 and -LB03 isolated from the same site, indicating that they may belong to a similar species group. This is consistent with the results of 16S rRNA gene analysis, in which identical sequences were obtained for these three morphospecies. Therefore, although the three morphospecies isolated from the Lebna dam (Tunisia) contained three colonies with different morphological characteristics, the remarkable 100% 16S rRNA gene sequence similarity, and the same type-B RFLP pattern indicate that these morphospecies might in fact be a single genotypic species (genospecies), but exhibiting more than one morphotype. Indeed, Otsuka et al. (2000) reported that morphological criteria are frequently variable, and depend on the environment. On the other hand, using the phycocyanin intergenic spacer and flanking regions, Bittencourt-Oliviera et al. (2001) reported that several different genotypes of *Microcystis* morphospecies were found in a single water body in Brazil. This means that DNA-DNA hybridization studies are required to confirm the taxonomic relationships between *Microcystis* morphospecies, particularly those occuring in the same water-body. The studies of Lachance (1981) revealed that two strains with as much as 99.8% rRNA gene similarity can share as little as 25% DNA-DNA reassociation, and so do not belong to the same species. In addition, Wayne et al. (1987) reported that a species is generally defined as a group of strains if they share approximately 70% or more DNA-DNA relatedness and 5°C or less DNA melting temperature (*T*m), and if their phenotypic characteristics are consistent with this definition.

Conclusion

As previously reported in the literature, this study confirms that the taxonomy of *Microcystis* based chiefly on morphological characteristics is not supported by phylogenetic analysis based on 16S rRNA sequences. Furthermore, based on the PCR-RFLP analysis of the 16S-23S rRNA spacer region, a similar pattern type was obtained for the three morphospecies (MCYS-LB01, -LB02 and -LB03) isolated from the same reservoir (Lebna dam, Tunisia), even though they had been classified as two distinct species, *M. aeruginosa* and *M. wesenbergii,* when morphological characteristics were used for identification. This means that, some of the morphological distinctions between the *Microcystis* morphospecies isolated within the same reservoir should be considered to be intraspecific or phenotypic variations. A highly polymorphic marker such as amplified fragment-length polymorphism (AFLP) may be useful to confirm this last hypothesis.

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