Description of *Gloeomargarita lithophora* gen. nov., sp. nov., a thylakoid-bearing, basal-branching cyanobacterium with intracellular carbonates, and proposal for *Gloeomargaritales* ord. nov.

David Moreira,¹,* Rosaluz Tavera,² Karim Benzerara,³ Fériel Skouri-Panet,³ Estelle Couradeau,¹,²† Emmanuelle Gérard,⁴ Céline Loussert Fonta,⁵ Eberto Novelo,² Yvan Zivanovic⁶ and Purificación López-García⁵

Abstract

A unicellular cyanobacterium, strain Alchichica-D10, was isolated from microbialites of the alkaline Lake Alchichica, Mexico. The cells were short rods (3.9±0.6 µm in length and 1.1±0.1 µm in width) forming biofilms of intense emerald green colour. They exhibited red autofluorescence under UV light excitation. UV-visible absorption spectra revealed that they contain chlorophyll a and phycocyanin, and electron microscopy showed the presence of thylakoids. The strain grew within a temperature range of 15–30 °C. Genomic DNA G+C content was 52.2 mol%. The most remarkable feature of this species was its granular cytoplasm, due to the presence of numerous intracellular spherical granules (16–26 per cell) with an average diameter of 270 nm. These granules, easily visible under scanning electron microscopy, were composed of amorphous carbonate containing Ca, Mg, Ba and Sr. A multi-gene phylogeny based on the analysis of 59 conserved protein markers supported robustly that this strain occupies a deep position in the cyanobacterial tree. Based on its phenotypic characters and phylogenetic position, strain Alchichica-D10 is considered to represent a new genus and novel species of cyanobacteria for which the name *Gloeomargarita lithophora* gen. nov., sp. nov. is proposed. The type strain is Alchichica-D10 (Culture Collection of Algae and Protozoa CCAP strain 1437/1; Collections de Cyanobactériennes et Microalgues Vivantes of the Museum National d’Histoire Naturelle in Paris strain PMC 919.15). Furthermore, a new family, *Gloeomargaritaceae*, and a new order, *Gloeomargaritales*, are proposed to accommodate this species under the International Code of Nomenclature for algae, fungi and plants.

Cyanobacteria thrive in a variety of aquatic and terrestrial habitats, where their ability, unique among bacteria, to carry out oxygenic photosynthesis makes them ecologically significant [1]. They are also important from an evolutionary point of view since they were responsible for the early oxygenation of the Earth’s atmosphere [2], and an ancestral cyanobacterium was the endosymbiont that gave rise to the chloroplasts of eukaryotic algae and plants [3]. In addition, cyanobacteria have a rich fossil record. The massive fossil stromatolites dating back to at least 2.7 billion years ago are considered to have been built by microbial communities dominated by cyanobacteria [4], and unequivocal calcified cyanobacterial fossils are common since the base of the early Cambrian, with *Girvanella* as the first undisputed occurrence at 700 million years ago [5]. The capacity of several cyanobacteria to precipitate calcium carbonate may have enhanced their preservation and explain, at least partly, their extensive fossil record. In fact, several species induce calcite carbonate precipitation by their photosynthetic activity [6, 7], which increases locally the concentration of CO$_2^-$ by the disproportionation of HCO$_3^-$ to CO$_2^-$ and CO$_2$, the latter being fixed by the enzyme ribulose-1,5-bisphosphate carboxylase-oxidase (RuBisCO). The export of alkalinity from the intracellular to the extracellular medium, by a mechanism that...
remains poorly known, raises the saturation index for carbonate minerals in the immediate cell environment and thus leads to mineral precipitation if free cations (e.g. Ca\(^{2+}\)) and nucleation sites are present. It has also been proposed that the cell surface, in particular the exopolysaccharidic matrix, may serve as a nucleation site for carbonates [8]. In all cases, the precipitation of carbonates by cyanobacteria has been regarded as an uncontrolled extracellular process.

However, we recently reported the discovery of a cyanobacterial species that contained intracellular carbonate inclusions [9]. It was the first time that this capacity had been found in cyanobacteria. More recently, this capability has also been found in a small number of other cyanobacterial taxa [10, 11]. Intracellular carbonates appear to be generally rare in bacteria since, up to the recent discovery in cyanobacteria, their occurrence had been described only in a single species, the proteobacterium *Achromatium oxaliferum* [12]. The new cyanobacterial strain *Alchichica-D10*, which we provisionally named *Candidatus Gloeomargarita lithophora* [9], was isolated from microbialite samples collected in the alkaline (\(\sim 43 \text{mM } \text{HCO}_3^-\), pH \(\sim 8.9\)) Lake Alchichica (Mexico) in 2007 and maintained alive in laboratory aquaria since then. Here, we describe formally this novel species and its phylogenetic position within the phylum Cyanobacteria.

