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A Review on Biology of Epitome Lichen Symbiotic Microalga

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Abstract

Two microalgal species, *Trebouxia jamesii* and *Trebouxia* sp. TR9, were detected as the main photobionts coexisting in the thalli of the lichen *Ramalina farinacea*. *Trebouxia* sp. TR9 emerged as a new taxon in lichen symbioses and was successfully isolated and propagated in *in vitro* culture and thoroughly investigated. Several years of research have confirmed the taxon *Trebouxia* sp. TR9 to be a model/reference organism for studying mycobiont–photobiont association patterns in lichen symbioses. *Trebouxia* sp. TR9 is the first symbiotic, lichen-forming microalga for which an exhaustive characterization of cellular ultrastructure, physiological traits, genetic and genomic diversity is available. The cellular ultrastructure was studied by light, electron and confocal microscopy; physiological traits were studied as responses to different abiotic stresses. The genetic diversity was previously analyzed at both the nuclear and organelle levels by using chloroplast, mitochondrial, and nuclear genome data, and a multiplicity of phylogenetic analyses were carried out to study its intraspecific diversity at a biogeographical level and its specificity association patterns with the mycobiont. Here, *Trebouxia* sp. TR9 is formally described by applying an integrative taxonomic approach and is presented to science as *Trebouxia lynnae*, in honor of Lynn Margulis, who was the primary modern proponent for the significance of symbiosis in evolution. The complete set of analyses that were carried out for its characterization is provided.

Keywords: Diversity • Genetics • Isolation • Morphology • Phylogeny

Introduction

Lichens are iconic examples of symbiotic interactions originated by the living together of heterotrophic ascomycetous or basidiomycetous fungi (i.e., the mycobionts) and populations of photosynthetic green microalgae (phycobionts) or cyanobacteria (cyanobionts) (i.e., the photobionts). Aside from these two major lichen symbionts that shape their unique symbiosis into a thallus, an indeterminate number of other microscopic organisms co-occur, intermingled in these associations [1].

Among the phycobionts, the genus Trebouxia Puymaly (Trebouxiaceae) is the most common and associates with a broad spectrum of lichen-forming ascomycetes worldwide. The number of species-level lineages recognized in Trebouxia increases as soon as new ecological niches or different lichen symbioses are investigated. However, only 30 Trebouxia species-level lineages have so far been formally described based on their cell morphology/ultrastructure and genetic diversity. Indeed, a reliable species identification and characterization is achieved only when Trebouxia cells grow *in vitro* outside the symbiotic state of the lichen thallus. Key diagnostic features of chloroplast morphology and pyrenoid ultrastructure develop at their best when the algae are axenically grown in cultures. Unfortunately, thus far, still too few species have been successfully isolated [2]. Furthermore, isolation and culture approaches for Trebouxia were only standardized a few years ago, while only recently, delivered a detailed morph anatomical characterization of pyrenoid and chloroplast structures. The authors recognized six types of pyrenoid ultrastructure and five main types of chloroplasts among the 20 Trebouxia species-level lineages to be used as a reference for species identification.

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Literature Review

In the past decades, several studies have proven the coexistence of multiple Trebouxia species-level lineages within a single lichen thallus, shedding light on diverse patterns of photobiont–mycobiont associations. The pioneer studies in this field revealed the coexistence of multiple Trebouxia taxa in the individual thalli of the lichen *Ramalina farinacea*. While most of these microalgae were identified as *Trebouxia jamesii* (the most abundant phycobiont in that thalli), the microalga numbered *Trebouxia* sp. TR9 was genetically and ultra-structurally different. The discovery of the presence of two photobionts in a single thallus made the lichen *R. farinacea* a model/reference system to study the photobiont coexistence in lichens. The application of the DNA met barcoding approach corroborated the coexistence of the two photobionts (*T. jamesii* and *Trebouxia* sp. TR9) in individual thalli of *R. farinacea*, but also highlighted much higher, unexpected microalgal diversity. Taking *R. farinacea* as a reference system, Molins pursued the reappraisal of the microalgal diversity in thalli from different ecologies by performing an ad hoc sampling design and an in-depth Illumina paired end met barcoding approach [3]. Their results show that in many cases, there is no balanced co-presence of *T. jamesii* and *Trebouxia* sp. TR9, as previously determined in *R. farinacea*.

