Genetic Diversity in Microcystis populations of a French storage reservoir assessed by sequencing of the 16S-23S rRNA intergenic spacer

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Genetic Diversity in *Microcystis* Populations of a French Storage Reservoir Assessed by Sequencing of the 16S-23S rRNA Intergenic Spacer

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Abstract

We compared the genetic diversity of the 16S-23S spacer of the rRNA gene (ITS1) in benthic and pelagic colonies of the *Microcystis* genus isolated from two different sampling stations with different depths and at two different sampling times (winter and summer) in the French storage reservoir of Grangent. In all, 66 ITS1 sequences were found in the different clone libraries. The nucleotide diversity of all the sampled isolates were in the same range (average number = 0.022) regardless of their origin, showing that several clones are involved in the summer bloom event and contribute to the high biomass production. Phylogenetic study and analysis of molecular variance (AMOVA) revealed no obvious genetic differentiation between the benthic and pelagic isolates. This finding confirms that the *Microcystis* genus in this lake is characterized by having both a benthic phase in winter and spring allowing this organism to survive in unfavorable environmental conditions, and a pelagic phase in summer and autumn when environmental conditions allow them to grow in the water column. Finally, comparing these sequences with those available in the GenBank database showed that some highly conserved genotypes are found throughout the world.

Introduction

The population genetics of cyanobacteria is still a relatively under-investigated field of research despite its great interest for helping us to understand the ecology of these microorganisms. Most of the papers using a molecular approach have focused on taxonomic or phylogenetic studies due to the difficulties in identifying these microorganisms [e.g., 14, 31], or on physiological studies because cyanobacteria are good models for working on processes such as oxygenic photosynthesis (for example, see the review of Blankenship and Hartman [4]). More recently, numerous papers on the genetic determinism of the toxicity of cyanobacteria have also been published [e.g., 7, 25]. However, in the past few years, some papers have focused on the genetic diversity of several cyanobacteria species. For example, Moore et al. [27], Urbach and Chisholm [38], and Urbach et al. [39] showed that the existence of two physiologically and genetically distinct populations of the cyanobacterium *Prochlorococcus* allows this microorganism to growth in a broad range of environmental conditions. These papers demonstrate the value of using a polyphasic approach including molecular characterization when trying to elucidate the ecology of this microorganism.

In both temperate and tropical areas, the cyanobacterium *Microcystis aeruginosa* is the dominant species in the phytoplanktonic communities of freshwater eutrophic lakes and ponds [28]. The life cycle of this species in temperate regions includes both pelagic and benthic stages. At the end of autumn, in winter and in spring, most of the colonies are found in the sediment and none in the water column, whereas in summer and in early autumn, pelagic colonies are very abundant in the water column [5, 6, 9, 34, 37]. During this pelagic phase, blooms can occur when environmental conditions are favorable to the growth of this species (high water temperature and high physical stability of the water column). These proliferations are hazardous for human populations because cyanobacteria are able to produce a wide range of toxins, which are known to have caused allergic reactions, poisonings, and deaths in several cases [20].

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In order to clarify the population dynamics of *M. aeruginosa* and what determines its proliferations in Lake Grangent (France), studies on the recruitment of this species from sediment to the water column have been performed [24]. At the end of the spring and at the beginning of summer, a close correlation was observed between the decrease in the abundance of colonies in the first 2 cm of the sediment layer and the increase of their abundance in the water column [24]. It was also noticed that numerous *M. aeruginosa* colonies were present in sediment all year round. In addition, cellular activity was detected in these benthic colonies, suggesting a switch from photoautotrophic to fermentative metabolism as previously described for this species by Moezelaar and Stal [26]. Our work was undertaken to find out whether there are two ecotypes (pelagic versus benthic) of *Microcystis* in Lake Grangent, or whether this species is characterized by having both a pelagic and a benthic phase. To do this, we performed a comparative study of the genetic variability of *Microcystis* colonies by sequencing of the 16S-23S spacer of the rRNA gene (ITS1) in colonies isolated from the sediment at two different depths (13 and 40 m) and from the water column.

