Molecular analysis of lichen-associated bacterial communities

Massimiliano Cardinale[1], Anna Maria Puglia[1] & Martin Grube[2]

[1]Dipartimento di Biologia Cellulare e dello Sviluppo, Università degli Studi di Palermo, Palermo, Italy and [2]Institute of Plant Sciences, Karl-Franzens-University Graz, Graz, Austria

Correspondence: Martin Grube, Institute of Plant Sciences, Karl-Franzens-University Graz, Holteigasse 6, A-8010 Graz, Austria. Tel.: +43 316 3805655; fax: +43 316 3809883; e-mail: martin.grube@uni-graz.at

Received 23 August 2005; revised 31 January 2006; accepted 31 January 2006.
First published online 13 April 2006.

DOI:10.1111/j.1574-6941.2006.00133.x

Editor: Riks Laanbroek

Keywords
lichens; symbiosis; bacterial communities; SSU rDNA; internal transcribed spacer polymorphism.

Abstract

The bacterial communities associated with 11 different lichen samples (belonging to eight different species) from different habitats were investigated. The culturable aerobic-heterotrophic fraction of the bacterial communities was isolated from nine lichen samples on protein-rich and sugar-rich/N-free media. Thirty-four bacterial isolates were purified and pooled into groups (phylotypes) by analysis of the ribosomal internal transcribed spacer polymorphism. Twenty five phylotypes were identified, each comprising between one and three isolates. One isolate of each phylotype was partially sequenced and the resulting 16S rRNA gene sequences were compared in a phylogenetic analysis. Three genera of Firmicutes, four of *Actinobacteria* and three of *Proteobacteria* were identified. Two phylotypes, belonging to the phyla *Actinobacteria* and *Proteobacteria*, respectively, were not identified at genus level. Some bacterial taxa were retrieved frequently in different lichen species sampled in the same or different sites. *Paenibacillus* and *Burkholderia* phylotypes seem to be common in lichens. *Luteibactor rhizovicina* was found in three different lichens of two different regions. In a cultivation-independent approach, total DNA was extracted from 11 lichen samples. Molecular fingerprints of the bacterial communities were obtained by PCR-amplification of the internal transcribed spacer region, and sequencing of selected bands indicated the presence of additional bacteria.

Introduction

The genomic exploration of microbes from poorly studied ecological niches has become an exciting endeavour in recent years. Niches as diverse as soil, water and air are being studied by various techniques, ranging from culture techniques to metagenomic approaches (e.g. Venter *et al.*, 2004). This is a promising perspective for the investigation of further, biologically rich habitats. Among these, diverse groups of cryptogams including lower plants and fungi could be particularly suitable as hosts for bacterial communities. A recent investigation of bryophyte-associated bacteria revealed numerous bacterial phylotypes (Opelt & Berg, 2004), some of which could not be assigned to genera. Another interesting habitat is certainly provided by fungal symbioses. Whereas the involvement of bacteria in mycorrhizal symbioses is well-studied (Garbaye, 1994), we focus here on another important symbiotic life style of fungi, the lichen association.

Of all fungal symbioses, lichens are a rather particular case. The lichen association of fungi and algae contributes to a substantial evolutionary radiation of the mycobionts (Gargas *et al.*, 1995; Lutzoni *et al.*, 2001), and also allows their algal partners to grow well under environmental situations that would usually not be favourable in biological isolation. This evolutionary successful lifestyle apparently arose early in the evolution of ascomycetes (Lutzoni *et al.*, 2001), before the Lower Devonian (Taylor *et al.*, 1995). The lichen symbiosis is maintained by approximately one fifth of all known extant fungi, and by more than 42% of known Ascomycota. Lichens are ubiquitous and are found from sub-polar ranges to tropical rainforests. They are also able to grow on diverse substrates and sometimes under extreme ecological conditions. Since most lichens are exceptionally drought-tolerant and slow-growing organisms, they provide an unusual and long-living ecological niche for additional microorganisms, which may include other fungi (well-studied lichen parasites, as well as less known epi- and endobionts (Petrini *et al.*, 1990; Prillinger *et al.*, 1997; Miadlikowska, personal communication)) and prokaryotes.

