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A Metagenomics Insight in the Cyanosphere of Edible Andean Macrocolonies (Llayta)

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Abstract

It is well stablished that some cyanobacterial biomasses have nutritious ingredients and have been consumed for centuries. In South America, macrocolonies of filamentous diazotrophic species, order Nostocales, known as Llayta, can be found at Andean wetlands and have been consumed since pre-Columbian times. *Cyanocohniella* sp. LLY has been identified as a major cyanobacterial member of the Llayta cyanosphere, however, the microflora colonizing these macrocolonies has been poorly explored. Genomic DNA from Gentamycin-treated cyanobacterial filaments purified from the rehydrated biomass of Llayta macrocolonies, were subjected to metagenomics studies to identify resilient members of the accompanying heterotrophic bacterial flora. Here, we report a metagenomics-based identification of five prominent bacterial members belonging to the genera *Mesorhizobium*, *Microvirga*, *Paracoccus*, *Aquimonas*, and *Blastomonas*, tightly adhered to Llayta trichomes. Their metagenome-assembled genomes and information on putative genes and genes clusters involved in primary and secondary metabolism is also provided. We expect this information on Llayta cyanosphere would help to further explore adaptive responses and role of cyanobacteria macrocolonies in ecosystem processes under the stressful environmental conditions prevailing at the Atacama Desert highlands.

Keywords: Andes wetlands; Cyanosphere; Edible cyanobacterial macrocolonies; Llayta; Metagenomics; Metal resistance genes; Microbiota; Nostocales

Introduction

Consumption of edible macrocolonies of filamentous cyanobacteria, locally known as Llayta, is a centenary Andean alimentary practice in South America [1-3]. Llayta macrocolonies can be found at Andean wetlands above 3,000 m of altitude, at sites not always readily accessible [4,5], but it is available nowadays as a dried natural product at food markets in northern Chile and southern Peru [6]. Also, Llayta is considered a safe natural food ingredient based on its biochemical content, absence of cyanotoxins, ethnographic testimonies, and absence of negative epidemiological evidence [4-7].

Filamentous diazotrophic cyanobacteria are ubiquitous to almost any ecosystem on Earth, including some under severe environmental conditions. Trichomes containing vegetative cells and N_2-fixing heterocyst develop during the life cycle of diazotrophic *Nostoc* species and can form green to brown sheet-like or spherical macrocolony, depending up-on the species, dehydration state, and location [8,9]. In natural environments, colonial and filamentous cyanobacteria can be colonized by microorganisms emplaced around, within or outside the host colony, with varied composition [10-14], and involved in biogeochemical cycles, ecosystem services, and algicidal or stimulatory effects [10,14-16].

Recently, Vilo et al. [16] reported an improved metagenomics analysis identifying *Cyanocohniella* sp. LLY as the potential cyanobacterium responsible for forming Llayta macrocolonies at the Andes wetlands, providing the first Metagenome-Assembled Genome (MAG) for this genus. Nonetheless, Llayta cyanosphere is still a pending issue. The identi-fication and draft genome of a *Bacillus* bacterium from the microbiota associated to Llayta colonies [17] was the first approach to address physiological relationships within the Llayta microbiota and to gain insights into the survival and adaptive strategies to dryness, metals, metalloids, and UV radiation, among other prevalent extreme environmental con-ditions at the Andes wetlands.

Metagenomics is proper culture-independent tool that allows insights into the composition and genomic capabilities of the microbial community from natural settings [18-20], and prospection of natural products [18-21]. To improve our under-

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standing on Llayta cyanosphere, we focused our metagenomics-based study on the resilient bacterial microflora associated with isolated, Gentamycin-treated Llayta filaments. Here we present the identification of microbial taxa tightly adhered to Llayta trichomes, the genome recon-struction of five prominent bacterial members, the identification and annotation of functional genes, and an insight into their metabolic capabilities.

