DOI 10.1002/pmic.201000679 *Proteomics* 2011, *11*, 2752–2756

DATASET BRIEF

Structure and function of the symbiosis partners of the lung lichen (*Lobaria pulmonaria* L. Hoffm.) analyzed by metaproteomics

Thomas Schneider[1], Emanuel Schmid[2], João V. de Castro Jr.[3], Massimiliano Cardinale[3], Leo Eberl[1], Martin Grube[4], Gabriele Berg[3] and Kathrin Riedel[5,6]

[1] University of Zurich, Institute of Plant Biology, Zürich, Switzerland
[2] ETH Zurich, Institute of Molecular Systems Biology, Zürich, Switzerland
[3] Graz University of Technology, Institute of Environmental Biotechnology, Graz, Austria
[4] Karl-Franzens University of Graz, Institute of Plant Sciences, Graz, Austria
[5] Braunschweig University of Technology, Institute of Microbiology, Braunschweig, Germany
[6] Helmholtz-Centre of Infection Research, Braunschweig, Germany

Received: October 22, 2010
Revised: February 11, 2011
Accepted: April 11, 2011

Environmental proteomics, also referred to as metaproteomics, is an emerging technology to study the structure and function of microbial communities. Here, we applied semi-quantitative label-free proteomics using one-dimensional gel electrophoresis combined with LC-MS/MS and normalized spectral counting together with fluorescence in situ hybridization and confocal laser scanning microscopy to characterize the metaproteome of the lung lichen symbiosis *Lobaria pulmonaria*. In addition to the myco- and photobiont, *L. pulmonaria* harbors proteins from a highly diverse prokaryotic community, which is dominated by *Proteobacteria* and including also *Archaea*. While fungal proteins are most dominant (75.4% of all assigned spectra), about the same amount of spectra were assigned to prokaryotic proteins (10%) and to the green algal photobiont (9%). While the latter proteins were found to be mainly associated with energy and carbohydrate metabolism, a major proportion of fungal and bacterial proteins appeared to be involved in PTMs and protein turnover and other diverse functions.

Keywords:
Lichens / Metaproteomics / Microbial ecology / Microbiology

Lichens are ecologically obligate, self-sustaining symbioses between a fungal partner (the mycobiont) and an inhabitant population of extracellularly located unicellular or filamentous algal or cyanobacterial cells (the photobiont) [1]. The emergence of the lichen habit coincides with a substantial primary evolutionary radiation of ascomycetous fungi and allows both partners to develop complex and exposed thallus structures under environmental situations that would usually not be favorable for them in biological solitude [2]. Even under rather hostile circumstances, the composite organisms or thalli can take thousands of years to develop [3].

In the last years it was shown that lichens form mini-ecosystems, which harbor in addition highly abundant and diverse bacterial communities [4–6]. They form biofilm-like structures and show a high degree of species specificity [7]. Although the structure and composition of lichen-associated bacterial communities have been studied by molecular tools (reviewed in [8]) it is difficult to analyze their function. Investigations on bacterial isolates and functional genes suggested that they are involved in nitrogen fixation and nutrient cycling [6]. For a deeper understanding of the

Correspondence: Professor Kathrin Riedel, Institute of Microbiology, Braunschweig University of Technology, Inhoffenstrasse 7, D-38124 Braunschweig, Germany
E-mail: ak.riedel@tu-bs.de
Fax: +43-531-391-5854

Abbreviation: FISH-CLSM, fluorescent in situ hybridization confocal laser scanning microscopy

Colour Online: See the article online to view Figs. 1 and 2 in colour.

lichen symbiosis, and in particular to learn more about the bacterial community and ecosystem functioning, integrated approaches are needed.

Thus, we combined a metaproteomics approach (reviewed in [9]), which allows to study many protein functions and responses simultaneously with fluorescence in situ hybridization and confocal laser scanning microscopy (FISH-CLSM) to analyze the structure and key functions of the symbiosis partners of *Lobaria pulmonaria* (Fig. 1A). This lichen forms impressive foliose thalli on trees and widely occurs in the northern hemisphere and south-eastern Africa. *L. pulmonaria* is a valued indicator of ecological continuity and species richness in forest ecosystems [10]. Because of its high sensitivity to air pollution, it is today considered endangered in many parts of central Europe and other industrialized regions. Apart from its ecological significance, *L. pulmonaria* has also been used in traditional medicine and is recently regaining pharmacological interest [11].

