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Fungal communities associated with *Evernia prunastri*, *Ramalina fastigiata* and *Pleurosticta acetabulum*: Three epiphytic lichens potentially active against *Candida* biofilms

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ABSTRACT

Fungal communities associated to three epiphytic lichens active against *Candida*, were investigated using culture-based methods We hypothetized that associated fungi would contribute to lichens activities. The ability of specific fungi to grow inside or outside lichens was investigated. To detect biogenesis pathways involved in the production of secondary metabolites, genes coding for nonribosomal peptide synthetase (NRPS) and polyketide synthase I (PKS I) were screened by PCR from fungal DNA extracts. Both endo and epilichenic communities were isolated from two fructicose (*Evernia prunastri* and *Ramalina fastigiata)* and one foliose (*Pleurosticta acetabulum*) lichens. A total of 86 endolichenic and 114 epilichenic isolates were obtained, corresponding to 18 and 24 phylogenetic groups respectively suggesting a wide diversity of fungi. The communities and the species richness were distinct between the three lichens which hosted potentially new fungal species. Additionally, the endo- and epilichenic communities differed in their composition: Sordariomycetes were particularly abundant among endolichenic fungi and Dothideomycetes among epilichenic fungi. Only a few fungi colonized both habitats, such as *S. fimicola, Cladosporium* sp1 and *Botrytis cinerea*. Interestingly, *Nemania serpens* (with several genotypes) was the most abundant endolichenic fungus (53% of isolates) and was shared by the three lichens. Finally, 12 out of 36 phylogenetic groups revealed the presence of genes coding for nonribosomal peptide synthetase (NRPs) and polyketide synthase I (PKS I). This study shows that common lichens are reservoirs of diverse fungal communities, which could potentially contribute to global activity of the lichen and, therefore, deserve to be isolated for further chemical studies.

1. Introduction

Lichens are symbiotic organisms formed with a fungal partner called mycobiont (generally an Ascomycete) and a photoautotrophic partner such as agreen algae, a cyanobacterium or both, called photobiont. A third partner linked to lichen cortex was recently characterized as basidiomycete yeast (Spribille et al., 2016). Lichen thallus is also a suitable environment for other kinds of microorganisms that live exclusively there, such as bacteria and fungi which are called endobionts when they live inside the thallus or epibionts when they live on its surface (Arnold, 2007; Bates et al., 2011; Grube and Berg, 2009; Honegger et al., 2013; Parrot et al., 2015). Both members of Ascomycota and Basidiomycota have been detected in lichens, and interestingly were often closely related to endophytes of mosses (U'Ren et al., 2012, 2010). Lichenicolous fungi form non-specific obligate associations with lichens. They are described as symptomless and grow within the thallus associated with the photobiont especially in the alga layer (Arnold et al., 2009; Honegger, 2012). They can also grow on the thalli as parasites of living lichens or as saprotrophs on dead lichen thalli (Lawrey and Diederich, 2003). Thus, as supported by data from phylogenetic studies, lichen thalli appear to be a consortia with unknown numbers of participants (Grube and Wedin, 2016; Honegger, 1992).

Studies of endolichenic fungi (ELF) showed that they were present in all biomes from the Arctic to the tropics (Arnold et al., 2009; Li et al., 2007a; Zhang et al., 2016). While associated bacteria are described to contribute to several functions in the lichen symbiosis, interactions between lichen-associated fungi and the mycobiont are poorly investigated and their functional role in the lichen symbiosis is unknown.

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Bacteria play a role in the fixation and delivering nitrogen, the defense against pathogens and feeders, the growth by producing hormones, and the biomass mobilization (Grube and Berg, 2009). Epilichenic fungi (EPF) growing on the surface of lichen thallus remain unstudied.

As lichens are producers of defense metabolites against pathogenic fungi, bacteria and parasites (Boustie et al., 2011; Shukla et al., 2010; Stocker-Wörgötter, 2008; Zambare and Christopher, 2012), the question remains whether ELF could contribute to this activity, as endophytes do for plants (Arnold, 2007). Since the first investigation on endolichenic metabolites produced by *Corynespora* sp. (Paranagama et al., 2007), there has been growing interest in the bioactive compounds produced by these microorganisms. For example, Suryanarayanan et al. revealed that ELF can produce antifungal and anti-bacterial compounds (Suryanarayanan et al., 2017) and it was shown that they biosynthesize a wide range of metabolites such as alkaloids, polyketides, terpenoids, steroids and cyclic peptides (Kellogg and Raja, 2016; Singh et al., 2017). However, the link between lichen's antimicrobial activity and the ability of ELF to produce secondary metabolites is scarcely demonstrated, while it could help to search for potentially active isolates and molecules.