Lake Alchichica microbialites are mostly composed of hydromagnesite \([\text{Mg}_3\text{(CO}_3)_2\text{(OH)}_2\cdot 4\text{(H}_2\text{O)}]\) and aragonite \((\text{CaCO}_3)\), and the microbial community inhabiting them is largely dominated by very diverse cyanobacteria [13, 14]. After several years of growth in laboratory aquaria, the microbialites collected in 2007 were still inhabited by a large diversity of cyanobacteria similar to that found in the Lake, which suggests that this community is highly resilient [13]. Microbialites and the aquaria walls were covered by extensive biofilms. These biofilms contained different cyanobacterial morphotypes, with a particularly abundant one consisting of small rod-shaped cells with granular cytoplasm, noticeable under optical microscopy (Fig. 1). To enrich this cyanobacterial species, we disrupted a biofilm sample and filtered the detached cells through an isopore filter of 3 µm pore size. We then inoculated a 96-well plate containing BG11 medium (http://www.cyanosite.bio.purdue.edu/media/table/BG11.html) with the filtered cells. After 1 month of incubation at 21 °C applying a diel cycle, we observed growth of the targeted morphotype in 6 wells. Sequencing of the 16S rRNA gene from these six cultures yielded identical sequences ([9]; accession number JQ733894). We further purified this cyanobacterium by growth on BG11-agar plates and single colony isolation. This allowed us to obtain cultures with this single cyanobacterial species. However, sequencing of 16S rRNA genes amplified with universal bacterial primers revealed the presence of a contaminant alphaproteobacterium closely related to several species of the genus *Sandarakinorhabdus* (with 97% 16S rRNA gene sequence similarity). We have been unable to eliminate this contaminant from our cultures, partly due to the very slow growth rate of the cyanobacterium. Interestingly, some of these species of the genus *Sandarakinorhabdus* have also been reported in association with other cyanobacteria, such as the strain *Sandarakinorhabdus* sp. A14 that is found in cultures of *Microcystis aeruginosa* [15]. Nevertheless, observations using epifluorescent optical microscopy and scanning electron microscopy showed that the cultures were largely dominated by the cyanobacterium and that the contaminant appeared to be rare. Thus, even if non-axenic, the cultures were suitable for the description of the novel cyanobacterial species using a variety of techniques.

Cyanobacterial cells belonging to the novel *Alchichica-D10* strain grown in BG11 medium measured 3.9±0.6 μm in length and 1.1±0.1 μm in width. The most conspicuous feature of those cells observed under scanning electron microscopy (using secondary electron mode) was the presence of numerous bright intracellular spherical granules (3–19 per cell) measuring between 60 and 380 nm in diameter (Fig. 2a). As previously determined by Benzerara et al. [10] using energy-dispersive x-ray spectrometry, these inclusions were composed of calcium carbonate. In addition, cells grown in BG11 contained a relatively small number of larger and darker inclusions rich in P that corresponded to polyphosphate granules (Fig. 2b). When grown in the aquarium water, highly alkaline and rich in Ca, Mg and other cations but poor in P, the cells contained more carbonate granules (16 to 26 per cell) with an average diameter of 270 nm. In contrast with cells grown in BG11, the chemical composition of these inclusions contained Mg, Ba and Sr as major elements in addition to Ca, and polyphosphate granules were rare (Fig. S1, available in the online Supplementary Material). Interestingly, Ba/Ca and Sr/Ca atomic ratios in the inclusions were 1370 and 86 times higher, respectively.

![Fig. 1.](image-url)
than those measured in the aquaria solution [9], although Ca, Sr and Ba are usually supposed to be incorporated relatively conservatively by carbonates. This suggested that the cells controlled the chemical composition of the inclusions.