The microbial community associated to *L. pulmonaria* was analyzed by FISH using EUB338I, II and III, universal bacterial probes, and ALF968, a specific probe for the *Alphaproteobacteria* (for detailed protocol see Supporting Information). Microscopic inspection revealed that the surface of the lichen thallus was densely colonized by bacteria, which formed a thin biofilm-like layer on the fungal hyphae (Fig. 1B and C). Cells belonging to *Alphaproteobacteria* showed a clear predominance; this confirms our findings for the reindeer lichen *Cladonia arbuscula* [5] and other lichen species [7] as well as recent data obtained for different foliose green algal lichen species collected from granite rock outcrops in northern Colorado [6].

A metaproteomics approach was then used to analyze structure and function of the symbiotic consortium. Proteins extracted from two lichen samples of *L. pulmonaria* (replicates I and II; sampled in April 2009 in Switzerland) were analyzed by one-dimensional gel electrophoresis (1-D SDS-PAGE) combined with LC-MS/MS and the resulting MS and MS/MS data were searched against a database consisting of protein sequences obtained from the public UniRef100 database (for detailed description of extraction, MS and data analyses see Supporting Information).

In total, 463 unique proteins or protein clusters were assigned to different phylogenetic and functional groups. Detailed information is given in three Supporting Information Tables: Table S1 presents an overview of representative proteins assigned to different phyla and functional categories, Table S2 shows all proteins belonging to the different clusters as well as the respective peptide sequence and charge state information, and Table S3 depicts mass spectra of proteins or protein clusters that were assigned by only one unique peptide. Moreover, MASCOT raw data were uploaded to the PRIDE database (http://www.ebi.ac.uk/pride/; accession number 16470) [12]. A semi-quantitative

