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A polyphasic approach leading to the revision of the genus *Planktothrix* (Cyanobacteria) and its type species, *P. agardhii*, and proposal for integrating the emended valid botanical taxa, as well as three new species, *Planktothrix paucivesiculata* sp. nov.[ICNP], *Planktothrix tepida* sp. nov.[ICNP], and *Planktothrix serta* sp. nov.[ICNP], as genus and species names with nomenclatural standing under the ICNP

Virginie Gaget[a,b,*], Martin Welker[c,1], Rosmarie Rippka[a], Nicole Tandeau de Marsac[a,2]

[a] *Institut Pasteur, Unité des Cyanobactéries, Centre National de la Recherche Scientifique (CNRS) Unité de Recherche Associée (URA) 2172, 75724 Paris Cedex 15, France*
[b] *Centre d'Analyse Environnementales, Bât. Dufy, 1 place de Turenne, 94417 Saint-Maurice Cedex, France*
[c] *AnagnosTec GmbH, Am Mühlenberg 11, 14476 Potsdam-Golm, Germany*

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ABSTRACT

Twenty strains of *Planktothrix* and five of '*Oscillatoria*' were characterized by a polyphasic approach, for clarification of their taxonomic relationships. Emphasis was given to the strains (17) of the Pasteur Culture Collection of Cyanobacteria (PCC). Phenotypic characters analyzed comprised morphology, phycobiliprotein composition, temperature and salinity tolerance. The *gvpA* gas vesicle gene was detected by PCR in all strains, and transmission electron microscopy confirmed gas vesicle formation in the strains of '*Oscillatoria*'. MALDI-TOF mass spectrometry revealed 13 chemotypes, nine of which produce microcystins. A multi-locus sequence typing (MLST) analysis was conducted using individual and concatenated nucleotide sequences of the 16S rDNA, internal transcribed spacer (ITS), *gyrB*, *rpoC1* and *rpoB*. The results highlighted an unexpected diversity within the genus *Planktothrix*, showing that the five strains of '*Oscillatoria*' need to be included in this taxon. Consequently, the genus consists of seven phylogenetic clusters, three of which represent new species, named *Planktothrix paucivesiculata* sp. nov.[ICNP] (type strain: PCC 8926[T]), *Planktothrix tepida* sp. nov.[ICNP] (type strain: PCC 9214[T]) and *Planktothrix serta* sp. nov.[ICNP] (type strain: PCC 8927[T]). These, together with the emended genus *Planktothrix* and its type species *P. agardhii*, valid taxa under the ICN, are described/re-described for gaining nomenclatural standing under the ICNP.

Introduction

Cyanobacteria have a long evolutionary history (2.45–2.32 billion years) [51], and form a distinct but morphologically heterogeneous group among eubacteria. Their classification has originally been established based on morphological and ecological criteria. Their nomenclature was, and still is, governed by the International Code of Botanical Nomenclature (ICBN) [26], recently renamed the International Code of Nomenclature for algae, fungi, and plants (ICN) [46]. Following the proposal of Stanier et al. [59], these oxygenic photosynthetic prokaryotes have also been added to the list of organisms, whose nomenclature is governed by the provisions of the International Code of Nomenclature of Bacteria (ICNB) [42], now the International Code of Nomenclature of Prokaryotes (ICNP) [17]. Consequently, cyanobacterial taxa started to be named according to either of the Codes, leading to a lot of confusion in the literature. The criteria of identification, however, remained the same, i.e., primarily morphological. Unfortunately, such criteria rarely reflect the evolutionary history of these microorganisms, and some characters are variable depending on growth

* Corresponding author. Current address: University of Adelaide, Ecology and Environmental Sciences, School of Biological Sciences, Benham Building, North Terrace Campus, South Australia 5005, Australia. Tel.: +61 08 8313 5292.
E-mail address: virginie.gaget@adelaide.edu.au (V. Gaget).
[1] Current address: bioMérieux La Balme, R & D Microbiology, 3 route de Port Michaud, La Balme-Les-Grottes 38390, France.
[2] Current address: Aix-Marseille University, Laboratoire de Chimie Bactérienne (LCB), CNRS Unité Mixte de Recherche (UMR) 7283, 13402 Marseille Cedex 20, France.

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conditions, thus their evaluation may be subjective. Furthermore, as for the majority of cyanobacterial taxa, none of the genus and species names cited in this manuscript have nomenclatural standing under the ICNB/ICNP [48]. To avoid the numerous quotation marks by which these taxa would need to be indicated in a study intending effective (or valid) publication of genus/species names under the provisions of ICNB/ICNP, they will be presented throughout the major part of this manuscript according to their validity status under the ICBN/ICN, or as proposed previously [61].

During the last two decades, the number of phylogenetic studies increased steadily with the support of accessible DNA sequence data, facilitating inference of the precise relationships between a few taxa. For cyanobacteria, the most commonly used genetic marker remains the 16S rRNA gene coding for the small ribosomal sub-unit, but several other genes, such as the house-keeping genes *rpoC1*, *rpoB* coding respectively for the gamma and beta sub-units of the RNA polymerase, and *gyrB* coding for the beta sub-unit of the DNA gyrase, have been increasingly used for these prokaryotes [12,57,63]. At present, one of the preferred approaches, along with whole genome sequencing, is the multi-locus sequence typing (MLST), a sequence analysis of multiple genes [24,45,63]. In most cases, house-keeping genes have been proven to be efficient genetic markers for these analyses [24,28,55].

