Applied Soil Ecology 68 (2013) 20–25

Contents lists available at SciVerse ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Localization of bacteria in lichens from Alpine soil crusts by fluorescence *in situ* hybridization

Lucia Muggia[a,b,*], Barbara Klug[a], Gabriele Berg[c], Martin Grube[a]

[a] Institute of Plant Sciences, Karl-Franzens-University Graz, Holteigasse 6, 8010 Graz, Austria
[b] Department of Life Science, University of Trieste, Via L. Giorgieri 10, Trieste, Italy
[c] Institute of Environmental Biotechnology, Graz University of Technology, Petersgasse 12, 8010 Graz, Austria

ARTICLE INFO

Article history:
Received 26 July 2012
Received in revised form 5 March 2013
Accepted 14 March 2013

Keywords:
Acidobacteria
Alphaproteobacteria
Confocal microscopy
FISH
Thallus structures

ABSTRACT

Lichens are prominent components of many biological soil crusts. Owing to their persistence, lichen thalli create microhabitats for other microbes. Here, the structure of bacterial communities at the thallus–soil interface in lichen soil crusts was studied by using fluorescence *in situ* hybridization (FISH), confocal laser scanning microscopy (CLSM) and 3D image reconstruction. Terricolous lichen thalli above the tree-line in open habitats of the Austrian Alps were sampled. We selected six lichen species associated with green algal photobionts: *Arthrorhaphis citrinella*, *Baeomyces placophyllus*, *B. rufus*, *Icmadophila ericetorum*, *Psora decipiens* and *Trapeliopsis granulosa*. Alphaproteobacteria and Acidobacteria are predominant in these soil crust lichens, where the latter are frequently present in the lower part of lichen thalli and in the hypothallosphere. In the inconspicuous thallus structures of *Arthrorhaphis citrinella*, *Baeomyces rufus*, *Icmadophila ericetorum* and *Trapeliopsis granulosa* we observed association of bacteria with algal cells in soil particles and on the outer surface of the mycobiont–photobiont aggregates. We found bacterial cells intermixed with photobiont cells in the lower part of the lichen thalli and as small colonies on the surface of the squamules of *Baeomyces placophyllus* and *Psora decipiens*. Moreover, technical issues of performing FISH and confocal microscopy with biological soil crusts are discussed.

1. Introduction

Biological soil crusts are widely present in the open vegetation of arid and semiarid environments, as well as in arctic tundras and at high elevations in Alpine regions. By aggregating soil, biological soil crusts reduce or prevent soil surface erosion by wind and water (Asta et al., 2001; Belnap et al., 2003; Jones et al., 1997). They also contribute to nitrogen and carbon fixation (Belnap, 2002; Beymer and Klopatek, 1991) and enhance the mineral nutrient content of soils (Harper and Belnap, 2001). Especially in habitats where plant growth is hampered by abiotic constraints, biological crusts may even dominate the landscapes.

Soils crust communities are complex and composed of diverse microorganisms, such as algae, bacteria (incl. cyanobacteria), mosses, lichen-forming and not lichenized fungi. The macroscopic structures of these communities vary principally with climatic parameters. Thin soil crusts composed by lichens are typical for dry and hot habitats such as deserts (Lalley et al., 2006), whereas the vertical profile of soil crust becomes more pronounced with increasing humidity and lower temperatures of higher latitudes. This trend correlates also with differences in bacterial biomass and species diversity (Harper and Belnap, 2001; Garcia-Pichel et al., 2003). Biological soil crusts are therefore characterized as different types that correspond with their predominant organisms (Redfield et al., 2002). Algal soil crusts have a typical green cast structure on moist soils, whereas cyanobacterial crusts are dark and moss soil crusts form greenish fur-like carpets (Wu et al., 2011). The highest morphological diversity is present in lichen soil crusts (Grube et al., 2010), since lichens not only suffuse the first millimetres of soil, but they can form thick cushions and complex three-dimensional structures. Lichen dominated soil crusts are fairly common in habitats of the Alps above the tree line (Türk and Gärtner, 2003), where lichens find suitable conditions with steep changes of hydration and temperature. Under cool and freezing temperatures the respiration is limited but photosynthesis is still accomplished. Thanks to their poikilohydric life style, many lichens are fairly resistant to damage from dehydration. In alpine heights, the crusts form perennial communities at windswept places, in soil-filled fissures of rocks, in gaps between higher plant vegetation, on bare soil along mountain paths and along windswept ridges, or at the fluvoglacial deposits in periglacial areas (Türk and Gärtner, 2003).

* Corresponding author. Current address: Institute of Plant Sciences, Karl-Franzens-University Graz, Holteigasse 6, 8010 Graz, Austria. Tel.: +43 3163805661; fax: +43 3163809883.
E-mail addresses: lucia.muggia@uni-graz.at, lucia_muggia@hotmail.com (L. Muggia).

