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Life in Hot Spring Microbial Mats Located in the Trans-Mexican Volcanic Belt: A 16S/18S rRNA Gene and Metagenomic Analysis

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ABSTRACT

The geothermal system of the Araró region, located in the Trans-Mexican Volcanic Belt of México, hosts various hot springs with unique physicochemical characteristics, including temperatures ranging from 45°C to 78°C. The microbial diversity in these hot springs has been explored only by culture-dependent surveys. In this study, we performed metagenomic Illumina MiSeq, and 16S and 18S rRNA pyrosequencing analysis of the microbial life are residing in the microbial mats of the springs called "Tina–Bonita". Our results show the presence of 186 operational taxonomic units, 99.7% of which belong to bacteria, 0.27% to eukaryotes, and 0.03% to archaea. The most abundant bacterial divisions are the Proteobacteria, Chloroflexi, and Cyanobacteria, which include 105 genera. The ecological indexes indicate that the microbial mats have moderate microbial diversity. An abundant group of genes that participate in photosynthesis, including photosynthetic electron transport, as well as photosystems I and II, were detected. Another cluster of genes was found that participates in sulfur, nitrogen, and methane metabolism. Finally, this phylogenetic and metagenomic analysis revealed an unexpected taxonomic and genetic diversity, expanding our knowledge of microbial life under specific extreme conditions.

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KEYWORDS
Bacterial diversity; hot spring microbial mats; metagenome; thermophiles

Introduction

Microbial mats are vertically stratified communities of microorganisms embedded in an exopolysaccharide matrix (Bolhuis et al. 2014). They are actively influenced by physicochemical gradients resulting from microbial activity (Paerl et al. 2000). Their main inhabitants are bacteria and archaea (Ward et al. 1998), although some eukaryotes are also present at low abundances (Casamayor et al. 2002). The architecture of the laminated community (e.g., in photosynthetic microbial mats) reflects the ecology of its inhabitants and the physiology of all the microbial interactions (Chan et al. 2016). Since mats are almost exclusively formed by prokaryotes, they have been proposed as biological models for microbial communities for very different kinds of studies, like evolution, microbial ecology, and astrobiology (Des marais 2003; Franks and Stolz 2009; Ward et al. 1998). As hot springs can exhibit diverse, extreme environmental conditions, it is evident that they can host numerous microbes, including thermophiles, acidophiles, and halophiles. Thus, it may be possible to find better or novel enzymes with applications in diverse industries, medicine, or agriculture by studying hot springs (Prieto-Barajas et al. 2018).

Microbial mats are present around the world and in a broad range of extreme environments, like low-temperature environments (De los Ríos et al. 2015; Peeters et al. 2012; Taton et al. 2003; Tytgat et al. 2014; Varin et al. 2011; Vincent et al. 2000), acid pools (Beam et al. 2016; Bond et al. 2000), coastal zones (Armitage et al. 2012; Bolhuis et al. 2013; Bolhuis and Stal 2011; Dijkman et al. 2010), high salinity ponds (Harris et al. 2013; Jonkers et al. 2003; Kunin et al. 2008; Ley et al. 2006; Lun Wong et al. 2015) and hot springs, where photosynthetic microbial mats are formed (Amin et al. 2017; Coman et al. 2013; Huang et al. 2011; Lacap et al. 2007; Mackenzie et al. 2013; Portillo et al. 2009; Thiel et al. 2016).

To assess the bacterial diversity in microbial mats, both cultivation-dependent and cultivation-independent approaches have been employed. Although both methodologies are useful, their research goals can be different. Culture-independent methodologies, such as PhyloChip microarray, massive parallel sequencing techniques, in combination with metagenomic analysis, have comprehensively expanded our understanding, even at millimeter-scale, of the microbial life in extreme environments (Amin et al. 2017; Kunin et al. 2008; Thiel et al. 2016, 2017).

The many functional microbial groups that have been associated with microbial mats include photosynthetic bacteria, aerobic and anaerobic heterotrophs, nitrifying and sulfate-reducing bacteria, and methanogenic archaea (Van Gemerden 1993; Thiel et al. 2017). In particular, photosynthetic, thermophilic mats contain a great abundance of cyanobacteria, filamentous bacteria, and unicellular groups, depending on the temperature gradient (Mackenzie et al. 2013). Other phyla

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present are Chloroflexi, Proteobacteria, Firmicutes, and Deinococcus–Thermus.

