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An isolated lichenicolous fungus forms lichenoid structures when co-cultured with various coccoid algae

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Abstract

We present experimental data on a lichen-inhabiting fungus which forms sterile dark coloured mycelia on thallus areoles of *Lecanora rupicola*. The lichenicolous fungus, tentatively named ALr-1, grows readily in solid media and is able to develop lichenoid structures when co-cultured with various coccal algal species *in vitro*. After an initial formation of phototropic hyphal bundles through initially free-living algal colonies, the lichenoid structures develop as a fungal surface layer, an algal rich layer, and lower layer formed exclusively by hyphae. Scanning electron micrographs show close contacts between the fungus and the algae by formation of appressoria. Re-inoculation of the host species resulted in a more detrimental interaction, in which a dense brown mycelium extends through the upper layers of the lichen. This phenotype has not yet been observed in nature and likely results from the particular experimental conditions. The fungus is not closely related to any other known parasitic species known on the native host species. A phylogenetic hypothesis of LSU and SSU rDNA indicates a relationship of ALr-1 basal to *Chaetothyriomycetidae*. The lichenoid structure of ALr-1 establishes more rapidly *in vitro* than re-synthesised lichen bionts and could represent an interesting model to study mutualistic interactions between fungi and algae.

Keywords: Lichens, endolichenic fungi, lichenoid associations, symbiosis, phototropic hyphae, rDNA

1. Introduction

Lichen symbioses, formed by fungi with eukaryotic or prokaryotic photoautotrophs, produce long-living and slow growing joint thalli, which represent a diverse ecological niche for other microorganisms. About 1500 lichen-inhabiting (or lichenicolous) fungi are so far described and characterised by their fertile or vegetative diagnostic structures (Lawrey and Diederich, 2003). The biological relationships of lichenicolous fungi with their hosts are manifold and range from rather aggressive destruction of theirs hosts (e.g. *Athelia arachnoidea* or *Marchandiomyces corallinus*), to a more or less commensalic habit with hardly any detrimental symptoms. In some cases, the hyphae of lichenicolous fungi can be stained selectively (e.g. Grube and Hafellner, 1990).

This procedure can be used to show which of the host symbiont (i.e. the algal or the fungal partner) is the primary target of an infection. Lichenicolous fungi so far studied in this respect, which are phylogenetically placed in major lichenised lineages (*Lecanoromycetidae* or *Arthoniomycetidae*), often tend to have a preference for the algae of their hosts, while others (e.g. members of *Hypocreales*; de los Rios et al., 2002) are mycoparasites of the host symbiont. Although some descriptive work and culturing was done on lichenicolous fungi (Sikaroodi et al., 2001; Hawksworth, 1975), for the great majority of these fungi such data are missing.

Many recent studies suggest that the number of lichen-associated fungi is much higher than previously thought (Girlanda et al., 1997; Petrini et al., 1990; Prillinger et al., 1997; Miadlikowska, pers. comm.), even though many of these accessory fungi, which are usually obtained by culture approaches, do not provoke infection symptoms in the colonised lichens. There is no experimental work about the

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biological properties of these fungi, and the mere culturing from the host lichens does not reveal whether they grow more or less cryptically or merely persist as resting diaspores. Any attempt to study their biology is complicated by lacking applicable staining methods, and problems to cultivate lichens and lichenicolous fungi. Culturing of previously known lichenicolous fungi has thus been achieved only in few cases (e.g. Crittenden et al., 1995; Hawksworth and Jones, 1981; Tibell, 1991). Recently Lawrey (1997) studied their behaviours experimentally by investigating the effect of a cultured lichenicolous fungus on autoclaved fragments of the host. Yet, this does not satisfactorily pay attention to interactions among the living partners of a lichenicolous interaction. One approach proposed in this study is the co-culture of a lichenicolous fungus with a range of algae including those isolated from its host lichen and others that are non-host species or strains.

Lecanora rupicola is a widespread crustose lichen, which occurs primarily on siliceous rocks. The lichen serves as the host for several known host-specific lichenicolous fungi and lichenicolous lichens. Known lichenicolous lichens are *Rhizocarpon inimicum*, *Rimularia insularis*, or *Rinodina insularis*. They establish independent lichenised thalli which grow specifically and locally on thalli of *Lecanora rupicola*, which apparently delivers algae for the initial thallus development of the parasites. Among the non-lichenised lichenicolous fungi, *Arthonia glaucomaria* inhabits the fertile structures of the host, while others grow on the vegetative thallus parts (*Opegrapha glaucomaria*, *Sclerococcum montagnei*; Leuckert and Poelt, 1989; Hafellner, 1996). In the course of our ongoing studies of *Lecanora rupicola*, we screened numerous specimens and found several additional lichenicolous fungi (Blaha, J., pers. comm.). In this paper we present the discovery of a yet undescribed lichenicolous hyphomycete, which was then co-isolated in culture experiments with the lichen mycobiont of *Lecanora rupicola*. As this fungus grows well in vitro we were able to study its biology in more detail by performing co-culture experiments with diverse coccal algae, thereunder *Trebouxia impressa*, the photobiont of the genuine lichen *Lecanora rupicola*.

2. Material and Methods

Material

Austria, Styria, Rennfeld, 9. May 2002, Leg. Julia Blaha 265. The specimen is stored in the herbarium of the Institute of Plant Sciences Graz (GZU).