Since then, *Trebouxia* sp. TR9 and *Trebouxia jamesii* were recurrently found in the thalli of both *R. farinacea* and other lichen species using culture isolations and Sanger sequencing. These species have been successfully maintained as viable *in vitro* culture for over 10 years. Several studies performed with *Trebouxia* sp. TR9 have generated an exhaustive knowledge on this alga, aiming at its characterization. This microalga presents a pyrenoid impressa-type and a shallowly lobed-type of chloroplast [4]. It is photosynthetically better performing at higher temperature and irradiance, and shows novel inducible responses against abiotic stresses. In particular, *Trebouxia* sp. TR9 responds well against oxidative stress, which is a crucial challenge for lichens exposed to cyclic desiccation and rehydration events, nitric oxide (NO), osmotic and saline stresses, and photo oxidants or heavy metal accumulation. The better physiological performance of *Trebouxia* sp. TR9 under oxidative conditions than that of the coexisting *T. jamesii* may reflect its greater capacity to undertake key metabolic adjustments including increased non-photochemical quenching, higher antioxidant protection, and the induction of repair mechanisms. Additionally, *Trebouxia* sp. TR9 generates peaks of

NO-end-products in suspension and show high rates of photo bleaching and reactive oxygen species (ROS) production under NO inhibition. NO is indeed a key molecule, conferring stress tolerance in lichens during early stages of thallus rehydration. *Trebouxia* sp. TR9 does not significantly increase the abscisic acid (ABA) levels and ABA-related gene expression until the external NaCl concentration is raised to 3 M NaCl. Furthermore, the responses of *Trebouxia* sp. TR9, *Asterochloris erici*, and *Chlorella vulgaris* to osmotic and saline stresses were compared and *Trebouxia* sp. TR9 had an extraordinarily higher tolerance to osmotic and saline stress than the other two species. This suggests that *Trebouxia* sp. TR9 may have developed alternative molecular pathways to cope with highly saline environments [5].

Another property of *Trebouxia* sp. TR9 is the capacity to immobilize most metals extracellularly such as when exposed to Pb, while in *T. jamesii*, the amount of intracellular Pb accumulation is three times higher than in *Trebouxia* sp. TR9. Both phycobionts adopt two different strategies against Pb stress (*Trebouxia* sp. TR9 forms extracellular aggregates, while *T. jamesii* has a lower wall Pb retention capability), in which the integration of distinct anatomical and physiological features affords similar levels of Pb tolerance. Related to this result, cell walls and extracellular polymers from *T. jamesii* and *Trebouxia* sp. TR9 were studied. The proportion of cell walls on the overall cell biomass was 2.6 times higher in *Trebouxia* sp. TR9 than in *T. jamesii*. At the ultrastructural level, four clearly differentiable layers in the *T. jamesii* cell wall were observed, whereas *Trebouxia* sp. TR9 showed a more diffuse structure in which only three layers could be distinguished. In general, cell wall biomass, monosaccharide composition, and extracellular polymers of *Trebouxia* sp. TR9 and *T. jamesii* showed clear differences, suggesting close associations between the differential ultrastructure and Pb-retention capabilities.