0929-1393/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.apsoil.2013.03.008

Species diversity of soil lichens is highly correlated with the bedrock type and the exposition. Shady or Northern exposures which have a higher water-holding capacity of upper soil layers and are covered by snow for longer time are suitable for gelatinous lichens and other species which require either liquid water or extensive humidity. Southern exposures tend to be dryer and promote desiccation-tolerant species (Türk and Gärtner, 2003). These microclimatic changes proved to influence the bacterial diversity associated with the lichen thalli (Cardinale et al., 2011).

Because of their role in the prevention of erosion, soil crusts are being studied more carefully for their composition of organisms (Bates et al., 2011; Garcia-Pichel et al., 2003; Redfield et al., 2002). Studies of the composition of lichen soil crusts mostly rely on microscopic and cultivation-independent molecular analyses. The lichen–soil interface was previously analysed with an electron microscopy approach by Asta et al. (2001), who showed that interactions of fungal hyphae with soil particle differed among the studied lichen species. The authors also noticed that the complex lichen–soil relationships could also include the presence of bacterial colonies. The distribution of bacterial groups, however, was not studied in greater depths. To localize bacterial communities in lichen thalli, fluorescence *in situ* hybridization (FISH) combined with confocal microscopy (CLSM) was used previously (Cardinale et al., 2008, 2011; Grube et al., 2009), but the soil interface of lichens has not yet been studied with this technique. As we experienced, such an approach is complicated by technical challenges. Superficial thallus structures are formed by tightly conglutinate fungal hyphae whereas the soil layers, suffused by fungi, are crumbly and hard to prepare for microscopic studies. Here we propose an approach to analyse the biologically complex soil crusts by FISH and CLSM.

2. Materials and methods

2.1. Sampling and species description

Six species of terricolous lichens were collected in the Austrian Alps, in alpine vegetation on acidic substrate (siliceous bedrock) above the tree-line, on south-east exposed localities during the first half of June 2009 [Austria, Styria, Wölzer Tauern, Pusterwald, Hinterminkel Valley, 150 hundred meters below the Pölseckjoch, 2020 m a.s.l., *L. Muggia & J. Hafellner*, 09.VI.2009; Koralpe range, Handalm, 1850 m a.s.l, *L. Muggia & J. Hafellner*, 15.VI.2009]. The selected species are *Arthrorhaphis citrinella*, *Baeomyces placophyllus*, *B. rufus*, *Icmadophila ericetorum*, *Psora decipiens* and *Trapeliopsis granulosa.* All of them are associated with green algae photobionts and reproduce sexually (through meiospores produced in apothecia). Two species, *Baeomyces placophyllus* and *Psora decipiens*, form compact squamules on the soil surface, the remaining four species have a granulose thalli with crumbly structure which is profoundly intermixed with the substrate. *A. citrinella*, *B. placophyllus*, *B. rufus*, *I. ericetorum* and *T. granulosa* are typical terricolous lichens on acidic and loamy naked soil. In other habitats, where outcrops of calcareous bedrock occur, *Psora decipiens* can become a rather common species among the soil crust lichens. All the selected species are, however, not restricted to Alpine habitats: *Baeomyces placophyllus* can be found since the collinean belt; *B. rufus* is common at all altitudes except in the Mediterranean region; *Icmadophila ericetorum* is very common also in montane forests; *Psora decipiens* grows on calcareous soil at all altitudes and is very common in the Mediterranean region; *Trapeliopsis granulosa* is distributed from the Mediterranean region to the highest elevations (Wirth, 1995; Van Haluwyn et al., 2012).

We sampled three small intact undisturbed soil blocks (at least 3 cm^2) of each lichen soil crust species. The blocks were collected with gloves, transferred into sterile tubes and fixed on the same day of the sampling.

2.2. Fluorescence in situ hybridisation (FISH)

Entire soil blocks of lichen material were fixed immediately after sampling in 4% paraformaldehyde (PFA) for 8 h. Material was washed three times with 1× PBS in order to remove paraformaldehyde. The fixed material was stored in 1× PBS and 96% EtOH (1:1) at −20 °C prior to subsequential procession. Small fragments of the soil block of about 1 cm^2 (lichen thallus together with the underneath soil) were embedded in water or in the O.C.T. Tissue Tek embedding solution (Sakura, Finetech Europe BV, Zoeterwoude, Netherlands) and cut with a cryotome (Leica CM 3000, Heidelberg, Germany) into 30 μm thick sections. The frozen sections were transferred with a sterilized and cooled needle in 1× PBS/96% EtOH (1:1) solution. Up to 8 tubes were prepared for each lichen sample and stored at −20 °C. The amount of stored section is sufficient to perform up to 10 replicates, corresponding to 10 hybridization experiments with different sets of FISH probes.

