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Article

Metagenome-based Exploration of Bacterial Communities Associated with Cyanobacteria Strains Isolated from Thermal Muds

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Abstract: Cyanobacteria constitute pioneer colonizer of specific environments whom settlement in new biotopes precedes the establishment of composite microbial consortia. Some heterotrophic bacteria constitute cyanobacterial partners that are considered as their cyanosphere, being potentially involved in mutualistic relationships through exchange and recycling of key nutrients, and sharing of common goods. Several non-axenic cyanobacterial strains have been recently isolated along with their associated cyanosphere from the thermal mud of Balaruc-les-Bains (France) and the biofilms of the retention basin where they develop. The community structure and relationships among members of the isolated cyanobacterial strains were characterized using a metagenomic approach combined with taxonomic and microscopic description of the microbial consortia. Results provide insights into the potential role and metabolic capabilities of microorganisms of thermal mud-associated cyanobacterial biofilms. Thus, the physical proximity, host-specificity and complimentary functions advocate for their complementarity between cyanobacteria and their associated microbiota. Besides these findings, our results also highlight the great influence of the reference protein database chosen when performing functional annotation of the metagenomes from organisms of the cyanosphere and the difficulty of selecting one unique database that appropriately cover both autotroph and heterotroph metabolic specificities.

Keywords: cyanobacteria; cyanosphere; heterotroph bacteria; metagenomics; functional redundancy

1. Introduction

Cyanobacteria belong to an ancient group of photosynthetic prokaryotes presenting a broad range of cellular strategies, physiological capacities and adaptations that support their occupation of diverse environments worldwide [1]. Cyanobacteria thus constitute pioneer colonizers of specific environments, and their settlement in new biotopes precedes the establishment of complex microbial consortia [2-3]. Indeed, these autotrophic micro-organisms rarely live in isolated populations but thrive into complex communities where intricate interactions take place [4].

Cultures of isolated cyanobacterial strains typically contain coexisting heterotrophic bacterial partners that are considered as their phycosphere, so-called cyanosphere [5]. Although axenic cultures of cyanobacteria can be achieved, cyanobacterial cultures comprising associated coexisting bacterial populations are more stable and have more robust and longer lifespans than axenic ones [6-7]. Interactions between photoautotroph and heterotroph microorganisms in both natural environments and laboratory conditions are

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thought to be driven by mutualistic relationships through exchange and recycling of key nutrients, and sharing of common goods [8]. However, these interaction networks comprise diverse heterotrophic bacteria associated with few photoautotrophic taxa, among which a myriad of inter-species relationships probably rather range from cooperative to competitive, all not being necessarily synergistic. Exploring the structure and role of phototroph-associated microbiota is becoming a topic of major interest, with the potential to reveal relevant emerging properties of these entangled communities, including in the production of molecules of interest [9].

Mud from thermal baths has long been recognized as a healing treatment for arthro-rheumatic diseases, and is colonized by cyanobacterial mats presumed to produce bioactive compounds that can significantly contribute to the health benefits of the cure [10]. Interestingly, in the absence of mud, there is usually no development of a cyanobacterial biofilm in the aquatic substrate despite the addition of nutrients, suggesting that this biofilm occupies a highly specific ecological niche [5]. Cyanobacteria occupy the top layer of said microbial mats, allowing them to harvest light for photosynthesis, and are essentially, but not exclusively, aerobic organisms. Most, if not all, of these cyanobacteria have the ability to secrete an exopolysaccharide sheath around groups of trichomes forming supracellular rope-like structures called bundles that can attach to mud particles [11]. By stabilizing and structuring this micro-habitat, biofilms can mature and become colonized by a diverse bacterial community constituting a specific cyanosphere [12-13]. Microbial mats retrieved in temperate temperature thermal muds occur in geographically distant locations and mostly include non-heterocytous filamentous cyanobacteria, predominantly *Microcoleus* and *Oscillatoria*, together with small spherical *Chroococcus*-like cyanobacteria and some eukaryotic micro-algae (mainly Diatomophyceae) [13-16]. Little is still known about the heterotrophic bacteria associated with cyanobacterial mats from thermal muds, despite that this biofilm-forming microbial community is characterized by high primary productivity and high rates of N_2 fixation [17-18].

Recently, studies have investigated the structure of cyanosphere communities using omics-based methods such as metagenomics, and have suggested the occurrence of intertwined metabolic pathways between cyanobacteria such as *Microcystis* and surrounding bacteria [19]. Few studies have yet provided metagenomics-based analyses of the cyanosphere in freshwater ecosystems, and observed a high complementarity between the metabolic potential of the cyanobacterial and the surrounding bacterial partners [20].

In the present study, we aimed to characterized the community structure and relationships among members of a cyanobacterial biofilm, using a metagenomic approach combined with taxonomic and microscopic description of the microbial consortia constituting the cyanosphere. Nine non-axenic cyanobacterial strains were used, recently isolated with their associated cyanosphere from biofilms occurring in the retention basin of the thermal mud of Balaruc-les-Bains (France) [16]. Results provide insights into the potential role and metabolic interaction of bacteria of thermal mud-associated cyanobacterial biofilms.

2. Materials and Methods

2.1. Field sampling and cyanobacteria isolation

Mud and biofilm samples were collected in 2014 between the 28thApril and 13th October twice monthly (Fig. 1) from the mud maturation basin Balaruc-Les-Bains's Thermes (43°26'44.0"N; 3°40'29.6"E) for phytobenthic community analysis and cyanobacterial strain isolation (Fig. 1), as previously described [16].

The retention basin maintains a water level above the mud and the continuous evacuation of the water surplus to the outside to avoid any risk of overflow (Figure 1d). The conditions of temperature, light and nutrient inputs are met to ensure that the process of mud maturation takes place. Two types of sampling were carried out: *i*) from biofilm covering the mud in the maturation basin, and *ii*) from epilithic biofilm on the walls of mud basin with the more extensive algae development.

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Samples collected the 07-24-2014 and the 08-04-2014 were inoculated on solid medium (5 or 10 g.L^{-1} of agar) with medium Z8 and Z8-salt [21]. Isolations were carried out by repeated transfers of single cells or filaments, on solid or liquid media (at least three times) under an inverted microscope (Nikon ECLIPSE TS100). Growing clones were then cultured in 25 cm^3 culture flasks (Nunc, Roskilde, Denmark) containing 10 mL of Z8. Cyanobacteria strains were maintained in the Paris Museum Collection (PMC) [22] at 25°C, using daylight fluorescent tubes providing an irradiance of 12 μmol photons.cm^{-2}.s^{-1}, with a photoperiod of 16h:8h light:dark. Isolated strains and cultures were all monoclonal and non-axenic. A full taxonomical analysis of the nine cyanobacterial strains was described elsewhere [16] and correspond to: *Planktothricoides raciborskii* PMC 877.14, *Laspinema* sp. PMC 878.14, *Microcoleus vaginatus* PMC 879.14, *Lyngbya martensiana* PMC 880.14, *Nostoc* sp. PMC 881.14, *Aliinostoc* sp. PMC 882.14, *Leptolyngbya boryana* PMC 883.14, *Dulcicalothrix* sp. PMC 884.14, and *Pseudochroococcus coutei* PMC 885.14. Cultures were transplanted in fresh Z8 liquid medium every six weeks to be maintained in growing phase when sampled for analyses.