The ultrastructure of the cells was studied by transmission electron microscopy. In addition to the typical Gram-negative double cell membrane, an important structural feature was the presence of thylakoids, clearly visible as several concentric layers parallel to the cell periphery (Fig. 3). The occurrence of thylakoids clearly differentiates Gloeomargarita lithophora gen. nov., sp. nov. from the deep-branching genus Gloeobacter, which lacks these endomembrane structures [16]. Intracellular structures were observed in the same cells with a contrast different from that of carbonate or polyphosphate inclusions (see arrows in Fig. 3) but with morphological and contrast similarities with carboxysomes found in other cyanobacteria (e.g. [17]).

Cells observed by confocal laser scanning microscopy under UV light (405 nm) excitation showed intense red autofluorescence (Fig. S2). The absorption spectrum of pigments extracted with 90 % acetone showed peaks at wavelengths of 620 and 664 nm, indicating the presence of phycocyanin and chlorophyll a (Fig. S3), which are typical pigments of cyanobacteria.

Colonies of strain Alchichica-D10 grew very slowly on agar plates. They exhibited an intense emerald colour and were surrounded by a thick mucilaginous cover (Fig. S4a). Individual cells appeared to be able to glide on the plate surface to initiate the growth of new peripheral colonies (Fig. S4b). This phenomenon can lead to the formation of migration fronts that provide a stratified structure to the margins of mature colonies (Fig. S4a). To determine the optimal growth conditions in the laboratory, we combined three different buffered pH values (8.0, 8.5 and 9.0), five temperatures (15, 20, 25, 30 and 37 °C), and three light intensities (photon flux of 5, 10 and 41 µmoles m⁻²s⁻¹) in both liquid and solid BG11 media buffered with HEPES. Growth was extremely slow at 15 °C and did not occur at 37 °C. Thus, we focused on the intermediate temperatures. In all cases, growth was slow and took at least 6 weeks to become noticeable by the development of visible colonies on solid medium or by the appearance of green colour in the liquid cultures. In these liquid cultures, the highest cell densities were observed at pH 8.0 and 8.5 at 25 and 30 °C, whereas in solid medium the optimal conditions appeared to be at a pH of 8.5 with low light intensity (photon flux of 5–10 µmoles m⁻²s⁻¹) and a temperature of 25–30 °C (Fig. S5).

Preliminary phylogenetic analyses based on 16S rRNA gene sequences suggested the proximity of strain Alchichica-D10 to the basal order Gloeobacterales [9]. However, this relationship was not strongly supported and was based on unrooted phylogenetic trees. In fact, a 16S rRNA gene rooted tree published later showed that Gloeomargarita lithophora gen. nov., sp. nov. did not branch as a sister of the species of the genus Gloeobacter but as the second branch to diverge within the phylum Cyanobacteria after the genus Gloeobacter, although still with weak statistical support [18]. To resolve this uncertainty, we carried out a multi-gene phylogenetic analysis. For this purpose, we extracted genomic DNA from Gloeomargarita lithophora gen. nov., sp. nov., which was sequenced using the Illumina Genome Analyzer II technology, which yielded 2.1 Gbp of DNA sequences (with a G+C content of 52.2 mol %). Among these sequences, we fetched 59 conserved genes involved in transcription and translation (Table S1). Their translated protein sequences were aligned with the respective homologous sequences found in all completely sequenced cyanobacterial genomes and those of several other bacteria included as outgroups. Alignments were trimmed to eliminate ambiguously aligned regions and concatenated to build a 7220

![Fig. 2](https://example.com/image2.png)

*Fig. 2.* Electron microscopy analyses of Alchichica-D10 cells grown in BG11. (a) Scanning-transmission electron microscopy image in high angle annular dark field (STEM-HAADF) mode: calcium carbonates appear as brighter round-shaped inclusions, while polyphosphate granules are darker, sometimes bigger globules. (b) Scanning-transmission electron microscopy energy dispersive X-rays spectrometry (STEM-EDX) map of the same area: calcium is in red, phosphorus in green and carbon in blue; as a result, calcium carbonates appear in red and polyphosphate granules in green. Bars, 1 µm.