Materials and Methods

Isolation of Llayta Filaments, Growth Conditions and Antibiotic Treatment

Dry biomass of Llayta macrocolonies were obtained during 2015 at the major farmer´s food market in Arica, Chile. Filament isolation procedures have been previously described [17]. Briefly, rehydrated Llayta samples were grown and enriched in liquid, ni-trogen-free Arnon mineral medium [4,5,22]. Aliquots were seeded in agar plates, isolated filaments were retrieved under a stereo microscope, grown in fresh medium as above, harvested after 3 weeks at the exponential growth phase, washed with fresh medium, and recovered as a pellet by centrifugation at 4,000 x g for 5 min, at room temperature. Our metagenomics studies were focused on genomic DNA recovered from bacteria closely associated to isolated Llayta trichomes. Then, metagenomics analyses were conducted on trichomes previously incubated with Gentamycin in the presence of carbon sources to stimulate growth of heterotrophic bacteria and in darkness to slow down cyanobacterial metabolic activity. Approximately, 10-15 mL of cultured Llayta filaments were collected at the exponential growth phase, washed with fresh medium, and suspended in 20 mL of fresh Arnon medium containing Gentamycin (1 mg/mL; Sigma Aldrich, Chile), Casamino acids (1.6 mg/mL; Sigma Aldrich, Chile), and D-glucose (0.8 mg/mL; Sigma Aldrich, Chile), and incubated for 48h in darkness at 30°C, and gently stirred (120 rpm). After this treatment, microbial contamination of Llayta filaments decreased by nearly four orders of magnitude (Gentamycin was the most efficient antibiotic tested). The biomass was recovered by centrifugation and used to extract total genomic DNA.

DNA Extraction and Sequencing

Total genomic DNA from the filament pellets was extracted with Ultra Clean Micro-bial DNA isolation kit (MoBio Labs. Inc., Carlsbad, CA, USA), following the manufactur-er´s instructions. DNA quality was evaluated by electrophoresis in 0.8 % agarose gel and quantified photometrically at 260 nm. The Llayta metagenome was sequenced via MiSeq sequencing technology using shotgun paired-end libraries, with an average insert size of 250 bp. Reads had an average length of 300 bp, with good quality scores, as evaluated by the FastQC program (version 0.10.0). The sequencing produced a total of 17,137,246 reads. Sequencing reads are available at the Sequence Read Archive (SRA) with accession num-ber SRR17916224. The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAKOMP000000000, JAKOMQ000000000, JAKOMR000000000, JAKOMS000000000, JAKOMT000000000, and JAKOMU000000000.

Bioinformatic Analysis

The metagenomic sequences were submitted to the Rapid Annotation using Subsys-tems Technology for Metagenomes (MG-RAST) web server [23] for a taxonomic and func-tional assignment using default parameters. In addition, metagenomic assembly was done using MEGAHIT assembler v.1.2.9 [24], and binning was conducted using the PATRIC web server [25]. The complete genome was annotated using the Rapid Annotations using Subsystem Technology (RAST) server version 4.0. Secondary metabolites were searched with PRISM version 4.4.5 [26] and AntiSMASH version 6.1.1 software [27]. In addition, in-house BLAST analysis was done against customized metal resistance genes databases.

Results and Discussion

During our work, an abundant microflora with high bacterial titers was detected in attempts to isolate and purify axenic Llayta filaments; however, incubation of isolated Llayta filaments with Gentamycin decreased such titers by nearly four orders of magnitude. Then, we focused our metagenomics-based study on the resilient bacterial microflora associated with isolated and Gentamycin-treated Llayta filaments to improve our understanding on Llayta cyanosphere.

Genomic DNA from Gentamycin-treated cyanobacterial filaments, purified from the rehydrated biomass of Llayta macrocolonies, were subjected to metagenomics studies to identify resilient members of the accompanying heterotrophic bacterial flora, construct the corresponding metagenomic-assembled genomes, and gain insights into their functional metabolic capabilities. We identified the antibiotic-resilient bacterial community associ-ated to Llayta filaments, reconstructed the genomes of five prominent bacteria, and de-tected putative biosynthetic genes related to primary and secondary metabolism and adaptive stress strategies.

Microbial Diversity in Llayta Trichomes

Taxonomic assignments obtained from metagenomics analyses of shotgun meta-genome showed that the microbiota associated with Llayta filaments was dominated by bacteria (99%), while representatives of the domains Archaea (0.2%) and Eukarya (0.1%) were present at a much lower extent. Predominant bacteria in Llayta trichomes belong to the phyla Proteobacteria (82%) and Cyanobacteria (16%) (Figure 1). Dominant genera were *Xanthomonas* (38%), *Stenotrophomonas* (15%), *Methylobacterium* (9.3%), *Nostoc* (40%), *Anabaena* (26%), and *Nodularia* (21%). Thus, *Xanthomonas* (38%) and *Nostoc* (40%) were the primary genera (Figure 1).