2.2. Morphological and ultrastructural analyses of cyanospheres

Morphological analyses of the cyanosphere were carried out using an Axio Imager M2 microscope (ZEISS) equipped with an AxioCam MRc Color camera and the ZEN software (ZEISS).

The cyanospheres of each strain were analyzed by transmission electron microscopy (TEM) as described by Parveen et al. [23], with few modifications [16]. Sample strains were centrifuged, fixed in glutaraldehyde/formaldehyde buffer, washed with Sorensen phosphate buffer, post-fixed with osmium tetraoxide, dehydrated in a graded ethanol series, the samples were embedded in EPON resin and sectioned at 0.5 μm, strained with uranyl acetate and placed on copper plate for observation on TEM (HitachiHT- 7700, Japan). Images were taken using a digital camera (Hamamatsu, Japan).

Each cyanobacterial strains were also analyzed by scanning electron microscopy (SEM). Cultured cyanobacterial cells and/or filaments were centrifuged (10 min; 15,000 rpm), and fixed, dehydrated in a graded ethanol series (50, 70, 90, and 100%) and critical point-dried in liquid CO_2 (Emitech K850, Quorum Technologies), and coated with 20 nm of gold (JEOL Fine Coater JFC-1200) as previously described [16]. The samples were then examined with a Hitachi Scanning Electron SU3500 Premium.

2.3. Genomic DNA extraction

DNA extraction from the nine non-axenic cyanobacterial strains and five mud samples was carried out with a ZymoBIOMICS DNA mini kit (Zymo Research, CA) following manufacturer's protocol. Mechanical lysis was carried out using a bead-beater (TissueLyser II, Qiagen) for 6 min at maximum speed. An extraction blank was performed as control. DNA quality and quantity was checked with Qubit (Thermo) apparatus.

2.4. Molecular phylogeny of cyanobacterial strains

Amplification of the 16S rRNA-encoding gene of the 9 cyanobacterial strain cultures was done using primers and PCR programs as in Cellamare et al. [24], and sequenced by GenoScreen (Lille, France), as previously described in Duval et al. [16]. Briefly, partial 16S rRNA sequences of minimum 1,398 base pairs (bp) were assembled and corrected using MEGA7 version software, and aligned, including the new sequences and those available in GenBank belonging to Oscillatoriales, Synechococcales, Nostocales and Chroococcales. *Gloeobacter violaceus* PCC 7421 was chosen as outgroup (n = 129). Phylogenetic analyses were performed using maximum likelihood (ML), using the MEGA7 version software [25], with 1000 bootstrap replicates.

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2.5. Taxonomic composition of mud samples and cyanosphere

The nine cyanobacterial strains were cultured in Z8 medium [26] at 25°C in 250-mL Erlenmeyer's vessels, with a photon flux density of 12 μmol.m^{-2}.s^{-1} and a 12:12h light:dark cycle. Total DNA extraction was carried out using a ZymoBIOMICS DNA mini kit (Zymo Research, CA). The V4-V5 variable region of the 16S rRNA-encoding gene was amplified from extracted DNA of the nine non-axenic cyanobacterial cultures and five mud samples using 515F and 906R primers [27], and sequenced (Illumina MiSeq paired-end, 2x250 bp, GenoScreen, France). Paired-end reads (representing at least 14,865 raw reads per libraries) were demultiplexed, quality controlled, trimmed and assembled with FLASH [28]. The obtained sequences, representing at least 25,674 reads per library, were further analyzed using the QIIME 2 2020.11 pipeline [29]. Chimeras were removed and sequences were trimmed to 367 pb then denoised using the *DADA2* plugin, resulting in Amplicon Sequence Variants (ASVs) [30]. ASVs were affiliated from the SILVA database release 138 using the *feature-classifier* plugin and *classify-sklearn* module [31]. Sequences assigned as Eukaryota, Archaea, mitochondria, chloroplast and unassigned were removed from the dataset then the sample dataset was rarefied to a list of 1,818 sequences. Alpha- and beta-diversity analyses were performed using the MicrobiomeAnalysis platform [32]. Principal coordinates analyses (PCoA) based on unweighted UniFrac distances were performed to examine the dissimilarity of bacterial composition between groups. Among-group variance levels were compared using PERMANOVA (1,000 permutations).

2.6. Metagenome sequencing and assembly of cyanobacteria's strains microbial consortia

Total DNA extracts of the nine cyanobacterial strain cultures were sequenced using both 2x250bp Illumina MiSeq 2500 and Single-Molecule Real-Time PacBio RSII platforms (GenoScreen, France). Scaffolds were assembled from MiSeq and PacBio reads using SPAdes-based Unicycler hybrid-assembler, with default parameters [33-34]. Nodes from assembly graphs were clustered using MyCC (k-mer size=4, minimal sequence size=1000) and taxonomically annotated using Contig Annotation Tool [35]. 16S rRNA-encoding genes were extracted from these nodes using Metaxa 2 and annotated using ACT [36]. All contigs were pairwise-aligned using Megablast (E-value≤1.e^{-10}), and all sequences sharing a ≥98% similarity on the shortest sequence were considered as coming from the same genome. Congruent data between these diverse methodologies (binning with MyCC and Blast with ACT) allowed to characterize the genomes of each cyanobacterium and its associated heterotrophic bacteria.

The genome assemblies of the cyanobacteria and their respective main co-cultured heterotrophs were integrated in the MicroScope platform v3.14.1 (https://mage.genoscope.cns.fr/microscope/home/index.php) [37]. For each genome, their respective completeness and contamination, as well as their number of CDS and %GC were estimated with CheckM V.1.13 using default parameters [38]. Function annotation was performed using the MicroScope platform using KEGG and MetaCyc databases [39], and results of these annotations were analysed by PCA, volcano plot with penalized t-test and heatmap with hierarchical classification (Euclidean distance) using MetaboAnalyst 5 platform [40].

The sequences of Metagenome-Assembled Genomes (MAGs) have been deposited in GenBank under the Bioproject accession number PRJNA686238, PRJNA686242, PRJNA686244, PRJNA686257, PRJNA686258, PRJNA686260, PRJNA686261, PRJNA686262 and PRJNA686263 (Biosample numbers are specified in supplementary table S1). The nine PMC strains are available from the collection of Cyanobacteria and Microalgae (PMC-ALCP) located in the Muséum national d'Histoire Naturelle (Paris, France, https://mcam.mnhn.fr/fr/collection-de-cyanobacteries-et-microalgues-vivantes-pmc-alcp-470) [22].

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3. Results and Discussion

3.1. Cyanobacteria isolates and their associated bacterial heterotrophs

The thermal mud (a.k.a. the peloid) from Thermes of Balaruc-Les-Bains, one of the oldest thermal centers in France, was mostly colonized by cyanobacteria and microalgae (Fig. 1). To characterize the cyanobacteria living in these muds and their potential metabolites production, nine strains representative of the algal community of this environment [14-15] were isolated from the water column and biofilms of the retention basin collected the 07-24-2014 and the 08-04-2014 (Fig. 1).