The development of new techniques to study ELF communities allows to a more comprehensive understanding of lichen biology. Indeed, we could expect that active lichens might share active fungi, or at least specific fungi may grow in active lichens and contribute to this specific activity. Therefore, the isolation of fungi should distinguish between endo and epilichenic fungi, and avoid potential contaminants. The sterilization of the lichen surface allowed for example the isolation of endolichenic fungi from *Parmelia taractica* and *Peltigera praetextata* (Girlanda et al., 1997). Surface sterilization of the lichen thalli is a crucial step to better understand this fungal ecology, and considerable improvements have been made for this process (Girlanda et al., 1997; Li et al., 2007b; Suryanarayanan et al., 2005). A study conducted by Suryanarayanan et al. of associated fungi of five epiphytic lichens, leaf and bark tissues of their host trees revealed little overlap beetween endolichenic and endophytic fungi in spite of their proximity (Suryanarayanan et al., 2005).

As ELF communities depend on both lichens and habitats, it might be difficult to correlate a lichen activity with its ELF activity, except if some fungi are more specific and often found growing in the same lichen. Another approach could rely on the identification of ELF, and the comparison with the literature and public databases. Indeed, methods for identifying ELF have evolved in the past decades (culture-based and culture-independent). The first isolation of endolichenic fungi was performed by Petrini et al. from lichen belonging to genera *Cladonia* and *Stereocaulon* (Petrini et al., 1990) and at that time, morphological characters were used for identification. More recently, the barcoding of lichens through high-throughput techniques revealed a high diversity of the ELF (Wang et al., 2016), for example in Arctic lichens (Zhang et al., 2016, 2015). More generally, the use of Sanger sequencing either directly on lichens, or on isolates, has considerably improved the identification of ELF. Indeed, either the comparison of sequences to public databases, or through phylogenetic inference permits to compare sequences from different studies, and identify well-known fungal species as well as putative new ones to Science. We therefore used this latter technique to determine if active lichens host specific and potentially new fungal species.

In our search of bioactive compounds from lichens, a screening of thirty-eight lichen extracts was previously performed. Seven lichen extracts have been shown to be active against sessile *Candida albicans* yeasts and exhibited activities against different steps leading to biofilm formation (Millot et al., 2017). Further to these results, the current study aimed to investigate the fungal community associated to the thalli of active lichens in order to explore the possible involvement of biotic interactions within the lichen that may be related with this anti-*Candida* activity. We investigated fungal communities of the two most promising lichens, *Evernia prunastri* (L.) Ach. and *Ramalina fastigiata* (Pers) Ach which showed anti-maturation and anti-biofilm activities at very low concentrations ($IC_{50\_maturation} < 4\ mg\ ml^{-1}$ and $IC_{50\_biofilm} < 10\ mg\ ml^{-1}$) (Millot et al., 2017). In addition, *Pleurosticta acetabulum* (Neck) Elix & Lumbsch which is common foliaceous lichen found on tree barks with a non-significant anti-biofilm activity was also studied. These three selected lichens belong to Lecanorales, two of them belonging to Parmeliaceae (*E. prunastri*; *P. acetabulum*) and one to Ramalinaceae family (*R. fastigiata*). These lichens grew on tree barks such as *Acer* sp. Both endolichenic and epilichenic fungi were isolated and sequenced, to obtain specific fungi, and possibly new species. To our knowledge, no study comparing ELF to EPF has been published to date. Additionally, to detect potentially active fungi, the presence of genes coding for nonribosomal peptide synthetase (NRPs) and type I polyketide synthase (PKS I), key enzymes for the biosynthesis of secondary metabolites, was screened by PCR.

2. Materials and methods

2.1. Lichen sample collection

The lichen samples were collected from France at Verneuil sur Vienne (45°50'50.9"N and 1°07'37.0"E) for *Evernia prunastri* and *Ramalina fastigiata* in June and November 2015 (Fig. 1). *Evernia prunastri* was collected on the bark of *Acer pseudoplatanus* and *Ramalina fastigiata* was collected on the bark of *Acer negundo*. *Pleurosticta acetabulum* was collected in June 2016 growing on the bark of *Acer pseudoplatanus* in Limoges (45°48'50.1"N and 1°13'53.7"E) (Fig. 1).

Lichen samples were identified by thalline chemical tests. Their identification was checked by lichenologists from AFL, the French Association of Lichenology. Voucher herbarium specimens were deposited at the herbarium of our Laboratory under number HL-L06/15-01 and HL-L11/15-01(*Ramalina fastigiata*); HL-L06/15-02 and HL-L11/15-02 (*Evernia prunastri*) and HL-L06/16-02 (*Pleurosticta acetabulum*).

After harvesting, lichens were transported in paper bags, stored at 4 °C and processed within 24 h. Impurities such as debris from barks, sands and foams were removed. Symptomless and undamaged thalli were washed in running tap water and then rinsed with distilled water.

2.2. Isolation and culture conditions

Endolichenic fungi were isolated after surface sterilization which was carried out by successive immersion of thalli in baths for 2 min (70% ethanol, 0.5% sodium hypochlorite, 70% ethanol). The thalli

Fig. 1. Three lichen species studied. (**A**) *Evernia prunastri*, (**B**) *Ramalina fastigiata* and (**C**) *Pleurosticta acetabulum*.