The genetic characterization of *Trebouxia* sp. TR9 was completed by sequencing its nuclear and organelle genomes using the Illumina, 454, and Solexa sequencing technologies. The nuclear genome of *Trebouxia* sp. TR9 (59.7 Mb) covers 100% of the estimated genome size and has a completeness of 96.7%. The number of detected gene models was 15,905, and the functional annotation had been improved with a total of 7068 different GO terms, 1826 enzyme class terms, and 7581 different gene annotations [6]. The mitochondrial genome sequence of *Trebouxia* sp. TR9 was the first complete mtDNA genome sequence available for a lichen-symbiont microalga. It comprises 70,070 bp and has a total of 61 genes; nine type I introns were detected in several genes. The chloroplast genome of *Trebouxia* sp. TR9, instead, comprises 303,323 bp, resulting in one of the largest known genomes among Chlorophyta. A total of 108 genes and 12 type I introns have been detected. The most remarkable characteristics are the presence of long intergenic spacers, the typical quadripartite structure of land plant chloroplasts with short inverted repeated sequences (IRs), a single gene of rbcL, and the loss of the rps4 gene, which was transferred from the chloroplast to the nucleus. Currently, whole genome sequencing offers new ways to study lichen populations and their interaction with their environments. Furthermore, phylogenomics analyses can help in resolving closely related or recently diverged lineages.

Lichens are poikilohydric organisms and limit the photosynthetic CO_2 assimilation to relatively short periods of time when their thalli are sufficiently hydrated, and the photobionts are metabolically active. Isotopic discrimination is a widely used technique in cyanobacteria and microalgae to determine the presence or absence of a carbon-concentrating mechanism (CCM). A CCM can provide a rapid response mechanism in environments where light and CO_2 availability are limited. Moreover, the activity of a CCM may also be related to the nitrogen economy of the organism. Lichen with a CCM may need to invest less in both the carboxylating enzyme Rubisco, and an enzyme involved in the recovery of assimilates during photorespiration. Traditionally, physiological studies of isotopic discrimination in algae or bryophytes have indicated that pyrenoids are related to CCM. The identification of proteins involved in carbon uptake suggests that *Trebouxia* sp. TR9 may possess carbon concentration mechanisms similar to C3 and C4/CAM.

Pure cultures of symbiotic algae are essential for species delimitation following an integrative taxonomic approach in which different analytical methods, combining morphology and genetic diversity, are considered. Indeed, particularly for the genus Trebouxia, the lack of axenically cultured species has been, thus far, one of the major reasons for the unbalanced proportion between the genetically identified species-level lineages and the formally described species. The feasibility of isolating lichen phycobionts was also hindered by the lack of standardized protocols, which instead have nowadays been established and published. These have so far mainly been applied to study species diversity for the two most frequent lichen-forming genera Asterochloris and Trebouxia, but can be applied alike for the isolation of other less common phycobionts. This method can be successfully applied for the isolation of many other coccoid, symbiotic green microalgae (such as Asterochloris, Myrmecia, Symbiochloris or Vulcanochloris), photobionts of lichens with diverse growth forms. However, the success rate of microalgae isolation is in general rather low, and it mostly depends on the species-specific requirements of the taxon to be isolated axenically *in vitro* [7]. Several attempts are usually required, and multiple inocula are set before the targeted strain/taxon is successfully isolated. The isolated strains must be genetically identified before proceeding with further analyses; indeed, many morphological traits can be shared by more than two species. In this context, *Trebouxia lynnae* represents an exception: it did not require any ad hoc setup to be isolated and it represents a taxon that also grows easily on very poor media (such as BBM, on which it is usually isolated).