Commonly-employed cyanobacteria isolation techniques from field samples, such as those presently performed, decrease the number of associated microorganisms, but also preserved microbes strongly attached to the cyanobacterial sheaths [41]. Thus, isolation of cyanobacteria usually results in non-axenic cultures, consisting of reduced microbial consortia, that can be considered as "*in vitro* blooms" in the sense that the culture conditions provide essential nutrient, temperature and light supplies for cyanobacteria to survive, growth or massively proliferate and dominate their microbial environment, as it occurs in nature [42]. Given this, it is relevant to investigate heterotrophic bacteria that are co-isolated with the cyanobacteria in cultures to question their rather opportunistic or mutualistic relationships.

A

Morphological taxa	Mud	Biofilm
Chlorophyceae (n=3)		
Cosmarium sp.		x
Scenedesmus spp.		x
Sphaerocystis sp.		x
Euglenophyceae (n=1)		
Euglena sp.	x	
Diatomophyceae (n=2)		
Navicula sp.1	x	x
Navicula sp.2		x
Cyanobacteria (n=9)		
Calothrix sp.*a		x
*Chroococcus minutus*b		x
Geitlerinema sp.		x
*Leptolyngbya foveolara*c	x	x
*Lyngbya martensiana*d	x	x
Nostoc spp.*e,f	x	x
*Phormidium autumnale*g		x
*Phormidium breve*h	x	
*Phormidium nigrum*i	x	
Total	7	12

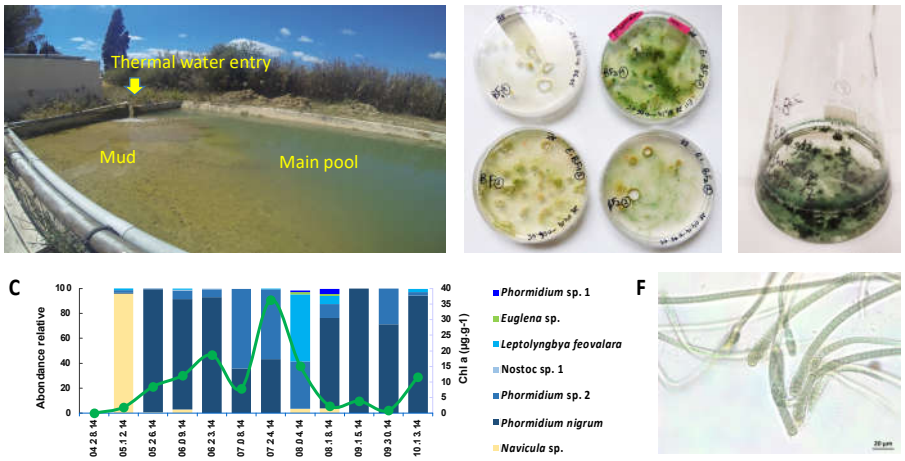

Figure 1. The algae and cyanobacteria community colonizing the Balaruc's thermal mud. A) the micro-algae and cyanobacteria present in surface biofilm and in the mud of the maturation basin of Balaruc's thermal mud. * indicates that this initial taxonomic identification was further re-evaluated based on polyphasic analyses of the nine strains, accordingly: a = *Dulcicalothrix* sp., b = *Pseudochroococcus coutei*, c= *Leptolyngbya boryana*, d = *Lyngbya martensiana*, e = *Nostoc* sp., f = *Aliinostoc* sp., g = *Microcoleus vaginatus*, h = *Laspinema* sp., i = *Planktothricoides raciborskii*. **B**) general view of the mud maturation basin during the sampling (07/24/2014). **C**) Monitoring of the mud algal and cyanobacterial succession during 2014 spring and summer seasons. **D**) Solid medium culture of various cyanobacteria collected from the Balaruc's thermal mud (07/24/2014). **E**) Liquid media culture of a monoclonal strain. **F**) Microscopic view of the *Dulcicalothrix* strain PMC 884.14.

Nine monoclonal strains belonging to the different cyanobacterial taxa that colonize Balaruc's thermal mud were selected for taxonomic characterization [16], together with the characterization of their associated microbial communities. Based on morphological, ultrastructural and 16S rRNA gene and 16S-23S internal transcribed spacer (ITS) sequence analyses, the nine cyanobacteria were firmly taxonomically identified, the phylogenetic trees obtained from the 16S rRNA gene sequences showing well supported nodes (Fig. 2). They are described as *Planktothricoides raciborskii* PMC 877.14, *Laspinema* sp. PMC 878.14, *Microcoleus vaginatus* PMC 879.14, *Lyngbya martensiana* PMC 880.14, *Nostoc* sp. PMC 881.14, *Aliinostoc* sp. PMC 882.14, *Leptolyngbya boryana* PMC 883.14, *Dulcicalothrix* sp. PMC 884.14, and *Pseudochroococcus coutei* PMC 885.14 [16].

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Interestingly, a similar set of cyanobacteria colonizes thermal mud in other areas, for example in Abano Terme in Italy [13], which also include OTU related to similar cyanobacterial taxa including the Microcolaceae, Chroococcales, Oscillatoriaceae, Leptolyngbyaceae orders and the *Phormidium* genus. We notice a global taxonomic similarity of the different cyanobacterial communities colonizing the thermal muds of Balaruc [16] and Abano [13], together with an apparent temporal stability in Balaruc cyanobacterial mud communities, since several genera were already identified decades ago [14-15]. This suggests that these thermal muds present comparable ecological conditions that select comparable, if not similar, species constituting a significant ecological determinant for these established microbial communities.

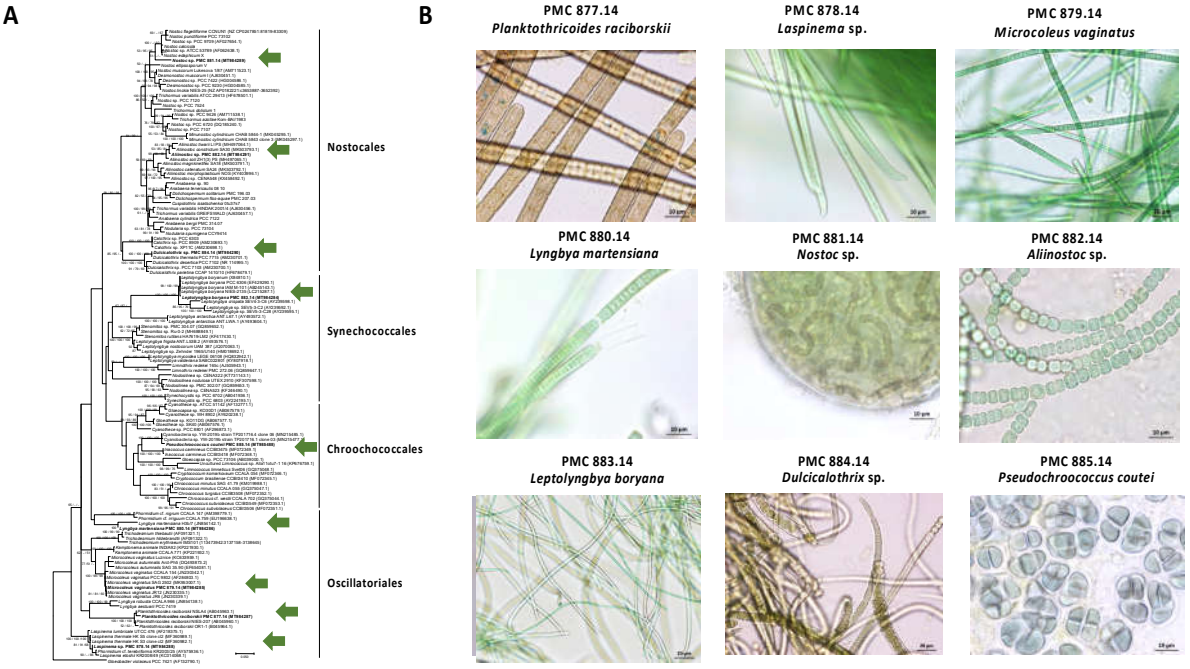

Figure 2. The 9 strains isolated from the Balaruc's thermal mud. A) Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences of representative cyanobacteria strains (129 sequences) belonging to the orders Oscillatoriales, Nostocales, Chroococcales, Synechococcales and the studied strains form the Thermes of Balaruc-Les-Bains (in bold with green arrows). Numbers above branches indicate bootstrap support (>50%) from 1,000 replicates. **B**) representative microscopic view of the 9 strains.

