Journal of Microbiological Methods 160 (2019) 1–10

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Review

Methods for extracting 'omes from microbialites

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ARTICLE INFO

Keywords:
Microbialite
Multiomics
Metagenomics
Metatranscriptomics
Viral extraction
Xanthogenate

ABSTRACT

Microbialites are organo-sedimentary structures formed by complex microbial communities that interact with abiotic factors to form carbonate rich fabrics. Extraction of DNA or total RNA from microbialites can be difficult because of the high carbonate mineral concentration and exopolymeric substances. The methods employed until now include substances such as cetyltrimethylammonium bromide, sodium dodecyl sulfate, xanthogenate, lysozyme and proteinase K, as well as mechanical disruption. Additionally, several commercial kits have been used to improve DNA and total RNA extraction. This minireview presents different methods applied for DNA and RNA extraction from microbialites and discusses their advantages and disadvantages. Moreover, extraction of all 'omes (DNA, RNA, Protein, Lipids, polar metabolites) using multiomic extraction methods (MPlex), as well as the state of art for extraction of viruses from microbialites, are also discussed.

1. Introduction

Microbialites, represent ancient micro-ecosystems of high microbial diversity and constitute a rich collection of genes for the study and understanding of bacterial evolution. They have become study models in the field of microbial ecology, since they represent self-sustained microbial communities, where the interactions between the many different components provide the basis for elemental cycling. Studies on microbialite ecology have helped us understand how microbes interact as well as their individual contributions to the cycling of nutrients. In the last 25 years the study of microbialites has been increased (Fig. 1).

Microbialites are defined by Burne and Moore (1987) as organo-sedimentary deposits formed by the metabolic activities of benthic microbial communities. This term describes the lithified product originated from the interactions between microorganisms and their environment (Allwood et al., 2006; Foster et al., 2009), where the actively growing layer of the microbialite is constituted by the microbial mat on the surface. The members of these microbial mats form a resistant and cohesive structure due to the production of extracellular polymeric substances (EPS), which are excreted by bacteria, mainly cyanobacteria (Foster et al., 2009). EPS constitutes a flexible extracellular matrix mainly composed of polysaccharides, lipids, amino acids and eDNA (Das et al., 2013; Klock et al., 2007) in which, the microorganisms are embedded and establish interactions and associations (Stal et al., 2010) (Fig. 2). Fossilized microbialites have been considered the oldest evidence of life on Earth, dated back to 3500 MYA in Australia (Schopf et al., 2002; Van Kranendonk et al., 2008) and 3700 MYA in the Isua supracrustal belt (ISB), southwest Greenland (Nutman et al., 2016).

Microbialites are lithified formations with a rocky appearance produced through precipitation, adhesion, cementation and accretion of sediment, minerals and organic matter by diverse metabolically and phylogenetically microbial communities (Paerl et al., 2001). The process of lithification occurs when the precipitation of minerals exceeds their dissolution (Dupraz and Visscher, 2005), which allows cementation and calcification. The precipitation mediated by microorganisms is not limited to carbonates, it also includes other minerals such as silicates and sulfates. Microbial communities have a fundamental role in the precipitation/dissolution process of carbonates, associated mainly to photosynthesis, sulfate reduction, sulfide oxidation and fermentation carried out by a great diversity of microbes including cyanobacteria, anoxygenic phototrophs, sulfate reducers and heterotrophs (Dupraz et al., 2009). Photosynthesis removes CO_2 from saturated water, with the subsequent elevation of pH and calcium carbonate ($CaCO_3$) precipitation (Burne and Moore, 1987) which favors the accretion of microbialites (Stal, 2000). Therefore, the EPS presence contributes to the accumulation of sediment and minerals that are subsequently cemented by the carbonate accretion (Neilan et al., 2002; Reid et al., 2000).

The production and decomposition of EPS is fundamental for the carbonates precipitation (López-García et al., 2005) and consequently for the formation of microbialites (Breitbart et al., 2009) since EPS are thought to be the main templates for mineral precipitation (Pacton et al., 2014). In microbial mats with high photosynthetic production,

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https://doi.org/10.1016/j.mimet.2019.02.014
Received 23 December 2018; Received in revised form 21 February 2019; Accepted 21 February 2019
Available online 12 March 2019

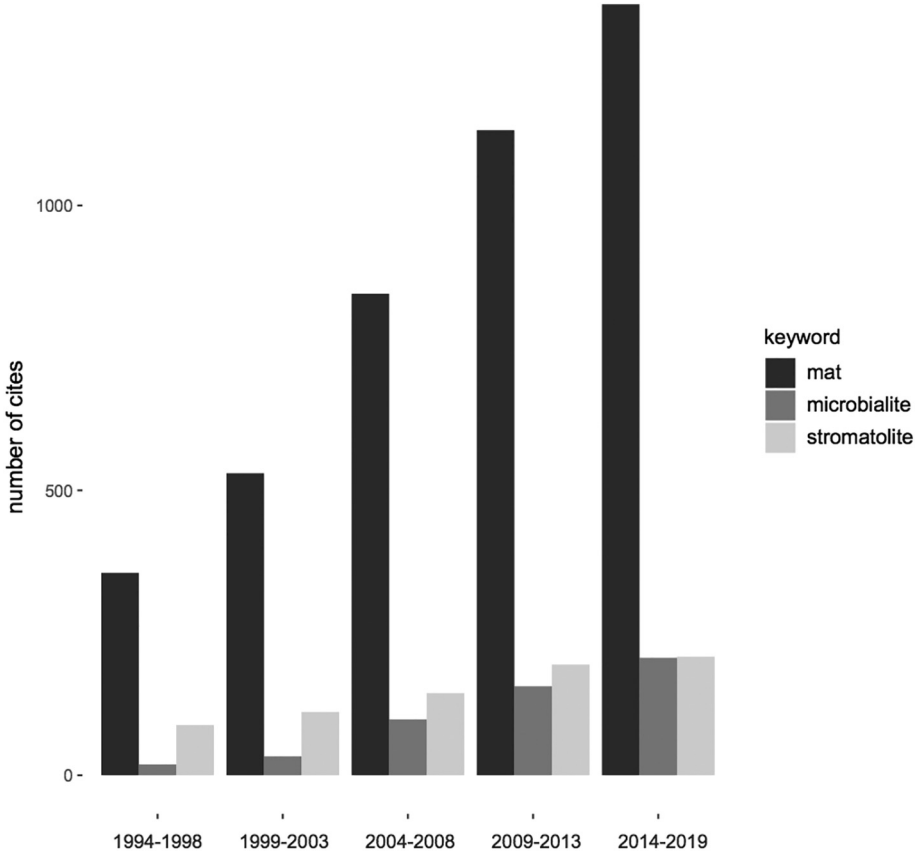

Fig. 1. Frequency of the papers cited in the last 25 years searched in pubmed.com of microbialites, stromatolites an microbial mats.