The Trans-Mexican Volcanic Belt represents an ancient, extensive series of volcanic structures (Ferrari et al. 2011), which now constitutes an excellent site for the formation of isolated, extremophile microbial communities associated with its particular geothermal characteristics (Brito et al. 2014; Medrano-Santillana et al. 2016). The geothermal zone of Araró, Michoacán is located within this area and has numerous thermal springs (Viggiano-Guerra and Gutierrez-Negrín 2005) where thermophilic, photosynthetic microbial mats are formed. The Araró geothermal zone is an independent system from Los Azufres, although they are separated by only 20 km. Most of the hot springs are located in the zone known as Zimirao (19°53'54" N, 100°49'50" W). There are more than 50 hot springs in the zone, many of which are employed for recreational activities. In particular, two hot springs, called "Tina–Bonita", have almost null human activity disturbance. Other interesting features of Bonita are its low water emission, constant water content during the year, and formation of colorful microbial mats (Prieto-Barajas et al. 2017).

In a previous study, only species belonging to the heterotrophic genera *Bacillus*, *Paenibacillus*, *Exiguobacterium*, *Aeromonas*, and *Pseudomonas* were recovered by microbial culture techniques (Prieto-Barajas et al. 2017). However, these communities have high photosynthetic, stress response, and potential methane metabolism elements that were not recovered by microbial cultures. We hypothesized that the microbial community is, by far, more diverse, and includes uncultured groups of archaea and eukaryotic microbes.

Materials and methods

Sample collection

The study site has been previously described (Prieto-Barajas et al. 2017). Briefly, the geothermal system of the Araró region is located in the central part of Mexico, inside the Trans-Mexican Volcanic Belt located in Michoacán State. The region is 20 km west of the Los Azufres geothermal field. The zone is known as Zimirao (19°53'54" N, 100°49'50" W) contains most of the hot springs, including the complex of the Tina–Bonita springs where the microbial mats were collected on 22 April 2016, during the spring/dry season. Three microbial mat samples (collected at a distance of ∼50 cm each) per hot spring were obtained at a depth of 1 or 2 cm in a simple random sampling. The Tina and Bonita springs are very close, separated by only 2 m, and have very similar physical parameters (Prieto-Barajas et al. 2017). The microbial mats were kept in darkness, and transported to the laboratory in refrigerated conditions using sterilized materials. They were later stored at −20 °C and processed on the same day.

Physicochemical parameters

The physicochemical parameters of water in the hot springs were measured during the sampling of the biological material, as previously reported (Prieto-Barajas et al. 2017). The parameters, including temperature (°C), pH, electrical conductivity, and dissolved oxygen were measured *in situ* with a Corning® Checkmate™ II modular meter system. Physicochemical water analyses, including fecal coliforms analysis, were performed in collaboration with the National Water Commission (CONAGUA-México). The measurement of fluoride was carried out with a conventional fluorometer. We measured the arsenic concentrations in the water samples of the Bonita and Tina hot springs by absorption spectroscopy using an atomic absorption spectrometer (Perkin-Elmer AAnalyst™ 200) with a hydride generation system.

Nucleic acid extraction

Metagenomic DNA was extracted from each of the microbial mat samples using the Mo Bio PowerSoil® DNA Isolation Kit, and purified with the Mo Bio PowerClean DNA Cleanup Kit. We subsequently quantified the DNA and assessed the quality of the material with a NanoDrop™ 2000 c spectrophotometer (Thermo Fisher Scientific), and we performed 1% agarose gel electrophoresis to determine the integrity of the genetic material. Subsequently, the samples were sent to the genomic services center at MR DNA (Shallowater, Texas, USA).

Data sequencing

We obtained the 16S/18S rRNA gene sequences by pyrosequencing of the V4 hypervariable region. Samples of 16S/18S DNA were sequenced using Roche 454 FLX Titanium instruments and reagents, according to the manufacturer's guidelines. The preprocessing reads were approximately 274 bp long. We removed the adapters and primers, sequences with ambiguous bases, and homopolymers of more than 6 bp. We generated operational taxonomic units (OTUs) and checked for the formation of chimeras. OTUs were defined by clustering with a divergence of 3%, or 97% similarity between sequences. For the final classification of OTUs annotation and their taxonomic identification, we used BLASTn (www.ncbi.nlm.nih.gov) against a mixed, curated database of the databases GenBank, Greengenes, and RDP-II (http://rdp.cme.msu.edu; DeSantis et al. 2006).

Statistical analysis of diversity

Statistical/ecological analyses consisted of measuring the alpha diversity with the Shannon, Simpson, and equitability ecological indexes, which compare diversity with other microbial communities. The sampling representativeness was measured with a rarefaction curve, in which the variables used were the number of observed OTUs by the number of obtained ribosomal gene reads (rRNA 16S/18S), using PAST software 3.15 (Paleontological Statistics).

Phylogenetic analyses

We performed a phylogenetic analysis of the 186 identified OTUs to investigate the diversity of the microbial lineages of the community, using the MEGA7 program (Kumar et al.

Table 1. Physicochemical parameters of the Tina–Bonita complex in Araró, Mexico.