3.2. Cyanosphere composition

One key metabolic feature of cyanobacteria is autotrophy. Their ability to photosynthesize organic molecules allows excess to be stored, or expelled as carbon-rich exudates consisting mostly of extracellular polysaccharides (EPS) and proteins [43]. In some case, this exudate can build up a sheath around the cyanobacterial cell, offering opportunities for physical anchoring and substantial food supply for heterotrophic bacteria [12].

Microscopic observation of the nine cyanobacteria (fig. 3 & 4) confirms the widespread presence of various morphotypes of bacteria, including different *cocci* and *bacilli*/rods, with or without prosthecae, located at the surface of the cyanobacterial cells, depending on the strains. Morphotypes appear firmly attached to external sheath (*e.g. Microcoleus vaginatus* PMC 879.14 or *Nostoc sp.* PMC 881.14), cellular junctions of filaments (*e.g. Leptolyngbya boryana* PMC 883.14) or to the extracellular polysaccharide mucilage (*e.g. Lyngbya martensiana* PMC 880.14, *Planktothricoides raciborskii* PMC 877.14, *Dulcicalothrix* sp. PMC 884.14 or *Pseudochroococcus coutei* PMC 885.14). Because our sample preparation procedure includes different rinsing steps, we assume that these bacteria cells observed on the surface of the cyanobacteria are durably attached or embedded to the extracellular polymeric matrix and may not represent a random association with free-living bacteria

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that could have contaminated the culture media. This tight physical relationship with cyanobacteria suggests close interactions with cyanobacterial surface components. Besides, the presence of bacteria that are directly attached or immediately adjacent to cyanobacterial cells suggests the possibility of intense and/or specific nutrient exchanges between these microorganisms as it has been observed in other microbial communities dominated by certain cyanobacteria [44].

The observation of heterotrophic bacteria associated with the surface of cyanobacteria had been first interpreted as evidence for symbiotic interactions. For example, the bacteria appeared more numerous and bioactive when positioned on heterocytes of *Anabaena* or *Aphanizomenon* compared to vegetative cells [45]. However, the metabolic interaction between cyanobacteria cells and attached bacteria remains difficult to demonstrate and direct evidence remains rare. Interestingly, the taxonomic comparison of the bacterial communities living attached to *Anabaena* and *Microcystis* cells shows clear differences with the communities occurring in the culture media suggesting that bacteria living in the immediate vicinity of cyanobacteria may present certain specificities potentially related with peculiar interaction with the cyanobacteria regardless to the cyanobacteria genus [46]. However, different dominant cyanobacteria may differ in many ways regarding their respective sheath and exudate production, including exopolysaccharides (EPS), that may vary in terms of their amount and composition. EPS are potentially composed of various substances, such as polysaccharides, lipopolysaccharides and glycoprotein heteropolymers and also a various set of metabolites including humic-like substances or secondary metabolites, such as scytonemins, mycosporine-like amino acids (MAAs) or carotenoids with potential photoprotective activities [47]. Although EPS may present some anti-microbial activities [48], it can also specifically serve as primary source of carbon for certain bacteria, such as several Flavobacteria or Roseobacter [49-50].

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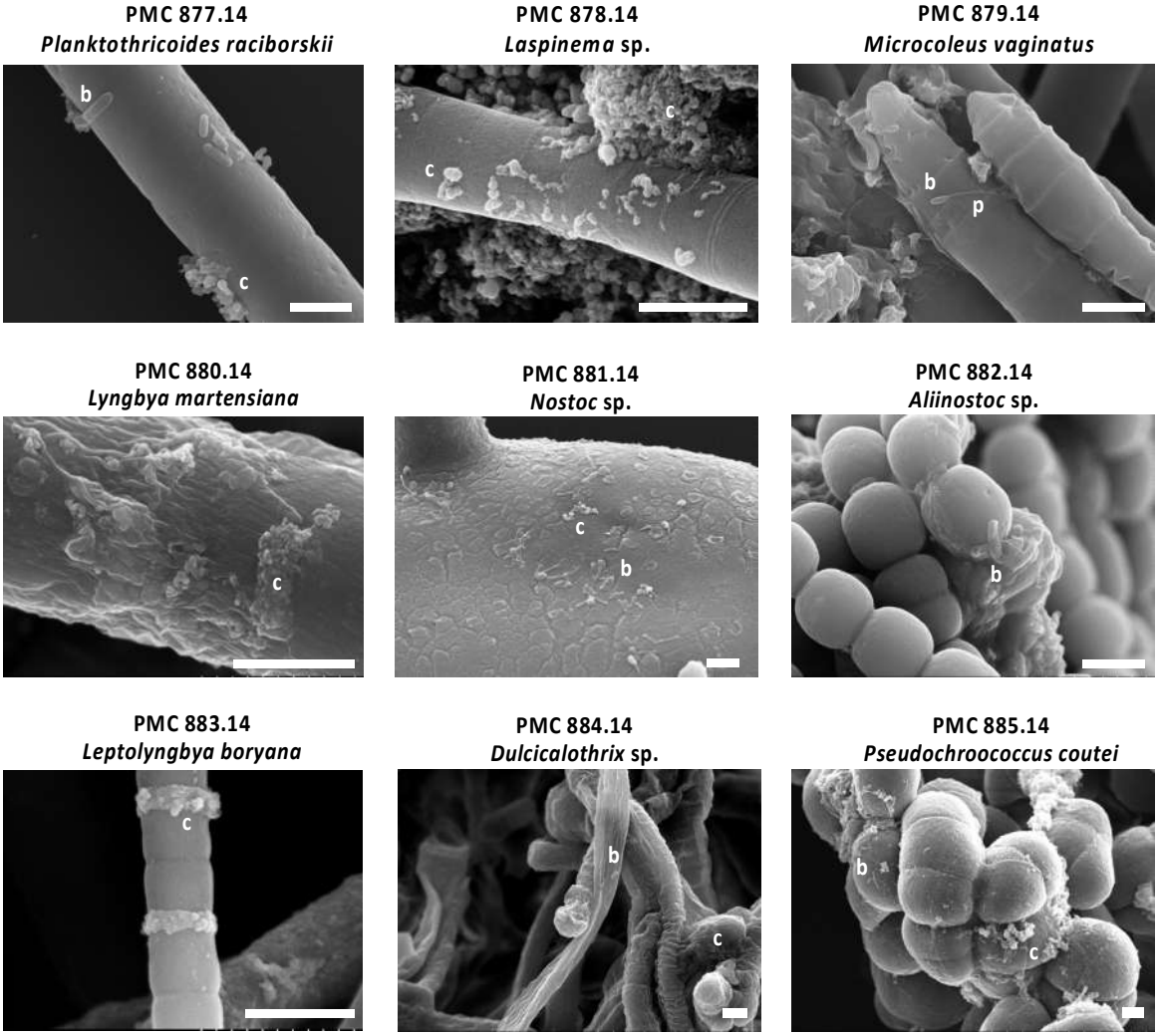

Figure 3. Representative scanning electron micrographs of the nine cyanobacterial strains isolated from Balaruc's thermal mud showing the presence of various bacteria morphotypes (including different *cocci* and *bacilli* and prosthecea indicated with c, b and p, respectively) been attached on the surface of the cyanobacterial cells. Scale bars represent 2 μm.

