**Chalicogloea cavernicola** gen. nov., sp. nov. (Chroococcales, Cyanobacteria), from low-light aerophytic environments: combined molecular, phenotypic and ecological criteria

M. Roldán,1,2 M. Ramírez,2 J. del Campo,3 M. Hernández-Marine⁠é2 and J. Komárek⁠4

**Correspondence**
M. Roldán
monica.roldan@uab.es

1Servei de Microscòpia, Universitat Autònoma de Barcelona, Edifici C, Facultat de Ciències, 08193 Bellaterra, Spain
2Dep. Productes Naturals, Biologia Vegetal i Edafologia, Unitat de Botànica, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain
3Institut de Biologia Evolutiva, CSIC-UPF, Passeig Marítim de la Barceloneta, 37–49, 08003 Barcelona, Spain
4Institute of Botany, Academy of Sciences of the Czech Republic, Dukelská 135, CZ-37982 Tréboň, Czech Republic

This work characterizes a unicellular cyanobacterium with nearly spherical cells and thin-outlined sheaths that divide irregularly, forming small packets immersed in a diffluent mucilaginous layer. It was isolated growing on calcite speleothems and walls in a show cave in Collbató (Barcelona, Spain). Spectral confocal laser and transmission electron microscopy were used to describe the morphology, fine structure and thylakoid arrangement. The pigments identified were phycoerythrin, phycocyanin, allophycocyanin and chlorophyll a. Three-dimensional reconstructions, generated from natural fluorescence z-stacks, revealed a large surface area of nearly flat, arm-like thylakoidal membranes connected to each other and forming a unified structure in a way that, to our knowledge, has never been described before. Phylogenetic analyses using the 16S rRNA gene sequence showed 95% similarity to strain *Chroococcus* sp. JJCM (GenBank accession no. AM710384). The diacritical phenotypic features do not correspond to any species currently described, and the genetic traits support the strain being classified as the first member of an independent genus in the order Chroococcales and the family Chroococcaceae. Hence, we propose the name *Chalicogloea cavernicola* gen. nov., sp. nov. under the provisions of the International Code of Nomenclature for Algae, Fungi and Plants. The type strain of *Chalicogloea cavernicola* is COLL 3T (≡CCALA 975T ≡CCAP 1424/1T).

**Abbreviations:** CLSM, confocal laser scanning microscopy; ML, maximum-likelihood; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain COLL 3T is JQ967037.

A supplementary figure is available with the online version of this paper.

Cyanobacteria are a dominant component of diversely structured photosynthetic biofilms living in low-light environments. Examples of these communities can be found in natural caves and artificially illuminated show caves and catacombs, in which they are affected by the physical, chemical and biological conditions (Hernández-Marine⁠é et al., 2001; Lamprinou et al., 2009; Roldán et al., 2004a; Roldán & Hernández-Marine⁠é, 2009; Urzí et al., 2010). These parameters are interdependent, and determine which organisms can thrive (Decho et al., 2010). Photosynthetic communities thrive mainly on the surfaces, which appear blue, greenish or grey and cover substrates irregularly. Since the famous study of green sickness (Lefèvre et al., 1964), which affected the prehistoric murals in the caves of Lascaux, France, the presence of photosynthetic micro-organisms on the walls and paintings of caves and monuments has been widely reported (Roldán & Hernández-Marine⁠é, 2009). Uncontrolled growth of these organisms can cause biodeterioration of and/or aesthetic damage to surfaces. Determining the roles they play requires knowledge of individual isolates, and the information gained can be used for protecting cultural heritage.

Molecular methods can be used to classify cyanobacteria taxonomically, especially for groups that have very few
morphologically differentiating characters (Wilmutte et al., 1992; García-Pichel et al., 1998; Komárek & Anagnostidis, 1998; Nybel et al., 2000; Abed et al., 2002; Berrendero et al., 2008; Oren, 2011). In addition, genetically identified items need to correlate with phenotypic and ecological data (Hoffmann et al., 2005; Johansen & Casamatta, 2005; Komárek & Kaštovský, 2003; Komárek et al., 2004; Komárek, 2010b; Komárková et al., 2010). The specific phenotypic traits used depend on the organism and the tools employed to characterize it. These traits can be used to differentiate among genera or morphospecies.