Physicochemical parameters		Hot springs	
		Bonita	Tina
Temperature	°C	60	58
pH		7.18	6.95
Arsenic content	mg/l	4	4.9
Electric conductivity	mS/cm	4.06	3.88
Total disolved solids	g/l	2.15	2.05
Total hardness (Ca, Mg)	mg/l	2752	2705
Total alkalinity	mg/l	524	520
Chlorides	mg/l	929	913
Sulphate	mg/l	225	221
Total coliforms	MPN/100	0	0
Coordinates		N 19°5357.5″ W 100°5001.0″	N 19°5356.1″ W 100°5002.0″

Temperature, pH, electrical conductivity, and total dissolved solids were measured *in situ*. Units: MPN/100: most probable number per 100 ml of sample. Coordinates at north (N) and west (W).

2016). We used the "Neighbor-Joining" method with bootstrap analysis with 1,000 repetitions. No external groups were employed. The generated tree was processed in iTOL (Letunic and Bork 2016).

Metagenome sequencing and analysis

We sequenced the shotgun metagenome using Illumina MiSeq® 2 × 250 in paired-end format. The Illumina MiSeq-generated sequence data were quality-checked using FastQC (Andrews 2015). Then, using Trimmomatic, we eliminated poor quality sequences and adapters (Bolger et al. 2014). The assembly of the sequences was analyzed with Velvet (Zerbino and Birney 2008). We annotated the sequences in the MG-RAST online server (http://metagenomics.anl.gov/) and the SEED databases (www.theseed.org/), which is a microbial gene ontology, and the Kyoto Encyclopedia of Genes (KEGG) for the metabolic pathways.

Results

Physicochemical parameters

Table 1 shows the physicochemical parameters of the hot spring complex Tina–Bonita. The temperatures ranged from 58 to 60°C. The arsenic contents ranged from 2.7 to 6.6 mg/L.