The cultured fungus ALr-1 is maintained in the culture collection of Georg Brunauer (University of Salzburg, Dept. of Organismic Biology).

Culture techniques

Culturing of ALr-1: Cultures with infected thallus parts were established using a method modified from that of Yamamoto (1990). Small pieces of the thalli were first cleaned mechanically using a forceps to remove attached debris and substrate. The fragments were then washed very carefully for 20 min in sterile double distilled water in an Erlenmeyer beaker placed on a magnetic stirrer. This step was repeated by transferring the lichen fragments to another tumbler containing, double-distilled water to which a drop of Tween 80 had been added (see also Bubrick and Galun, 1986). After another 30 minutes, the first washing step was repeated two times. After each step, the thallus fragments were rinsed with twice distilled water. The clean pieces were then homogenised in water (1–3 ml), using a mortar and a pestle. The resulting suspension contained many small pieces of the cortex, medulla and the algal layer. Finally, the suspension was filtered through two sieves of different mesh sizes, 500 µm and 150 µm. Pieces of about 150 µm diameter were picked up under a dissecting microscope with bamboo sticks and used for inoculation (Yamamoto, 1990). Each tube containing slanted agar was inoculated with only one fragment.

Co-culture experiments

The fungal isolations were grown in tubes on agar slants containing 5 ml LB-medium (Lilly and Barnett, 1951). To prevent algal growth in the fungal cultures, the tubes were kept in complete darkness (wrapped in aluminium foil) for 2–3 months. All cultures were kept in a growth chamber at 20°C, which was regarded as the mean temperature in the natural environment of this *Lecanora rupicola* during the summer season.

For co-culture experiments following algal strains were taken: A pre-established culture of *Coccomyxa* sp. (DQ660909), from the culture collection of ESW (maintained at Department of Organismic Biology, University of Salzburg) and the cultured photobionts of *Lecanora rupicola Trebouxia impressa* (DQ660908) and *Trebouxia* sp. (DQ660907) from *Protoparmeliopsis muralis*. The algal cultures were obtained according to the method of Yoshimura et al. (2002). Fungal colonies were first homogenised with sterile, double distilled water in an autoclaved mortar. Then a small amount of cells (about 10 µl) was taken from an axenic culture of coccal algae and added to the homogenised fungus under sterile conditions. The suspension containing the mixed symbionts was then transferred with a pipette to Petri dishes (110 × 15 mm) containing nutrient media (with the same composition as used for the isolation of fungi). Afterwards, the culture dishes were sealed with parafilm and kept in a culture chamber under the same conditions as mentioned above.

Host re-infection

Two drops of homogenised ALr-1 cultured mycelium was placed to two separate pieces of an uninfected *Lecanora rupicola* specimen (Austria, Salzburg, Schladminger Tauern, Preberkessel, 2100 msm on silicious rock, June 2005, leg. Roman Türk). The inoculated material was placed in a culture room and kept at room temperature for 2–3 months (8 hrs light/day). Every second to third day the thallus was sprayed with distilled water.

Sequencing

Total DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Vienna) according to the manufacturer's instructions. DNA-extracts were used for PCR-amplification of fungal nuclear ribosomal gene loci including ITS, 5' of SSU rDNA, and 5' end of the LSU rDNA. ITS rDNA was amplified with the primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al., 1990), for LSU rDNA we used LR0R and LR5 (Vilgalys and Hester, 1990), and for SSU rDNA we used primers SSU0054-5' and SSU1203-3' (Gargas and DePriest, 1996). To confirm the photobiont species of *Lecanora rupicola* we amplified and sequenced algal nuclear ITS rDNA using the primers ITS1T and ITS4T (Kroken and Taylor, 2000).

PCR was performed with a Gene Amp PCR System 2700 thermal cycler (Perkin Elmer, USA). The PCR mix contained 1 unit of Dynazyme II Taq polymerase (Finnzymes), 0.2 mM of each of the four dNTPs, 0.5 µM of each primer and 30–50 ng of genomic DNA (gDNA, quantified by OD_{260}). The PCR-conditions were as follows: 95°C for 1 min as initial denaturation step (30 cycles), 94°C (denaturation) for 30 secs, 56°C (annealing) for 30 secs and 72°C (extention) for 1 minute (2 mins for LSU rDNA).

The PCR products were purified using a QiaQuick PCR product purification kit (Qiagen, Vienna) and quantified by measurement of OD_{260} before the sequencing. Cycle sequencing reactions were set up with the same primers as for the PCR using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applera, Vienna). Parameters for the reactions were: 96°C denaturation step (20 s), 50°C annealing step (15 s), and 60°C primer extension (4 min). The cycle was repeated 30 times. Cycle sequencing products were run on an ABI 310 automated sequencer (Applera, Vienna).

Phylogenetic analyses

Sequences of LSU and SSU rDNA were aligned with a number of sequences from NCBI Genbank representing different groups of ascomycetes (see Table 1). The aligment was prepared utilising the CLUSTAL algorithm as implemented in BioEdit (Hall, 1999; http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html) and then optimised manually. The obtained alignment included 39 sequences and had an overall length of 2983 characters. Ambiguously aligned regions and intron sequences were excluded prior to analysis, which included 682 parsimony informative characters.