Features that are in agreement with phylogenetic relationships in unicellular cyanobacteria include the type of cell division and the thylakoid arrangement (Komárek & Anagnostidis, 1998; Komárek & Kaštovský, 2003). Photosynthetic pigments may also be used as a taxonomic marker in cyanobacteria (Bryant, 1982; Wilmutte, 1994; Roldán et al., 2004b), although this depends on whether information on the pigment type is available at the genus or species level, and whether there are changes in pigments at different life-cycle stages (Wolf & Schüssler, 2005) or under different environmental conditions (Ramírez et al., 2011).

During an intervention intended to clean speleothems in an illuminated tourist cave, colonies of a unicellular cyanobacterium, COLL 3T, were detected. Here, we report their isolation and taxonomic characterization.

Samples were collected from a speleothem illuminated during cave visits with white fluorescent bulbs. Samples were collected at the deepest part (length 549 m and slope 20 m) of the Salpetre Cave in Collbató (Catalonia, northeast Spain; 41° 34′ 31.72″ N 1° 50′ 10.14″ W). Salpetre first began to be exploited in the 16th century for potassium nitrate and, in 1934, the cave was artificially illuminated and made into a tourist attraction. The cave shows considerable micro-environmental stability throughout the annual cycle (data-logger Testo 177-H1). The mean air temperature was 14.7 °C, with an annual variation of 4.1 °C; the mean stone temperature was 15.5 °C, with an annual variation of 1.2 °C; the mean ambient humidity (94.0 %; range 90.5–96.9 %) was high in the sampling zone; and the mean environmental CO2 concentration was 588.2 p.p.m. (data-logger Testo 400). The photosynthetic photon flux density was <2.5 μmol photon m−2 s−1. Samples were placed immediately in Petri dishes on a 2 mm layer of BG11 medium (Stanier et al., 1971) solidified with agar (1 %; Merck). COLL 3T was isolated from natural biofilms and grown on 1 % agarized BG11 medium at 17 °C under 10 μmol photon m−2 s−1 with a light/dark cycle of 12:12 h.

Materials were examined using an Axioplan microscope (Carl Zeiss) and photographed with an AxioCam MRc5 digital camera system. Cell measurements were taken from photomicrographs.

Confocal images were taken with a Leica TCS-SP5 CLSM (Leica Microsystems) equipped with a 63 × 1.4 (oil HC × PL APO lambda blue) objective. Autofluorescence from photosynthetic pigments was viewed in the red channel (590–800 nm emission) using the 561 nm excitation laser line. Concanaavalin A–Alexa 488 (Molecular Probes) (0.8 mM) targets cell-associated mucilage and was recorded in the green channel (excitation, 488 nm; emission, 495–530 nm). Stacks were subsequently processed with the Imaris version 6.1.0 software (Bitplane AG Zürich) to obtain maximum-intensity projections. The isosurface module of Imaris was used to build 3D models. Lambda stacks (xy/z) were taken in order to determine the emission spectra of the photosynthetic pigments (chlorophyll a, phycoerythrin, phycocyanin, allophycocyanin) (Roldán et al., 2004b). The excitation wavelength used was the 488 nm line of an Ar laser. Emission detection was set from 520 to 780 nm. For each xy focal plane, confocal laser scanning microscopy (CLSM) was used to measure the emission variation every 10 nm (lambda step size 5.2 nm). The emission spectrum analysis was processed using the LAS AF software. To analyse cells, 50 regions of interest of 1 μm2 were delimited to calculate the mean fluorescence intensity in relation to the wavelength.

For transmission electron microscopy (TEM), samples were fixed in glutaraldehyde (2.5 %) and paraformaldehyde (2 %) in 0.1 M cacodylate buffer for 2–4 h, washed in this buffer, post-fixed in osmium tetroxide, dehydrated by a graded acetone series and embedded in Spurr’s resin. Ultrathin sections, cut from the block, were then stained with 2 % uranyl acetate and lead citrate and examined using a JEOL 1010 TEM at 100 kV accelerating voltage.