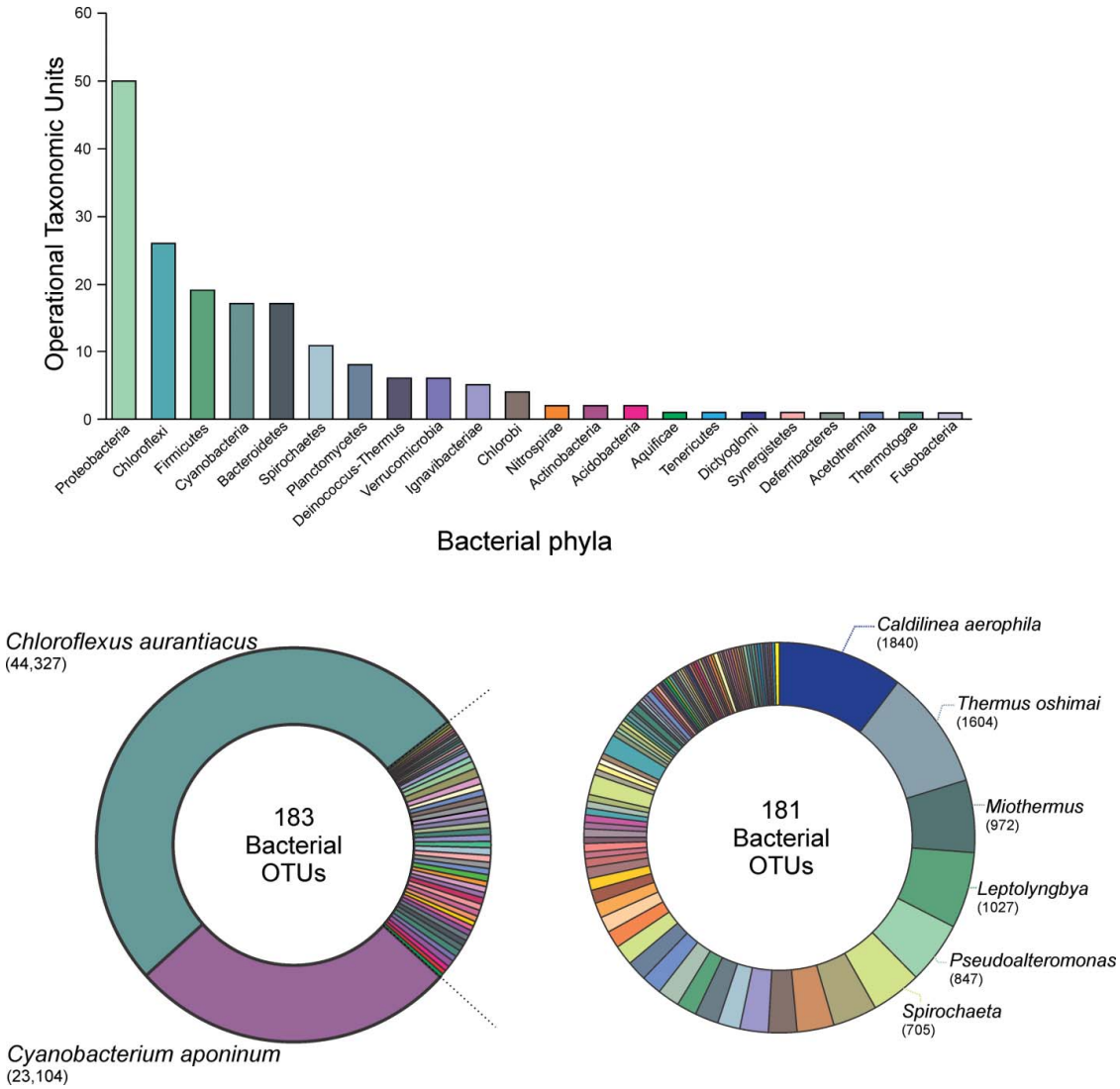

Figure 1. Diversity of the microbial OTUs detected in the thermophilic microbial mat complex. Top panel graph: Number of OTUs recruited in the bacteria phyla. The bottom left donut chart shows the 183 total OTUs found in the Tina–Bonita microbial mats, with *Chloroflexus aurantiacus* and *Cyanobacterium aponinum* species, the most abundant microbes in the ecosystem. The right donut chart represents the rest of the 181 OTUs with some of the most abundant species indicated. The number of reads per OTU detected by 16S/18S rRNA pyrosequencing is indicated in parentheses.

Also, the neutral pH, and sulphate and chloride contents, among others, of the springs were similar to those previously determined (Prieto-Barajas et al. 2017).

Microbial diversity and phylogenetic analysis

The microbial diversity analyses yielded 84,552 sequences, which formed 186 OTUs. The most diverse group of microorganisms was Bacteria, representing 99.7% of the reads (84,352) and 183 OTUs, whereas Eukarya represented 0.27% (174) and Archaea only 0.03% (26). The bacteria belonged to 22 bacterial divisions, 36 classes, 74 families, 105 genera, and 117 species. Proteobacteria, Chloroflexi, Firmicutes, Bacteroidetes, and Cyanobacteria were the most diverse divisions (Figure 1). However, the most abundant OTUs corresponded to the group of photosynthetic bacteria *Chloroflexus aurantiacus* (52.5%, 44,327 reads) and *Cyanobacterium aponinum* (27.4%, 23,104 reads) constituted 79.9% (67,431 reads) of the total sequences; other abundant bacteria included *Caldilinea aerophila*, *Thermus oshimai*, and *Leptolyngbya* sp. The sulphate-reducing bacteria *Desulfotomaculum* and *Desulforudis* (Firmicutes) and members of the Desulfobacteraceae and Syntrophobacteraceae families (*δ*-Proteobacteria) were also observed in the community. Archaea was only represented by 26 sequence reads and one OTU: *Methanomethylovorans* sp., a methanogenic Euryarchaeota. Algal species were represented by *Antithamnionella spirographidis* and *Ankylochrysis lutea* from the Rhodophyta and Ochrophyta divisions, respectively. Supplementary Figure 1 shows the phylogenetic analysis of the diversity of the OTUs found, which depicts the relationships between the groups of organisms and the lineages observed.

Ecological indexes

The microbial diversity of Tina–Bonita is moderate. The Shannon index showed a moderate-low biological diversity of 1.72 (range 0.5–5), the Simpson 1-D index was 0.644 (0–1), and the equitability (J) index was 0.356; a few microbial groups dominate, so the populations are not evenly distributed. To assess the representativeness of the sampling, we created a rarefaction curve. We found that the number of OTUs formed by the accumulated sequences of the ribosomal genes logarithmically increased until it reached the asymptote, indicating that the samples of the mat biologically represent the community (Figure 2).

Metagenomics and functional analysis

The metagenome of the Tina–Bonita microbial mats, obtained by sequencing on the MiSeq paired-end Illumina platform, was comprised of 6,871,964 readings, which were evaluated and edited. The reads were approximately 240 bp long, with a Phred quality index of Q36–38, and a wide range of GC contents (52% ± 11%).

A total of 2,999,158 hits were obtained in the SEED database. At the first level, genes for the synthesis of amino acids (293,494) and carbohydrates (375,611), and protein metabolism (283,167) were the best represented. At the second level, lysine, threonine, methionine, and cysteine biosynthesis related genes have 82,676 reads or 28.17% of amino acid synthesis, central carbohydrate metabolism (119,377) particularly glycolysis and gluconeogenesis (16,908), and protein biosynthesis (161,387) (Figure 3).