Materials and Methods

Site Description. Located near Saint-Etienne, in the upper part of the river Loire, the Grangent reservoir was created in 1957 (Fig. 1). It has an area of 365 ha with a length of 21 km, a maximum width of 400 m, a maximum depth of 50 m, and a capacity of 57.4×10^6 m^3. Lake Grangent constitutes a significant water reservoir for energy production and the irrigation of the Forez plain. This water body is also a recreational area used in summer for bathing and nautical activities. Since 1970, large external phosphorus loads have caused the hypereutrophication of the reservoir. This dystrophy led to the formation of *M. aeruginosa* blooms for several years [2].

Sampling the M. aeruginosa Colonies in the Sediment and in Water Column. Samples of sediment containing benthic colonies of cyanobacterium *M. aeruginosa* were collected using a core sampler in winter (from January to March 2001) at two stations characterized by different depths: 40 m depth for the Cam station and 13 m depth for the Gran station. Ten-mL aliquots of each sample of sediment were diluted with water (1/10) and filtered through a 50-μm filter. *Microcystis* colonies were roughly separated from splinters under a binocular microscope, rinsed in water, and then concentrated by centrifuging in an Eppendorf tube and stored at −25°C. Each tube contained at least 100 *Microcystis* colonies.

Cyanobacteria in the water column were sampled at the same two stations at the end of summer and early autumn (in August and at the beginning of October 2001) using an electric pump filtering 100 L of water through a 50-μm filter and concentrated in a 1-L bottle. In the laboratory, 100-mL aliquots of each sample were filtered once again through a 50-μm filter. Like sediment samples, *Microcystis* colonies were rinsed several times in water and then concentrated in an Eppendorf tube (>100 colonies in each tube) and stored at −25°C.

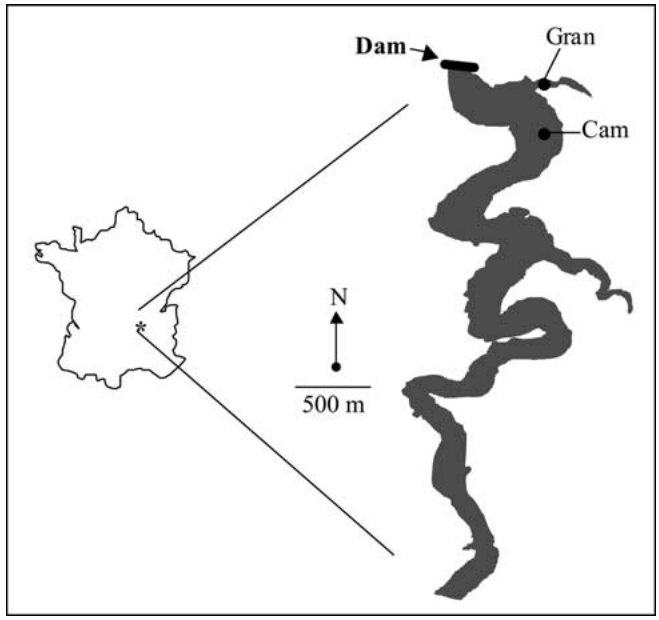

Figure 1. Location of the Grangent storage reservoir in France. Gran and Cam are the two sampling stations used in this study.

Molecular Methods. For each tube, DNA was extracted using the same protocol as described in Humbert and Le Berre [16]. Briefly, after centrifugation colonies were incubated in 750 μL cell lysis buffer [0.1 M Tris-HCl, 0.1 M NaCl, 50 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate] at 37°C for 1 h. Five mg proteinase K/mL was then added and the tubes were placed in a water bath at 40°C and left overnight. After a phenol–chloroform extraction and an ethanol precipitation, DNA was stored at −40°C until utilization. ITS1 of the rRNA operon was amplified in each DNA extract. The 25-μL PCR mixtures contained 60 ng of template DNA, a 120-μM concentration of each of the four dNTPs, 10× PCR reaction buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl; pH 9), 1 μM of each primer (forward primer: 5′-TGT AAA ACG ACG GCC AGT CCA TGG AAG YTG GTC AYG-3′; reverse primer: 5′-CCT CTG TGT GCC TAG GTA TCC-3′), and 1.5 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech). The DNA templates and a negative control were subjected to an initial denaturing step at 94°C for 1 min. The following 37 cycles consisted of a 50-s denaturing step at 92°C, a 50-s annealing step at 57°C and a 50-s extension