The phylogenetic hypothesis was constructed using a Bayesian approach as implemented in the program MrBayes 3.0 (Huelsenbeck and Ronquist, 2001). The general time reversible substitution model with among-site variation (GTR + I + Γ4) was used for likelihood calculations, according to the ModelTest evaluation of substitution models (under the Akaike criterion, Posada and Crandall 1998). The Metropolis coupled Markov Chain Monte Carlo (MCMCMC) analysis was run for 2.000.000 generations, with 12 chains starting from a random tree, and using the default temperature of 0.2. Every hundredth tree was sampled, and the first 3000 generations before stationarity of likelihood values was achieved were discarded as burn-in. A consensus phylogram showing mean branch lengths was calculated with the sumt command in MrBayes using the halfcompat option of the contype command. The topology of the tree with higher than 50% posterior probabilities of branches is presented in Table 1, with thicker branches for groups supported by 95% or higher posterior probability.

HPLC analysis

Dry lichen thalli and vacuum-dried mycobionts (removed from the medium; c. 1 cm^2 in diameter) were extracted for 4 hours with methanol for HPLC analysis. HPLC analysis was performed according to Stocker-Wörgötter (2002) using a Hitachi/Merck HPLC system with a Beckman 5C18 column and a DAD (Photodiode array detector; 190–800 nm wavelength range).

Scanning electron microscopy (SEM)

Thallus pieces were fixed with 1.2% glutaraldehyde (buffered at pH 6.5 with 0.1 M sodium cacodylate) for 2 h at room temperature. Post-fixation of the samples was performed with 1% osmium tetroxide (buffered at pH 6.5 with 0.1 m sodium cacodylate) for 2 h. The post-fixed probes were dehydrated in an ascending series of ethyl alcohol, critical-point-dried in a drying device CDP 030 (BAL-TEC, Balzers, Liechtenstein) and subsequently sputtered with gold (ca. 10 nm). The specimens were examined in an environmental scanning electron microscope ESEM XL30 (FEI Company, Philips, Eindhoven, Netherlands) operating at 15–20 kV.

Light microscopy

Light microscopic investigations were carried out with a LEITZ Axiophot (Leitz, Vienna). Sections were prepared

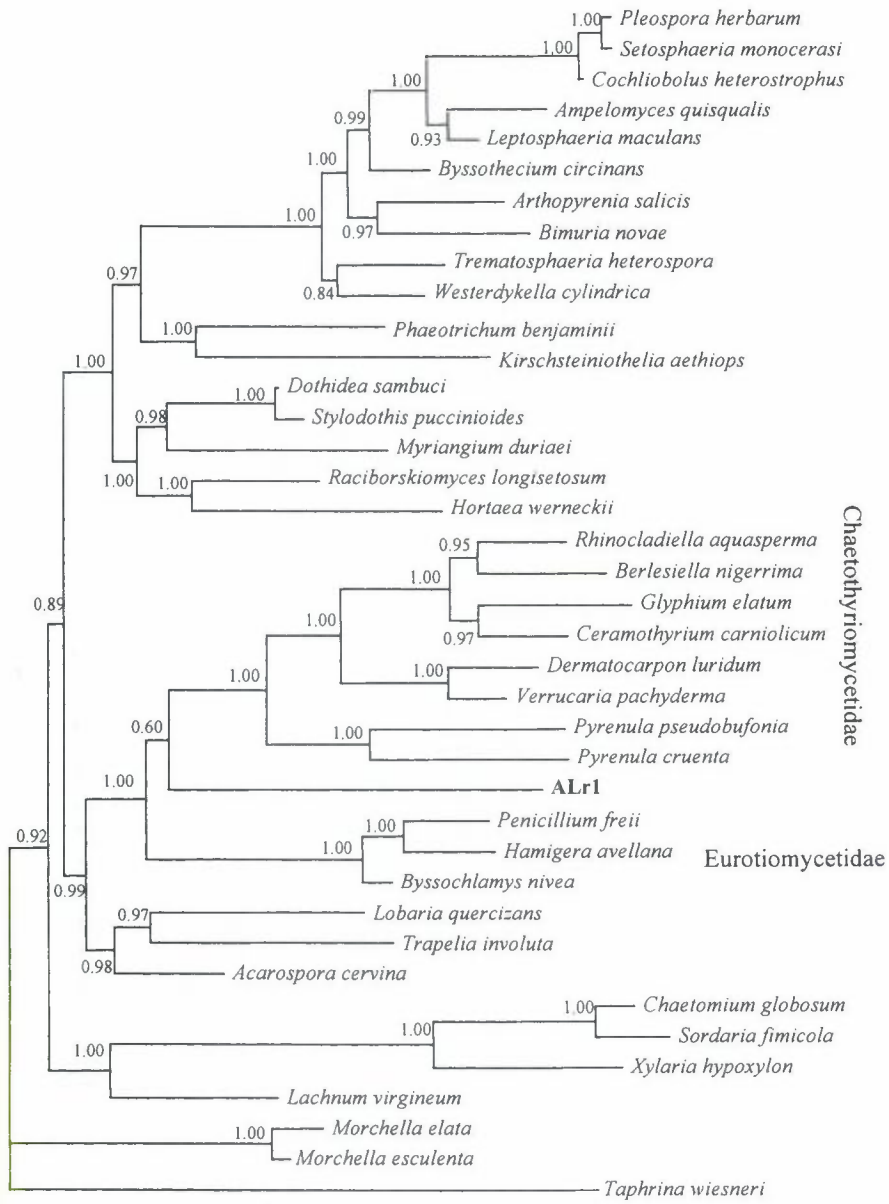

Table 1. SSU and LSU rDNA sequences of representatives of major known ascomycete lineages. ALr-1 locates basal to Chaetothyriales in this Bayesian consensus phylogram.

with a Cryotom (Leitz 1321 freezing microtome, Leitz, Vienna).