Genomic DNA for phylogenetic analysis was extracted from 15 ml cyanobacterial culture after centrifugation using 3 % cetyltrimethylammonium bromide (Doyle & Doyle, 1987). Samples were kept at −80 °C. 16S rRNA gene amplification and sequencing analyses were performed as described previously (Ferrera et al., 2004). 16S rRNA genes were amplified using the primers 27f and 1492r. Amplified 16S rRNA gene products were precipitated with ethanol and resuspended in 20 μl sterile water. The PCR product from the COLL 3T culture was sequenced completely using primers 358f, 517r, 907f and 1492r by MACROGEN Genomics Sequencing Services. The resulting sequence was double checked using CHECK_CHIMERA (Maidak et al., 2001) and by BLAST search with different sequence regions. Environmental 16S rRNA gene sequences of cyanobacteria were obtained from GenBank in a two-step screening. First, sequences found by the NCBI Taxonomy application were retrieved and checked by BLAST (Altschul et al., 1997) to confirm their placement. Second, we used these and other published sequences from cultures or environmental surveys that belong to the target groups (but are not labelled as such in GenBank) to retrieve additional sequences by BLAST. Neighbour-joining phylogenetic trees were reconstructed with wide taxon coverage to determine whether or not ambiguous divergent sequences belonged to a given group. Related sequences from cultured organisms were also retrieved from GenBank and pruned to keep only a few representatives for phylogeny.
16S rRNA gene sequences were aligned using MAFFT (Katoh et al., 2002) with a close relative as outgroup. Alignments were checked with Seaview 3.2 (Galtier et al., 1996) and highly variable regions of the alignment were removed using Gblocks (Castresana, 2000). Maximum-likelihood (ML) phylogenetic trees with complete sequences were reconstructed with RAxML (Stamatakis, 2006) using the evolutionary model GTRMIXI. Phylogenetic analyses were carried out in the freely available University of Oslo Bioportal (http://www.bioportal.uio.no). Repeated runs on different starting trees were carried out in order to select the tree with the best topology (the one with the best likelihood of 1000 alternative trees). A bootstrap ML analysis was carried out with 1000 pseudoreplicates and the consensus tree was computed with MrBayes (Huelsenbeck & Ronquist, 2001). Trees were edited with FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Optical observations (Figs 1 and 2) showed that the irregular colonies were immersed in a diffusible mucilaginous layer that grew on calcite speleothems and walls in Collbató, alone or intermixed with coccoid and filamentous cyanobacteria and moss protonemata. Cells were arranged in small, bright bluish-green aggregates, mostly consisting of two to sixteen cells. Envelopes followed the outline of individual cells or the siblings after the division (Fig. 1d). Under CLSM, some cells exhibited green concanavalin A fluorescence in the outer sheath layers (Fig. 2a, c), while others did not (Fig. 2b). Their thickness varied, apparently depending on the cell’s position within the colony, from a diffusible, slightly lamellate slime, up to 14 μm thick, to almost no apparent individual sheath. Cells divided irregularly in different planes and did not return to their original shape before the next division (Figs 1a, b and 2b). The cell shape was more or less spherical, slightly elongated in pre-divisional cells or irregular in outline, but never pear-shaped. The cells displayed different cell dimensions, ranging from 2.6 to 4.3 μm (mean 3.3 ± 0.4 μm; n=54) in diameter. Neither nanocyte formation nor resting stages were observed.

The cellular content of fluorescent pigment was not homogeneous (Fig. 2a–c). Three-dimensional models showed that the thylakoids form a structure consisting of interconnected layers, orientated at different angles, that leave elongated or star-shaped non-fluorescent areas (Fig. 2d). The in vivo pigment spectral profile determined from cultured material showed a small shoulder at 579 nm corresponding to phycoerythrin and an emission peak at 662 nm corresponding to phycobiliproteins and chlorophyll a (Fig. S1, available in IJSEM Online).