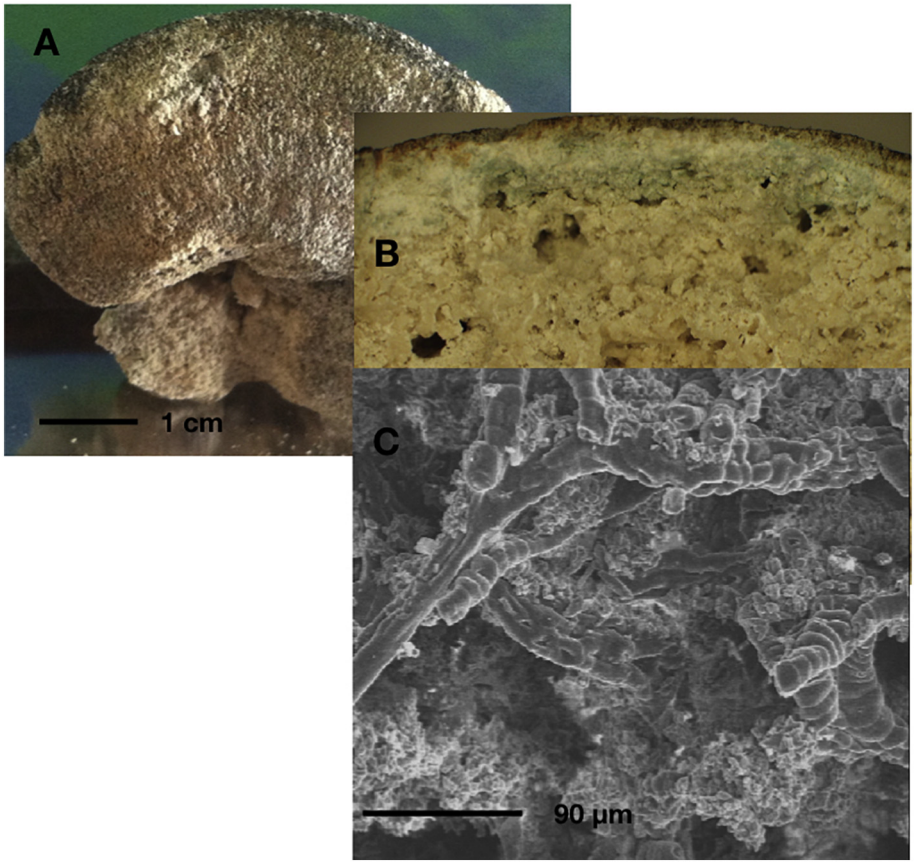

Fig. 2. Cross section of dry microbialite from Bacalar lagoon (A), showing stereoscopic image of surface growth layer (brown/green upper section) and internal carbonate core (B). Insert of SEM section showing cyanobacterial filaments embedded in minerals (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

there is a high EPS production. Therefore, if the EPS production exceeds its degradation, the microbialites have little degree of lithification (Wierzchos et al., 2006). In contrast, with fast EPS degradation by heterotrophic microorganisms, calcium ions are released and available for precipitation as $CaCO_3$ and formation of lithified layers (Decho et al., 2005).

The minerals precipitated in microbialites are varied and include calcite, aragonite, hydromagnesite, vaterite and dolomite among others (Zeyen et al., 2017; Valdespino-Castillo et al., 2018). Therefore, they can show different degrees of lithification, appearance, shape and growth (Pringault et al., 2005). The microbialites morphology depends on factors such as the microbial composition of the active growth portion (Papineau et al., 2005), the predominant metabolic pathways, the geochemical conditions of the environment (Havemann and Foster, 2008), as well as physical factors such as wave action, the depth of the system (Burns et al., 2004) and the topography (Gischler et al., 2011). Microbialites are classified according to the internal structure: Stromatolites, which typically have a thin flat and uniform lamination that can be wavy or rough (Havemann and Foster, 2008); thrombolites, characterized by a lumpy internal structure with fenestrations (Desnues et al., 2008; Planavsky and Ginsburg, 2009) and oncoliths, where the carbonates precipitation occurs in the form of concentric layers (Pringault et al., 2005; Wade and Garcia-Pichel, 2003) (Fig. 3).

Microbialites have been reported in almost all types of aquatic environments around the planet (Centeno et al., 2012; Gischler et al., 2008; Goh et al., 2009; Russell et al., 2014), including freshwater environments (with high carbonate content) (Burns et al., 2009), hypersaline, coastal lagoons, alkaline lakes, thermal waters, as well as in marine and brackish environments (Gischler et al., 2008; Pringault et al., 2005), such as Shark Bay (Western Australia), Highborne Cay (Bahamas), Pavilion Lake, (Canada), Lake Tanyangika (Africa), Lake Salda Golu (Turkey), Cuatro Cienegas (Mexico), Lake Alchichica (Mexico), Bacalar Lagoon (Mexico) (Fig. 3) Ruidera Pools (Spain) and Great Salt Lake (GSL), (United States) (Centeno et al., 2012; Lindsay et al., 2017).

Microbialites have been studied since the 1950s (Rezak, 1954), however, their studies have focused on mineral, biochemical or microscopic analyses (Coshell et al., 1998; Paerl et al., 2001; Reid et al., 2000). The study of the diversity and microbialites structure began using different microscopy techniques including light microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and laser confocal microscopy. Therefore, the identification of the microorganisms associated with these carbonate structures for a long time was based on morphological descriptions (Foster et al., 2009). The technological development of Next Generation Sequencing techniques (NGS) has allowed the characterization of many microbial communities using genes that are amplified directly from DNA from environmental samples (Centeno et al., 2012; Coman et al., 2015; Lindsay et al., 2017; Toneatti et al., 2017) facilitating the estimation of their genetic diversity and composition. The first report of DNA extraction and analysis from microbialites was by Steppe et al. (2001) which used a commercial kit for DNA extraction, followed by Neilan et al. (2002) who used a modified technique for DNA extraction from plants tissues (Supplementary Table 1).