In situ hybridization was performed directly on poly-L-Lysine-precoated microscope slides (Polysine microscope slides; VWR, Vienna; Klug et al., 2011). Up to 8 sections of paraformaldehyde-ethanol-fixed sample material were placed on the slide. The lichen thallus sections and the soil fragments were carefully flattened out on the slide under a dissecting microscope (Zeiss, Vienna) by using two sterile thin needles in order to preserve samples structure. The samples were dried for 15 min at 46 °C. In order to facilitate the penetration of the FISH probes in the bacterial cells the samples were treated with 1 mg/ml lysozyme (Sigma–Aldrich, Teinheim, Germany) for 10 min at room temperature. Thereafter, the slides were briefly rinsed with 500 μl of sterile water. An ethanol series (50%, 80%, 96% ethanol, each for 3 min) was applied to dehydrate the samples. The samples were subsequently dries for few minutes at 46 °C after that 100 μl of hybridization buffer (5 M NaCl, 1 M Tris/HCl, 2% SDS, formamide, water) containing fluorescent labelled oligonucleotide probes (100 ng/μl) was applied. In order to prevent the evaporation of the hybridization buffer, the slides were enclosed in an incubation chamber for two hours in the dark at 46 °C. Afterwards samples were washed for 15 min in washing buffer at 37 °C and rinsed briefly with cold water 2–3 times to remove residual washing buffer. The FISH probes required formamide concentrations of the hybridization buffer in a range between 10 and 45%, and only probes with the same stringency requirements were applied simultaneously. Probes requiring different stringency conditions were applied sequentially: the hybridization was performed first with the FISH probes requiring the highest stringency, and secondly, after the washing, with the probe requiring the lower stringency conditions. After the final washing with water, the slides were quickly air-dried with compressed air. Samples were mounted with the ProLong Gold antifadent solution (Molecular Probes, Vienna) to avoid the fading of the fluorescence of the probes. A cover slip was placed on top and the samples were stored overnight in the dark and then sealed with nail polish.

FISH experiments were performed using six group-specific probes: ALF968 binding Alphaproteobacteria (Neef, 1997), Bet42a binding Betaproteobacteria (Manz et al., 1992), Gam42a binding Gammaproteobacteria (Manz et al., 1992), EUB338-EUB338III (EUBmix) binding Eubacteria (Amann et al., 1990; Daims et al., 1999), LGC354A-C (LGCmix) binding Firmicutes (Meier et al., 1999), and SS-HOL binding Acidobacteria (Meisinger et al., 2007). Probes were labeled with fluorochrome Cy3, Cy5, 6-FAM and FITC. Negative controls of the FISH were performed with the NONEUB probe (Wallner et al., 1993) labeled with the same fluorochromes used

for the individual specific probes. Three FISH replicates were performed and analyzed by CLSM for each lichen sample.

2.3. Confocal laser scanning microscopy (CLSM)

The labelled samples were analysed with a Leica TCS SP confocal laser-scanning microscope (Leica Microsystem, Heidelberg, Germany) equipped with argon and helium/neon laser. Photomultipliers parameters were adjusted to achieve the maximum signal from each fluorochrome, but at the same time trying to keep the noise signal generated by the soil particles and by the algal cells as low as possible. Up to three FISH probes were applied in parallel on the samples and signals were measured simultaneously. The ranges of wavelength set to detect the emission signal of the fluorochromes labelling the FISH probes were the following: Cy3 at 560–610 nm, Cy5 at 660–700 nm, 6-FAM and FITC at 470–530. Stacks of 0.5 μm slices were scanned through the 30 μm thick section and their maximum projections were converted into 2D pictures. The 3D-reconstructions of 2D stacks and the different section levels to characterize the size and the position of the bacteria colonies were performed with the software AMIRA v. 5.2 (Visage Imaging GmbH). No image artifacts were present or needed to be corrected.

3. Results and discussion

3.1. Fluorescence in situ hybridization

The fragile nature of the soil crust specimens required a particularly careful handling of the samples during the preparation for the FISH experiments. The species *Arthrorhaphis citrinella*, *Baeomyces rufus*, *Icmadophila ericetorum*, and *Trapeliopsis granulosa* have granulose and poorly coherent thalli (Fig. 1a, c, d and f, first column), which easily disintegrate into small fragments when the material is dry. These thalli do not display the typical differentiation with an algal layer of defined thickness. The thalli of these species adhere tightly to the soil or to small plant or moss remnants. Moreover, the lack of an upper cortex of fungal origin in *I. ericetorum* and *A. citrinella* (Smith et al., 2009) may further increase the fragility of the thalli. *Baeomyces rufus* and *T. granulosa* (Fig. 1c and f) present an upper cortex of entangled hyphae but this layer disappears as ageing thalli develop soredia (small hydrophobic fungal congregations with internalized algae for asexual reproduction) on the surface; these two species can form very large aggregates of adjacent thalli. On the other hand, *Baeomyces placophyllus* and *Psora decipiens* (Fig. 1b and e first column) develop squamulose thalli with well delimited upper cortex and with well distinguishable algal layer and medulla (a fungal layer below the algal stratum). None of the analysed species form a lower cortex. The lowermost parts of the thalli intergrade with the soil, though details in structure are different among the species. Material therefore needs to be sampled as small blocks of several millimetres together with the underlying soil. In this way the easily fragmentation of the samples into tiny pieces is minimized and it is possible to cut sections of desired thickness. We chose to cut section of 30 μm for two reasons: (i) thinner section would break easier during the preparation, (ii) thicker sections would contain greater amount of mineral material which disturbs the probe signals during confocal scanning observation. Further, the thicker the section is, the higher is the number of slides required to analyse the sample in confocal microscopy. That increases the acquisition of noise signals with consequently loss of details. Soil debris and the parts of the lichen thallus, however, frequently fragmented as soon as frozen sections melted when transferred into 1× PBS/96% EtOH (1:1) solution or were pipetted on the slides for subsequent processing.