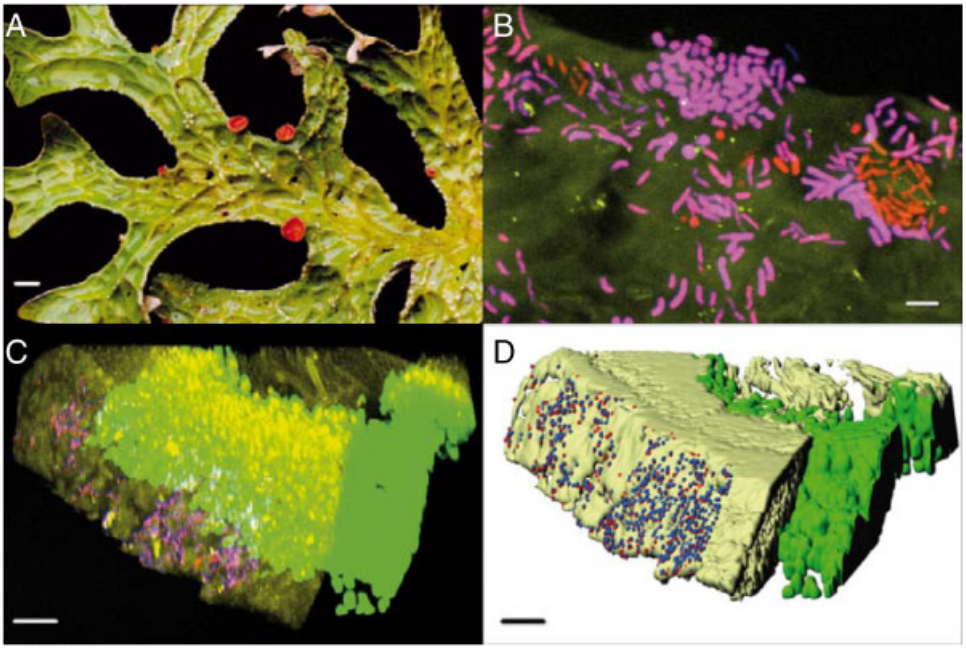

Figure 1. Bacterial colonization of *L. pulmonaria* and dominance of alphaproteobacteria. Thalli of *L. pulmonaria* (A) were sampled using sterilized forceps and cut in 30 μm-thick cryosections. The cryosections were stained by FISH-CLSM (B–D). Confocal stacks were acquired with a Leica TSC SP (Leica Microsystems, Germany). Three-dimensional reconstruction (D) was created with the software Imaris 6.4.0 (Bitplane, Switzerland). (B and C) Green: algal chlorophyll; yellowish: fungal tissues; pink: *Alphaproteobacteria*; red: other bacteria. (D) Green surface: algal chlorophyll; yellowish surface: fungal tissues; blue/red spots: *Alphaproteobacteria*; red spots: other bacteria. In (C and D) the fluorescent signals from bacterial and fungal tissues were partially cropped, to evidence the stratification of layers. Scale bars, (A) 1 cm, (B) 2 μm, (C) and (D) 20 μm. For details about the FISH-CLSM procedures, see Supporting Information.