The earliest reports about non-cyanobacterial prokaryotes in lichens were contradictory, and likely due to a misinterpretation of crystallized secondary compounds (Uphof, 1925; Suessenguth, 1926). Clear evidence for the presence of bacteria in lichens was then provided by a series of papers that appeared long before the emergence of molecular methods. Henkel & Yuzhakova (1936) and Iskina (1938) detected

nitrogen-fixing bacteria in lichens by cultivation on nitrogen-free Ashby medium, and they assigned these strains to *Azotobacter*. While Krasil'nikov (1949) could not confirm these results with the sample he studied, Scott, (1956) mentioned again the frequent finding of *Azotobacter* in lichens. Besides *Azotobacter*, other genera were previously reported from lichens, such as *Bacillus* (Henkel & Plotnikova, 1973), *Beijerinckia* (Panosyan & Nikogosyan, 1966), *Clostridium* (Iskina, 1938) and *Pseudomonas* (Henkel & Plotnikova, 1973). While an involvement in nitrogen fixation for lichens was repeatedly considered in these works, the finding of actinobacteria prompted Zook (1983) to suggest also a defensive role for bacteria in lichens. Moreover, Lenova & Blum (1983) suggested that up to millions of bacterial cells could be present per gram of a lichen thallus.

The analysis of bacteria in these works relied only on phenotypic methods using cultured isolates and therefore our knowledge about the taxonomic diversity of lichen-associated bacteria is still rather limited. However, a precise determination of strains using molecular data is required to gain further insights and to assess, for example, whether bacterial strains are selective for their host lichens or evolved resistances against the antimicrobial activity of lichen secondary metabolites (Ingólfsdóttir *et al.*, 1985). Only one study so far has characterized the diversity of actinomycetes in lichens using DNA fingerprinting (Gonzáles *et al.*, 2005). In this contribution we present a more general molecular approach to culturable lichen-inhabiting bacteria and study their diversity in selected lichens using DNA sequence data. Moreover, we show first results of a cultivation-independent technique suitable for studying all the bacterial communities associated with lichens.

Materials and methods

Lichen samples

We analyzed 11 different lichen samples, belonging to eight species, sampled at five different sites. For further details on the selected species, see Table 1. Each sample was taken with washed instruments and put in sterile bags. The samples were immediately frozen and conserved until further analysis. Sub-samples of approximately 0.3–0.5 g of the thalli were washed in sterile water and utilized for the isolation of bacteria and for direct extraction of total DNA.

Bacterial isolation

To isolate the external bacteria, nine subsamples of lichens were vortexed in 0.8% NaCl solution for 60 s and 100 μL of the solution was plated on both Tryptone-Yeast extract (TY) and sugar-rich/N-free media (5 g glucose, 5 g mannitol, 0.8 g K_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.15 g $CaCl_2$, 0.04 g $FeSO_4 \cdot 7H_2O$, 0.005 g $Na_2MO_4 \cdot 2H_2O$, 15 g agar with volume made up to 1 L with distilled water and pH adjusted to 7.0). The sub-samples were then surface-sterilized by immersion for 4 min in H_2O_2 (9%) and washed in sterile water prior to isolation of the internal bacteria. Thallus fragments were subsequently crushed in 500 μL of sterile water with a sterile scalpel and 100 μL of the resulting suspension were then plated on the same media as above.

The plates were incubated at 25 °C for 15 days. For each plate, the colonies showing distinct phenotypes were purified by plating them separately on new plates with the same media. The pure cultures were numbered according to the lichen sample and the origin (external surface or internal parts, respectively), as well as the isolation medium (e.g. Bint1: bacterium isolated on TY medium from the internal thallus of the lichen sample 'B', GestV2N: bacterium isolated on N-free medium from the external surface of the lichen sample 'G'). All isolates are stored at −80 °C in 20% glycerol at the Dipartimento di Biologia Cellulare e dello Sviluppo, Palermo, Italy.