3. Results

Examination of extensive, freshly collected material of *Lecanora rupicola* from the Alps revealed one specimen showing a fungal infection, which renders parts of the thallus darker than usual (Fig. 1). Closer inspection showed that a hyphomycete was involved which developed local blackish outgrowths, preferentially at the margins of the thallus areoles. As seen with the light microscope, the surface hyphae of this lichenicolous fungus are melanised, and form short hyphal filaments (c. 3 µm wide) or more often exhibit a meristematic growth, with irregularly shaped microcolonies (Fig. 2; cell sizes ranging between 3–7 µm). These tiny fungal colonies tend to detach from the lichen host, and likely represent multicelled asexual diasporas e.g. conidia. The host lichen does not appear by observation to be damaged by the infections.

Infected areoles of *Lecanora rupicola* were then used for culture experiments. Beside the actual lichen mycobiont, the lichenicolous fungus was also obtained in cultures of thallus fragments from *Lecanora rupicola* on LB-medium. This fungus is tentatively named here ALr-1 (Associate of *Lecanora rupicola*-1). The lichen mycobiont and the lichen-associated fungus can easily be distinguished from cultures of the mycobiont of *Lecanora rupicola*. The latter forms cream-coloured and compact mycelial bodies, whereas ALr-1 grows as a spreading brownish mycelium, which grows faster than the genuine lichen mycobiont. The lichen mycobiont needed about half a year to enter a stationary phase with no more visible growth, while ALr-1

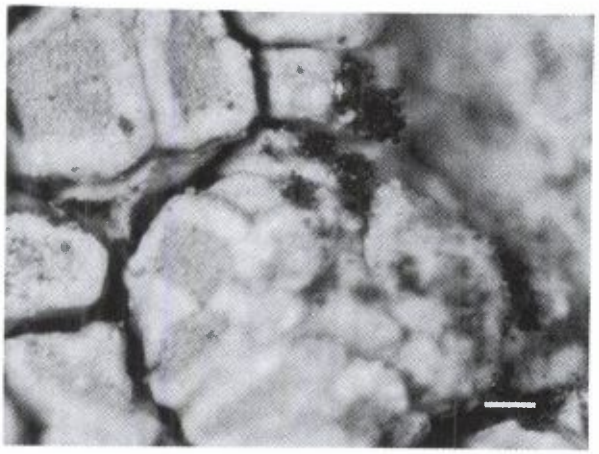

Figure 1. Growth habit of ALr-1 on the host lichen *Lecanora rupicola*. Scale = 0.5 mm.

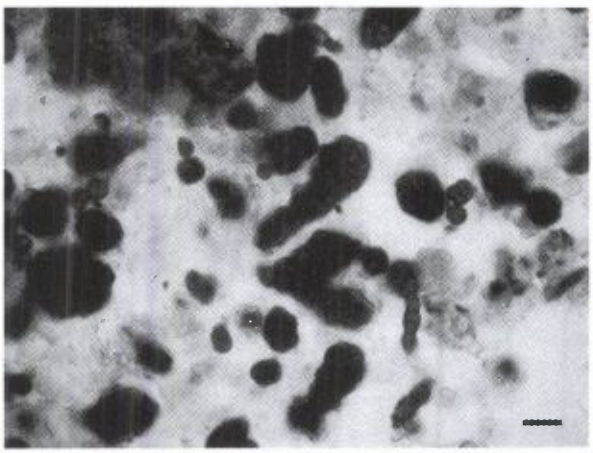

Figure 2. Meristematic growth of ALr-1. Squash mount of sectioned areole. Light microscopy. Scale = 20 µm.

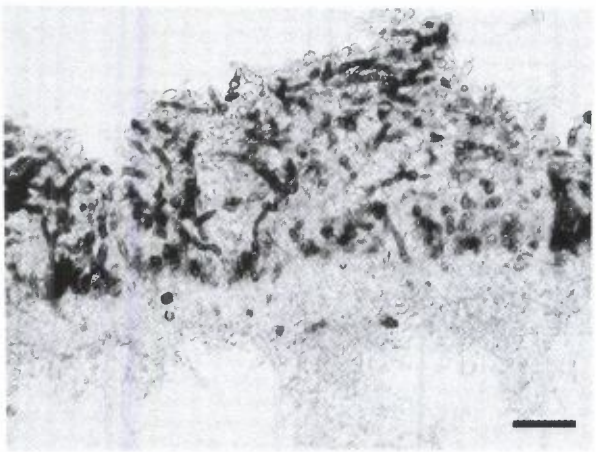

Figure 3. ALr-1 co-cultured with *Coccomyxa* sp. on LB medium. Light microscopical survey of a transversal section showing a thick melanised upper layer. Scale = 10 µm.