Table 1. Nucleotide diversity (estimated by sequence pairwise comparisons) in the various *M. aeruginosa* samples isolated from the sediment or from the water column at two sampling stations (Camaldule and Grangent) in the Grangent storage reservoir

	Number of sequences	*Number of haplotypes*	*Nucleotide diversity ± S.D.*[a]
Cam, sediment (31 Jan)	6	6	0.029 ± 0.018
Cam, sediment (13 Feb)	9	5	0.020 ± 0.011
Cam, sediment (27 Mar)	9	8	0.017 ± 0.010
Cam, water column (7 Aug)	6	4	0.013 ± 0.008
Cam, water column (21 Aug)	14	13	0.021 ± 0.011
Gran, water column (21 Aug)	9	5	0.027 ± 0.015
Gran, water column (1 Oct)	7	6	0.024 ± 0.015
Gran, sediment (31 Jan)	6	4	0.022 ± 0.014

[a]S.D.: Standard deviation.

step at 72°C. A final 10-min extension step was carried out at 72°C. Amplifications of the target region were checked by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. In every sample, a single band was observed each time by electrophoresis, which agrees with the findings of Janse et al. [18], who showed using a DGGE approach, that there was only one operon (or several identical operons) in the genus *Microcystis*. Positive PCR products were cloned in pGEM-T vector (Promega) according to the manufacturers' recommandations (1:1 molar ratio of the PCR products to the vectors).

At least 10 white colonies were randomly picked from each clone library and the ITS1 sequences were PCR amplified using commercial T7 (5′-TAC GAC TCA CTA TAG GGC GA-3′) and SP6 (5′-TAG GTG ACA CTA TAG AAT AC-3′) primers. Amplifications of the target region were checked by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Positive PCR products were purified before sequencing, using the Qiaquick PCR purification Kit according to protocol of the manufacturer. Sequencing was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and the Applied Biosystems 373 automated DNA sequencer (PerkinElmer) according to the manufacturers' instructions. The removal of unincorpored dye terminators from sequencing reactions was done using the DyeEx kit (Qiagen). The sequences were determined independently in both strands. We selected for further analyses only the sequences sharing >95% identity with the *Microcystis aeruginosa*, *M. wesenbergii*, *M. ichthyoblabe*, *M. novacekii*, and *M. viridis* sequences from GenBank. As recently suggested by DNA–DNA hybridization experiments [31], these species are in fact all conspecific.

The nucleotide sequences have been deposited in the GenBank-EMBL database under the accession numbers AY431034 to AY431099.

Data Analysis. The sequences were aligned using the Pileup module of the GCG package (Genetics Computer Group, Inc., Madison, Wisconsin) and edited manually on GeneDoc [29]. Three phylogenetic trees was constructed by the neighbor-joining (NJ) method on Jukes–Cantor pairwise distances, by maximum parsimony and by maximum-likelihood analyses using the PHYLIP Software Package [11]. The bootstrap option was used to run 500 replicates, and the trees were drawn using TreeView [32].

Estimations of nucleotide diversity, analyses of molecular variance (AMOVA), and pairwise F_{ST} calculations were performed using ARLEQUIN software v2.000 [35]. AMOVA is a method for studying molecular variation within a species. Like a hierarchical analysis of variance, it allows separation and testing of tiers of genetic diversity [8].