The community primarily used photosynthesis (27,610), or nitrogen (25,957), phosphorus (43,442), sulfur (43,442), or potassium (19,491) metabolism. Several genes are involved in stress responses (50,614) (Figure 4), and the metabolism of aromatic compounds (26,727).

The annotation in the KEGG database allowed to predict the central metabolism of the thermophilic community, which included photosynthesis (15,579 reads), nitrogen (5,781 reads), sulfur (10,823 reads), and methane (11,335 reads) metabolism (Figure 5). The complexity of these metabolisms, as well as the presence of the genes associated with them, is represented in the metabolic pathways, which are interconnected and contribute to the microbial mat physiology. The biosynthesis of antimicrobial compounds included streptomycin biosynthesis (22,004 reads), phenylpropanoid biosynthesis (9,008 reads), and penicillin and cephalosporin biosynthesis (6,432 reads).

Discussion

The neutral waters of the geothermal zone of Araró, with temperatures below 75°C, allow the proliferation of photosynthetic microbial mats. Several thick mats, and even filamentous streamers are formed in the efflux channels. These communities are molded by environmental conditions, like temperature, the presence of chemical compounds, and biological competition, which exert strong selective pressures. The physicochemical parameters, such as the temperature and arsenic content, in the Tina–Bonita complex correlated with the culturable bacterial diversity (Prieto-Barajas et al. 2017). However, the analysis of microbial communities by microbial culture can underestimate the

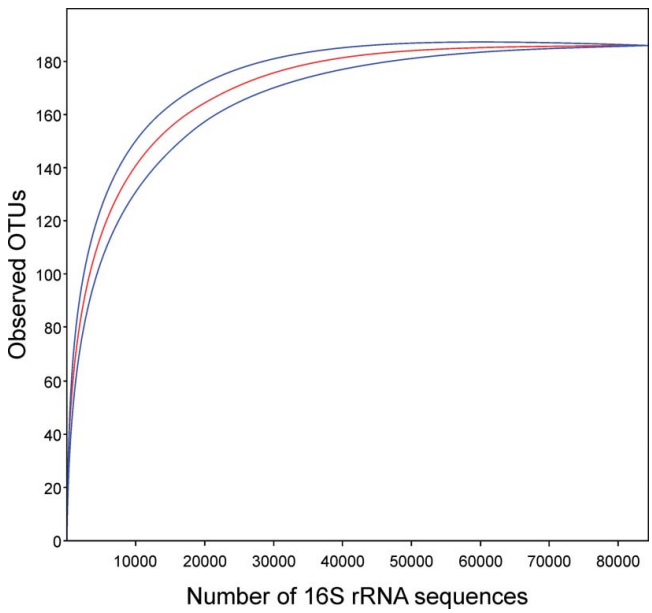

Figure 2. The rarefaction curves as ecological models of the representativeness of the Tina–Bonita microbial community. The curve in red represents the increasing number of rRNA 16S sequences per OTU observed as the line approaches the asymptote. The blue lines represent the 95% confidence interval of the analysis in PAST 3.12.