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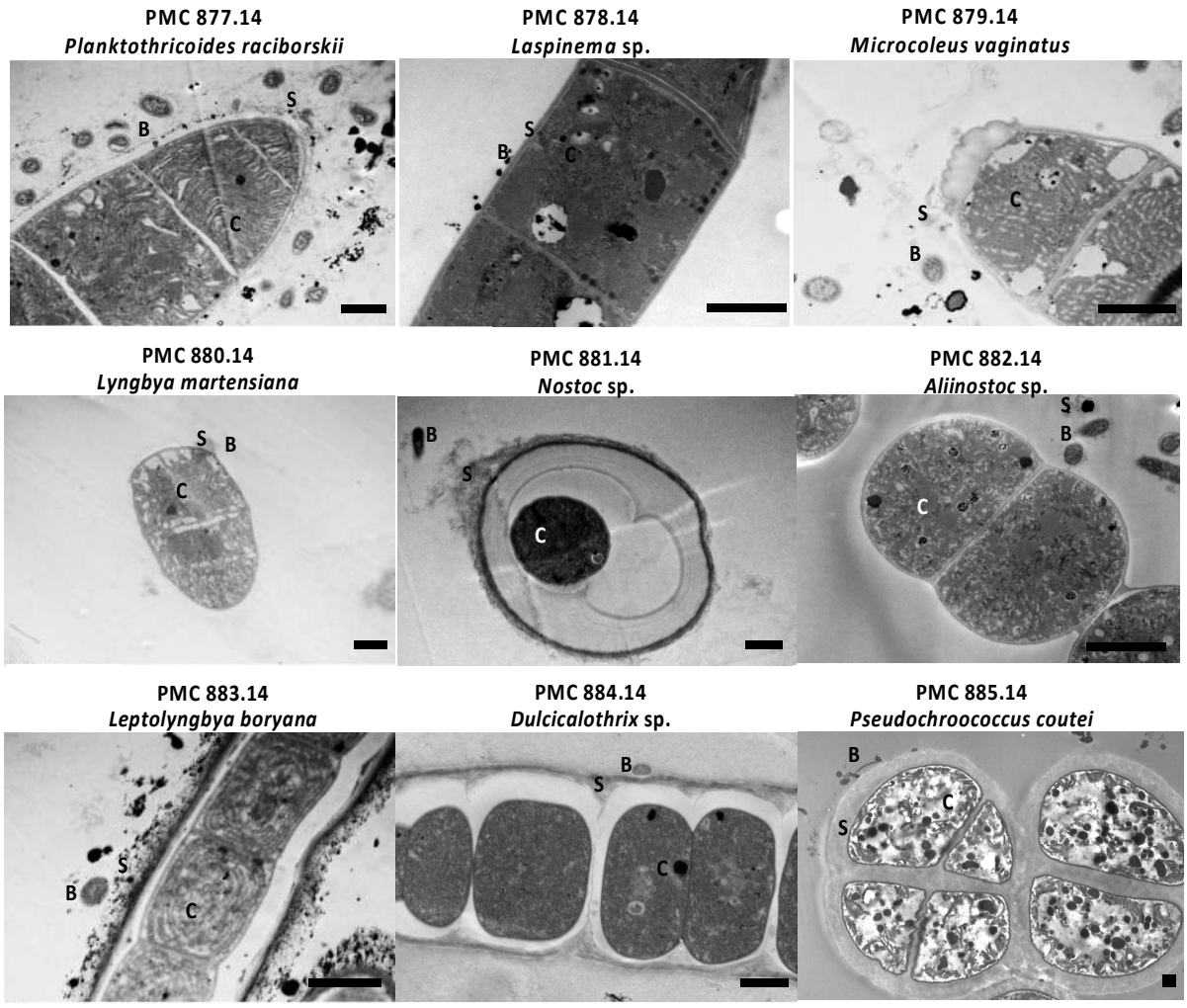

Figure 4. Representative transmission electron micrographs of the nine cyanobacterial strains from Balaruc's thermal mud showing the presence of bacteria at the vicinity of the cyanobacterial sheath or EPS (C, cyanobacteria; C, sheath; B, bacteria). Scale bars represent 1 μm.

The bacterial diversity present in Balaruc's thermal mud and cyanobacterial strains was described by 16S rRNA metabarcoding sequencing (Fig. 5). The thermal muds (n = 6) yield between 188 and 129 ASVs, when the strains (n = 9) present 14 to 37 ASVs. The mud communities exhibit higher diversity (illustrated by Shannon's index) and different community composition (Fig. 5A, B) compared with those detected to cyanobacterial strains. This observation is congruent with the general process of cyanobacterial strains isolation which is expected to reduce the associated diversity with regards to the complex microbial community retrieved in the different mud samples (Fig. 5C). It also shows that the different cyanobacteria strains exhibit only rare ASVs in common (Fig. 5D). Several heterotrophic bacteria taxa are shared among different cyanospheres (belonging to proteobacterial orders: burkholderiales, rhizobiales, sphingomonadales and caulobacteriales), or shared with mud samples, suggesting host-specific association with each cyanobacterial taxa in culture.

In a previous investigation of heterotrophic bacteria associated with non-axenic strains of *Microcystis*, *Dolichospermum*, *Planktothrix* or *Aphanizomenon*, authors reported the presence of Proteobacteria, (including Gammaproteobacteria), Sphingobacteria, Actinobacteria or Cytophagia that were retrieved in certain cultures [51]. Differently, Zheng and co-workers [8] report specificities of bacteria community living associated with *Synechococcus* cultures, showing a dominance of Bacteroidetes, Planctomycetes, Gamma- and Alphaproteobacteria, with a clear enrichment of certain Bacteroidetes in comparison with

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the free-living fraction of the cultures. Interestingly, the heterotrophic bacteria diversity associated with the lab culture of the diatom *Asterionella formosa* has also been investigated, yielding 50 different ASVs dominated by Proteobacteria (Beta-, Gamma- and Alphaproteobacteria), representing 90% of the reads, and to a lesser extent Bacteroidetes and Firmicutes [52].

Overall, the relative specificity and low-diversity of bacterial communities associated with cyanobacteria cultures suggest specific selective ecologic or metabolic interactions constrained by both the culture conditions and the vicinity of the cyanosphere itself [50]. However, the different factors determining the development of one bacterium or another within the culture remains to be explored, and could be either an opportunistic or a genuine mutualistic association.