Initially, the FISH protocol optimized by Cardinale et al. (2008) and Grube et al. (2009) for lichens was applied. Following this protocol the whole hybridization is performed in a 1.5 ml tube and only after the final washing step the sections are applied on the microscope slide. Cardinale et al. (2008) and Grube et al. (2009) performed their analyses with fruticose, foliose and crustose alpine lichens (these latter growing on rock), which develop a compact and coherent thallus structure. This protocol, however, is not applicable for lichens that directly suffuse into the soil because the handling during this FISH protocol leads to significant loss of samples. If the amount of starting material is increased in order to compensate the loss of the samples by pipetting, however, an additional problem arises: the sections precipitate all together with the soil particles at the bottom of the tube during each incubation time. This tight aggregation of the fragments prevents (i) the activity of the lysozyme on all the bacterial cells walls, (ii) the homogeneous dehydration during the ethanol series, (iii) the efficient penetration of the FISH probes in all the bacterial cells, (iv) the effective removal of lysozyme, of the hybridization buffers as well as of the washing buffers in the respective steps. We optimized, therefore, the *in situ* hybridization for the lichen soil crusts directly on poly-L-lysine-coated slides.

Cardinale et al. (2008) claimed the easy detachment of lichen thallus sections from the coated slide, probably because rather thick and long sections of lichens were used. Thick or long sections indeed are characterized by considerable tension forces due to fungal cell wall polymers. The much thinner sections of soil crust lichen material, on the contrary, attach permanently on the slide. Their small sizes also prevent their bending–which acts on longer sections–and from loosening during the staining process. Together with the small sections, however, also mineral matrix such as sand grains, fragments of soil and tiny crystals are applied on the slides. It is known that these impede the application of FISH to environmental samples (Shiraishi et al., 2008). The inevitable soil grains first disturb the sectioning by standard methods; secondly, once on the slide, they can false bind the negatively charge oligonucleotides probes falsifying the signal; thirdly, they have a strong autofluorescence which hinders the FISH probe signals in the confocal microscopy (Shiraishi et al., 2008). However, it is hardly possible to remove the mineral fragments because the scratching would damage the poly-L-lysine layer which would cause the loosing of the sections in the subsequent steps of the hybridization protocol (removal of these grains by a filtering step was not feasible as the sections were too small).

3.2. Bacterial colonies and structure of lichen soil crusts

Alphaproteobacteria and Acidobacteria were detected as predominant groups in the selected lichen soil crusts, which might correlate with the acidic substrate on which the lichen crusts develop, with the exception of *Psora decipiens*. The general probe for Eubacteria, Alphaproteobacteria and Acidobateria always gave a strong signal, whereas we could not detect Beta- and Gammaproteobacteria and Firmicutes with the specific probes. A low signal of Beta- and Gammaproteobacteria and Firmicutes probes, whenever present, has been undetected probably due to the disturbance caused by the mineral material present in the samples. This confirms that these bacterial groups occur at rather low frequency in certain lichens, as suggested in earlier studies (Cardinale et al., 2008; Grube et al., 2009; Pankratov, 2012).

The distribution of bacterial colonies on the lichen structures differs among the six soil crust species. Clusters of hyphae, algae and bacteria that are intermingled with the soil matrix are found in the species with granulose thalli: *Arthrorhaphis citrinella*, *Baeomyces rufus*, *Icmadophila ericetorum* and *Trapeliopsis granulosa*. In particular, bacterial colonies are more or less homogeneously distributed