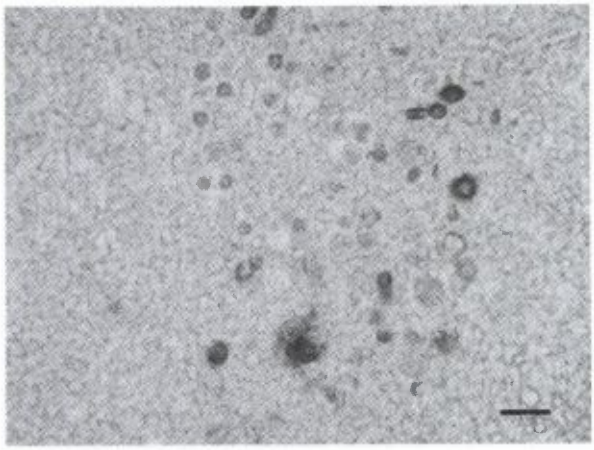

Figure 4. ALr-1 co-cultured with *Trebouxia* sp. on LBM. Light microscopy. Section showing melanised hyphae in algal-layer like stratum. Scale = 10 µm.

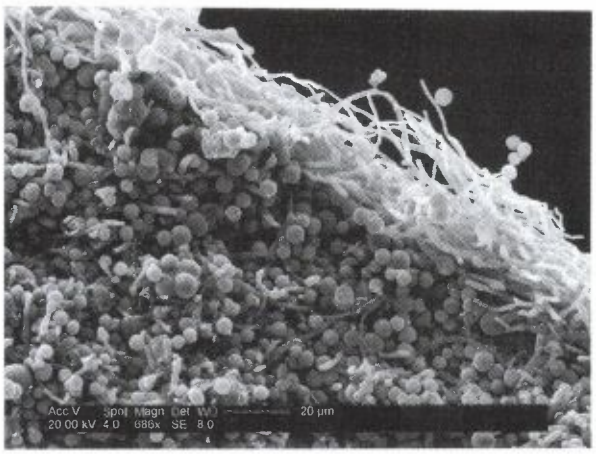

Figure 5. SEM micrograph of stratified co-culture of ALr-1 with *Trebouxia sp.* on PDA medium.

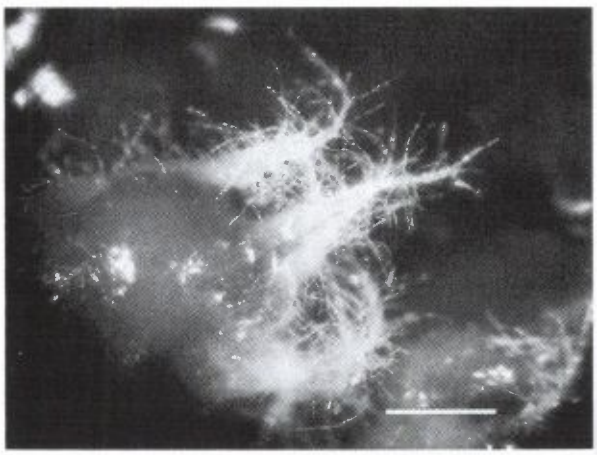

Figure 6. Phototropic hyphae of ALr-1 co-cultured with *Coccomyxa* sp. on PDA medium. Scale = 0.5 mm.

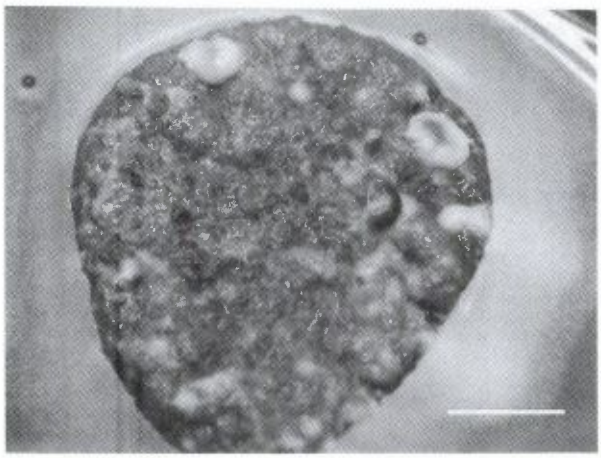

Figure 7. Developing and melanising upper layer of ALr-1 above cultured algal colony (*Coccomyxa* sp.) on PDA. Scale = 1 cm.

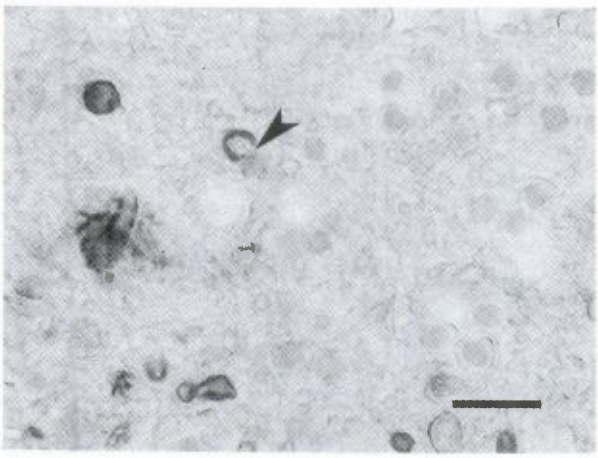

Figure 8. ALr-1 co-cultured with *Trebouxia impressa* on LBM showing melanised coccoid hyphae in algae-rich layer. Arrow marks section through a haustorium. Light microscopy. Scale = 10 µm.

reached this stage after 6 to 8 weeks. A later inspection of the herbarium collection at the Institute of Plant Sciences Graz (GZU) revealed two additional specimens of *Lecanora rupicola* with morphologically similar infections: One specimen was from France, Korsika, Golo Valley, 6. Nov. 2003, leg. Mayrhofer & Prügger (s.n.). The other lichen was from Austria, Styria, Gleinalpe, 12. Aug. 2001, Leg. Hafellner 56266. In the latter sample, the infection appears more massive than in other cases, and the merging lichenicolous colonies seem to detrimentally affect the lichen by screening off the light from the lichen photobiont beneath.