Molecular characterization of the isolates and identification of phylotypes

Bacterial genomic DNAs were extracted by the lysozyme-proteinase K-sodium dodecyl sulphate method (modified

Table 1. Lichens used in this work

Lichen sample	Lichen species	Sampling site and altitude	Date of sampling
A	*Cladonia digitata*	Austria, Styria, Rabenwald, 1200 m	10/2004
B	*Cladonia rangiferina*	Austria, Styria, Rabenwald, 1200 m	10/2004
C	*Cladonia coniocraea*	Austria, Styria, Botanical Garden of Graz, 365 m	10/2004
D	*Cladonia pyxidata*	Austria, Styria, Rabenwald, 1200 m	10/2004
E	*Cladonia coccifera*	Austria, Styria, Rabenwald, 1200 m	10/2004
G	*Cladonia pyxidata*	Austria, Styria, Plankogel, 1440 m	5/2005
H	*Pseudevernia furfuracea*	Austria, Styria, Plankogel, 1520 m	5/2005
J	*Hypogymnia physodes*	Austria, Styria, Plankogel (on moss), 1440 m	5/2005
K	*Hypogymnia physodes*	Austria, Styria, Plankogel (on bark) 1520 m	5/2005
R	*Roccella phycopsis*	France, Normandy, St. Malo, 10 m	3/2005
S	*Roccella fuciformis*	France, Normandy, St. Malo, 10 m	3/2005

from Sambrook *et al.* (1989) by increasing the reagent concentration to 2.4 mg mL^{-1} lysozyme, 0.5 mg mL^{-1} proteinase K and 0.8% sodium dodecyl sulphate). The bacterial isolates were assigned to groups with similar banding profiles of amplified ribosomal internal transcribed spacers between the 16S and 23S ribosomal genes (ITS 16S–23S). Because of the high level of polymorphism of the ribosomal spacer, the ITS 16S–23S profiles (showing one to numerous bands per profile) are regarded as specific for bacterial strains. This allowed us quickly to screen the bacterial diversity. The isolates with the same profile were considered to belong to the same bacterial phylotype. For the ITS 16S–23S amplification we used 0.3 μM of each of the optimized primers ITSF (5′-GTCGTAACAAGGTAGCCG-TA-3′) and ITSR eub (5′-GCCAAGGCATCCACC-3′) (Cardinale *et al.*, 2004) in a reaction volume of 25 μL, containing 100–150 ng template DNA, 2 units of Taq DNA polymerase (Sigma-Aldrich Corp., St. Louis, MO) and 0.2 mM of each dNTP. PCR was carried out for 30 cycles under the following conditions: 90 s at 95 °C, 60 s at 55 °C and 90 s at 72 °C in a thermal cycler T-personal (Biometra, Goettingen, Germany). An initial hot start (5 min at 95 °C) and a final extension (5 min at 72 °C) were also performed. Finally, 5 μL of PCR product was checked by agarose gel electrophoresis and ethidium bromide staining. The ITS profiles of different isolates were compared by aligning the bands using a DNA marker with bands between 100 and 10 000 nucleotides (DNA Ladder mix, FERMENTAS, St. Leon-Rot, Germany). Additionally, Amplified Ribosomal DNA Restriction Analyses (ARDRA; Urzi *et al.*, 2001; Lagacé *et al.*, 2004; Ntougias *et al.*, 2004) were in some cases performed with the endonucleases *Hinf*I, *Taq*I and *Alu*I to confirm the results of the ITS screening and to assign isolates with ambiguous ITS profiles to a particular phylotype.

Identification of the phylotypes

Representative isolates of all detected phylotypes were further characterized by 16S rRNA gene sequencing and phylogenetic analysis. The amplification of the 16S rRNA gene was performed with the primers fD1 and rD1 (Weisburg *et al.*, 1991) using the same PCR protocol described above for the ITS 16S–23S amplification. The PCR products were visualized by agarose gel electrophoresis and quantified by comparison with the bands of the DNA Ladder Mix marker (FERMENTAS). About 60 ng of each product were used for sequencing reactions, which were performed by the BMR laboratory of the University of Padova, Italy (http://bmr.cribi.unipd.it/). The sequence electropherograms were checked with the programs ABIVIEW (EMBOSS package, http://emboss.life.nthu.edu.tw/emboss/), EDITVIEW (http://www.appliedbiosystems.com/support/software/) and FINCHTV (http://www.geospiza.com/finchtv/index.htm). To identify similar sequences that are available in the NCBI Genbank, sequences were used in BLASTn searches (Altschul *et al.*, 1997, http://www.ncbi.nlm.nih.gov/blast/). The sequences from the isolates are available from Genbank/EMBL under the accession numbers AM062703 to AM062725.