Five bacteria associated with isolated Llayta filaments were identified by metagenomics analysis: two nitrogen-fixing Alphaproteobacteria (genera *Mesorhizobium* and *Microvirga*), one aerobic photoheterotrophic Alphaproteobacteria (genus *Blastomonas*), one Gammaproteobacteria (genus *Aquimonas*) and one nitrogen denitrifying bacterium (genus *Paracoccus*). The summary statistics for the assembled MAGs and the corresponding phy-logenetic analyses by the Maximum Likelihood

Table 1: Statistics for assembling and binning of the reconstructed metagenomes of prominent bacteria members associated with Llayta filaments.

Genus	Sequence size	Contigs number	N50 value	Completeness (%)	Contamination (%)
Aquimonas (gamma-proteobacterium)	4,464,829	80	155,856	98.3	1.4
Blastomonas (aerobic photoheterotrophic alphaproteobacteria)	3,312,218	641	7,513	98.1	15.2
Mesorhizobium (N_2-fixing alpha-proteobacterium)	4,745,523	165	427,465	91.3	11.1
Microvirga (N_2-fixing alpha-proteobacterium)	3,719,434	70	273,976	98.4	2.8
Paracoccus (nitrogen denitrifying bacterium)	4,116,456	107	139,894	100	0.0

Table 2: Number of genes associated with metal resistance in Llayta MAGs.

Metal resistance	*Blastomonas*	*Aquimonas*	*Mesorhizobium*	*Microvirga*	*Paracoccus*
Copper homeostasis and tolerance	10	5	13	12	11
Cobalt-zinc-cadmium resistance	6	4	6	7	8
Mercuric resistance	4	2	4	4	4
Resistance to chromium compounds	1	1	1	2	1
Arsenic resistance and detoxification	8	5	16	13	8

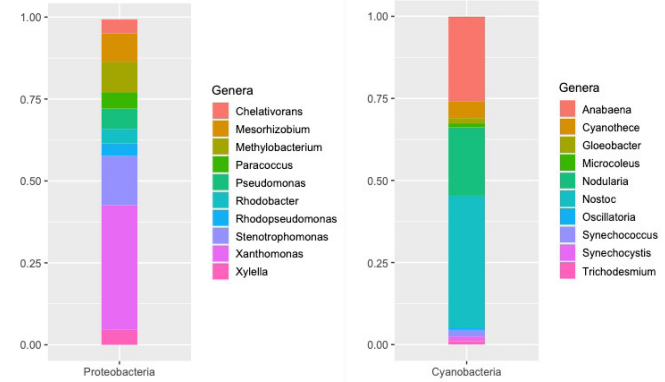

Figure 1: Genus-level relative abundance of the ten dominant Proteobacteria and Cya-nobacteria found in the Gentamycin-treated Llayta filaments.