1.1. Overview of conventional methods for DNA extraction from microbialites

The main objective of the DNA extraction method is to obtain the most suitable DNA, in terms of quality and quantity, for downstream analysis. In general, these methods have the following steps: breakage the cell wall and cell membranes, separation of hydrophobic cell debris and DNA precipitation. Moreover, depending on the type of sample, some methods include a purification step in order to eliminate any inhibitors for downstream applications such as PCR (Table 1). The following strategies have been used for DNA extraction from microbialites.

Extracting DNA from microbialites is technically challenging. In our own experience in the optimization of methods for extraction, it is necessary to pay attention to the following details: The first point to consider, when dealing with environmental samples, is the sample size. Some environmental samples have a large amount of chemical substances including humic acids and organic matter that can be co-extracted with DNA, these act as inhibitors for enzymatic reactions, and may damage DNA during long-term storage (Natarajan et al., 2016).

In addition, the presence of carbonate minerals and clays could interfere with extraction and characterization of nucleic acids, since clays and other minerals can bind to DNA (Herrera and Cockell, 2007).

Fig. 3. Natural study sites where microbialites exist in Mexico A) External view of columnar thrombolites from crater-lake Alchichica, Mexico, B) portion of active growth from thrombolites, C) Alchichica crater-lake, D) Bacalar Lagoon.

Table 1
Microbialite DNA clean-up, lysis and purification protocols.

Reference	Sample size	Pre-lysis	DNA purification
Neilan et al., 2002	10–100 mg	N	N
Abed et al., 2003	300–500 mg	N	N
López-García et al., 2005	NT	Rehydrated phosphate saline buffer	N
Papineau et al., 2005	0.25–0.35 g[a]	N	ChromaSpin1TE-1000 (Clontech Laboratories, Inc.)
Falcón et al., 2007	2 g	N	N
Havemann and Foster, 2008	75–85 mg	N	UltraClean Soil DNA kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA)
Breitbart et al., 2009	250 μL	N	Silica column from a Qiagen DNeasy kit (Qiagen; Valencia, CA)
Allen et al., 2009	1–3 cm^3	Washing with 0.5 M EDTA with shaking at 37 °C overnight	N
Foster et al., 2009	20–80 mg	N	From C4 solution step (MoBio PowerSoil DNA kit; Mo Bio Laboratories, Carlsbad, CA, USA).
Myshrall et al., 2010	50 and 80 mg	N	From C4 solution step (MoBio PowerSoil DNA kit; Mo Bio Laboratories, Carlsbad, CA, USA).
Santos et al., 2010	5 g	N	GENECLEAN Kit II (Bio 101)
Khodadad and Foster, 2012	100 mg	N	From C4 solution step (MoBio PowerSoil DNA kit; Mo Bio Laboratories, Carlsbad, CA, USA).
Mobberley et al., 2012	NT	N	From C4 solution step (MoBio PowerSoil DNA kit; Mo Bio Laboratories, Carlsbad, CA, USA).
Centeno et al., 2012	5 g	N	DNeasy Blood & Tissue kit (Qiagen, Valencia, CA)
Russell et al., 2014	2 g	N	N
Coman et al., 2015	2 cm^3	Washing three times with 0.5 M Na_2EDTA and shaking 30 min at 37 °C	25 mg Chelex100 (Bio-Rad,USA)
Saghaï et al., 2015	0.2 g[b]	Carbonates dissolved with 100 μL HCl 33% then neutralized with 1 mL of 1:1 PBS pH 7 and 0.5 M EDTA pH 9	Power Clean™ Pro DNA Clean-up Kit (Mo Bio, Carlsbad, CA, USA), to remove residual EPS
Casaburi et al., 2016	NT	Samples stored in RNA later	From C4 solution step (MoBio PowerSoil DNA kit; Mo Bio Laboratories, Carlsbad, CA, USA).

N: none; NT: Data not shown.
[a] DNA yield: 5 μg/g.
[b] DNA yield: 0.80–1.4 μg from 32 independent aliquots of 0.2 g (6.4 g total per pooled sample).

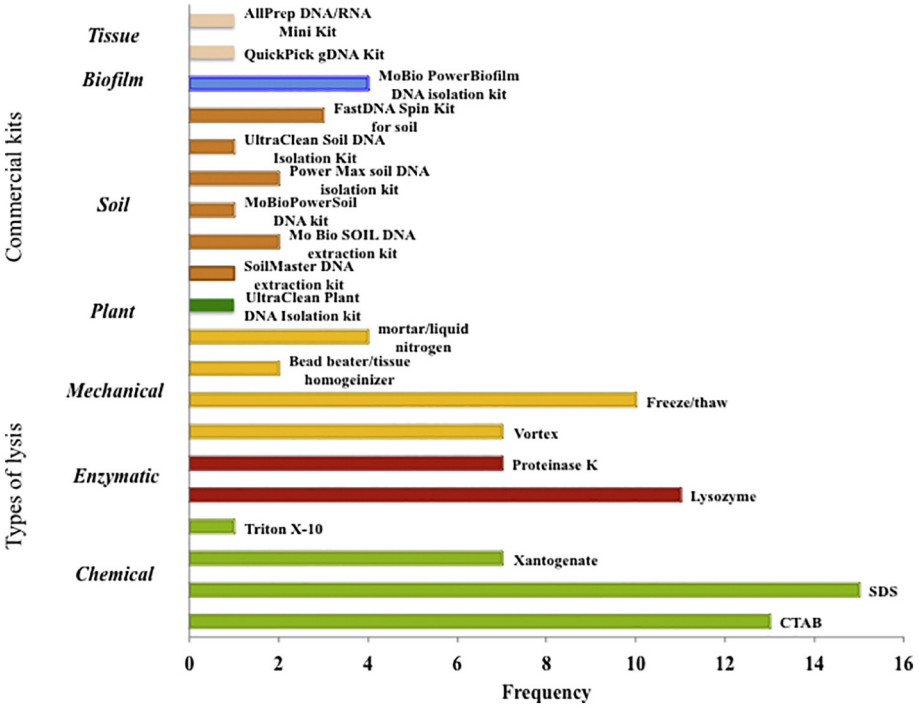

Fig. 4. Frequency of methods for DNA extraction from microbialites, different types of lysis and commercial kits.