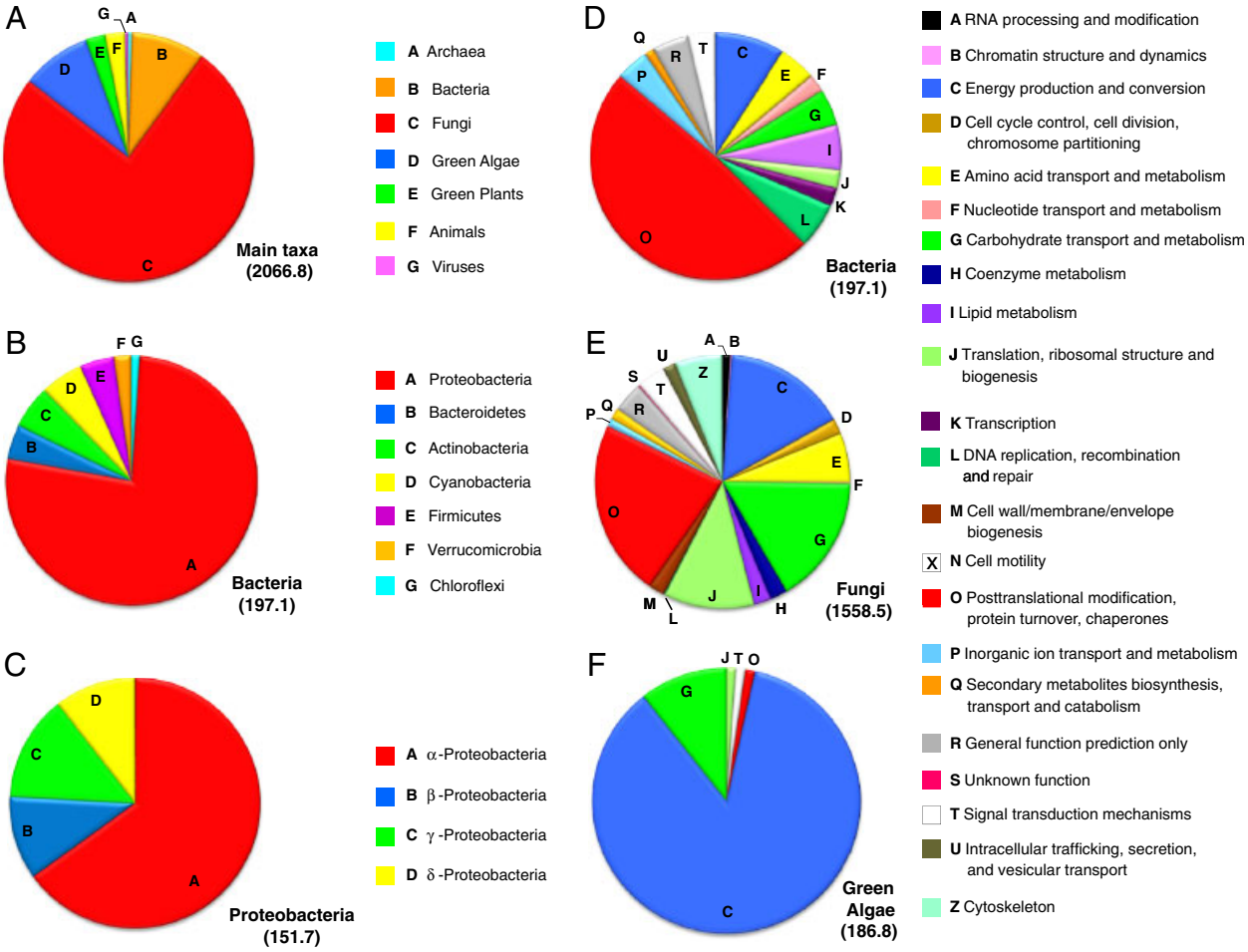

Figure 2. Assignment of spectra obtained by 1-D SDS-PAGE combined with LC-MS/MS to taxonomic groups associated to the lichen thalli (A–C) and to clusters of orthologous group (COG/KOG) functions (D–F). Numbers in brackets indicate total normalized numbers of spectra (calculated by the Scaffold software), which were assigned to the respective phylogenetic and functional group(s). For details about MS and data analyses, see Supporting Information; absolute values are given in Supporting Information Table S2.

analysis based on a normalized unique spectral counting by the Scaffold Software and the newly developed Perl-script-based automated bioinformatics workflow PROPHANE (Proteomics result Pruning & Homology group ANnotation Engine; described in detail in the Supporting Information and Methods I and II) demonstrated that the majority of spectra could be assigned to fungal proteins (75.4%), followed by green algal proteins (10%), bacterial proteins (9.5%) and proteins from *Archaea* (0.5%) (Fig. 2A; Supporting Information Table S4). Moreover, a significant number of spectra could be assigned to plant, animal and viral proteins; however, these proteins are most probably contaminations occurring prior to or during sampling. As expected from microscopic observations, fungi are the most abundant partner in the lichen symbiosis. The majority of fungal spectra belonged to proteins of the *Ascomycota* group (98.9%), spectra of the *Basidiomycota* fungi represented only a small proportion; they might belong to basidiomycete yeasts that have been reported repeatedly from lichens. The photobiont of *L. pulmonaria* is a monoculture of the green algae *Dictyochloropsis reticulata* [13]. Notably, *Bacteria* and *Archaea* contributed as many normalized numbers of spectra than green algae (207.3 versus 186.8); a fact underlining the importance of prokaryotes in the lichen symbiosis. To our knowledge, this is the first report providing evidence that *Archaea*, which are well-known inhabitants of other extremophilic ecological niches [14] and the hypothallosphere of intertidal maritime lichens [15] but not of thalli of inland lichens, could also play a role in lichen symbioses. LC-MS/MS analysis assigned proteins to seven phylogenetic groups of bacteria (Fig. 2B, Supporting Information Table S4). Most spectra (76.8% of all eubacterial spectra) were assigned to proteins from *Proteobacteria*. The dominance of *Alphaproteobacteria* (65.1% of all proteobacterial spectra) within *Proteobacteria* corroborated the FISH-CLSM results (Fig. 2C, Supporting Information

Table S4). The remaining eubacteria spectra were assigned to proteins of *Cyanobacteria* (5.5%), *Actinobacteria* (5.5%), *Firmicutes* (4.4%), *Bacteriodetes* (4.4%), *Verrucomicrobia* (2.2%) and *Chloroflexi* (1.1%). *Cyanobacteria* are well known as sole photosynthetic or as accessory N-fixing partners in different lineages of lichen-forming *Ascomycota* [16]. In *Lobaria*, cyanobacteria of the genus *Nostoc* form internal cephalopodia [17]. Members of *Firmicutes* were isolated from lichens [18] as well as found in cultivation-independent analyses of lichen-associated microbial communities [6, 7]. In a recent study of Bates et al., [6] members of *Actinobacteria*, *Bacteriodetes* and *Verrucomicrobia* were found in lichens isolated from granite rocks by employing a 16S rRNA sequencing approach. The metaproteomic data revealed a high diversity of the lichen-associated bacterial community; taxonomic diversity appeared even higher than found with microbial fingerprints on the basis of the 16S rRNA or functional genes for other alpine lichen species [7]. This could be explained by limitations of the methods, where the uncommon are often ignored [19].