![Fig. 3](https://example.com/image3.png)

*Fig. 3.* Bright-field transmission electron microscopy image of a thin section of Alchichica-D10 cells embedded in EPON resin. Several concentric thylakoid membranes are visible under the cell membrane. Arrowheads indicate structures that may correspond to carboxysomes. Bar, 1 µm.
Fig. 4. Bayesian phylogenetic tree based on the analysis of a concatenation of 59 conserved proteins (7220 amino acids) reconstructed using PhyloBayes MPI [24] with the CAT GTR model. Numbers at branches are posterior probabilities (only those >0.50 are shown). For space constraints, the outgroup and the Synechococcus/Prochlorococcus group have been replaced by triangles (for the complete tree see Fig. 5b). Bar, number of substitutions per position.
amo acids concatenation, which was analysed using Bayesian inference to reconstruct a phylogenetic tree. The resulting tree was highly supported and placed Gloeomargarita lithophora gen. nov., sp. nov. in an early-diverging position, as the third most basal cyanobacterial branch, just after the two available species of the genus Gloeobacter and a group containing the strain Synechococcus sp. PCC 7336 and the two thermophilic strains Synechococcus sp. JA-2-3B’s (2–13) and JA-3-3Ab isolated from Yellowstone (Figs 4 and S6). Therefore, the genera Gloeobacter and Gloeomargarita are not sister groups, contradicting our previous single-gene-based suggestion that strain Alchichica-D10 might be a divergent species belonging to the order Gloeobacterales. Indeed, the presence of thylakoids in strain Alchichica-D10 (see above) constituted a major difference with the thylakoid-lacking genus Gloeobacter [16], in agreement with their placement in independent branches in the multi-gene phylogenetic tree.

Although Gloeomargarita lithophora gen. nov., sp. nov. is the only species available in culture for this new genus, a large diversity of related environmental 16S rRNA gene sequences has been detected, indicating that it belongs to a diverse clade found in various environments, in particular freshwater microbialites and microbial mats [19]. Interestingly, several sequences have been retrieved from microbial mats thriving in continental hot springs from various locations, such as Yellowstone, central Tibet and Algeria [20–22]. Moreover, cells with morphological characteristics similar to those of Gloeomargarita lithophora gen. nov., sp. nov., including the presence of numerous carbonate inclusions in the cytoplasm, were observed by electron microscopy in the Algerian hot spring samples [19]. These results indicate that the different lineages related to the genus Gloeomargarita have adapted to a wide range of temperatures.

As Gloeomargarita lithophora gen. nov., sp. nov. does not belong to the order Gloeobacterales, the erection of a new genus, family and order to accommodate this novel cyanobacterial species is required (see below). As far as we know, the genus name Gloeomargarita has never been used in botanical literature, so it can be validly published as a new cyanobacterial genus under the International Code of Nomenclature for algae, fungi and plants [23].

DESCRIPTION OF GLOEOMARGARITA LITHOPHORA SP. NOV.

Gloeomargarita lithophora (li.tho’pho.ra. Gr. masc. n. lithos stone; Gr. masc. n. phoros carrier; N.L. fem. n. lithophora carrier of stones).

Exhibits the following properties in addition to those given in the genus description. Cells are 1.1 µm wide and 3.9 µm long on average. Growth occurs at 15–30 °C (optimum 25 °C) in alkaline freshwater and BG11 medium. The G+C content of the genomic DNA of the type strain is 52.2 mol%.

The type strain, Alchichica-D10 (=CCAP 1437/1=PMC 919.15), was isolated from microbialites of the alkaline Lake Alchichica (Mexico) preserved in laboratory aquaria at Orsay (France). The 16S rRNA gene sequence of the type strain is available in GenBank under accession number JQ733894.

The holotype of Gloeomargarita lithophora is the specimen PueAl-43a in the FCME Herbarium of the Faculty of Sciences at the UNAM.

Type locality: Alchichica Lake (Mexico).

Living cultures CCAP 1437/1 and PMC 919.15 are ex-holotypes.

DESCRIPTION OF GLOEOMARGARITACEAE FAM. NOV.

Gloeomargaritaceae (Gloe.o.mar.ga.ri.ta.ceæ. N.L. fem. n. Gloeomargaritaceae type genus of the family; suff. –aceæ ending to denote a family; N.L. fem. pl. n. Gloeomargaritaceae the family of the genus Gloeomargarita).

The description is the same as for the genus Gloeomargarita.

Type genus is Gloeomargarita gen. nov.

DESCRIPTION OF GLOEOMARGARITALES ORD. NOV.

Gloeomargaritales (Gloe.o’mar.ga.ri.ta’les. N.L. fem. n. Gloeomargaritales type genus of the order; suff. -ales ending denoting an order; N.L. fem. pl. n. Gloeomargaritales the order of the genus Gloeomargarita).

The description is the same as for the genus Gloeomargarita.

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Conflicts of interest
The authors declare that there are no conflicts of interest.
References