Experiments to culture ALr-1 with different types of green algae were carried out with two species of the genus *Trebouxia* and one of the genus *Coccomyxa* on two different media. In all cases, light-microscopical investigations and sections of the synthetic culture exhibited lichenoid structures with determined layers, as typical for lichens. With all tested algae, ALr-1 formed an upper cortex, an algal layer, and a medulla-like structure in the developing thallus (Figs. 3–5). Initially, bundled hyphae at the upper surface of ALr-1 show a phototropic orientation when co-cultured with algae and before the peculiar lichenoid thallus is developed. The phototropic conglutinated bundles of parallel hyphae break through the growing algal colonies and then grow with their tips towards the light source of the culture chamber, while side branches are also developed (Fig. 6). At the surface of the algal colonies, these phototropic hyphal bundles then start to form a nearly algal-free and horizontal cortex structure which becomes brownish after some time (Fig. 7). The upper cortical layer reaches a thickness of at least 20 µm. It contained only few algal cells and exhibited a vertical arrangement of conglutinated hyphae as it is also found in the upper cortex of many lichens. Below this surface layer, the algae concentrated in a layer resembling an algal layer of lichens, where the hyphae densely enlaced the algal cells. In this layer melanised, short-celled and ball-shaped, nearly coccoid hyphae can also be found (Fig. 8). As the lowermost layer, an extensive algal free zone is formed, in which the hyphae do not show any determined orientations. Differences in density and colour of this lichenoid structure formed by ALr-1 were observed and seem to correlate both with the species of algae involved and the culture medium composition (Fig. 9a–d). Co-cultures on LB-medium produced a more compact thallus organisation than those formed on PDA.

Fungal-algal contacts were studied in more detail by SEM, which also reveals alga-specific differences. The fungus seems to establish loose contacts with the *Coccomyxa*-algae as shown by the SEM micrographs, but the association with the *Trebouxia* species is much tighter than with *Coccomyxa* sp. In most of the cases the *Coccomyxa* algae were contacted by short side branches of hyphae, similar to those previously described for lichens by Tschermak (1941) and other *Coccomyxa* cells were found to be attached to protrusions of the hyphal cell wall (Fig. 10). These contacts were generally established with the narrow edge of the ellipsoid algal cells. The interaction may be harmful, as it was observed occasionally that hyphae lead to degradation of *Coccomyxa* cells (Fig. 11). Contacts to algae of the *Trebouxia* species appeared to be different. Hyphae indent the algal cell wall, resembling intraparietal haustoria formed by many lichen mycobionts (Fig. 12). In this interaction, the outer layers of the algal cells were likely penetrated.

HPLC analyses of methanolic extracts of the fungus did not reveal production of any secondary secondary metabolites known to be formed by obligately lichenized fungi.