Among cyanobacteria, the order *Oscillatoriales* comprises filamentous, non-heterocystous and non-branching morphotypes. The monophyletic status of the taxon '*Oscillatoriales*' has repeatedly been challenged but, in the absence of an alternative widely accepted new taxonomic system, this higher taxon still remains in use [9,10,37]. Within the genus *Oscillatoria*, several new genera have been created [1], whose justification by phylogenetic inference, however, was only obtained some years later [61,76]. This is the case for the genus *Planktothrix*, which was separated from other *Oscillatoria* species on the basis of ecological considerations and phenotypic characters such as the presence of numerous gas vacuoles distributed throughout the entire cell, easily visible by phase contrast microscopy [1,68]. The genus *Planktothrix* owes its name to the capacity of its members to form planktonic water blooms in freshwater ecosystems [37,56].

In the past decade a few taxonomic studies have been dedicated to this genus. Suda et al. [61] revised the taxonomy of 75 water bloom-forming *Oscillatoriales* strains. On the basis of 16S rRNA gene sequences, seven phylogenetic groups were distinguished. These groups were further divided into sub-groups based on phenotypic characters, chemical properties and DNA-DNA hybridization data. As a result, the authors emended the definitions of the genera *Planktothrix*, *Tychonema* and *Limnothrix*. Moreover, two new species, *P. pseudagardhii* and *P. mougeotii*, and a new genus, *Planktothricoides*, were described. In 2010, Lin et al. [43], have used morphological and molecular criteria to define four clades including four different species of *Planktothrix*. Liu et al. [44] recently described the new species *P. spiroides*, a coiled morphotype proven to be part of the genus *Planktothrix* based on 16S rRNA gene sequence data and fatty acid composition. All these studied strains belonging to the *Planktothrix* genus form a monophyletic group.

The water bloom-forming *Planktothrix* strains of the common species *P. agardhii* and *P. rubescens* are known to produce a large variety of toxic or bioactive secondary peptide metabolites [75]. Among these, anabaenopeptins (syn. oscillamides), aeruginosins (syn. aeruginosides) and microcystins are most frequently found [41,72,75]. Furthermore, many *Planktothrix* strains produce microcystins, cyanobacterial hepatotoxins, which are considered as a risk for public health [13,18]. Given that the microcystin content of *Planktothrix* blooms is generally higher than that of *Microcystis* blooms [13,19], the detection and identification of these toxigenic cyanobacteria is of major importance for risk assessment and management.

The PCC collection (Pasteur Culture Collection of Cyanobacteria) contains 13 axenic *Planktothrix* strains, including two new strains isolated in the course of the present study. All of them share certain morphological features with some of the *Oscillatoria* strains from the collection. A recent study has shown that these *Oscillatoria* strains might belong to the genus *Planktothrix* [21]. The present work aimed to characterize this group of strains and clarify their taxonomic position by applying a polyphasic approach. Towards this goal, phenotypic characters were examined such as trichome width, growth under different temperatures and salinity conditions, and the production of secondary metabolites. Along with these characters, genetic information obtained by sequencing five molecular markers allowed the examination of potential correlations between pheno- and genotypes leading to the definition of three new species within the genus *Planktothrix*.

Materials and methods

Strains and culture conditions

Twenty *Planktothrix* and five *Oscillatoria* strains (Table 1) have been examined in the present study: 18 from the Pasteur Culture Collection of Cyanobacteria (PCC), four from the Microbial Culture Collection (MCC) at the National Institute for Environmental Studies (NIES), and three from the Culture Collection of Algae and Protozoa (CCAP). All are axenic, except for the CCAP strains and strain NIES-1266, which are clonal but contain bacterial contaminants. Ten of the PCC strains (Table 1) are more recent accessions. With one exception (strain PCC 9329), these were isolated and purified by successive plating of isolated filaments as described previously [53]. Four of them (strains PCC 9625, PCC 9631, PCC 9637 and PCC 9801) were isolated in collaboration with L. Via-Ordorika, whose contribution is gratefully acknowledged. Strains PCC 9702, PCC 10106 and PCC 10110, from samples in Sweden and the Marne river (France), and strains PCC 10606 and PCC 10607, from blooms in two French freshwater reservoirs (Table 1), were isolated by authors of the present communication (R. Rippka and V. Gaget, respectively). Strain PCC 9239 has kindly been provided as an axenic culture by K. Sivonen (Department of Applied Chemistry and Microbiology, University of Helsinki) under the designation *Oscillatoria* sp., strain 18 (green). The origin of all other PCC strains has been described earlier [52] (Table 1). The cultures were maintained in liquid media as described previously [21,52].

Temperature and salinity maxima for growth

Growth responses to different temperatures were examined in 150 ml Erlenmeyer flasks containing 40 ml of liquid medium inoculated with 10% (vol/vol) of culture. Replicates of these cultures were incubated either at 25 °C (control), 30 °C, 35 °C or at 37 °C for 3 weeks, under the same light conditions [21]. Salinity experiments were conducted in test tubes (18 mm diameter) in a final volume of 6 ml. Aliquots (1 ml) of stock liquid culture at $OD_{750} = 0.9$ (or equivalent cell material) was transferred to the appropriate liquid medium supplemented with Turks Island Salt solution (Merck Index No. 9954), in order to obtain the following final salinities: 0%, 0.4%, 0.9% and 1.9%. Each condition was tested on two biological duplicates and two technical duplicates. Control cultures with no addition of Turks Island Salt solution were incubated under the same conditions.