Hence, the nucleic acids adsorption to mineral matrixes can result in low extraction efficiencies (Direito et al., 2012).

Moreover, microbialites can produce large amount of EPS, depending on the microbial community composition as mentioned above. EPS can affect post-DNA extraction techniques, such as amplification by polymerase chain reaction (PCR) because the mucus can capture Mg^{2+} ions and affect the enzymatic reactions. Therefore the larger the sample size the more inhibitory substances could be extracted.

According to this, the amount of sample could be a crucial point in any DNA extraction method. Moreover, Santos et al. (2010) reported that the amount of stromatolite sample to extraction buffer ratio is important in order to obtain enough good quality nucleic acids. Previous work shows that DNA extraction from microbialites can be made with very small amounts of sample such as 10 mg (Neilan et al., 2002) to large amounts such as 10 g (White III et al., 2015) (Table 1).

The second point to consider is cellular lysis, in order to break down

cell walls and membranes to get the DNA from all types of microorganisms present in the sample, without fragmenting the genomic DNA. Cellular lysis can be made by: a) mechanical disruption, such as grinding the sample using a sterile mortar and pestle after freezing with liquid nitrogen (Coman et al., 2015; Falcón et al., 2007) or by subjecting the samples to continuous periods of vortex-shaker agitation (Casaburi et al., 2016; Mobberley et al., 2012); b) Physical lysis by freeze-thaw cycles (e.g −20 °C) (Allen et al., 2009; Centeno et al., 2012; Santos et al., 2010) or by sample sonication (Pacton et al., 2014) c) Chemical lysis using chemical agents such as cetyltrimethylammonium bromide (CTAB) (Casaburi et al., 2016), sodium dodecyl sulfate (SDS) (Coman et al., 2015), Triton X-100 (Neilan et al., 2002) or Xanthogenate (Havemann and Foster, 2008), which can break down the cell wall and allow the release of all cellular content; d) Enzymatic lysis using Proteinase K (Coman et al., 2015) and Lysozyme (Chan et al., 2014) (Supplementary Table 1; Fig. 4). These enzymes are able to digest proteins and hydrolyze peptidoglycan of bacterial walls respectively. Methods for DNA extraction from microbialites use a combination of the different cellular lysis techniques mentioned above (Supplementary Table 1).

Cellular debris which include proteins and lipids are eliminated with phenol-chloroform which is a standard method widely used in environmental samples (Renshaw et al., 2015). This method separates DNA from lipids and proteins due to their solubility in organic solvents, while DNA is soluble in the aqueous phase. Abed et al. (2003) is the first report of phenol-chloroform DNA extraction from microbialites, and after that it has been widely used (Allen et al., 2009; Breitbart et al., 2009; Centeno et al., 2012; Coman et al., 2015; López-García et al., 2005; Papineau et al., 2005; Santos et al., 2010). DNA precipitation is facilitated with alcohols such as ethanol and isopropanol (Neilan et al., 2002; Santos et al., 2010).

1.2. Alternative methods for DNA extraction from microbialites with a pretreatment of the sample

Carbonate dissolution. Wade and Garcia-Pichel (2003) reported three methods for disrupting the carbonate matrix and allowing the cellular lysis to obtain DNA suitable for downstream analysis free of carbonates: the first pulverized the microbialite with sterile mortar and pestle; the second separated the microbes from the carbonate matrix through dissolution with HCl at different pH; and the third, proposed the carbonates dissolution by chelation of divalent cations with ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) (Sigma Chemical Co., St. Louis, MO, USA) at different concentrations. These authors found that acid dissolution is not appropriate to nucleic acid extraction since DNA was not recovered. Instead, they proposed the carbonates dissolution with EDTA followed by DNA extraction with commercial kits which allows to obtain high molecular weight DNA. Wade and Garcia-Pichel (2003) conclude that acid dissolution is not an appropriate method for DNA extraction. However, Couradeau et al. (2011) dissolved carbonates with HCl and obtained DNA as well as Saghaï et al. (2015) (Table 1). This could be due to the type of sample and the amount of carbonates. Besides, carbonate dissolution with EDTA has been used by Allen et al. (2009), Coman et al. (2015) in microbialites made of calcite and Corman et al. (2016) (Table 1, Table 2) with suitable DNA for downstream analysis. EDTA is widely used in pre and post DNA extraction methods such as cell membrane lysis, carbonate dissolution and conservation of DNA for storage. EDTA chelates divalent metal cations, for example magnesium, that is cofactor to nucleases. Therefore it is used to minimize metal ion contamination as well as to prevent enzymatic activity. EDTA is used to prevent the growth of contaminating organisms (Barra et al., 2015; Lahiri and Schnabel, 1993). However, it is necessary to pay attention to the concentration used due to the chelating effect of EDTA that could inhibits certain further enzymatic reactions (e.g., blunting, ligation, PCR) if present in high concentration. Most protocols specify EDTA concentration near ≤1 mN to prevent the inhibition.

Exopolymeric substances elimination. Exopolymeric substances are produced by microorganisms present in microbialites such as cyanobacteria which sequester cations including Ca^{2+} and Mg^{2+} (Couradeau et al., 2011). These could be eliminated by enzymatic treatment with Viscozyme (Sigma-Aldrich Buchs, Switzerland) as reported by Couradeau et al. (2011). Viscozyme is a multi-enzyme complex containing carbohydrases (i.e. cellulase, hemicellulase, xylanase, arabinase and β-glucanase) that can help to hydrolyze exopolymeric substances. It has been used for DNA extraction from organisms with rigid cell wall (i.e. dinoflagellates, cryptophytes and diatoms) (Auinger et al., 2008) and phytoplankton (Mäki et al., 2017). Additionally, to remove residual EPS from DNA extractions from microbialites the Power Clean™ Pro DNA Clean-up Kit (MoBio, Carlsbad, CA, USA) has been reported by Saghaï et al. (2015). Moreover, xanthogenate can be used for DNA and RNA extraction free of enzyme inhibitors or nucleases from cyanobacteria without enzymatic or mechanical cell disruption. Xanthogenate is a non toxic agent that solubilizes polysaccharides, since cyanobacteria contain chlorophyll and photosynthetic pigments (i.e. phycocyanin and phycoerythrin) and produce mucilaginous polysaccharides which seldomly complete cellular lysis (Tillett and Neilan, 2000). This method was first used by Tillett and Neilan (2000) in cyanobacterial cultures and then was widely used for microbialite DNA extraction (Casaburi et al., 2016; Foster et al., 2009; Goh et al., 2009; Havemann and Foster, 2008; Khodadad and Foster, 2012; Mobberley et al., 2012; Myshrall et al., 2010) (Supplementary Table 1).