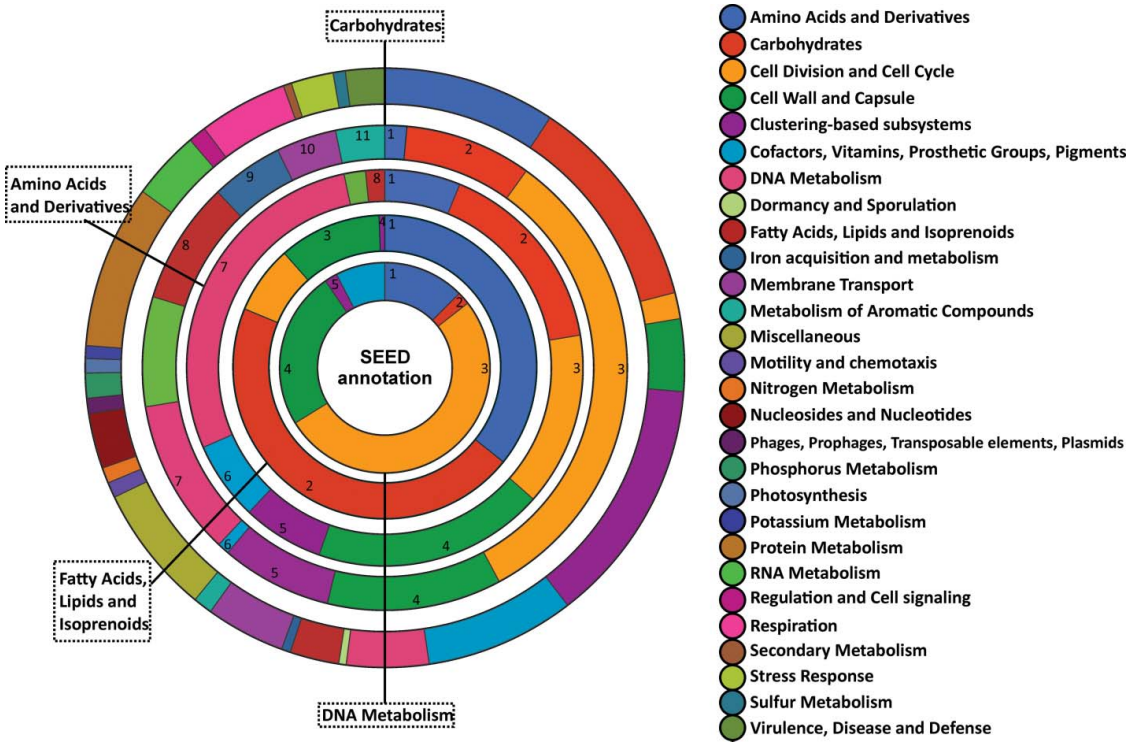

Figure 3. Metagenome annotation in the SEED database through the MG-Rast online server. The circles are numbered from the largest, outer circle to the inside. First circle: general or first level annotation into 28 subsystems. The colors, clockwise, correspond to the subsystems as described in the legend. Second circle: annotation of genes for carbohydrates, including those for 1. amino sugars, 2. CO_2 fixation, 3. central carbohydrate metabolism, 4. di- and oligosaccharides, 5. fermentation, 6. glycoside hydrolases, 7. monosaccharides, 8. one-carbon metabolism, 9. organic acids, 10. polysaccharides, and 11. sugar alcohols. Third circle: genes for amino acids and derivatives, including 1. alanine, serine, and glycine; 2. arginine; 3. aromatic amino acids; 4. branched-chain amino acids; 5. glutamine, glutamate, and aspartate; 6. histidine; 7. lysine, threonine, and methionine; and 8. Proline. Fourth circle: genes for fatty acids, lipids, and isoprenoids, including those for 1. Fatty acids, 2. Isoprenoids, 3. Phospholipids, and 4. Triacylglycerols. Fifth circle: genes for DNA metabolism, including 1. CRISPs, 2. DNA recombination, 3. DNA repair, 4. DNA replication, and 5. DNA uptake and competence. Fields without numbers are null categories.

microbial diversity of such mat communities (Handelsman 2004). In the previous study by Prieto-Barajas et al. (2017), a few groups of culturable bacterial genera were isolated and characterized during the four seasons of the year, in which only Firmicutes, Actinobacteria, and Proteobacteria were detected. In the current study, we also detected those groups, which were among the most abundant groups of bacterial divisions.

Many known thermal environments contain a large proportion of photosynthetic organisms, and the presence of cyanobacteria has been widely reported in microbial mats (Mackenzie et al. 2013; Lacap et al. 2007; Ward et al. 2006). Araró is characterized by the dominance of photosynthetic bacteria, both anoxygenic Chloroflexi and oxygenic photosynthetic bacteria of the Cyanobacteria division. At a proportion of 2:1, *Chloroflexus aurantiacus* and *Cyanobacterium aponinum* constituted almost 80% of the total microorganisms inhabiting the mats. The interaction between Chloroflexi and Cyanobacteria is well-known. Portillo and collaborators (2009) hypothesized a mutualistic relationship between these two bacterial divisions. Chloroflexi–Cyanobacteria microbial mats are developed at concentrations of sulphide not greater than 100 μM and at temperatures up to 72°C, at which *Chloroflexus* is usually photoheterotrophic and yields its position in the upper layers of the laminated mat to the cyanobacteria and, in return, receives nutrients (Hanada and Pierson, 2006). A representative of this symbiotic interaction, *Synechococcus* from the Cyanobacteria division and *Roseiflexus* from the Chloroflexi division have