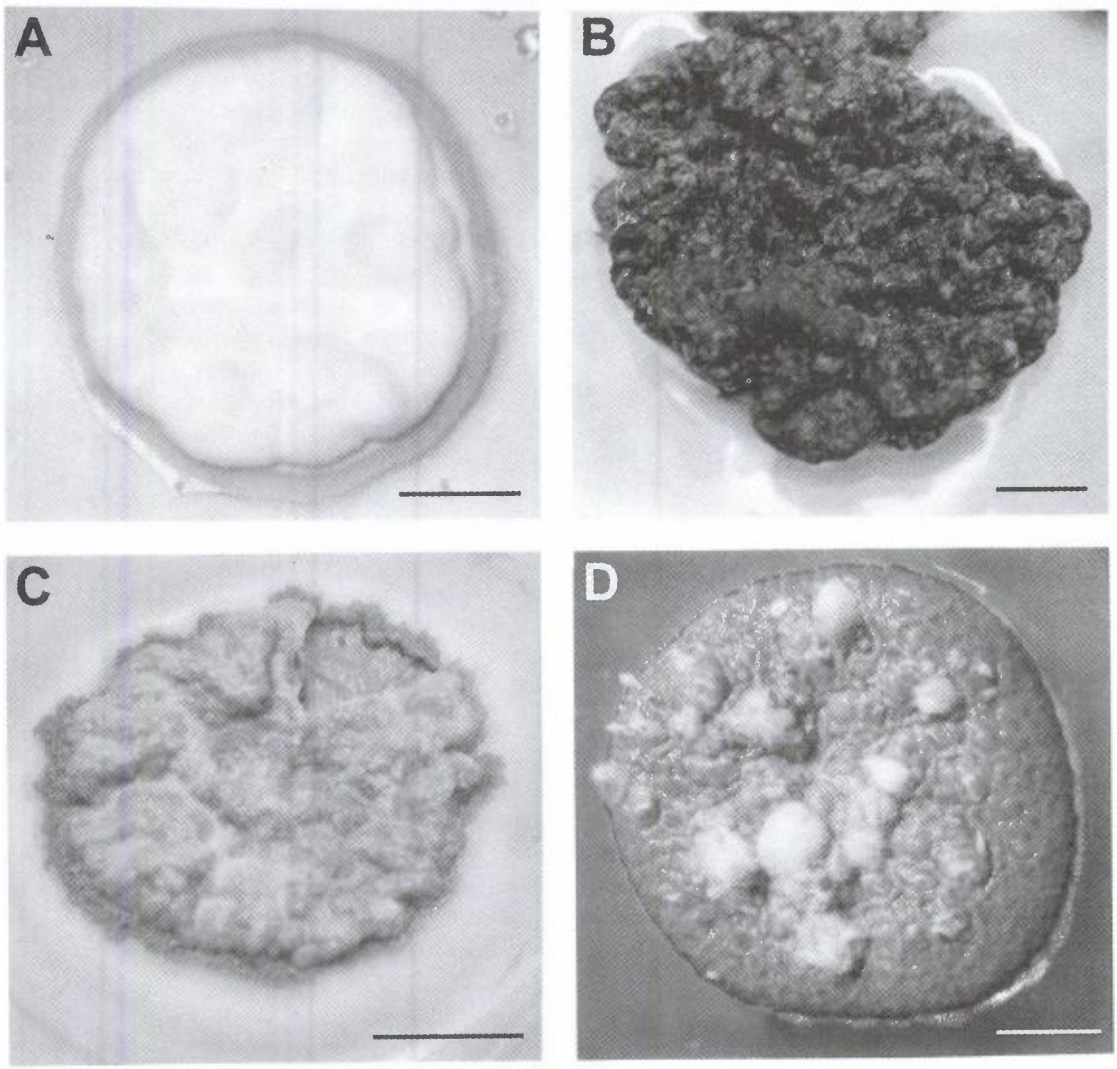

Figure 9. Culture morphology – macroscopically. a) ALr-1 co-cultured with *Trebouxia impressa* on LB-medium. b) ALr-1 with *Coccomyxa* sp. on LB-medium. c) ALr-1 with *Trebouxia impressa*: PDA-medium. d) ALr-1 with *Coccomyxa* sp. on PDA-medium. Scale = 1 cm.

Figure 10. SE micrograph. Magnitude: 8238x. ALr-1 in close contact with *Coccomyxa* sp. grown on PDA medium.

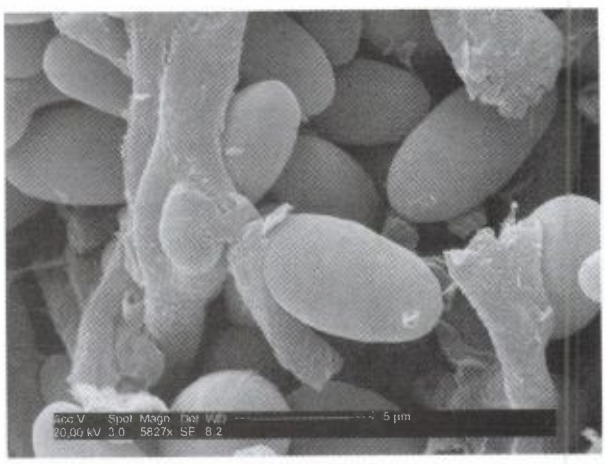

Figure 11. SE micrograph. Magnitude: 5827x. ALr-1 co-cultured with *Coccomyxa* sp. on PDA.

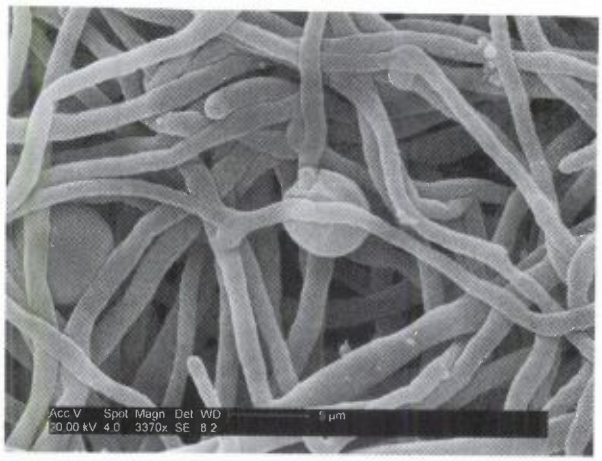

Figure 12. SE micrograph. Magnitude: 3370x. ALr-1 in co-culture with *Trebouxia impressa* on PDA.

As BLAST searches using the ITS sequence of ALr-1 did not reveal any clear relationship with any so far sequenced fungal group, except for. "*Ascomycete* sp. ZGZII03060" (DQ124138) from an unpublished study of helicosporous hyphomycetes from China we sequenced conserved ribosomal DNA loci. The phylogenetic analyses of combined SSU and LSU rDNA data do not suggest that ALr-1 is closely related to any known group of lichens, rather the fungus is phylogenetically located at the basis of *Chaetothyriomycetidae* (Table 1).