1.3. Commercial kits for DNA extraction

Commercial kits are widely used for DNA extraction since these allow efficient and quick methods compared with conventional strategies. Kits are based on detergent agents and use silica-based columns or magnetic beads for purification. A large amount of commercial kits are available for DNA extraction from environmental samples (soil, plant, water, sediments, and biofilm) however, for microbialites there is not a specific kit to date. Kits designed for other environmental samples specially biofilms and soil are commonly used i.e. MoBioPowerSoil DNA kit (MoBio Carlsbad, CA, USA) (Couradeau et al., 2011), QuickPick™gDNA Kit (Bio-Nobile, Parainen, Finland) (Couradeau et al., 2011), UltraClean Plant DNA Isolation Kit, Mo Bio Laboratories, Inc., Solana Beach, CA, USA) (Wade and Garcia-Pichel, 2003), SoilMaster DNA extraction kit (Epicentre) (López-García et al., 2005), Mo Bio Soil DNA extraction kit (Mo Bio Solano Beach, CA) (Breitbart et al., 2009), FastDNA Spin Kit for Soil (MP Biomedicals) (Baumgartner et al., 2009; Lindsay et al., 2017), AllPrep DNA/RNA Mini Kit (Qiagen Inc., Valencia, CA, USA) (Nitti et al., 2012), MoBio Power Biofilm DNA isolation kit (MO BIO Laboratories, Carlsbad, USA) (Saghaï et al., 2015; Schneider et al., 2013), Power Max soil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) (White III et al., 2015; White III et al., 2016b), UltraClean Soil DNA Isolation Kit, (MoBio Laboratories, Carlsbad, CA, USA) (Paul et al., 2016), FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) (Corman et al., 2016) (Table 3; Fig. 4). The most widely used kit is Mo Bio Soil DNA extraction kit (Mo Bio Solano Beach, CA) and MoBio PowerBiofilm DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA) (Ruvindy et al., 2016; Wong et al., 2015; Wong et al., 2017, 2018) which also has been used for DNA extraction from microbial mats. Microbial mats are considered non-lithified analogs of microbialites and have a diverse microbial community (Valdespino-Castillo et al., 2018; Wong et al., 2018).

1.4. Which is the best method for DNA extraction from microbialites?

It is well known that microbialites are formed by aragonite, calcite, hydromagnesite and gypsium, among other minerals, however the content of each mineral in microbialites could be different. Knowing the microbialites mineralogical composition before DNA extraction could

Table 2
Mineral composition of microbialites and study site.

Reference	Study site	Mineral composition
Breitbart et al., 2009	Cuatro ciénegas, Coahuila, Mexico	Calcite
Coman et al., 2015	Oil drilling, Romania	Calcite
Couradeau et al., 2011	Alchichica crater lake, Puebla, Mexico	hydromagnesite
López-García et al., 2005	Lake Van, Turkey	Aragonite, calcite
Paul et al., 2016	Storr's lake, the Bahamas	Some made of Mg-calcite and aragonite Others only Mg-calcite
Saghaï et al., 2015	Alchichica crater lake, Puebla, Mexico	Hydromagnesite and aragonite
Santos et al., 2010	Ruidera Pools Natural Park, Spain	Calcite
Schneider et al., 2013	Lake 21, Islands of Republic of Kiribati	Gypsium, aragonite
White III et al., 2016a,b	Pavilion lake, British Columbia, Canada	Calcite
Valdespino-Castillo et al., 2018	Cayo Sabinal, Northern, Cuba	Calcite, hexahidrite, aragonite, gypsium, quartz
	Bacalar Lagoon, Quintana Roo, Mexico	Calcite, plagioclase, kaolinitesiderite
	Alchichica crater lake, Puebla, Mexico	Aragonite, calcite, siderite
	Alchichica crater lake, Puebla, Mexico	Hydromagnesite, aragonite, siderite
	Pozas azules, Cuatro Ciénegas, Coahuila, Mexico	Calcite, quartz, siderite
	Muyil Lagoon, Quintana Roo, Mexico	Calcite, hydromagnesite, pyrite, aragonite

help to do a better selection of the DNA extraction method. As reported by Valdespino-Castillo et al. (2018), the mineral composition and abundance of chemical elements in microbialites are different even in those taken from the same sampling site, moreover, the microbial composition of microbialites depends on the physicochemical characteristics of the study site.

There is a wide variety of techniques for DNA extraction from microbialites. These techniques can be very complex or very fast, the selection of a method will depend on the degree of the microbialite lithification, the presence of minerals and the EPS amount, that can difficult the access to microorganisms that are embedded in this matrix, preventing the release of their DNA during extraction methods. As mentioned above, conventional methods for DNA extraction that do not include any pretreatment of the sample and purification steps exist (Abed et al., 2003; Neilan et al., 2002) (Supplementary Table 1), as do conventional methods with a sample pretreatment (Allen et al., 2009; Coman et al., 2015) (Table 1) and those with a DNA purification step (Breitbart et al., 2009; Havemann and Foster, 2008; Papineau et al., 2005) (Table 1). Moreover, commercial kits have been widely used and few of them require a previous carbonates dissolution step (Corman et al., 2016; Couradeau et al., 2011; Saghaï et al., 2015) or EPS elimination with viscozyme (Couradeau et al., 2011) (Table 3).

Therefore, the selection of DNA extraction method must be done carefully and taking into account that the same method could not be

Table 3
Commercial kits use for metagenomic DNA extraction of microbialites.