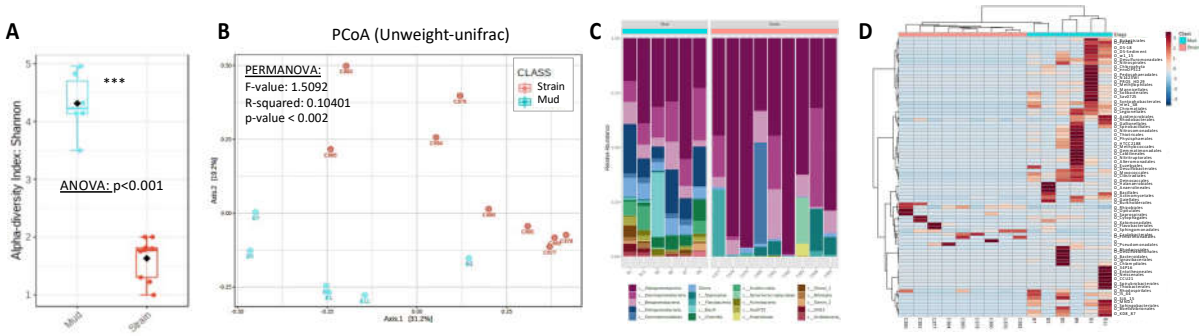

Figure 5. Bacterial diversity of the Balaruc's thermal muds (n=6, turquoise) and cyanobacterial strains (n=9, pink) by 16S rRNA metabarcoding. A) Alpha diversity analyzed with Shannon's index. B) Beta diversity analyzed on PCoA performed with unweight-Unifrac distance and Permanova analysis. C) relative proportion of all principal bacterial orders. C877-C885 indicates PMC 877-885 strains. D) ASV heatmap (<0.1%) with hierarchical classification (Euclidean distance) with color scale representing respective abundancies.

3.3. Cyanosphere's main actors revealed by metagenomics

Synergistic scaffold binning approach based on both *k*-mer and taxonomic affiliation allows to characterize between two (*Laspinema* sp. PMC 878.14) and five (*Lyngbya martensiana* PMC 880.14) high quality MAGs in each culture sample (size > 2,000,000 pb and completeness > 92%). All sequencing and assembly statistics are summarized in Table 1. As expected, all cultures present one single cyanobacterial MAG together with other heterotrophic bacteria MAGs, comprising at least one member of the Proteobacteria, and in some cultures, members of the Bacteroidetes, Gemmatimonadetes, Planctomycetes, Armatimonadetes or Chloroflexi. Although the MAGs corresponding to Cyanobacteria each fit to a single full genome of a monoclonal genotype, the MAGs of all Proteobacteria, on the other hand, show inaccurately numerous sequences with very high contamination score (up to 300%) indicating that these MAGs each correspond to different genomes that have been artificially binned within the same MAG because of the global taxonomic affiliation obtained for their respective contigs. These should thus be considered as metagenomes.

This metagenomic approach yields a much lower bacterial diversity compared to metabarcoding in terms of taxa. This apparent discrepancy is consistent with the fact that metagenomics is based on the direct sequencing of abundant DNA, when metabarcoding sequences are obtained after targeted amplification of a 16S rRNA fragment. Thus, metagenomics tends to emphasize the most active and numerous bacteria present within the cultures, when metabarcoding may easily access rarer, and even dead bacteria because of the eDNA resistance and DNA amplification process of the metabarcoding procedure [53]. For this reason, we assume that metagenomic approach provides a more accurate *vista* of the most active community of microorganisms within the cultures and is of course most relevant for functional investigations [54].

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Heterotrophic bacteria MAGs identified in the different cyanobacterial strains isolated from Balaruc's mud cultures belong to taxa already reported to be commonly associated with cyanobacteria in culture or in the environment. Halary and co-workers (2021) have recently shown that the cyanospheres associated to four *Aphanizomenon* strains also exhibit Proteobacteria, Bacteroidetes and Gemmatomonadetes. In addition, metagenomic investigation of various *Microcystis* colonies collected in nine lakes around the world [19] and those of 46 *Microcystis* strains collected from 18 north-American lakes [7] have shown the presence of the same higher-rank bacterial taxa (Proteobacteria/Alphaproteobacteria and Bacteroidetes/Cytophagales) within their respective cyanospheres. Interestingly, Perrez-Carrascal and co-workers [20] have recently shown a remarkably stable bacterial composition among the cyanospheres of 109 single *Microcystis* colonies analyzed by metagenomics, suggesting this as evidence for symbiotic relationships between *Microcystis* and its microbiota, supported by functional complementation among partners based on gene annotation analyses.

Table 1. Cyanosphere associated-MAG statistics and taxonomic annotations (cyanobacteria in bold).

Strain__MAG (16S phylum annotation)	nb_contigs	Size (pb)	GC%	nb CDSs	CheckM Completeness (missing genes)	CheckM Contamination (duplicated genes)	MicroScope Taxonomy
PMC 877__Bacteroidetes	8	2,940,501	32.92	2,707	99.65 % (1)	0.17 % (1)	Bacteria > Bacteroidetes/Chlorobi group > Bacteroidetes
PMC 877__Cyanobacteria	**15**	**7,391,557**	**43.50**	**7,130**	**99.89 % (1)**	**0.89 % (6)**	**Bacteria > Cyanobacteria > Oscillatoriales > Microcoleaceae > *Planktothricoides***
PMC 877__Proteobacteria	7	3,625,685	64.63	3,616	99.64 % (2)	1.40 % (9)	Bacteria > Proteobacteria > Alphaproteobacteria > Sphingomonadales
PMC 878__Cyanobacteria	**69**	**7,336,742**	**47.37**	**5,829**	**99.32 % (4)**	**1.16 % (10)**	**Bacteria > Cyanobacteria > Oscillatoriales > Microcoleaceae > *Laspinema***
PMC 878__Proteobacteria	45	12,013,276	65.81	11,940	100 % (0)	220.83 % (55)	Bacteria > Proteobacteria
PMC 879__Cyanobacteria	**34**	**6,900,942**	**45.68**	**6,400**	**99.607 % (3)**	**0.22 % (1)**	**Bacteria > Cyanobacteria > Oscillatoriales > Microcoleaceae > *Microcoleus***
PMC 879__Proteobacteria	11	10,840,780	67.92	10,627	100 % (0)	195.83 % (56)	Bacteria > Proteobacteria
PMC 880__Bacteroidetes	1803	14,271,057	48.22	14,012	100 % (0)	156.82 % (92)	Bacteria > Bacteroidetes/Chlorobi group > Bacteroidetes
PMC 880__Cyanobacteria	**132**	**6,444,104**	**39.60**	**5,904**	**97.15 % (14)**	**1.19 % (6)**	**Bacteria > Cyanobacteria > Oscillatoriales > Oscillatoriaceae > *Lyngbya***
PMC 880__Gemmatimonadetes	25	4,542,236	65.84	4,112	98.90 % (1)	3.30 % (3)	Bacteria > Gemmatimonadetes
PMC 880__Planctomycetes	216	6,159,044	53.18	5,068	96.40 % (4)	3.53 % (3)	Bacteria > Planctomycetes
PMC 880__Proteobacteria	2151	21,578,483	64.87	22,250	100 % (0)	375.53 % (56)	Bacteria > Proteobacteria
PMC 881__Cyanobacteria	**648**	**7,938,353**	**41.66**	**7,711**	**99.48 % (3)**	**1.19 % (8)**	**Bacteria > Cyanobacteria > Nostocales > *Nostoc***
PMC 881__Proteobacteria	1146	27,227,936	67.65	25,649	100 % (0)	348.73 % (56)	Bacteria > Proteobacteria
PMC 882__Armatimonadetes	5	7,360,037	54.92	6,711	100 % (0)	95.83 % (55)	Bacteria > Terrabacteria group > Armatimonadetes