Phylogenetic position of the isolates

The 16S rRNA gene sequences were aligned using CLUSTALX (Thompson *et al.*, 1994), and a distance matrix was calculated by the DNADIST software of the PHYLIP package (Felsenstein, 2004; http://evolution.genetics.washington.edu/phylip.html) using the F84 algorithm (Felsenstein & Churchill, 1996). The Neighbour-Joining (Saitou & Nei, 1987) phylogenetic tree was constructed with the NEIGHBOR software of the PHYLIP package. The topology of the tree was statistically tested by performing 1000 bootstrap resamplings of the data with the program SEQBOOT and a Majority Rule consensus tree was obtained by the program CONSENSE of the PHYLIP package. The tree of Fig. 1 was prepared with the TREEVIEW program (Page, 1996; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Cultivation-independent analysis of the lichen-associated bacterial communities

After external sterilization by immersion in H_2O_2 (9%) for 4 min, total DNA was extracted from approximately 0.3–0.5 g of lichen thalli using the CTAB/chloroform-isoamyl alcohol method as previously described (Cubero *et al.*, 1999). The regions of DNA including the 16S ribosomal gene, the internal transcribed ribosomal spacer and approximately 100 nucleotides of the 23S ribosomal gene were amplified using 5 μL of crude extracted DNA as template, 0.4 μM of each fD1 and FGPL 132–38 primers (Quatrini *et al.*, 2002), 0.2 mM of each dNTP, 2.5 units of Taq DNA polymerase (Sigma-Aldrich) and 2 mM $MgCl_2$, according to the PCR-protocol previously described for 16S rRNA gene and ITS 16S–23S amplifications. A second nested amplification was performed to amplify the ITS 16S–23S region of all bacterial communities using 10 μL of the first PCR product as template and the primers ITSF and ITSReub. The concentrations of other reagents were the same as above. About 200 ng of PCR products were visualized in both ethidium bromide-stained agarose gel (2.5% NuSieve agarose, USB, Cleveland, OH) and in silver stained (Bassam *et al.*, 1991), native acrylamide gel (5% acrylamide : bisacrylamide, Sigma-Aldrich). The banding patterns were compared by aligning the bands with the DNA size marker. The lengths of detectable bands ranged between about 150 and 1200 nucleotides. The fluorescence intensity relative to the overall intensity of each profile was optically evaluated and the data were saved in a Microsoft© EXCEL matrix. This