Results

Sequence Analysis. The length of the entire ITS1 sequences ranged from 356 to 365 bp. The conserved domains (D1, D1', D2, D3, D4, D5 and box A) described by Iteman et al. [17] and one tRNA gene, $tRNA^{Ile}$, were found in all 66 sequences. A total of 37 haplotypes were identified among the 66 sequences, but the nucleotide diversity was low in all the samples studied (Table 1). No polymorphism was found in the D1, D1', D3, D4, and D5 domains and only a very small number of point mutations in the D2 domain (2 mutations in one sequence), in box A (one mutation in two sequences). Concerning the $tRNA^{Ile}$ gene, two sequences differed from the other 64 by the same point mutation (position 46 in this gene). In nonconserved domains, most of the polymorphism was due to point mutations and to small indels (fewer than five nucleotides). The alignment of all sequences revealed 56 variable positions (14.4%) including indels, and 45 variable positions (12.4%) excluding indels.

Comparison of the Genetic Diversity at the Different Sampling Points. There was no significant difference in the nucleotide diversity of *Microcystis* colonies isolated from the sediment at depths of 13 or 40 m or from the water column sampled either from Cam or Gran stations (Table 1). Similarly, the distribution of the 66 sequences in the phylogenetic tree (Fig. 2) did not reveal any obvi-