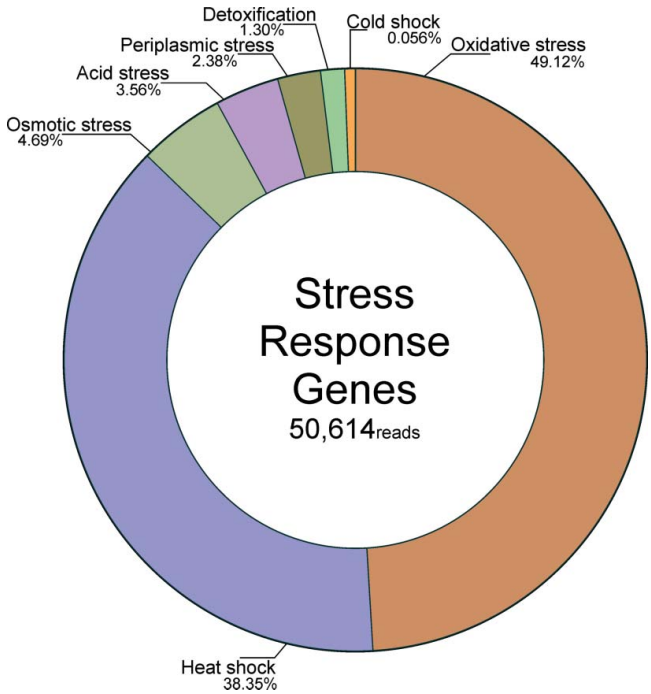

Figure 4. Donut chart showing more than 50,000 reads associated with stress response genes. Oxidative stress and heat shock were among the most abundant reads from the Tina–Bonita microbial mats.

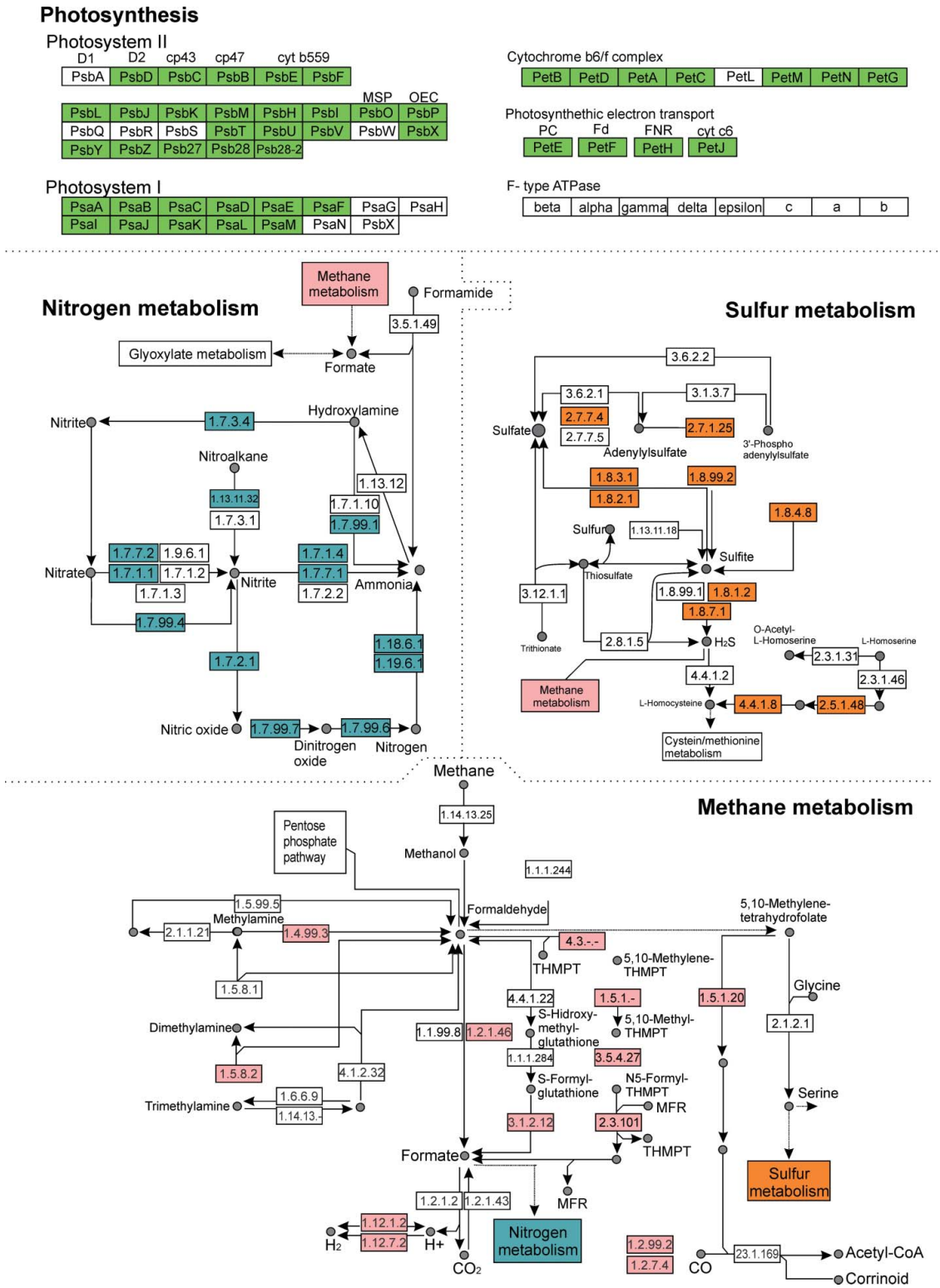

Figure 5. Schematic of some metabolic pathways found in the Tina–Bonita microbial mats. The picture shows the nitrogen, sulfur, and methane metabolic genes, as well as genes involved in photosynthesis, according to the KEGG pathway analysis. The different colored squares indicate that such genetic elements were detected in the mat metagenome.