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PMC 882__Cyanobacteria	**46**	**8,132,153**	**41.34**	**7,582**	**98.22 % (9)**	**0.22 % (1)**	**Bacteria > Cyanobacteria > Nostocaceae > *Trichormus***
PMC 882__Proteobacteria	41	9,857,661	66.03	9,926	100 % (0)	161.46 % (56)	Bacteria > Proteobacteria
PMC 883__Armatimonadetes	4	3,575,512	55.83	3,399	92.13 % (9)	1.39 % (2)	Bacteria > Armatimonadota > Fimbriimonadales
PMC 883__Chloroflexi	6	5,641,484	58.03	5,032	98.18 % (2)	0.91 % (1)	Bacteria > Terrabacteria group > Chloroflexi
PMC 883__Cyanobacteria	**5**	**6,694,777**	**46.94**	**6,313**	**99.41 % (3)**	**1.02 % (5)**	**Bacteria > Cyanobacteria > Synechococcales > *Leptolyngbya***
PMC 883__Proteobacteria	72	6,645,423	67.63	6,660	100 % (0)	84.48 % (95)	Bacteria > Proteobacteria
PMC 884__Bacteroidetes	4	2,942,276	39.52	3,054	99.51 % (1)	0.50 % (1)	Bacteria > Bacteroidetes/Chlorobi group > Bacteroidetes
PMC 884__Cyanobacteria	**46**	**13,243,152**	**38.60**	**11,176**	**99.27 % (5)**	**1.35 % (8)**	**Bacteria > Cyanobacteria > Nostocales > *Calothrix***
PMC 884__Proteobacteria	132	11,961,935	68.16	12,136	100 % (0)	200 % (56)	Bacteria > Proteobacteria
PMC 885__Bacteroidetes	30	7,094,005	39.52	6,347	100 % (0)	100 % (56)	Bacteria > Bacteroidota > Flavobacteriales > *Flavobacterium*
PMC 885__Cyanobacteria	**137**	**5,858,006**	**35.29**	**5,328**	**98.51 % (10)**	**1.31 % (5)**	**Bacteria > Cyanobacteria > Chroococcales > *Chroococcus***
PMC 885__Proteobacteria	52	18,208,582	65.84	17,483	100 % (0)	300 % (56)	Bacteria > Proteobacteria

3.4. Functional genomics of the cyanosphere

The functional annotation of the 27 MAGs obtained from the metagenomic dataset was achieved by searching orthologs of protein involved in reference metabolic pathways. To this end, both KEGG and MetaCyc pathway databases were used to better explore the metabolic capabilities of the cyanosphere [19]. The Principal Component Analysis (PCA) based on KEGG and MetaCyc pathway completeness (in %age) displayed the taxon-related fractions of the metagenomes in distinct well-distinguished clusters (Figure 6A-B). These clusters clearly separated cyanobacteria from heterotrophic bacteria, especially Proteobacteria, indicating divergences in their functional gene contents related to the cyanobacterial taxa. The nine points corresponding to the cyanobacteria MAGs were more tightly packed together than those corresponding to Proteobacteria and other heterotrophs, suggesting less variation among pathway contents. Differently, volcano plots generated from molecular pathway completeness show that heterotrophic bacteria, taken together, display much more complete functional pathways than cyanobacteria when using KEGG, while MetaCyc presents the opposite landscape, with much higher completeness scores in the pathways from cyanobacteria metagenomes (Figure 6C-D). These observations were further confirmed on heatmap visualization, as KEGG indicates significantly more complete pathways in heterotrophs, when MetaCyc shows much more complete pathways in cyanobacteria MAGs (Figure 6E-F).

The KEGG and MetaCyc projects (237 and 296 super pathways, respectively) have developed large metabolic pathway databases that are used for a variety of applications including genome analysis and metabolic engineering [39]. On one side, MetaCyc contains many pathways not found in KEGG, from plants (such as many photosynthesis-related processes), fungi, metazoan, and actinobacteria, when, on the other side, KEGG contains pathways not found in MetaCyc, for xenobiotic degradation, glycan metabolism, and metabolism of terpenoids and polyketides [39]. Surprisingly, the functional exploration of the cyanosphere through metagenome functional pathway annotation illustrates this obvious discrepancy. Indeed, it suggests that KEGG would be more appropriate for

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illustrating the metabolism of heterotrophs, when MetaCyc seems to present a better coverage of the specific metabolism of autotrophs, such as cyanobacteria.

Cook and co-worker [19] were recently able to investigate the functional metagenomes of bacteria associated with *Microcystis* colonies using KEGG interrogation. Although they established that the numerous key pathways involved in anaerobiosis were present in heterotroph metagenomes, suggesting that anaerobic process may play an important role in the nutrient recycling within the cyanosphere, they also pointed out that the KEGG database does not contains many *Microcystis* functions relevant to photosynthesis processes. They also suggest that future effort concerning the functional annotation of the cyanosphere should investigate multiple databases to cover as many genes and pathways as possible, as no unique current database seems to contain all metabolic pathways potentially present in complex microbial assemblages, such as those suspected to occur within the cyanosphere.

Perez-Carrascal and co-workers [20] also investigated gene functions in both *Microcystis* and its cyanosphere using orthologous search based on KEGG interrogation. According to their respectively more similar functional gene contents, phylogenetically related MAGs tend to cluster together. We presently observe a similar pattern for cyanobacteria and proteobacteria clusters that discriminate from other heterotroph bacteria. As expected for distantly related bacteria, cyanobacteria and associated heterotrophs exhibit different functional gene repertoires, some of which could be complementary and mutually beneficial. This observation also suggests that the different members of these clusters (*i.e.* cyanobacteria, proteobacteria and other bacteria) may present a certain functional redundancy because of their respective global similarity in terms of functional gene contents.