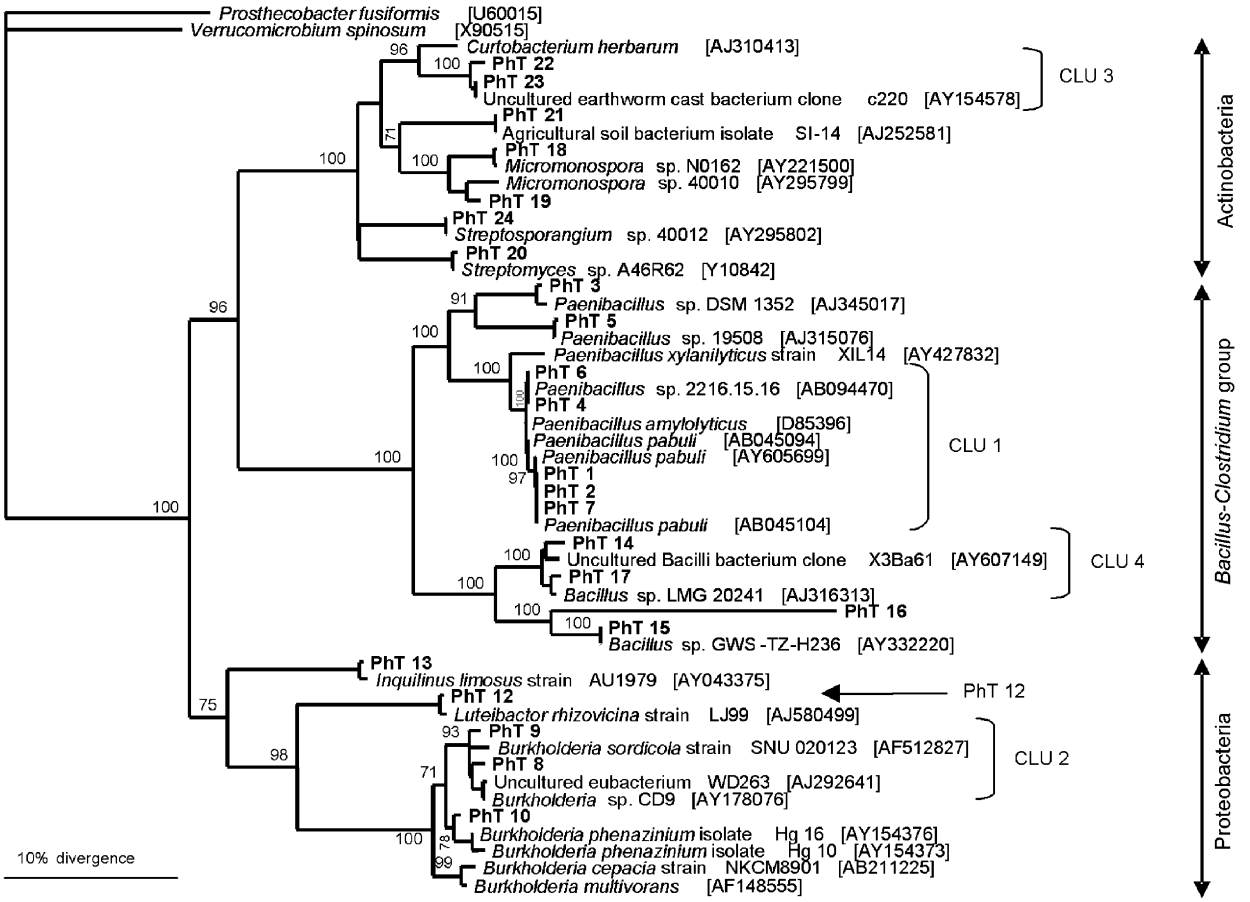

Fig. 1. Neighbour-joining tree, obtained using an alignment of 600 nucleotides of the 16S rRNA gene sequences, showing the phylogenetic relationships of the lichen-associated bacteria. Numbers on the internal nodes are bootstrap support values of 1000 re-samplings. Clusters (CLU1 – CLU4) are explained in the text.

matrix was used for the statistical analysis of the similarity between the profiles by using the NTEDIT and NTSYS2.0 software (Rohlf, 1998). The tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm, based on the average taxonomic distances [Dist coefficient (Sneath & Sokal, 1973), Dice similarity index (Atlas & Bartha, 1993)]. The genetic diversity of the bacterial communities was evaluated by the ecological diversity index of Shannon-Wiener ($H = -\Sigma P_i \log P_i$) (Atlas & Bartha, 1993) and the dominance index of Simpson ($D = \Sigma P_i^2$) (Hunter & Gaston, 1988), where P_i is the ratio between the intensity of fluorescence of each *i*th band and the total fluorescence intensity of the profile (normalized as percentage). Commonly present or otherwise intense bands were excised from the NuSieve gel and the DNAs were recovered by using the GFX^{TM} Purification Kit (Amersham Biosciences, Little Chalfont, UK). The ITS region of four bands was sequenced and the sequences are available from Genbank/EMBL under the accession numbers DQ343830 to DQ343833.

Results

Bacterial isolation

Thirty four morphologically distinct bacteria were isolated from nine lichen samples (between one and eight from each lichen). Eighteen were from the external surfaces and 16 from the internal thallus parts (Table 2). Twenty-four bacteria were isolated on Tryptone-Yeast extract medium (TY) and 18 on Glucose-Mannitol-N-free medium. Generally, few colonies (between 1 and 26) of each phenotype grew on the isolation plates; only the phenotypes represented by CestV1, Dint1, Eint1b, GestV1, GestL2 (Table 2) formed more than 100 (up to thousands) colonies on each plate. Ten of the 24 isolates on TY medium were able also to grow efficiently on N-free medium.

Screening of the isolates

The analysis of the ribosomal internal spacer polymorphism was used to group the 34 isolates to 25 distinct phylotypes.