The majority of bacterial, fungal and green algal spectra could be assigned to functional categories according to clusters of orthologous groups of proteins (COG/KOGs); however, the distribution of protein functions varied strongly for each phylogenetic group (Fig. 2D–F). Spectra assigned to proteins involved in PTMs, protein turnover and chaperones are highly abundant in bacteria (42.7% of all spectra) and fungi (20.9% of all spectra). Furthermore, spectra assigned to proteins involved in energy production and conversion (7.9% of bacterial and 14.5% of fungal spectra) were dominating in both groups. Various proteins involved in transport processes and metabolism can be differentiated. Carbohydrate metabolism appears to play a major role in fungi: 15.1% of the fungal spectra were assigned to this functional category. Protein and carbohydrate turnover functions would agree with previous findings of lytic activities found with cultivable lichen bacteria [7]. Proteins responsible for transport of amino acids, nucleotides, coenzymes and lipids are well present in bacteria and in fungi. This agrees well with reports of amino acid release of lichen-associated bacteria [20], and supports the theory that reallocation of resources in the lichens ecosystem is assisted by bacterial functions. Numerous fungal but also bacterial spectra assigned to proteins involved in biosynthesis of secondary metabolites were found. The mycobiont of *L. pulmonaria* is known to produce melanin as sun-screening polymer [21]. So far no sun-protecting compounds are known from the bacterial partners in *L. pulmonaria*; notably, we could assign bacterial spectra to the phytoene dehydrogenase CrtI, an enzyme that mediates the conversion of phytoene into colored carotenoids, known to protect bacteria against reactive oxygen species [22].

The protein profiles of the photosynthetic green algal symbiosis partner showed a completely other picture. Spectra were assigned to proteins belonging to only five functional groups. Proteins involved in energy production and conversion strongly dominate the protein fraction of green algae (83.6%). This was followed by proteins responsible for carbohydrate transport and metabolism (10.3%) and confirms the main and well-known function of algae in the symbiosis: photosynthesis and provision of carbohydrates.

The analysis of the *L. pulmonaria* metaproteome provides new insights into the structure and function of the lichens symbiosis and highlights the importance of the bacterial community in the symbiotic ecosystem – the number of prokaryotic spectra was as high as the number of normalized spectral counts of the green algal photobiont. Furthermore, they are phylogenetically more diverse than found in the previous studies using other techniques and investigating alpine lichen species. Our study provided a first hint that *Archaea* could also be associated with lichens; however, this finding has to be verified by complementary approaches. Functional analysis confirmed the symbiotic function of the photobiont and multiple functions for the bacterial partner. This first metaproteome data set creates new baseline information for future studies on the influence of environmental parameters on the endangered lung lichen and, more generally, the coordinated interaction of the partners in the lichen symbiotic system.

The complete MASCOT data set is provided on the PRIDE database [12]. The link is http://www.ebi.ac.uk/pride/; the accession number is 16470.

The authors thank Silke Werth (WSL Birmendorf, Switzerland) for sampling. Thomas Schneider was financially supported by the Austrian Research Network MICDIF (project number S 10004). Furthermore, we would like to thank the Functional Genomics Center Zurich for technical support of the project. This research was funded by a grant from the Austrian Science Foundation (FWF) to M. G. and G. B.

The authors have declared no conflict of interest.

References

[1] Hawksworth, D. L., Honegger, R., The lichen thallus a symbiotic phenotype of nutritionally specialized fungi and its response to gall producers, in: Williams, M. A. J. (Ed.), *Plant Galls*, Clarendon Press, Oxford 1994, pp. 77–98.

[2] Lutzoni, F., Pagel, M., Reeb, V., Major fungal lineages are derived from lichen symbiotic ancestors. *Nature* 2001, *411*, 937–940.

[3] Grube, M., Hawksworth, D. L., Trouble with lichen: the re-evaluation and reinterpretation of thallus form and fruit body types in the molecular era. *Mycol. Res.* 2007, *111*, 1116–1132.

[4] Cardinale, M., Puglia, A. M., Grube, M., Molecular analysis of lichen-associated bacterial communities. *FEMS Microb. Ecol.* 2006, *57*, 484–495.