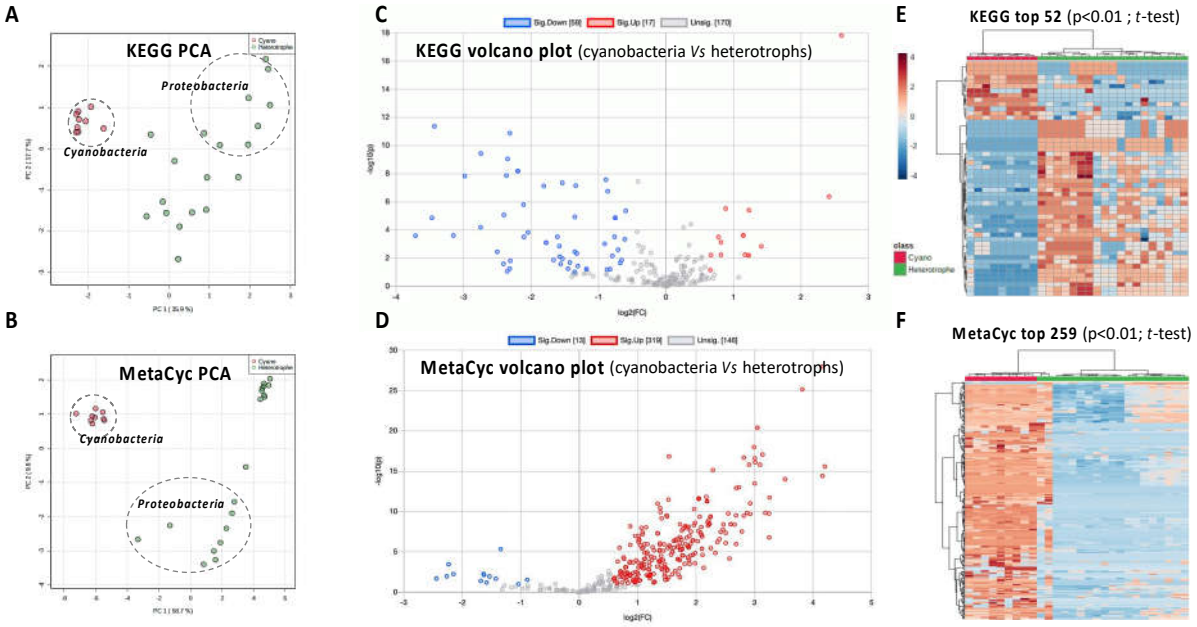

Figure 6. Functional metagenomics of the cyanosphere from the nine cyanobacterial strains from Balaruc's thermal mud according to KEGG (A, C and E) and MetaCyc (B, D and F) database interrogation. A) Principal Component Analysis based on KEGG pathway completeness (%age) in cyanobacteria and heterotroph metagenomic sequences. B) Principal Component Analysis based on MetaCyc pathway completeness (%age) in cyanobacteria and heterotroph metagenomic sequences. Dotted ellipse indicates Cyanobacteria and Proteobacteria metagenomes from the nine cyanobacterial strains. C) Volcano plot of individual KEGG pathways completeness (%age) when comparing cyanobacteria and heterotroph bacteria metagenomes taken together. D) Volcano plot of individual MetaCyc pathways completeness (%age) when comparing cyanobacteria and heterotroph bacteria metagenomes taken together. Functional pathway significantly over- and under-represented in cyanobacteria are indicated in red and blue, respectively. E) Heatmap with hierarchical classification of KEGG pathway completeness (relative %age indicated by color scale). F) Heatmap with

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hierarchical classification of MetaCyc pathway completeness (%age). Cyanobacteria and heterotroph metagenomes are indicated in red and green, respectively.

Taken together, these functional annotation data (supplementary table S2 and S3) claim for a clear distinction between metabolic pathways found in cyanobacteria and in their heterotrophic partners, and a global homogeneity with little variations in the metabolic potential within these respective groups, that seems mostly related to specificities of autotrophic versus heterotrophic metabolisms. Specifically, certain functional pathways display higher completion levels in heterotrophs (considered together) compared to cyanobacteria including the amino acid, carbohydrate (except the gluconeogenesis) and energy metabolisms (except oxidative phosphorylation). On the contrary, cyanobacteria appear more enriched in protein functions related to energy production (except the lipid degradation), nitrogen fixation, and biosynthesis of amino acids, carbohydrates, electron carriers, secondary metabolites and co-factors, such as various carotenoids, vitamin E and vitamin K2.

Recently, Pascault and co-workers [55] have explored the respective metabolic roles of the cyanobacteria and their bacterial microbiota in a day-night environmental metatranscriptomic analysis of the cyanosphere supported by KEGG annotations. This study revealed that the functional expression of the active community was overall driven by their respective trophic modes. Indeed, the autotrophs (*Dolichospermum* and *Microcystis*) exhibited functional enrichments in photosynthesis, CO_2 fixation, carbohydrate metabolism, oxidative phosphorylation, N_2 fixation, phosphorus and glutamate metabolisms, when heterotrophs mostly presented enrichments in carbohydrate metabolism, TCA cycle, glycolysis/gluconeogenesis, pyruvate metabolism and transcription. Taken together, this approach exquisitely illustrated the global metabolic complementation that exists between autotrophs and heterotrophs within the cyanosphere and the global functional complementarity between the different taxa.

Photosynthesis-derived carbon produced by cyanobacteria is exported and forms a thick extracellular polymeric substance (EPS) that likely fuels the growth of heterotrophic bacteria that make use of these compounds [56]. Indeed, numerous pathways of carbohydrate degradation have been retrieved in the metagenomes of the bacteria living within *Microcystis* colonies maintained with extracellular polysaccharidic mucilage [19]. Nitrogen is also considered as a limiting nutrient in aquatic ecosystems and the capability of fixing atmospheric N_2 by certain cyanobacteria may support higher production of the cyanosphere communities under limiting conditions [55]. Carotenoids may also constitute a remarkable example of common good for the cyanosphere community as they could be beneficial for the cyanobacteria by broadening the photosynthetic light absorption spectrum, acting as an accessory pigment, while also contributing to the photoprotection of heterotrophic bacteria against oxidative stress [20,57].

Taken together, these observations suggest that the potential metabolic association of cyanobacteria and heterotrophic bacteria within the cyanosphere might be driven by the reciprocal exchange of common goods, including carbon and nitrogen sources, as well as various vitamins and co-factors.

4. Conclusion

The nine non-axenic cyanobacterial strains isolated from the thermal muds of Balaruc-les-Bains revealed host-attached bacteria that include different bacterial ASVs depending on the host. As for planktonic cyanobacteria, members of the cyanosphere are heterotrophs that rely on photosynthetic biomolecule production by cyanobacteria. Their metagenomes encode some functions that are complimentary and may promote the growth of cyanobacteria, including the production of common goods. Thus, the physical proximity, host-specificity and complimentary functions advocate for mutualist relationship between cyanobacterial hosts and their associated microbiota.

The cyanospheres of Balaruc's mud cyanobacterial cultures were composed of distinct microbiomes but with similar functional potential highlighting functional

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redundancy, which could constitute an advantage for the respective culture fitness through metabolic exchanges and recycling. Besides these findings, our results also highlight the great influence of the reference protein database chosen when performing functional annotation of the metagenomes from organisms of the cyano-/phyco-sphere and the difficulty of selecting one unique database that appropriately cover both autotroph and heterotroph metabolic specificities.

By controlling nutrient cycling and biomass production at the base of the food web, interactions between cyanobacteria and bacteria represent a fundamental ecological relationship in aquatic environments including thermal muds. The importance of the cyanosphere has been postulated for four decades, yet only recently have new technological and conceptual frameworks made it possible to start revealing the specificities of this microbial consortia.

Author Contributions: B.M., A.R. and C.B. designed experiment. J.D., S.H., C.D. and B.P. performed sample analysis. B.M., S.H. and S.D. analysed data. B.M., S.H., S.D., J.D. and C.B. wrote the manuscript. All authors have given approval to the final version of the manuscript.

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