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Diversity of biocrust-forming cyanobacteria in a semiarid gypsiferous site from Central Spain

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ABSTRACT

Cyanobacteria are a key constituent of biocrusts, communities dominated by lichens, mosses and associated microorganisms, which are prevalent in drylands worldwide and that largely determine their functioning. Despite their importance, there are large gaps in our knowledge of the composition and diversity of cyanobacteria associated with biocrusts, particularly in areas such as the Mediterranean Basin. We studied the diversity of these cyanobacteria in a gypsiferous grassland from Central Spain using both morphological identification after cultivation and genetic analyses with the 16 S rRNA gene. Nine different morphotypes were observed, eight corresponding to filamentous, and one to unicellular cyanobacteria. We found cyanobacterial genera typical of biocrust communities, such as *Microcoleus* and *Trichocoleus*, and N-fixing cyanobacteria such as *Scytonema* and *Nostoc*. Genetic information allowed us to identify cultures belonging to recently described genera such as *Roholtiella*, *Nodosilinea* and *Mojavia*. We also describe two new phylotypes of *Microcoleus* and *Scytonema*, which are key genera contributing to ecosystem functioning in biocrust-dominated ecosystems worldwide.

1. Introduction

Cyanobacteria are a key component of biocrusts, soil surface communities also formed by lichens, mosses, liverworts and other microorganisms that are a prevalent biotic feature of drylands worldwide (Büdel et al., 2016). Cyanobacteria are present in virtually all biocrust communities due to their capacity to adapt to a wide range of ecological conditions (Tamaru et al., 2005). Early successional biocrusts are dominated by filamentous pioneer cyanobacteria, which are the first colonizers of bare ground areas in drylands (Garcia-Pichel and Wojciechowski, 2009). These organisms secrete an exopolysaccharide (EPS) matrix that promotes soil stabilization and enhances microhabitat conditions for colonization of other cyanobacteria and the remaining biocrust constituents (Mager and Thomas, 2011). Cyanobacteria with heterocysts (heterocytes) are also important contributors to nitrogen fixation in oligotrophic ecosystems such as drylands (Belnap, 2002). Together with the other components of cryptogamic covers, heterocyst-forming cyanobacteria contribute to the fixation of nearly half of the total amount of biologically fixed nitrogen worldwide (Elbert et al., 2012).

Detailed studies of the composition and biogeography of cyanobacteria have been carried out in North America, Asia, Africa, Europe and Australia (e.g. Garcia-Pichel et al., 2013; Dojani et al., 2014; Kumar and Adhikary, 2015; Williams and Büdel, 2012; Williams et al., 2016). However, to date, few studies have analyzed the cyanobacteria associated to biocrusts in gypsum habitats (Garcia-Pichel et al., 2001; Steven et al., 2013), even though they are hotspots of botanical diversity (Escudero et al., 2014) and harbor very conspicuous biocrust communities dominated by lichens (Castillo-Monroy et al., 2010; Martínez et al., 2006). We studied biocrusts in a gypsiferous semiarid site from Central Spain to advance our knowledge of cyanobacterial communities associated gypsum biocrusts. We used a combination of molecular and morphological information because this increases the number of identified sequences in molecular databases, which is a major concern in the study of cyanobacterial diversity nowadays (Thomazeau et al., 2010; Weber et al., 2016).

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2. Materials and methods

2.1. Field site

This study was carried out in the Aranjuez Experimental Station, located in Central Spain (40°01′55.7″N - 3°32′48.3″W and 590 m above sea level). The climate is semiarid, with an intense summer drought lasting from June to September. Mean annual temperature is 15 °C and annual precipitation is 349 mm. Soils are rich in gypsum, and are classified as *Gypsiric Leptosols* (IUSS Working Group WRB, 2014; see Castillo-Monroy et al., 2010 for a physico-chemical characterization). The vegetation cover is sparse and dominated by herbaceous plants such as *Macrochloa tenacissima* (L.) Kunth. and shrubs such as *Retama sphaerocarpa* (L.) Boiss. and *Helianthemum squamatum* (L.) Dum. Cours. The soil in open areas located between plant patches is covered by a well-developed biocrust community dominated by the squamulous lichens *Diplochistes diacapsis* (Ach.) Lumbsch., *Squamarina lentigera* (Weber) Poelt. and *Psora decipiens* (Hedwig) Hoffm.; with patches of acrocarpous mosses *Pleurochaete squarrosa* (Brid.) Lindb. and *Tortula revolvens* (Schimp.) G. Roth. (see Maestre et al., 2013 for a full list of lichens and mosses found in the site).

2.2. Soil collection and morphological characterization of cyanobacteria

We randomly selected eight 50 × 50 cm plots in areas with a well-developed biocrust community in July 2013. At each plot, we collected five samples (0-1 cm depth), which were pooled and taken to the laboratory. Lichens and mosses were removed, and soil was sieved through a 2 mm sieve and kept dry in the dark.

Cyanobacterial strains were isolated using a modification of the procedure described in Loza et al. (2013). Aliquots of ~1 g of soil were mixed with 1.5 ml of cyanobacterial culture media and distributed uniformly over different solid media (1.5% agar concentration). We used four common culture media for cyanobacteria: BG11, $BG11_0$ (Rippka et al., 1979), modified CHU 10, and modified CHU 10 without addition of N (Gómez et al., 2009). These media allowed the growth of cyanobacteria by providing a range of nutrient richness with and without N, which is important to isolate both N-fixing and non-N-fixing cyanobacteria. To avoid fungal contamination, we added cycloheximide (0.1 mg/ml). Cultures were incubated in a growth chamber at constant light and temperature (20-50 μmol photons m^{-2} s^{-1} and 28 °C) three to four weeks until colonies grew without overlapping. Cyanobacterial colonies were isolated under a dissecting microscope (Leica, Leica Microsystems, Wetzler, Germany) as described in Gómez et al. (2009). Cultures were kept in the same medium and conditions both in agar plates and in liquid medium to further promote their growth.