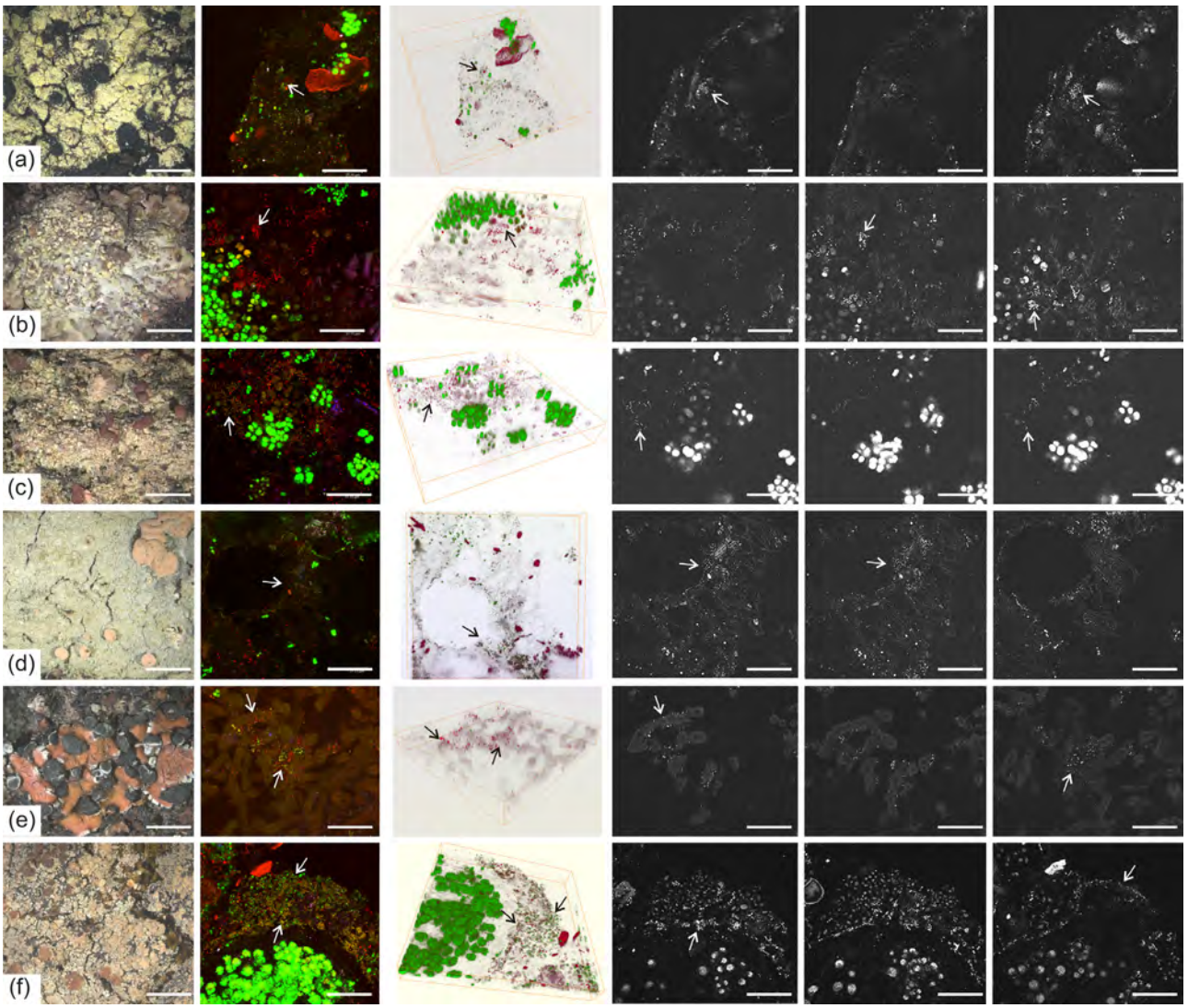

Fig. 1. Localization of bacteria in lichen soil crusts and 3D reconstructions. From left to right: habit of lichen thallus, 2D-overlay of 3D-CLSM optical slices, 3D-reconstructions, localization of bacterial communities at three different section levels in the lichen soil crust. *Arthrorhaphis citrinella* (a), *Baeomyces placophyllus* (b), *Beomyces rufus* (c), *Icmadophila ericetorum* (d), *Psora decipiens*, detail of hyphae from a thallus fragment (e), and *Trapeliopsis granulosa* (f). In 2D-overlay algae are in green, Alphaproteobacteria in yellow, Eubacteria in red. In the 3D reconstruction fungal hyphae are in grey, algal cells in green, Eubacteria are in red and Alphaproteobacteria are the tiny green dots. In the three different section levels fungal hyphae are in grey and bacterial cells are the white dots and rod-shape dots; algal cells are the white bigger and brighter spots. Arrows indicate corresponding bacterial colonies in the 2D-overlay and in the different section levels. For macrophotographs, bar = 2 mm; for 2D-overlay of 3D-CLSM data and section levels microphotographs, bar = 31 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

in the soil matrix beneath *A. citrinella*, *B. rufus* and *I. ericetorum*. In these crusts, with rather crumbly structure, the bacteria colonies form continuous layers at different depths, as visible in optical sections (Fig. 1a–f second column). In some samples the bacterial cells are visibly attached to the fungal hyphae (e.g., *I. ericetorum* and *Psora decipiens*; Fig. 1d and e second column). Rod-shaped bacteria were observed in the preparations of *B. rufus* particularly in the visualization of different section levels (arrow Fig. 1c in second, fourth and sixth column). Only in few sections of *I. ericetorum* and *T. granulosa* we detected abundant bacterial colonies of Acidobacteria, Alphaproteobacteria and Eubacteria on the upper surface of the thallus (Fig. 1d and f second column; Fig. 2c, d and f). On the other hand, bacterial colonies are always conspicuous on the upper surface of the distinctly squamulose species *B. placophyllus* and *P. decipiens* (Fig. 2b and e) which also show a clear internal stratification of functional layers. In these species, the bacterial colonies form a thick layer which also contains unspecified epithalline algae (Fig. 2a and f). In *B. placophyllus* and *I. ericetorum* we observed a gradient of bacterial abundance, with an increasing frequency from the lowest layer of the mycobiont hyphae (medulla) towards the soil beneath (Fig. 2b–d). *P. decipiens* presents conspicuous thick medullary hyphae which are evident both in transversal and horizontal sections. The bacteria are here located preferentially in angles of the hyphal net, forming aggregates of variable sizes or individual bacterial cell are directly attached to the fungal hyphae (Fig. 1e, second column). Alternatively, bacterial cells are also distributed in well compacted colony aggregates on the upper cortex of the squamules (Fig. 2e). In *B. placophyllus* dense Alphaproteobacteria colonies are present at the boundary of algal layer and the medulla, whereas both Alphaproteobacteria and Eubacteria colonies are scattered towards the lower part of the medulla in contact with the soil (Fig. 1b, second column). Acidobacteria were detected in great amounts in rhizine-like structures of *I. ericetorum* (Fig. 2c and d). Rhizines are variably shaped root-like structures of agglutinated hyphae for attachment of lichen thalli with the substrate. To what extent these specific accumulations of Acidobacteria are involved in nutritional functions of the lichens, however, remains unclear for the time being. At the same time,