Reference	Sample size	Previous lysis treatment	Commercial kit
Wade and Garcia-Pichel, 2003	70–200 mg	1) Frozen in liquid nitrogen pulverized with a mortar and pestle.[a] 2) acid dissolution of the carbonate with HCl[b] 3) dissolution of the carbonate with EDTA.[c]	"Bead Solution" tubes of the commercial kit UltraClean Plant DNA Isolation Kit, Mo Bio Laboratories, Inc., Solana Beach, CA, USA) and 7 to 10 freeze-thaw cycles
López-García et al., 2005	NT	Rehydrated dry samples with sterile phosphate saline buffer	SoilMaster DNA extraction kit (Epicentre)
Breitbart et al., 2009	5 g	N	Mo Bio SOIL DNA extraction kit (Mo Bio; Solano Beach, CA)
Baumgartner et al., 2009	0.19–0.25 g	Mechanically homogenized with a pipette tip	FastDNA Spin Kit for Soil (QBiogene)
Couradeau et al., 2011	NT	Carbonates dissolved with HCl 33% and neutralized with PBS pH 7, 0.5 M EDTA pH 9	1) QuickPick™gDNA Kit (Bio-Nobile, Parainen, Finland) previously incubation for 3 h at 56 °C with Proteinase K and ViscozymeH 2) MoBioPowerSoil DNA kit (MoBio, Carlsbad, CA, USA) previous incubation with ViscozymeH (Sigma-Aldrich,Buchs, Switzerland)
Nitti et al., 2012	30 mg	Samples stored in RNAlater.	AllPrep DNA/RNA Mini Kit (Qiagen Inc., Valencia, CA, USA)
Schneider et al., 2013	100 mg	Frozen with liquid nitrogen	MoBio PowerBiofilm DNA isolation kit (MO BIO Laboratories, Carlsbad, USA) with an extra purification step[a]
Russell et al., 2014	2 g	N	MoBio PowerBiofilm DNA isolation kit (MO BIO Laboratories, Carlsbad, USA), cell lysis was done using FastPrep-24 Cell Homogenizer for 40 s
White III et al., 2015	10 g	N	Ground with mortar and pestle with liquid nitrogen and Power Max soil DNA isolation kit (MoBio Laboratories, Inc.,Carlsbad, CA, USA)
Saghaï et al., 2015	0.2 g	Carbonates dissolved with 100 µL HCl 33% then neutralized with 1 mL of 1:1 PBS pH 7 and 0.5 M EDTA pH 9[d]	Ground with mortar and pestle and Power Biofilm ™ DNA Isolation Kit (MoBio,Carlsbad,CA,USA) with an extra purification step
Corman et al., 2016	0.34 ± 0.16 g	Washing with Na_2EDTA buffer at pH 5 overnight	FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA
White III et al., 2016a,b	5 g	N	PowerMaxR Soil DNA Isolation Kit (Mobio, Carlsbad, CA, USA)
Paul et al., 2016	NT	N	UltraClean Soil DNA Isolation Kit, MoBio Laboratories, Carlsbad, CA, USA
Lindsay et al., 2017	NT	N	FastDNA Spin Kit for Soil (MP Biomedicals).
Toneatti et al., 2017	0.2 g	N	Power BiofilmTM DNA Isolation Kit (MO BIO Laboratories, Inc.) and the inhibitor removal Technology R (IRT)
Johnson et al., 2018	0.2 g	N	Mo Bio SOIL DNA extraction kit (Mo Bio; Solano Beach, CA)

N: none; NT: Data not shown.
[a] DNA yield: 3.5 µg/g.
[b] Not DNA obtained.
[c] DNA yiled: 7.8 µg/g.
[d] DNA yield: 0.80–1.4 µg from 32 independent aliquots of 0.2 g (6.4 g total per sample) and pooled.

efficient for DNA extraction from the same study site. A clear example is the work of Breitbart et al., (2009) who needed different DNA extraction strategies for microbialites from Cuatro Cienegas, i.e. DNA from oncolites was extracted with MoBio Soil DNA extraction kit (Mo Bio; Solano Beach, CA) (Table 3) while thrombolites was done with freeze/thaw, CTAB, phenol method, using only the surface living layer, and included an additional purification step (Table 1). Authors suggest that the need for different strategies was due to the differential abundance of EPS in the samples.

The use of commercial kits can be very fast, however it can not be successfully applied to all samples. Corman et al. (2016) reported that with commercial kits for DNA extraction they did not obtain high molecular weight genomic DNA suitable for PCR amplification. They suggest an extraction protocol that uses a chelating agent and microwave radiation. In addition, Couradeau et al., 2011 used a commercial kit with an extra step for DNA extraction. This protocol includes the use of MoBio PowerSoil DNA kit (MoBio Carlsbad, CA, USA) and a previous step with Viscozyme (Sigma-Aldrich Buchs, Switzerland) which has a better extraction yield than Quick Pick™ gDNA Kit (Bio-Nobile, Parainen, Finland) with proteinase K and Viscozyme incubation (Sigma-Aldrich Buchs, Switzerland) (Couradeau et al., 2011) (Table 3).

Xanthogenate method could be used in microbialites with high content of EPS (Foster et al., 2009) (Supplementary Table 1), including incubation with Viscozyme (Sigma-Aldrich Buchs, Switzerland) (Couradeau et al., 2011) or with a purification step after DNA extraction with commercial kits that can eliminate inhibitors from samples (Breitbart et al., 2009; Centeno et al., 2012; Havemann and Foster, 2008; Papineau et al., 2005; Santos et al., 2010) (Table 1).

Finally, Valdespino-Castillo et al. (2018) used an extraction method that includes a chemical disruption with CTAB and SDS, enzymatic lysis using lysozyme and proteinase K, grinding in mortar with liquid nitrogen, phenol chloroform extraction and a purification step with DNeasy Blood & Tissue kit (Qiagen, Valencia, CA). These authors managed to obtain DNA from microbialites with very different mineral compositions suitable for PCR amplification. This method has been previously used for DNA extraction of microbialites from Mexico (Centeno et al., 2012; Falcón et al., 2007; Valdespino-Castillo et al., 2014, 2017).