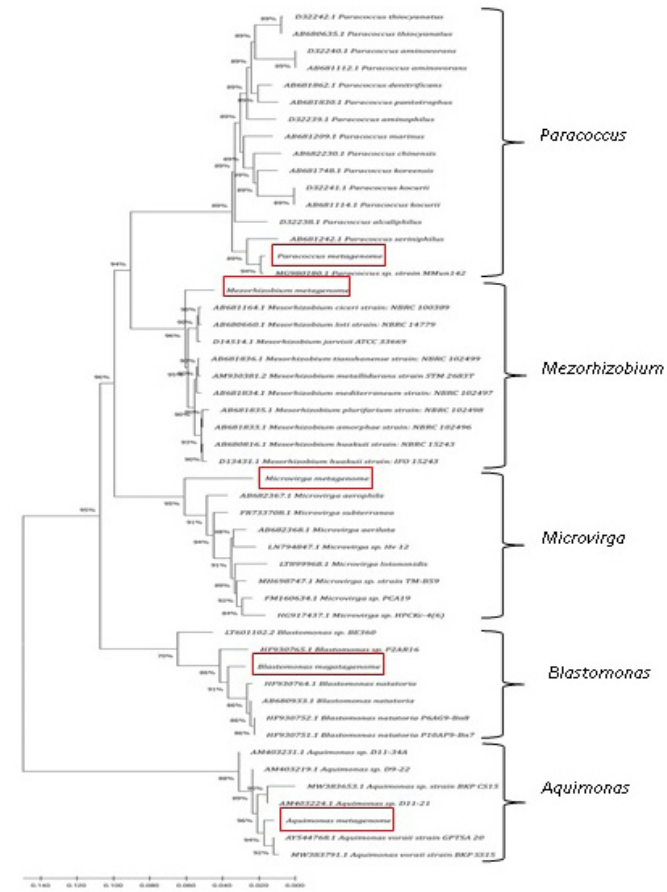

Figure 2: Phylogenetic tree of 16S rRNA gene depicting clades of the five assembled bacteria genera, *Aquimonas*, *Blastomonas*, *Microvirga*, *Mesorhizobium*, and *Paracoccus*.

method based on 16S rRNA gene se-quences are shown in Table 1 and Figure 2, respectively.

Paracoccus was the only bacterial genus associated with Llayta filaments that coincides with *Paracoccus* strain B50 found as a culturable bacterial isolate associated to *Nodularia spumigena* filaments, which negatively affected the cyanobacterium growth [14]. We also identified a member of the genus *Anabaena* on the resilient microflora accompanying the Llayta cyanosphere, in agreement with previous observations reported on *Nostoc* colonies from Parinacota wetlands [12]. Recent studies have reported on the diversity and em-placement of heterotrophic bacteria in *Nostoc* macrocolonies. Secker et al. [11] showed ab-sence of bacteria at the inner matrix of *Nostoc* macrocolonies from ephemeral wetlands in New Zealand, while high bacterial diversity was found at their external surface, enriched with members of the genus *Sphingomonas*. Conversely, a different bacterial composition was found at the inner matrix, outer layer, and the littoral zone of *Nostoc* macrocolonies from Chungará Lake in northern Chile [12]. Comparatively, and using metagenomics, multiple taxonomic markers, and microscopic approaches, Satjarak et al. [13] reported high taxonomic diversity (cyanobacteria, microalgae, and anoxygenic bacterial genera) on the accompanying epimicrobiota of macroscopic dark-brown sheets of *Nostoc* from standing water pools at Parinacota, Lauca National Park, in northern Chile.

Metal Resistance Capabilities of the Llayta MAGs

The reconstructed Llayta MAGs were analyzed with the RAST server for genome an-notation based on subsystem annotations. A total of 156 putative metal resistance genes was also observed in the Llayta microbiota (Table 2) indicating the putative adaptability of microbial communities to the volcanic-rich activity of the Andes Mountains. We observed genes for copper homeostasis and tolerance to mercury and chromium compounds (Table 2). All MAGs analyzed showed the presence of components of the cop operon associated with cytoplasmic copper levels control [28]. *Blastomonas* MAG contains genes coding the copper resistance protein B, which showed a 79% identity with *Blastomonas* sp. protein (accession number WAC22814.1). Copper resistance protein B has been described to promote copper sequestrating at the outer membrane. *Microvirga* and *Paracoccus* MAGs had genes coding for protein CopG associated with copper binding [28]. Microvirga MAG showed a 100% identity with conserved domain DUF411 from *Microvirga* sp. protein (accession number MCG6123028.1), which has been classified as a CopG-like protein [29]. *Paracoccus* MAG showed three copies of CopG gene, with 100% identity to *Paracoccus* sp. DUF411 domain-containing proteins, and metal binding proteins (accession numbers: MCG6113278.1, MCG6112398.1, and MCG6112409.1). Genes coding for proteins of the cut family involved in copper homeostasis (uptake, storage, delivery, and efflux) [28] were observed. MAGs from *Blastomonas*, *Microvirga*, *Mesorhizobium*, and *Paracoccus* contained genes coding for CutE protein related to copper storage and transport [30] (100% identity with *Blastomonas* sp., accession number MCG6119876.1; 100% identity with *Microvirga* sp., accession number MCG6121847.1; 100% identity with *Mesorhizobium* sp., accession number MCG6114269.1; and 100% identity with *Paracoccus* sp., accession number MCG6110613.1, respectively). All MAGs showed the presence of czc operon associated with cobalt-zinc-cadmium resistance and export pathways for Cd^{2+}, Zn^{2+}, and Co^{2+} ions [31]. Finally, all MAGs had putative capabilities for coding CzcD protein involved in reg-ulating the Czc system and CzcA protein, a metal cation efflux transporter [30,31]. Regarding arsenic resistance, arsC gene and arsenical-resistance protein ACR3 were present all MAGs. The arsC gene translates into arsenate reductase enzyme, which catalyzes the

reduction of arsenate to arsenite to be extruded from the cell by arsenite permeases such as ACR3 [32,33].