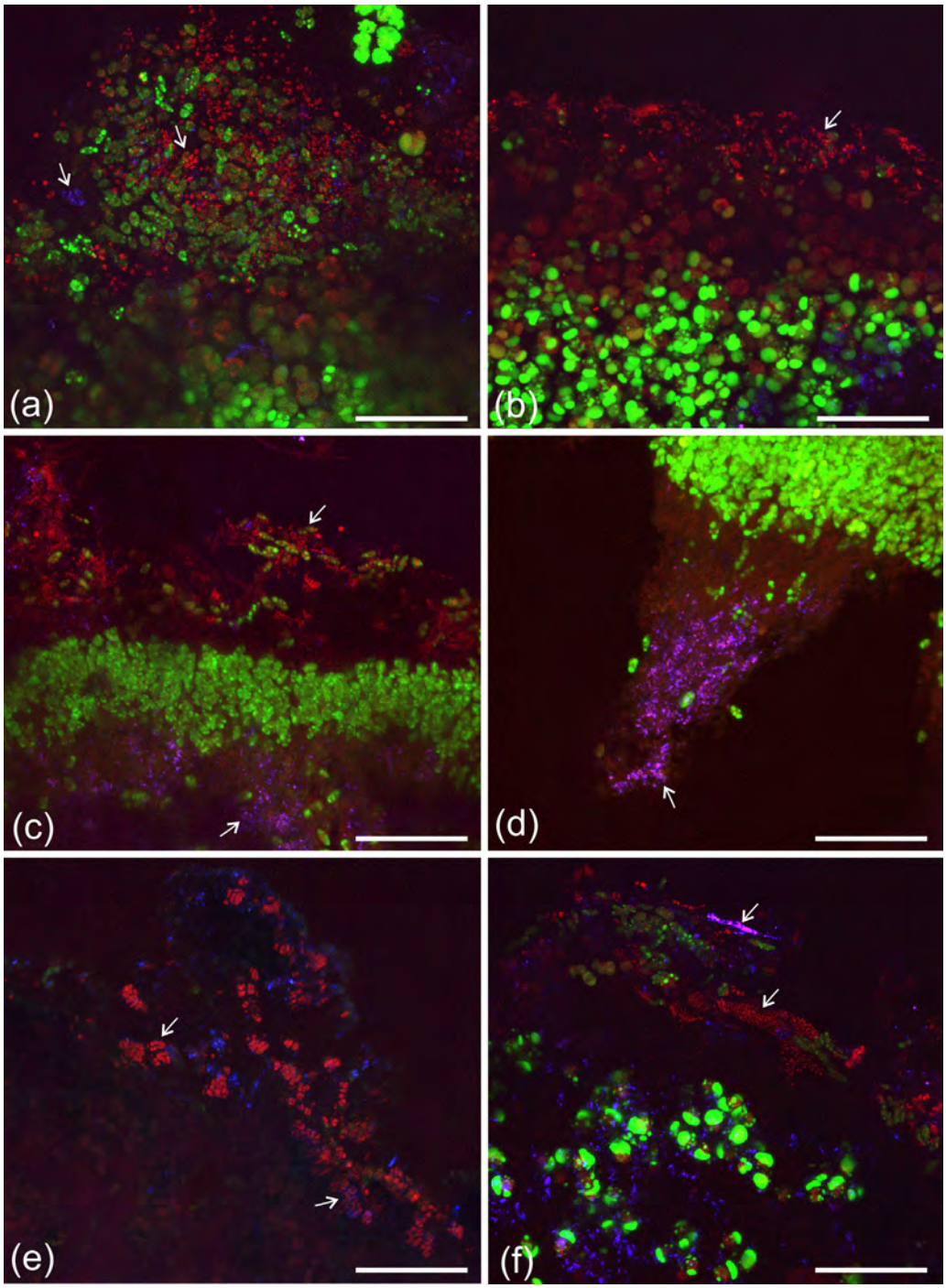

Fig. 2. FISH-stained bacteria in lichen soil crust: Eubacteria in red, Acidobacteria in pinkish-violet, green spots are algal cells (lichen photobiont as well as other smaller algae present in the soil crusts), navy blue dots are noises generated by mineral fragments. (a) *Arthrorhaphis citrinella*: unstructured distribution of algae and Eubacteria; (b) *Baeomyces placophyllus*: structured distribution of Eubacteria on the upper cortex of the thallus, algal layer distinguishable by the autofluorescence of the photobiont cells; (c and d) *Icmadophila ericetorum*: structured distribution of Eubacteria in the upper part of the thallus, Acidobacteria distributed in the lower part of the fungal layer (c) and in a rhizine-like hyphae structure (d); (e) *Psora decipiens*: upper cortex of the squamules colonized by Eubacteria distributed in well delimited colonies; (f) *Trapeliopsis granulosa*: Eubacteria and Acidobacteria are distributed on the corticated upper part of the thallus, Acidobacteria are further randomly intermixed with photobiont cells beneath. Arrows indicate bacterial colonies. Bar = 31 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

we are still unable to quantify the bacterial colonies present in the analyzed lichen soil crusts. The fixation of the samples and the protocol applied for the FISH experiments require several washing steps, both during the fixation and the hybridization, during which part of the original amount of bacterial cells is probably removed. Additionally, the noise signal generated by the mineral fragments present in the samples and the high density of some bacterial colonies detected with the EUB probes can bias a quantitative analysis.

Biological soil crusts represent highly complex life forms at the interface of soil with the atmosphere. Among these life forms, lichens represent a special case. The symbiosis of fungi with algae has evolved a unique structural organization in the fungal kingdom, the lichen thallus, which is primarily a fungal construction for

cultivation of photosynthetic partners, green algae or cyanobacteria (in 90% and 10% of lichen mycobiont species, respectively). The coherence of symbiotic thallus structures differs among species of soil crust lichens, as do their attachment structures to the supporting soils. These differences may also affect the structural organization of bacteria in the hypothallosphere, the substrate layers influenced by the covering lichen thalli (Bjelland et al., 2010). Lichens with poorly coherent structures are characterized by a more or less homogenous bacterial colonization of the underlying soil layers, whereas compact thalli including those with rhizine-like structures, show a higher degree of organization, aggregation of hyphae and associated bacteria. The dominance of *Acidobacteria* associated to the rhizines of lichens let us speculate about