Our research group has worked with samples of different types of microbialites, extracting nucleic acids with a combination of the extraction methods mentioned above. In our experience, choosing which is the most appropriate extraction technique depends on the particular characteristics of each sample (e.g., lithification degree, EPS amount among others) as well as the research goals. A point of great importance that must be taken into account is the quality of the extracted DNA for downstream applications including NGS techniques that require high DNA amounts. Another issue to consider is the DNA fragmentation during the extraction process, a problem that is frequently given with the different methods of cellular disruption required; which, could limit the study of certain genes for downstream analysis, i.e. third-generation sequencers such as PacBio RS II (Pacific Biosciences, Menlo Park, California) require high-quality and high-molecular-weight DNA ($>$ 50 kb). This sequencing technology is based on single DNA molecules producing several kilobases, which can improve genome assemblies (Mayjonade et al., 2016). Moreover, in third-generation sequencing there is no DNA amplification, and the DNA sample must be absent of chelating agents, detergents, divalent metal cations, denaturants, RNA, or any contaminants from starting material (quality assessment: OD260/280 ratio of 1.8 to 2.0 and an OD260/230 ratio of 2.0 to 2.2), and pH between 6 and 9 (Endrullat et al., 2016). A method reported for the obtention of high molecular weight DNA from microbial mats suitable for third-generation sequencing consists in separating the cells from the microbial mat matrix with several NaCl (1 M) washes. Cells are further lysated using SDS, β-mercaptoethanol, and freeze-thaw cycles; the DNA is precipitated with isopropanol and polyethylene glycol (PEG) (Bey et al., 2010). In metagenomic studies, large DNA molecules provide more information. Thus, obtaining high-molecular-weight DNA (35–50 kb) (Bey et al., 2010) is an important requirement since long-read sequencing protocols (e.g., PacBio, Nanopore, Illumina, among others) analyze single DNA molecules. Therefore success depends on both high-quality DNA samples and long DNA fragments. In addition, prior library preparation, DNA should be always assessed and depending on the application using 30–50 ng of DNA (Endrullat et al., 2016).

It is important to take into account the amount of DNA extracted depending on the purpose of the study, i.e. Papineau et al. (2005) reported 5 $\mu g\, g^{-1}$ of DNA after chemical, enzymatic lysis and mechanic lysis; and a purification step with ChromaSpin1TE-1000 (Clontech Laboratories, Inc.) (Table 1). Wade and Garcia-Pichel, 2003reported different results by using different extraction methods: pulverized with liquid nitrogen (3.5 μg/g), carbonate dissolution with EDTA (7.8 μg/g), while acid dissolution of the carbonate with HCl did not recover DNA. Nonetheless in another study system, Saghaï et al., 2015 dissolved carbonates with HCl 33%, and DNA extraction was done with the Power Biofilm™ DNA Isolation Kit (MoBio, Carlsbad, CA, USA) with an extra purification step using Power CleanTM Pro DNA Clean-up Kit (Mo Bio, Carlsbad, CA, USA), obtaining a DNA yield of 0.80–1.4 μg from 32 independent aliquots of 0.2 g (6.4 g total per simple). This seems to be the less efficient method for DNA extraction. In cases where larger quantities of DNA are required as in a metagenomic library construction, it is important to select the method that contains the highest DNA amounts with high quality. Alternatively, several extractions of independent subsamples can be done and pooled to obtain the desired quantity of DNA (Saghaï et al., 2015). In addition, if the purpose is to make a library for gene sequencing where an amplification step is done, a low yield is compensated by amplification.

Several different methods have been used for DNA extraction of microbialites from different geographical origin, and choosing the most appropriate will depend on the characteristics of the sample. In this review, all methods used to date were included, although it is necessary to evaluate more thoroughly how the mineral composition and concentration of EPS can affect the DNA extraction yield.

1.5. RNA extraction from microbialites

Although DNA based approaches provide information regarding the genetic composition of microbialites, it can not shed light on the metabolically active microorganisms and gene expression. Little research on microbialites translation of mRNA/rRNA has been done, probably due to the difficulty of recovering high quality RNA by the action of ribonucleases, which are abundant in the environment. Microbialites have high contents of organic material including EPS, thus few work has attempted to characterize the patterns of RNA diversity. In the following text, we mention the methods and strategies used up until now for RNA extraction from microbialites.

1.5.1. RNA later

Preservation of samples prior to an RNA extraction is fundamental for the success of metatranscriptomic or gene expression experiments where high-quality and quantity of RNA are essential. Liquid nitrogen is widely used for this purpose, however, sampling in remote regions or sites with difficult access or poor infrastructure have depended on commercial reagents that preserve RNA (e.g. RNA later, Thermo Scientific, Waltham, MA, USA). RNA later contains high concentrations of quaternary ammonium sulfates and cesium sulfates which denature RNases, DNases and proteases to prevent the RNA degradation, in consequence, it is effective in the preservation of nucleic acids (Kruse et al., 2017). RNA later has been used for the RNA preservation during sampling of microbialites for transcriptomic studies (Edgcomb et al., 2014; Louyakis et al., 2018) since mRNA can be degraded by nucleases within seconds.

1.5.2. RNA extraction with phenol

Phenol is a common and efficient reagent used in protocols for RNA extraction, due to its denaturing capabilities of nucleases. In microbialites, Santos et al. (2010) reported a method where 5 g of the surface, greenish layer of a stromatolite were sampled and chemical lysis was carried out with SDS and phenol–chloroform–isoamylalcohol (PCI) 25:24:1. Nucleic acids were precipitated with ethanol. RNAzol protocol has been used by Mobberley et al. (2015). This commercial reagent contains phenol and guanidine thiocyanate in a monophase solution which dissolves DNA and RNA and eliminates proteins and nucleases. It is widely used in biological samples including plant, animal, bacterial and viral. The protocol used by Mobberley et al. (2015), used 1 g of thrombolitic mat ground in a mortar with liquid nitrogen that was divided into 80–100 mg aliquots mixed with RNAzol reagent. RNA Precipitation was done with 1 μL of RNA precipitate carrier (MRC, Cincinnati, OH) and ethanol. The precipitate carrier is a solution of acryl polymer used for the isolation of small amounts of RNA (< 10 μg) and large RNA (> 200 bases), this can increase the recovery of total RNA.