Secondary Metabolism of the Llayta MAGs

Secondary metabolism analysis of the five reconstructed MAGs showed specific adaptations to overcome some of the environmental conditions found at the Atacama Desert highlands. *Paracoccus* MAG contained the gene sequence associated with ectoine synthase [34], showing 100% identity to ectoine synthase gene from *Paracoccus hibiscisoli* (accession number WP_136857538). *Blastomonas* MAG showed genes coding for lasso peptides B1 and B2. MAGs from *Aquimonas*, *Mesorhizobium*, and *Paracoccus* showed the presence of genes coding for phytoene synthase and lycopene cyclase.

On putative carotenoid biosynthetic capabilities [35], lycopene cyclase and phytoene synthase coding genes in *Aquimonas* MAG showed 100% identity to lycopene cyclase fam-ily protein and phytoene synthase family protein from *Aquimonas* sp. (accession numbers MCG6118585 and MCG6118584, respectively). Phytoene synthase gene sequence from *Mesorhizobium* MAG showed 100% identity to phytoene/squalene synthase family protein from *Mesorhizobium* sp. (accession number MCG6115296). In *Paracoccus* MAG, the phy-toene synthase and lycopene cyclase coding gene showed 100% sequence identity to phy-toene synthase protein, and lycopene beta cyclase CrtY reported for *Paracoccus* sp. (acces-sion numbers MCG6110761 and MCG6110759, respectively).

The inferred functional capabilities of the Llayta microbiota from their metagenomic content may be important to improve our understanding on the adaptive responses of this microbial community to survive and proliferate under the stressful environmental conditions found in the Andean wetlands. For instance, we found evidence of ectoine production on *Paracoccus* MAG, indicating specific adaptations to high salinity and dessication. Ectoine is a known natural compound that has been associated to osmoregulation in bacteria [34]. Also, carotenoids biosynthesis was observed in *Aquimonas*, *Mesorhizobium*, and *Paracoccus* MAGs, indicating the need for protection against UV radiation, a well-known hazard in the Atacama Desert, particularly on the Andean plateau [36,37]. In addition, metalloid resistance seems to be a common trait among Atacama Desert microorganisms, which may be critical for those microbial communities to acquire arsenic reduction and extrusion, copper sequestration, and cobalt, zinc, and cadmium outer extrusion.

Conclusions

Members of the gentamycin-resilient microbial community associated with cyanobacterial macrocolonies of Llayta filaments were identified using a culture-independent approach. Metagenomics analyses allowed the reconstruction and draft genomes for five prominent bacteria. This information provides an insight into microbial functional capabilities, biosynthetic pathways, and adaptive strategies to the environmental conditions at high-altitude Andean wetlands. Beyond the well-known, centuries-old consumption of Llayta macrocolonies, their cyanosphere opens new opportunities for biotechnological applications. Finally, the cyanosphere associated to filaments of edible Llayta macrocolonies is another example of the microbial richness present at the Atacama Desert; such microbiome deserves extensive research to better understand its role in drylands ecosystem processes [38,39].

Author Statements

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Data Archiving

The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAKOMP000000000, JAKOMQ000000000, JAKOMR000000000, JAKOMS000000000, JAKOMT000000000, and JAKOMU000000000.

Data Availability

Data will be made available on request.

Credit Authorship Contribution Statement

Benito Gómez-Silva: Conceptualization, Investigation, Writing-original draft preparation, Writing-review and editing, Project administration, Funding acquisition. **Claudia Vilo**: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing-original draft preparation, Writing-review and editing. **Alexandra Galetovic**: Methodology, Validation, Investigation, Data curation, Writing-review and editing. **Qunfeng Dong**: Validation, writing-review and editing. All authors have read and agreed to the published version of the manuscript.

Declaration of Compiting Interest

The authors declare no conflict of interest.

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