analogies with the endophytic root-inhabiting bacteria. The differences in bacterial colonization suggest that lichens could be particularly effective in controlling microbial structures, either by influencing hydration or by more complex mechanisms such as secondary metabolite production (lichens are well-known for their richness of secondary metabolites). Alternatively, the presence of certain bacteria groups in the lichen soil crusts may explain the installation of the lichens in different biotopes, since the species here analyzed occur also in different habitats.

Adequate control could also be an important trait to maintain microbial integrity in robust and long-living biological soil crusts in harsh environments.

4. Conclusion

In this contribution we report about analyses of bacterial communities in six species of alpine lichen soil crusts by applying FISH and CLSM. We discussed the challenges concerning the preparation of soil crust materials. We propose here an improved protocol for fluorescence *in situ* hybridization to obtain adequate results in confocal laser scanning microscopy. The performance of fluorescence *in situ* hybridization on poly-L-lysine coated slides prevents loss of lichen material to the best possible and improves penetration of the probes into the sample. By applying probes for the major bacteria groups of Alpha-, Beta- and Gammaproteobacteria (Eubacteria), Firmicutes and Acidobacteria, we detected that Alphaproteobacteria and Acidobacteria predominate the lichen structures, whereas Beta- and Gammaproteobacteria and Firmicutes could not be visualized.

Acknowledgments

This research is funded by the Austrian Science Foundation (FWF P19098, I799). We thank Massimiliano Cardinale (Graz) for valuable comments.

References

Amann, R.I., Binder, B.J., Olson, R.J., Chrisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analysing mixed microbial population. Appl. Environ. Microbiol. 56, 1919–1925.

Asta, J., Orry, F., Toutain, F., Souchier, B., Villemin, G., 2001. Micromorphological and ultrastructural investigation of the lichen–soil interface. Soil Biol. Biochem. 33, 323–337.

Bates, S., Nash III, T.H., Sweat, K.G., Garcia-Pichel, F., 2011. Fungal communities of lichen-dominated biological soil crust, diversity, relative microbial biomass, and their relationship to disturbance and crust cover. J. Arid Environ. 74, 1192–1199.

Belnap, J., 2002. Impacts of off-road vehicles on nitrogen cycles in biological soil crusts, resistance in different U.S. deserts. J. Arid Environ. 52, 155–165.

Beymer, R.J., Klopatek, J.M., 1991. Potential contribution of carbon by microphytic crusts in pinyon-juniper woodlands. Arid Soil Res. Rehabil. 5, 187–198.

Belnap, J., Büdel, B., Lange, O.L., 2003. Biological soil crusts, characteristics and distribution. In: Belnap, J., Lange, O.L. (Eds.), Biological Soil Crusts: Structure, Function and Management. Springer, Berlin, pp. 3–30.