Phylogenomics data generated from pure algal cultures are preferred today, rather than met genomics DNA extracted from lichen thalli to reconstruct robust phylogenies (thereby avoiding amplification of co-existing algae). However, previous multi-locus phylogenetic reconstructions have been essential to uncover the relationships among species-level lineages and their geographic and symbiotic origins in Trebouxia. Indeed, *T. lynnae* is known to associate with lichen fungi belonging to phylogenetically distant families such as Parmeliaceae, Caliciaceae, Lecanographaceae, Megasporaceae, and Ramalinaceae and from different continents and ecologies. Furthermore, *T. lynnae* is the primary phycobiont of lichens that develop diverse growth forms such as the crustose genera Lecanographa and Protoparmelia, and the fruticose Ramalina, and was detected as a minor symbiotic partner in the terricolous crustose Buellia and vagrant Circinaria from Spain. In general, *T. lynnae* is a cosmopolitan species of phycobiont, as it was reported in lichens from Sweden, Poland, North America, New Zealand, Madeira, Cape Verde, and Spain (including the Iberian Peninsula, Canary and Balearic Islands). The wide versatility of associations and the geographic breadth of *T. lynnae* are consistent with other taxa placed in clade A of Trebouxia. In fact, this clade is the one gathering the greatest Trebouxia diversity in terms of mycobiont-species associations, geographic origins, and morphological and ultrastructural diversity. Clade A indeed was recently inferred to be the clade that originally, exclusively, or partially, occupied forested habitats, and was subsequently extended to occupy regimes characterized by cooler and drier habitats, and is now the clade showing the widest distribution among lichen symbioses [8].

More advanced techniques have been also applied to better investigate the cellular structure: recently, it was shown that fast-freeze electron microscopy techniques (such as cryo-SEM) capture organisms at high resolution in their living state, offering novel views of the cellular ultrastructure, organization, and differentiation. Although these techniques bypass possible ultrastructure modifications due to preparation procedures, they have not been extensively used with lichens and their axenically isolated phycobionts. In the present study, we applied for the first time the cryo-SEM method to the analysis of isolated lichen-forming microalgae. This method allowed us to observe the presence of eisosomes in *T. lynnae* for the first time. Eisosomes are defined as plasma membrane invaginations filled with protein complexes that are thought to be involved in the desiccation-dehydration processes in eukaryotic organisms with cell walls. These structures have been described for Trebouxia cells analyzed within the lichen thallus, and their detection serves as a further confirmation of the desiccation-dehydration tolerance typical of lichens and their phycobionts in particular [9].

A further novelty of the present species description is the extensive analysis of the flagellar apparatus of the zoospores. In general, the ultrastructure of the flagellar apparatus is similar to the previously described zoospores of *Asterochloris erici*, *A. glomerata*, *A. pyriformis*, *Myrmecia*

israelensis, and *Trebouxia impressa*. Although zoospore formation in cultured Trebouxia has been reported several times, the formation of zoospores in culture is not as common as the formation of autospores. Indeed, in culture, autospore formation is the main reproductive strategy adopted by Trebouxia. Obtaining zoospores from *T. lynnae* was a very laborious process, and required different treatments (glucose, darkness, etc.), thus the present detailed characterization of their ultrastructure significantly complements the description of the species. Culture growth conditions for inducing the production of zoospores of symbiotic microalgae have not been standardized yet, and future experiments should be centered on this aim.

In this study, researchers measured and compared the isotopic discrimination of *T. lynnae* and its sister species *T. jamesii*. The results confirm that the pyrenoid is a CCM structure and that *T. lynnae* could be considered to have both C3 and C4 metabolism, as it presents intermediate values. This result matches the data obtained by the nuclear genome, in which sequences of proteins involved in carbon uptake mechanisms have been identified. Some lichenologists have analyzed isotopic discrimination in whole lichen thalli, but these measurements resulted from the photosynthetic characteristics of the photobiont in combination with any potential process happening while lichenized with the mycobiont. *T. lynnae* showed different values than *T. decolorans*. These preliminary results need further confirmation, as the observed differences may be due to the growth condition of *T. lynnae* used for this experiment [10]. Indeed, the isotopic discrimination analysis requires a lot of biomass, and therefore *T. lynnae* was grown on a medium supplemented with glucose and casein, which induced a faster growth during the standardized period of time (i.e., 21 days) set for sampling the algae for any analyses. Here, the isotopic discrimination analysis was applied for the first time to characterize a lichen-forming microalga to show its potential as the method could be set up using the BBM culture medium and standardized for future studies.

Conclusion

In conclusion, the extensive description provided in this study well supports the new species *Trebouxia lynnae* Barreno as one of the most prominent lichen phycobionts, and stands as a promising model and reference for forthcoming research.

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