4. Discussion

In this study a peculiar lichenoid association of the cultured fungus ALr-1 is presented, which was investigated by light-microscopy and SEM. In co-cultures with various algae, the fungus develops a stratified thallus-like structure on different media. In contrast to most resynthesis experiments with lichen symbionts, the lichenoid association of ALr-1 grows significantly faster (see result section). A rather unusual aspect of the association with algae was the initial presence of phototropic hyphal bundles, which first penetrated the non-lichenised algal colonies and then started to form a fungal surface which becomes pigmented after a while. Phototropism is known to be mediated by of photoreceptor systems in fungi (e.g. Idnurm et al., 2006), but has never before observed in hyphomycetous ascomycetes. The ontogenetic pattern leading to lichenoid associations of ALr-1 is different from the typical pattern found in more typical lichenised fungi, first by the overall dark coloured mycelium and second by the looser structure of the peripheric layers. Moreover, phototropic hyphal bundles have never been observed in developing lichen initials. They were also absent from the infected original lichen. In ALr-1, the photobionts are densely entangled by the contacting hyphae, which form algal-free peripheric layers early in the ontogeny, and then often proceed with the production of secondary metabolites in the developing stratified structure. The morphogenetic capacity in the infected lichen seems to be low, as, apart from the dark coloured surface hyphae, the thallus of the host does not show any macroscopically obvious indication of the presence of ALr-1 inside the thallus. Moreover, since anything resembling the lichenoid structure of ALr-1 has never been found in nature, we assume that the lichen host is required for establishment. However, we have found that the re-infection of the host results in a more or less detrimental interaction with invasive growth of ALr-1. We assume that this behaviour might be due to either the experimental conditions or the concentrated inoculum. We are therefore now conducting experiments in natural habitats of the host lichen, using dilution series to investigate infective processes in greater detail.

In lichen symbioses, a typical thallus can only differenciate in the presence of an alga from a specific set of appropriate, more or less related algal lineages. In contrast, ALr-1 is able to develop similarly structured lichenoid association also with different lineages in the algal class *Trebouxiophyceae*. Nevertheless, the different types of contact with the algal cells by the fungi can be attributed to genetically determined symbiont selectivity patterns. The tighter contacts with *Trebouxia* could indicate that members of this genus are preferred partners of cryptic symbioses in the natural lichen.

Previous work on lichen-associated fungi revealed a considerable number of additional fungi in culture experiments. While Petrini et al. (1990) focused on fungal organisms adhering to the surface of lichens, Girlanda et al. (1997) also showed that such fungi readily occur inside the thallus. Anyway, these studies not reveal insights into the biology of the lichen-inhabiting fungi. Another study indicated that certain non-lichenised fungi can develop lichenoid association (Gorbushina et al., 2005), but such fungi were not retrieved from lichens directly and it was not clear whether these associations are also present in nature. However, Turian (1977) previously described a peculiar dematious hyphomycete, *Coniosporium aeroalgicola*, which seems to be an ubiquitous component of aereo-algal communities and which – similar to ALr-1 – is able to form lichenoid structures. The relationships of ALr-1 with fungi assigned to *Coniosporium* still need to be elucidated. Peculiar interactions between other normally free-living fungi and coccale green algae were recently studied using culture experiments. Watanabe et al. (2005) analysed consortia of *Chlorella sorokiniana* and found an *Acremonium*-like hyphomycete in co-cultured material (beside bacteria). The hyphae of this fungus were found to be tightly attached to the hyphae, suggesting a symbiotic relationship, also because the chlorophyll content of

Chlorella was not diminished during a period of seven months. Interestingly an *Acremonium*-like fungus was previously also isolated from lichens (Girlanda et al., 1997). Our study, nevertheless, reports for the first time that a lichenicolous fungus may undergo specific relationships with the algae of the host lichen, and also with algae that that are free-living and may occur frequently on the surface of the host.

According to available molecular or phenotypic data, ALr-1 does not seem to represent any known lichenicolous fungus on *Lecanora rupicola*, although the phylogenetic position of *Sclerococcum* is unknown so far. *Sclerococcum* seems to detrimentally parasitise the algal partner of the host and is phenotypically distinct from ALr-1 in forming well-delimited sporodochia and a hyaline basal sporodochial layer of more or less rounded cells (Grube, unpublished observation). We however, refrain from a formal description of this new species, mainly due to the lack of molecular data about the relationships of the known lichenicolous hyphomycete genera. It is not known so far whether ALr-1 can also be found in other lichen species beside *Lecanora rupicola*, or may in fact even be a widespread inhabitant of lichens, though similar localised infections with dematious hyphae are also observed in other crustose lichens. The preference for the host's algal partners, which are common in lichens of similar habitats, would at least be in agreement with a wider occurrence of this species in other lichens.

From an evolutionary perspective, the phylogenetic position of ALr-1 is quite interesting. According to Lutzoni et al. (2001), lichenisation is a rather early and principal achievement in the phylogeny of ascomycetes, while several important non-lichenised lineages may have evolved from potentially lichenised ancestors. This is clearly supported by the phylogenetic topology within the *Chaetothyriomycetidae*, where lichenised lineages (*Pyrenulales*, *Verrucariales*) appear to represent basal lineages from which non-lichenised *Chaetothyriales* (including several human pathogens) have emerged. The position of ALr-1 suggests that basal lineages already acquired the potential to form lichenoid associations but have not evolved a high selectivity for particular algal partners. It will be highly interesting to study whether fungi of likewise basal position to *Chaetothyriomycetidae*, whether or not associated with lichens, display a similar biological behaviour.

We assume that other lichen-associated fungi which may develop similar lichenoid association in cultures could be more frequent than estimated so far. Apart from this, the ready and comparatively rapid formation of apparently mutualistic associations of ALr-1 with algae suggest that this fungus could be useful to investigate processes involved in morphogenetics of fungal-algal associations and in the recognition of partners.

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