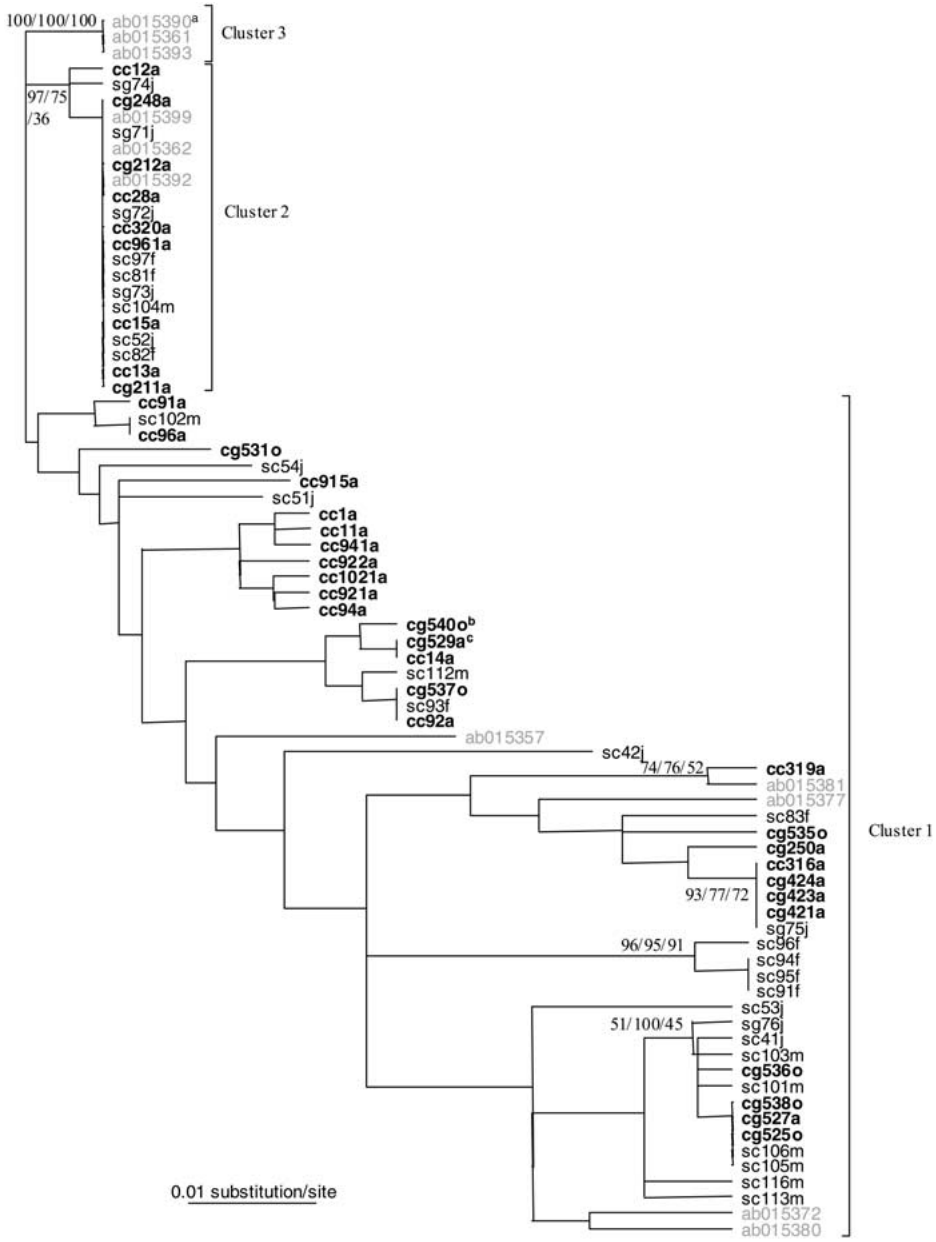

Figure 2. Phylogenetic relationships between *Microcystis* 16S-23S rRNA sequences. This maximum-likelihood tree was based on *Microcystis* sequences from the Grangent storage reservoir and from clones isolated by Otsuka et al. [30] in Asia. Neighbor-joining, maximum parsimony, and maximum-likelihood bootstrap values >70% are respectively given at the nodes. [a]In gray, accession number in GenBank of the sequences from Otsuka et al. [30]. [b]sc42j: sequence from *Microcystis* colonies collected in sediment (versus water column), cam sampling station (versus gran sampling station), 42 clone number, sampling in January (versus sampling in February, March, August, or October). [c]In bold type: sequences from colonies collected in water column.

ous segregation between samples isolated from the sediment and samples isolated from the water column or, between samples isolated from the Cam or the Gran sampling stations. For example, in cluster 2, there were 7 sequences from the Gran sampling stations and 11 from Cam, and the same number of sequences from clones isolated from the sediment and the water column (Fig. 2).

In addition to this first graphical analysis, the genetic structure of the *Microcystis* population was assessed using analysis of molecular variance (AMOVA), which makes it possible to test a particular genetic structure in defined groups of populations. In a first analysis, two groups of populations were defined based on whether the colonies had been isolated from the water column or from the sediment without taking the sampling station into account; in a second analysis, the two groups of populations were defined with regard to the sampling site, Cam or Gran, without taking into account whether they had been isolated from the sediment or the water column. For these two hierarchical levels of analysis, <0.3% of the variance was due to the group structure (sediment versus water column and Cam versus Gran), and the two fixation indices were <0.003 (not significantly different from 0).

Discussion

All these findings showed that in Lake Grangent, there are no genetically differentiated populations of *Micro-*

cystis in the sediment and in water column, or at the Cam and Gran sampling stations. This means that hydrodynamic processes homogenize the phytoplanktonic community in this lake, and that there is no reduction in the genetic diversity during the summer proliferation of *M. aeruginosa* in the water column. Concerning this last point, there is very little information available on the genetic diversity of cyanobacterial species during a bloom event. Kondo et al. [19] found a low level of variation in 16S sequences obtained from a *Microcystis* bloom sample, but the choice of the 16S gene as the PCR target does not make it possible to obtain a good assessment of the intraspecific genetic diversity because of the high conservation of this sequence. On the other hand, using the phycocyanin intergenic spacer and flanking regions allowed Bittencourt-Oliviera et al. [3] to show that different genotypes were found in a single water body in Brazil. In the same way, Kurmayer et al. [21, 22] and Fastner et al. [10] showed in Lake Wannsee in Germany that there was genetic (in microcystin-producing genotypes) and phenotypic (in bioactive oligopeptides) diversity in the *Microcystis* genus during bloom events. In the two dominant cyanobacterial species in the Baltic Sea phytoplanktonic community in summer (*Aphanizomenon* and *Nodularia spumigena*), Barker et al. [1] and Hayes and Barker [15], by studying the phycocyanin intergenic spacer (PC-IGS) and partial flanking coding regions of *cpcB* and *cpcA*, found that there was a low level of genetic variability in *Aphanizomenon* in contrast to *Nodularia spumigena*. Concerning *Aphanizomenon* species, Laamanen et al. [23] used ITS1 sequencing and found very low genetic diversity in the Baltic Sea but high diversity in the lakes flowing into this sea, which suggests that natural selection reduces the genetic diversity in the Baltic Sea population of *Aphanizomenon*.