been reported in Yellowstone National Park in the United States and Boekleung microbial mats from Western Thailand (Portillo et al. 2009; Ward et al. 2006). Although, in other springs, Chloroflexi bacteria are prevalent (Wang et al. 2013). At some hydrothermal springs, Chloroflexi and non-cyanobacteria have been found to form mats (Skirnirdottir et al. 2000). *Cyanobacterium aponinum*, the second most abundant taxon in this thermal microbial community, has been retrieved and characterized from microbial mats of the Euganean thermal springs, located in Padua, Italy (Moro et al. 2007), but it is also dominant in mesophile springs in Iceland (Gudmundsdottir et al. 2015). Among other thermal springs, *Synechococcus* is the

dominant unicellular cyanobacteria (Ward et al. 1998). The primary production of the photosynthetic microbial mats of Araró is probably driven by Chloroflexi, Cyanobacteria, Chlorobi, and α-Proteobacteria (*Rhodobacter*), whereas the Yellowstone National Park mats additionally include photosynthetic Proteobacteria and Acidobacteria (Klatt et al. 2013).

Sulphate-reducing bacteria are predominant inhabitants of microbial mats of both photosynthetic and non-photosynthetic natures (Baumgartner et al. 2006; Michaelis et al. 2002). Their role in the cycling of sulfur compounds, as well as their ecological role as anaerobic heterotrophs, makes them indispensable in the carbon and sulfur cycles (Fike et al. 2008). In Araró, the two families of bacteria of the δ-Proteobacteria subdivision, two genera of Firmicutes, and *Chlorobium* from the Chlorobi division may contribute to sulphate reduction which is an important ecological task. Current efforts in our lab are carrying out to isolate and characterize genetic elements from sulfate-reducing bacteria.

On microbial mats, the close interactions between the microorganisms of different metabolisms couple them within the same biogeochemical cycles. In our study, the genes required to carry out photosynthesis were detected from three categories: photosystem I, photosystem II, and the electron transport chain. Photosynthesis has a major role in the photosynthetic community, and it can be affected by environmental parameters, such as temperature and geochemical composition (Inskeep et al. 2013). Chloroflexi, Cyanobacteria, Chlorobi, and α-Proteobacteria contribute to photosynthesis by producing organic matter and, in some cases, reducing sulfate.

The nitrogen cycle is a process that requires the interplay of many microorganisms (Chan et al. 2015). Therefore, the nitrogen cycle is well-represented in this mat community; genes involved in almost every step of the pathway are present, similar to the findings in microbial mats reported by Bonilla-Rosso et al. (2012) and Chan et al. (2015). The annotation in the KEGG Orthology database showed that the key elements for atmospheric nitrogen fixation to ammonia and several elements for nitrate and nitrite transformation were represented. Many cyanobacterial species are capable of fixing dinitrogen, separating temporarily this processes photosynthesis and fixation (Waterbury 2006).

One of the major metabolic systems in microbial mats involves a large variety of sulfuric chemical compounds (Des marais 2003) derived from the characteristic H_2S gradient that increases considerably from the surface layers to the bottoms of the mats (Harris et al. 2013). The functional microbes responsible for reducing sulfur compounds are the sulfate-reducing bacteria, which are essential for the sulfur cycle (Frigaard and Dahl 2009). Additionally, the metagenome analysis suggests the presence of genes for the transformation of sulfites, sulfates, and hydrogen sulfide, as well as the metabolism of the amino acids cysteine and methionine.

There are other exciting genes annotated in the SEED database in this microbial community. The presence of enzymes for the degradation of aromatic compounds, as well as the synthesis of antibiotics, represents fertile territory for future investigation. No less importantly, several genes coding for stress response proteins were found, and oxidative stress (49.12%) and predicted heat shock genes (38.35%) were the most highly abundant among them. The microbial diversity of the community is relevant for its survival under extreme, stressful conditions, as it facilitates mutualistic, symbiotic, and intimate interactions among its members.

Conclusion

The photosynthetic microbial mats from Araró, situated in the Trans-Mexican Volcanic Belt of México, comprise complex communities represented mainly by photosynthetic microorganisms, in addition to archaea and eukarya. The mats exhibit moderate diversity because of their inequitable distribution of microbes, driven by selective environmental factors, which do not, however, prevent the formation of stable communities. Understanding and comparing the microbial diversity and ecological roles of the hot spring microbial mats from Araró, México, with other spring mats from other regions of the world, will eventually expand our knowledge of microbial life in specific, extreme environments, and highlight the urgency to preserve such ecosystems.

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