Bjelland, T., Grube, M., Hoem, S., Jorgensen, S.L., Daae, F.L., Thorseth, I.H., Øvreås, L., 2010. Microbial metacommunities in the lichen–rock habitat. Env. Microb. Rep. 3, 434–442.

Cardinale, M., Vieira de Castro, J., Müller, H., Berg, G., Grube, M., 2008. In situ analysis of the bacteria community associated with the reindeer lichen *Cladonia arbuscula* reveals predominance of alphaproteobacteria. FEMS Microbiol. Ecol. 66, 63–71.

Cardinale, M., Steinová, J., Rabensteiner, J., Berg, G., Grube, M., 2011. Age, sun, and substrate: triggers of bacterial communities in lichens. Environ. Microbiol. Rep. 4, 23–28.

Daims, H., Brühl, A., Amann, R., Schleifer, K.H., Wagner, M., 1999. The domain-specific probe EUB338 is sufficient for the detection of all Bacteria. Development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22, 434–444.

Garcia-Pichel, F., Johnson, S.L., Youngkin, D., Belnap, J., 2003. Small-scale vertical distribution of bacteria biomass and diversity in biological soil crusts from arid lands in the Colorado Plateau. Microbiol. Ecol. 46, 312–321.

Grube, M., Cardinale, M., Vieira de Castro, J., Müller, H., Berg, G., 2009. Species-specific structural and functional diversity of bacterial communities in lichen symbiosis. ISME J. 3, 1105–1115.

Grube, M., Rabensteiner, J., Grube, U., Muggia, L., 2010. Architecture of biocomplexity: lichen-dominated soil crust and mats. In: Seckbach, J., Oren, A. (Eds.), Microbial Mats. Springer, London, pp. 343–357.

Harper, K.T., Belnap, J., 2001. The influence of biological soil crusts on mineral uptake by associated vascular plants. J. Arid Environ. 47, 347–357.

Jones, C.G., Lawton, J.H., Shachak, M., 1997. Positive and negative effects of organisms as physical ecosystem engineers. Ecology 78, 1946–1957.

Klug, B., Rodler, C., Koller, M., Wimmer, G., Kessler, H.H., Grube, M., Santigli, E., 2011. Oral biofilm analyse palatal expander durch fluoreszenz in situ hybridisierung und konfokale laser scanning mikroskopie. J. Visualized Exp.: JoVE 56, http://dx.doi.org/10.3791/2967.

Lalley, J.S., Viles, H.A., Henschel, J.R., Lalley, V., 2006. Lichen-dominated soil crusts as arthropod habitat in warm deserts. J. Arid Environ. 67, 579–593.

Manz, W., Amann, R., Ludwig, W., Wagner, M., Schleifer, K.H., 1992. Phylogenetic oligonucleotide probes for the major subclasses of Proteobacteria: problems and solutions. Syst. Appl. Microbiol. 15, 593–600.

Meier, H., Amann, R.I., Ludwig, W., Schleifer, K.H., 1999. Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G+C content. Syst. Appl. Microbiol. 22, 186–196.

Meisinger, D.B., Zimmermann, J., Ludwig, W., Schleifer, K.H., Wanner, G., Schmid, M., Bennet, P.C., Engel, A.S., Lee, N.M., 2007. In situ detection of novel Acidobacteria in microbial mats from a chemolithoautotrophically based cave ecosystem (Lower Kane Cave, WY, USA). Environ. Microbiol. 9, 1523–1534.

Neef, A., 1997. Anwendung der in situ Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. Doctoral Thesis (Technische Universität München).

Pankratov, T.A., 2012. Acidobacteria in microbial communities of the bog and tundra lichens. Exp. Art. 81, 51–58.

Redfield, E., Barns, S.M., Belnap, J., Daane, L.L., Kuske, C.R., 2002. Comparative diversity and comparison of cyanobacteria in three predominant soil crusts of Colorado Plateau. FEMS Microbiol. Ecol. 40, 55–63.

Shiraishi, F., Zippel, B., Neu, T.R., Arp, G., 2008. In situ detection of bacteria in calcified biofilms usin FISH and CARD-FISH. J. Microbiol. Methods 75, 103–108.

Smith, C.W., Aptroot, A., Coppins, B.J., Fletcher, A., Gilbert, O.L., James, P.W., Wolseley, P.A., 2009. The Lichens of Great Britain and Ireland. British Lichen Society, London.

Türk, R., Gärtner, G., 2003. Biological soil crusts of the subalpine, Alpine and nival areas in the Alps. In: Belnap, J., Lange, O.L. (Eds.), Biological Soil Crusts: Structure, Function and Management. Springer, Berlin, pp. 67–73.

Van Haluwyn, C., Asta, J., Boissière, J.C., Clerc, P., 2012. Guide des lichens de France. Lichens des sols. Belin, p. 224.

Wallner, G., Almann, R., Beisker, W., 1993. Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry 14, 136–143.

Wirth, V., 1995. Flechtenflora 2. In: Auflage. Ulmer, Stuttgart.

Wu, L., Lan, S., Zhang, D., Hu, C., 2011. Small-scale vertical distribution of algae and structure of lichen soil crusts. Soil. Microbes 62, 715–724.