1.5.3. Commercial kits

These methods are efficient and quick. Kits for RNA extraction from soils have been used for microbialites as well, due to the complex composition of the sample. One of the most widely used kits is the PowerSoil® Total RNA Isolation kit (Mo Bio Laboratories). This kit is successful due to a solution that separates molecules in the sample. Proteins are eliminated with phenol and inhibitors. The nucleic acids are precipitated with isopropanol which works well for small amounts of RNA, however, it can co-precipitate inhibitors and salts, which is why there is a purification step in which DNA and RNA are bound to a column, and phenolic compounds, pigments and humic substances are eliminated. Moreover, in this step, DNA and RNA are eluted selectively with a chaotropic solution. While RNA is eluted, DNA remains bound to the purification column, and is eluted afterwards. This kit was used by Alcántara-Hernández et al. (2017) and Valdespino-Castillo et al. (2017) with successful results using large samples (6 g) allowing for mRNA of specific genetic regions, particularly associated to the N and P cycles, to be explored. Recovery of the largest proportion of microorganisms embedded in the microbialite matrix (Fig. 2) is very important in order to get all the RNA present in the sample. This can be done including freeze-thaw cycles with liquid nitrogen and bead solution from the PowerSoil® Total RNA Isolation kit (Mo Bio Laboratories) (Alcántara-Hernández et al., 2017). Moreover, microbialites are rich in salts and carbonates, in consequence, an extra purification step with the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) could be necessary (Alcántara-Hernández et al., 2017; Valdespino-Castillo et al., 2017). DNA can contaminate RNA extractions, thus incubations with DNase are common (DNase I, Qiagen) to avoid false positives in gene expression studies. Another commercial kit is FastRNA Pro Soil-Direct Kit (MP Biomedical, LLC, Solon, OH, USA) used by Edgcomb et al. (2014). This protocol eliminates nucleases with phenol, and uses a solution for inhibitor removal in an RNA MATRIX Slurry for the elimination of humic acids. This kit does not include separation of RNA from DNA, however, DNA can be eliminated with a Turbo DNase (Ambion) treatment before the RNA Matrix cleanup included in the extraction kit (Edgcomb et al., 2014). Powerbiofilm RNA Isolation Kit (Qiagen, Carlsbad, CA,USA) used by Louyakis et al., (2018), has been used for RNA extraction from microbial mats (Cardoso et al., 2017; Thiel et al., 2018). This protocol included β-mercaptoethanol in the lysis step to eliminate nucleases. In addition, this protocol includes a step to eliminate EPS and a purification step in which nucleic acids are bound to the column. Residual inhibitors are discarded in the flowthrough, and this kit also includes a DNA digestion step. Louyakis et al. (2018) extracted microbialite RNA from 100 mg samples, and DNA digestion was made with the Turbo DNA-free Kit (Ambion, Applied Biosystem Business, CA); samples were purified using RNA Clean and Concentrator-5 (Zymo Research, Orange, CA, USA).

1.6. Which is the best method for RNA extraction?

Although there are few works of RNA extracted from microbialites, the methods reported have been efficient for studies of metatranscriptomic and gene expression. When we select a RNA extraction method it is very important to take into account two points: first, RNA samples must be free of inhibitors for the success downstream applications and second, it is fundamental to achieve high-quality RNA, measured through the value of the RNA integrity number (RIN) (Schroeder et al., 2006). This value must be near to 10 and reflects good integrity of RNA (Schroeder et al., 2006), meaning that fragmentation of RNA was not significant throughout the extraction.

1.7. Viral extraction from microbialites

Viruses (principally phages) are living entities with cosmopolitan distribution in aquatic and terrestrial environments (Desnues et al., 2008). Viruses are involved in microbial growth rates, adaptation, genetic exchange and evolution (Desnues et al., 2008). Viral extraction in environmental samples can either be completed directly by isolation of viruses from samples or indirectly via computation. The indirect method uses clustering, denovo assembly and annotation to reconstruct viruses directly from metagenomic samples (Paez-Espino et al., 2016). These indirect methods have some limitations including direction of low abundant viruses but are one way to elucidate viral communities. The direct method consists of recuperating the viral particles from a portion of stromatolite resuspended in buffer, which is then filtered through 0.22 μm to eliminate the cellular fraction. The viral particle fraction is purified using a cesium chloride density gradient centrifugation. Finally, viral DNA is extracted with formamide/CTAB extraction (Desnues et al., 2008; White III et al., 2018). Further direct methods for the extraction of viruses from microbialites are needed to further our understanding of viral-microbial host interactions in microbialites.

1.8. Extraction of the 'multiome,' in microbialites

In the era of less expensive sequencing and mass spectrometry, the entire 'multiome,' can be elucidated from DNA, RNA, Protein, Lipid and Polar metabolite (White III et al., 2016a). The newest challenge is how can you extract all these 'omes,' from single samples in a robust and standardized method?. The single-sample metabolite, protein and lipid extraction (MPLEx) method has recently been used for integrative multi-omics analysis, providing information of proteomics, metabolomics and lipidomics from the same sample (Nakayasu et al., 2016). This method enables a comprehensive understanding of complex biological systems and can be very useful when samples are difficult to obtain (Nakayasu et al., 2016). The protocol is based on chloroform-methanol-water solution extraction, which separates into three different phases: the upper aqueous phase containing hydrophilic metabolites; the interphase containing proteins and the bottom phase containing lipids (Nakayasu et al., 2016). This method is interesting for the thorough understanding of nutrient cycling and the molecular changes in microbial communities due to environmental perturbations (Nicora et al., 2018). For instance, soil samples have been studied with this method (Nicora et al., 2018) and a similar method has been applied in microbial mats research (Kim et al., 2015 Another method for the multiome study is the single pot for DNA, RNA, proteins and metabolite extraction from a single sample, with methanol/chloroform extraction followed by phenol/chloroform extraction, has been successfully applied in eukaryotic cells (Vorreiter et al., 2016). However, there are no reports of these methods applied in microbialites. While we mentioned a multiplex and single pot method for extraction of the 'multome,' this will be an ongoing challenge for many sample types including microbialites.

Supplementary data to this article can be found online at https://

doi.org/10.1016/j.mimet.2019.02.014.

Acknowledgements

E.S.G.A. received a postdoctoral grant from *Dirección General de Asuntos del Personal Académico*, Universidad Nacional Auntónoma de México (DGAPA, UNAM). We acknowledge César Guzmán "Celacanto" for the photo from Alchichica crater-lake. We thank Laboratorio de Estudios Ecogenómicos, Unidad Académica de Yucatán, UNAM for use of their facilities. Technical support is acknowledged to Joanna M. Ortíz Alcántara and Osiris Gaona. Funding for research at the Bacterial Ecology Laboratory, UNAM is provided through UCMEXUS CN116 and UNAM PAPIIT 202016-3.

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