All colonies were characterized morphologically using a dissecting microscope and an Olympus BH2-RFCA (Olympus, Tokio, Japan) photomicroscope. Identification and morphological characterization of cyanobacteria were conducted considering the following attributes: colony morphology, trichome shape, presence of sheaths, details of cell morphology, number of trichomes per filament and end cell characteristics. Taxonomy was based on Geitler (1932), Anagnostidis and Komárek (1999), Komárek and Anagnostidis (2005) and Komárek (2013).

2.3. Genotypic characterization

DNA was extracted with the Ultraclean Microbial DNA Isolation Kit (Mobio, Carlsbad, CA, USA) following the manufacturer's instructions. A prior step was added at the beginning of the procedure, as samples were homogenized and exposed to three cycles of thermal shock using alternating immersion in liquid N and heating to 60 °C to break the protective EPS that covers the surface of many cyanobacteria (Loza et al., 2013). PCR amplifications were performed using the bacterial 16S rRNA primers 27F and 1494R (Neilan et al., 1997). The PCR mixture (25 μl) contained 2.5 μl Buffer 10X, 1.5 mM $MgCl_2$, 50 μM dNTP, 10 pmol of each primer, BSA 1 mg/ml, 5 μl TaqMasterTM PCR Enhancer 5x (Eppendorf, Germany), 0.75 U Ultratools DNA polymerase (Biotools, Spain) and 10 ng DNA. Amplification took place in a thermocycler PCR Eppendorf Mastercycler (Eppendorf, Viena) with the reaction conditions described by Gkelis et al. (2005). Success in PCR was checked with agarose gel 1.5% using 1 Kb Gene Ruler (MBL Biotools, Spain) and fluorescent DNA stain GelRed™. PCR products were purified with Real Clean Spin Kit (Real, Durviz, Spain) and sequenced at Centro Nacional de Investigaciones Oncológicas (Madrid, Spain). When sequences had low size (< 200 bp) or quality (low confidence on % base assignation in sequence chromatograms), PCR products were cloned into pGEM-T vectors with the pGEM Easy Vector system (Promega, US) according to manufacturer recommendations and sequenced according to vector information and primers. Sequences were obtained for both strands independently.

We compared our results with sequences from the National Center for Biotechnology Information (NCBI) database to complement identifications. For phylogenetic analysis, sequences were aligned using ClustalW (Thompson et al., 1994) with the software Bioedit 7.2.5 (Ibis Biosciences, Carlsbad, CA). We obtained the most similar sequences and reference strains of the closest species from the NCBI database with BLAST (*blast.ncbi.nlm.nih.gov*) and then performed multiple alignment with all sequences (Altschul et al., 1990). Phylogenetic trees were generated with the MEGA7 software (Tamura et al., 2013) using the Maximum Likelihood, Neighbor Joining (Saitou and Nei, 1987), and Maximum Parsimony methods and the Tajima Nei matrix (Tajima and Nei, 1984) to calculate pairwise distances. The alignment was checked and corrected manually with the Bioedit software (Ibis Biosciences, Carlsbad, CA). All sequences obtained had an expected length ranging from 1046 to 1459 bp, except AR11, which had 532 bp. Independent phylogenetic trees made with and without AR11 produced similar clustering, therefore this sequence was included in the phylogenetic analysis. Phylogenetic trees were carried out using our nineteen 16S rRNA sequences together with 53 cyanobacterial sequences from the NCBI database. There were a total of 879 positions in the final dataset. Statistical significance was performed with the bootstrap test described in Felsenstein (1985), using 1000 and 500 replicates for the Neighbor Joining tree and both Maximum Parsimony and Maximum Likelihood trees, respectively. Sequence similarity between our sequences and those from the NCBI database was determined as 100*(1-P-distance), and was carried out using the MEGA7 software (Tamura et al., 2013).

Cultures were named after the site (Aranjuez, AR-) and were included in the culture collection of the Universidad Autónoma de Madrid (UAM). The nucleotide sequences obtained in this study were uploaded to the Genbank (NCBI) database (accession numbers: MF002044 - MF002062).

3. Results

Macroscopic and microscopic evaluation of cultivated cyanobacteria yielded nine different cyanobacterial morphotypes and the successful isolation and sequencing of 12 strains. Three main types of morphologies were found: filamentous and heterocyst-forming cyanobacteria, filamentous cyanobacteria without heterocysts, and unicellular cyanobacteria (Fig. 1; Table 1). The three methods used to obtain the phylogenetic tree (Maximum Likelihood, Neighbor Joining and Maximum Parsimony) produced similar clustering. Therefore, we show only the Maximum Likelihood tree, with the indication of the bootstrap values for all three approaches (Fig. 2).

Cluster I –*Roholtiella.* Sequences of isolated strains AR2 to AR6 were included in this cluster together with sequences of *Roholtiella* Bohunická, Pietrasiak & Johansen., a recently described genus (Bohunická et al., 2015) from the Nostocaceae family. This genus includes some species formerly identified as *Tolypothrix* Kützing ex Bornet