[5] Cardinale, M., Castro J. V., Jr., Müller, H., Berg, G. et al., *In situ* analysis of the bacterial community associated with the reindeer lichen *Cladonia arbuscula* reveals predominance of *Alphaproteobacteria*. *FEMS Microb. Ecol.* 2008, *66*, 63–71.

[6] Bates, S. T., Cropsey, G. W., Caporaso, J. G., Knight, R. et al., Bacterial communities associated with the lichen symbiosis. *Appl. Environ. Microbiol.* 2011, *77*, 1309–1314.

[7] Grube, M., Cardinale, M., Castro, J. V., Jr., Müller, H. et al., Species-specific structural and functional diversity of bacterial communities in lichen symbiosis. *ISME J.* 2009, *3*, 10051015.

[8] Grube, M., Berg, G., Microbial consortia of bacteria and fungi with focus on the lichen symbiosis. *Fungal Biol. Rev.* 2010, *23*, 72–85.

[9] Schneider, T., Riedel, K., Environmental proteomics: analysis of structure and function of microbial communities. *Proteomics* 2010, *10*, 785–798.

[10] Gauslaa, Y., *Lobaria pulmonaria*, an indicator of species-rich forests of long ecological continuity. *Blyttia* 1994, *52*, 119–128.

[11] Suleyman, H., Anti-inflammatory and anti-ulcerogenic effects of the aqueous extract of *Lobaria pulmonaria* (L.) Hoffm. *Phytomedicine* 2003, *10*, 552–557.

[12] Vizcaíno, J. A., Côté, R., Reisinger, F., Foster, J. M. et al., A guide to the Proteomics Identifications Database proteomics data repository. *Proteomics* 2009, *9*, 4276–4283.

[13] Tschermak-Woess, E., *Dictyochloropsis splendida* (Chlorophyta), the correct phycobiont of *Phlyctis argena* and the high degree of selectivity or specificity involved. *Lichenologist* 1995, *27*, 169–187.

[14] Auguet, J. C., Barberan, A., Casamayor, E. O., Global ecological patterns in uncultured Archaea. *ISME J.* 2009, *4*, 182–190.

[15] Bjelland, T., Grube, M., Hoem, S., Jorgensen, S. L. et al., Microbial metacommunities in the lichen–rock habitat. *Environ. Microbiol. Rep.* 2010, *3*, 2.

[16] Miadlikowska, J., Kauff, F., Hofstetter, V., Fraker, E. et al., New insights into classification and evolution of the Lecanoromycetes (Pezizomycotina, Ascomycota) from phylogenetic analyses of three ribosomal RNA- and two proteincoding genes. *Mycologia* 2006, *98*, 1088–1103.

[17] Hill, D. H., Hawksworth, D. L., *The Lichen-Forming Fungi*, Blackie, London 1984

[18] González, I., Ayuso-Sacido, A., Anderson, A., Genilloud, O., Actinomycetes isolated from lichens: evaluation of their diversity and detection of biosynthetic gene sequences. *FEMS Microbiol. Ecol.* 2005, *54*, 401–415.

[19] Bent, S. J., Forney, L. J., The tragedy of the uncommon: understanding limitations in the analysis of microbial diversity. *ISME J.* 2008, *2*, 689–695.

[20] Liba, C. M., Ferrara, F. I. S., Mangio, G. P., Fantinatti-Garboggini, F. et al., Nitrogen-fixing chemoorganotrophic bacteria isolated from cyanobacteria-deprived lichens and their ability to solubilize phosphate and to release amino acids and phytohormones. *J. Appl. Microbiol.* 2006, *101*, 1076–1086.

[21] McEvoy, M., Gauslaa, Y., Solhaug, K. A., Changes in pools of depsidones and melanins, and their function, during growth and acclimation under contrasting natural light in the lichen *Lobaria pulmonaria*. *New. Phytol.* 2007, *175*, 271–282.

[22] Giuliano, G., Pollock, D., Scolnik, P. A., The Gene *crtI* mediates the conversion of phytoene into colored carotenoids in *Rhodopseudomonas capsulate*. *J. Biol. Chem.* 1986, *261*, 12925–12929.