TEM (Fig. 3) showed that the sheath near the cell consisted of thin, poorly compacted fibres. The cell wall had a total width of about 33 nm and, as is usual for cyanobacteria, consisted of three layers, in which the electron-dense peptidoglycan layer measured about 6.6 nm. The thylakoids were arranged in irregular arrays that intersected at several angles (Fig. 3b). The thylakoid arrays crossed each other, leaving an irregular central area, depending on the position of the thylakoids and the orientation of the image, where the nucleoid was located. Binary fission occurred via a constrictive pinching mechanism (Fig. 3b). The division

![Fig. 1. (a, b) Confocal 3D images of Chalicogloea cavernicola gen. nov., sp. nov. COLL 3T. General views of colonies, showing the irregular division planes. The cells were solitary or arranged in amorphous colonies composed of a few cells. Neither nanocyte formation nor resting stages were observed. (c, d) Optical photomicrographs of strain COLL 3T. (c) Cells or siblings enveloped by concentric sheaths. (d) Sheaths stained with methylene blue. Bars, 10 μm.](image-url)
process was initiated by a symmetrical invagination of both the cytoplasmic membrane and the peptidoglycan layer, while ingrowing of the outer membrane was delayed (Fig. 3c, d). Sections through cells also showed carboxysomes, numerous glycogen granules between thylakoid membranes and polyphosphate bodies.

Fifty chroococcalean cyanobacterial sequences from the public domain were added to the analysis for reference purposes; the 16S rRNA gene sequence of an unidentified strain of *Pseudomonas aeruginosa* (GenBank accession no. AB364957) was used as an outgroup to root the phylogenetic tree (Fig. 4). In the ML phylogenetic tree, COLL 3 T showed the highest similarity to *Chroococcus* sp. JJCM (95%). The 16S rRNA gene sequence of COLL 3 T was divergent enough from that of any other cyanobacterium available in GenBank to suggest that it represents a novel and differentiated taxon. Here, we propose the name *Chalicogloea cavernicola* (M. Roldán, M. Hernández-Marín & J. Komárek) gen. nov., sp. nov., to be published under the provisions of the International Code of Nomenclature for Algae, Fungi and Plants. The isolate *Chalicogloea cavernicola* COLL 3 T is deposited at the Culture Collection of Autotrophic Organisms as CCALA 975 T and at the Culture Collection of Algae and Protozoa as CCAP 1424/1 T.

**Description of Chalicogloea, gen. nov.**

*Etymologia:* Chalicogloea (Cha.li.co.gloe’a. Gr. n. chalix chalk; Gr. n. gloios glutinous substance; N.L. fem. n. Chalicogloea glutinous substance growing on chalk).

aerophytic and subaerophytic communities on stony and rocky substrates. It is defined by a separate position in the phylogenetic tree, which is also supported by cytological specificities. The recognized phylogenetic clusters of cyanobacteria (mostly on a generic level) are also commonly separated by distinct autapomorphic features, and can also be identified by optical microscopy. The genus *Chalicogloea* differs from phenotypically similar generic taxa by the following morphological markers.

(i) It is separated from all genera that reproduce facultatively or obligatorily by baeocytes due to the total absence of multiple fission. This important marker is also supported by the large distance of these generic units from *Chalicogloea* in the 16S rRNA gene phylogenetic tree. All baeocytic species have a position among more complicated genera. This covers, for example, the genera *Stanieria* (aquatic, mostly marine), *Chroococcidiium* (submersed, never forming compact packets) and especially *Chroococcidiopsis*, known from various extreme habitats.

(ii) Of the non-baeocytic types, *Chalicogloea* can be confused phenotypically with genera that have the following diacritical markers.

**Gloeocapsa.** The cells are in colonies that are more or less distant from one another, each enveloped by their own mucilaginous sheath. They are usually coloured by sheath pigments. The cells divide regularly into three perpendicular planes in subsequent generations and the cells grow in the original, more or less spherical shape and size before the next division (Golubić, 1967). *Gloeocapsa* belongs to a different family than *Chalicogloea*, and these genera are distinctly separated phylogenetically.

**Cyanosarcina.** This genus is mostly similar to *Chalicogloea* in its uncoloured, firm, thin sheaths, but, after the irregular division, the cells remain in polyhedral-rounded form in aggregated, dense packets. The cell morphology is different, and the colonies usually have a more irregular shape. Almost all *Cyanosarcina* species have a different ecology. They occur in aquatic habitats, sometimes in mineral and thermal waters. The only exception is *Cyanosarcina parthenonensis*, described from aerophytic limestone substrates in Greece, but this one species could belong to the genus *Chalicogloea* (cf. Anagnostidis & Pantazidou, 1991).