The lack of genetic differentiation between benthic and pelagic *Microcystis* populations suggests that there is no genetically differentiated ecotype (pelagic versus benthic) in the *Microcystis* genus of the Lake Grangent corresponding to different functional populations. In contrast, it has, for example, been demonstrated using the same genetic marker (16S-23S rRNA) that there are several depth-related ecotypes in *Synechococcus* populations in a 68°C hot spring mat community [12]. Thus, in Lake Grangent, *Microcystis* is able to occupy very different environmental conditions, ranging from the sediment to the water column, and to maintain a reduced enzymatic activity at a depth of 40 m [24]. At this depth, there is never any light, and the water temperatures range from 4 to 8°C, depending on the season. Benthic overwintering of *Microcystis* colonies allows this species to survive during unfavorable environmental conditions without any obvious loss of genetic variability due to natural selection.

In order to compare the genetic diversity of *Microcystis* populations in Lake Grangent with those obtained from other lakes separated by geographic distance as well as by physical and chemical parameters, 11 of the 47 sequences obtained by Otsuka et al. [30] from *Microcystis* strains isolated in Asian lakes were added to the analysis. These 11 sequences were randomly selected in each of the three clusters defined by Otsuka et al. [30]. Three main clusters can be identified among these 77 sequences (Fig. 2). Two of them (clusters 2 and 3) were highly supported by the bootstrap analyses, in contrast with cluster 1. This cluster, which is defined by default in regard to the two other clusters, contains heterogeneous sequences but also some groups of sequences supported by bootstrap resampling. The same kind of phylogenetic structure was observed by Otsuka et al. [30]. The first two clusters (clusters 1 and 2) contained sequences from both Lake Grangent and the Asian lakes, whereas the third cluster did not contain any sequences from Lake Grangent but only nontoxic strains isolated from three lakes in Japan [30]. This absence of sequences belonging to this third cluster does not seem to be due to a sampling effect because we have many more sequences from Lake Grangent than did Otsuka et al. [30] in their study. It would be very interesting to find out whether this cluster is found in other French lakes or only in Asian regions as suggested by this finding.

In contrast with sequences from cluster 1, ITS1 sequences from cluster 2 were homologous, regardless of their geographical origin (Lake Grangent or Asian lakes). Interestingly, this cluster defined by Otsuka et al. [30] contained only toxic *Microcystis* strains isolated in Japan and in China. Two hypotheses can be suggested to explain this high degree of sequence conservation in cluster 2. The first is that this genotype has only differentiated very recently, and that this was followed by its rapid dispersal throughout the world. The second hypothesis is that it is a natural selection that has maintained a high degree of homology in these ITS1 sequences. It is not yet possible to choose between these two hypotheses, but with regard to the second hypothesis, it has previously been demonstrated that there is no clear relationship between rRNA gene phylogeny and microcystin production in the *Microcystis* genus [36] or in any other cyanobacterial genus [14] and so other genes (and functions) could be involved. Considering more particularly the microcystin synthetase genes, it was very recently suggested by Rantala et al. [33] that these genes were originally present in all of the genus *Microcystis* and that their current sporadic distribution in this genus is due to repeated losses.

To conclude this article, the fact that some *Microcystis* genotypes seem to be distributed worldwide was also previously reported for bacterioplankton species [40]. In this study, Zwart et al. suggested that this can be

explained by the fact that these organisms, which are distributed worldwide, possess unique functional capabilities enabling them to compete successfully in a wide range of freshwater environments. In the same way, Finlay [13] gives complementary arguments. According to him, the large abundance of individuals in microbial species (in relation to their small body size) allows the explanation of the global dispersal of these species.

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