**Pseudocapsa.** Members of this genus form more or less irregular, packet-like colonies with colourless sheaths, but the cells first divide radially and form characteristic colonies (especially younger stages) with radially arranged cells (e.g. Kováčik, 1988). There are both aquatic and aerophytic types in *Pseudocapsa*, although they are not yet sequenced. However, the form of the cells and their organization is distinctly different from other similar genera.

**Chlorogloeocystis.** This is an aquatic genus that occurs in mineral waters and has colonies impregnated by ferric precipitates (Brown *et al.*, 2005). The spherical cells in colonies are organized more or less in irregular rows, without coloured envelopes. This genus has a known
phylogenetic position that is very distant from that of *Chalicogloea*.

*Gloeocapsopsis*. This genus is similar to *Chalicogloea* in its ecology, the form of its colonies and the special envelopes around single cells; however, the cells are more irregular and the laminated sheaths are usually intensely coloured by sheath pigment (yellow–brown, reddish or violet). The phylogenetic position of *Gloeocapsopsis* is not yet well established, and certain differences are visible within the genus between the marine types and aerophytic populations usually described from wet stony walls. Moreover, *Gloeocapsopsis crepidinum* (Thuret) Geitler ex Komárek, the type species, is halophilic and lives on coastal marine rocks (Silva e Silva et al., 2005, Ramos et al., 2010).

The thylakoid arrangement was rarely considered before the availability of TEM (Lang & Whitton, 1973; Komárek, 2010a), although it is one of the phenotypic characteristics that is consistent with molecular criteria (Komárek & Kaštovský, 2003). A large number of coccoïd cyanobacteria have a concentric-like thylakoid arrangement, although few studies to date have employed advanced bioimaging protocols to evaluate fine spatial arrangements in cellular organization (Mullineaux, 1999; Nevo et al., 2007; Liberton et al., 2011). It is difficult to observe the thylakoid organization by optical microscopy or even with TEM; nevertheless, optical images and TEM figures provide a valid approach to determining the internal structure, which could be studied better using CLSM serial sections to reconstruct entire cells. The 3D reconstructions generated from natural fluorescence z-stacks revealed a large surface area of nearly flat, arm-like thylakoidal membranes connected to each other and forming a unified structure in a way that has, to our knowledge, never been described before. This membrane arrangement in *Chalicogloea* accounted for the changing directions of the thylakoids seen in TEM and can be used as an autapomorphic
character for comparison with organisms with molecular similarities (Komárek & Kaštovský, 2003) or for separation from strains that show parietal thylakoidal arrangements. Moreover, such a tight organization within a small cell volume could be an advantage for living in dim caves.

Although not discussed here, phycoerythrin enables organisms to adapt to different qualities of light and to absorb extra energy in a low-light environment (Albertano & Hernández-Mariné, 2001) and has been identified as part of the present description. The pigments detected can be used to monitor biodeteriogens in dim caves (Roldán et al., 2006).

The closest related sequence to COLL 3T is that of Chroococcus sp. JJCM, a packet-forming organism isolated from plankton from a reservoir, which was originally determined to be Chroococcus cf. minor and later assigned to Eucapsis sp. (Komárková et al., 2010). There is 95 % 16S rRNA gene sequence similarity between COLL 3T and Chroococcus sp. JJCM, which is good evidence of an independent evolutionary history (Johansen & Casamatta, 2005; Stackebrandt & Goebel, 1994). COLL 3T has the same morphology and habitat as a cyanobacterium reported under the name Gloeocapsa NS4 (Cox et al., 1981), which was isolated at Juentica Cave (Asturias, Spain). Although strain COLL 3T and Gloeocapsa NS4 have the same type of cell division, thylakoid arrangement and ecology, there is no molecular support to classify them under the same genus.

Strain COLL 3T was identified here with an integrated approach that brings together morphological characters and molecular phylogeny and which is based on analysing the complete 16S rRNA gene sequence. It should be highlighted that diacritical characters, and the advanced bioimaging protocols used to determine these characters, are very important for identifying the species within the Chroococcales. Overall, the polyphasic approach suggests that strain COLL 3T belongs to a new genus and represents a novel species of this genus. In addition, it should be classified as an independent taxon in the order Chroococcales and family Chroococcaceae.

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