Table 2. Bacterial isolates obtained from lichen samples

Phylotype	Isolate	Host lichen	Most closely related sequence (Genbank accession number)	16S rRNA gene sequence similarity in % (no. of nucleotides)	Taxonomical position of isolate (most related species)	Notes/reference of most related sequence
PhT 1	Bint1[†]	*Cladonia rangiferina*	*Paenibacillus pabuli* (AB045104)	99.3 (893)	Firmicutes, *Paenibacillaceae* (*Paenibacillus pabuli*)	Chitinolytic aerobic endospore-forming bacteria
	BestV1	*Cladonia digitata*				
	Aint*1					
PhT 2	BestV3	*Cladonia rangiferina*	*Paenibacillus pabuli* (AB045104)	99.4 (933)	Firmicutes, *Paenibacillaceae* (*Paenibacillus pabuli*)	Chitinolytic aerobic endospore-forming bacteria
PhT 3	Eint3	*Cladonia coccifera*	*Paenibacillus* sp. DSM 1352 (AJ345017)	98 (978)	Firmicutes, *Paenibacillaceae* (*Paenibacillus agarexedens*)	Agarolytic aerobic endospore-forming soil bacteria Uetanabaro *et al.* (2003)
PhT 4	Eint1a	*Cladonia coccifera*	Bacterium H17 (AY345554)	99.9 (860)	Firmicutes, *Paenibacillaceae* (*Paenibacillus amylolyticus*)	Isolated from "microbial hotspot" in the Hawaiian Archipelago
PhT 5	Hint1N	*Pseudevernia furfuracea*	*Paenibacillus* sp. 19508 (AJ315076)	99.1 (810)	Firmicutes, *Paenibacillaceae* (*Paenibacillus mendelii*, *Paenibacillus phyllosphaerae*)	Isolated from biodeteriorated mural paintings in a necropolis tomb Heyrman & Swings (2001)
PhT 6	*Jint1*	*Hypogymnia physodes* (on moss)	Bacterium H17 (AY345554)	100 (742)	Firmicutes, *Paenibacillaceae* (*Paenibacillus amylolyticus*)	Isolated from "microbial hotspot" in the Hawaiian Archipelago
PhT 7	*JestL1*	*Hypogymnia physodes* (on moss)	*Paenibacillus pabuli* (AB045104)	99.8 (803)	Firmicutes, *Paenibacillaceae* (*Paenibacillus pabuli*)	Chitinolytic aerobic endospore-forming bacteria
PhT 8	*Dint1*	*Cladonia pyxidata* (from Rabenwald)	uncultured eubacterium WD263 (AJ292641)	98 (1003)	*Betaproteobacteria*, *Burkholderiaceae*	Isolated from polychlorinated biphenyl-polluted soil Nogales *et al.* (2001)
	DestL1				(*Burkholderia glathei*, *Burkholderia sordidicola*)	
	BestV4	*Cladonia rangiferina*				
PhT 9	*GestV1N*	*Cladonia pyxidata* (from Plankogel)	*Burkholderia sordidicola* strain SNU 020123 (AF512827)	97.9 (948)	*Betaproteobacteria*, *Burkholderiaceae* (*Burkholderia sordidicola*, *Burkholderia glathei*)	Isolated from the white-rot fungus *Phanerochaete sordida* Lim *et al.* (2003)
	HestL1N	*Pseudovernia furfuracea*				
	JestL1N	*Hypogymnia physodes* (on moss)				
PhT 10	*EestV2*	*Cladonia coccifera*	*Burkholderia phenazinium* isolate Hg 10 (AY154373)	98.5 (729)	*Betaproteobacteria*, *Burkholderiaceae* (*Burkholderia phenazinium*)	Component of naphthalene-degrading soil guilds Wilson *et al.* (2003)
PhT 11	*GestV2N*	*Cladonia pyxidata* (from Plankogel)	nss[‡]	nss	*Betaproteobacteria*, *Burkholderiaceae*	–

PhT 12	*CestV1*	*Cladonia coniocraea*	*Luteibactor rhizovicina* isolate OUCZ70 (AY785744)	99.9 (718)	*Gammaproteobacteria*, *Xanthomonadaceae* (*Luteibactor rhizovicina*)	PCB-degrading bacterium associated with plant roots
	BestL2	*Cladonia rangiferina*				
	DestL2	*Cladonia pyxidata (from Rabenwald)*				
PhT 13	*BestL3*	*Cladonia rangiferina*	*Inquilinus limosus strain* AU430 (AY043373)	99.4 (712)	*Alphaproteobacteria*, *Rhodospirillaceae* (*Inquilinus limosus*)	Isolated from clinical sample
PhT 14	*EestL2*	*Cladonia coccifera*	*Bacillus* sp. isolate IDA216	99 (686)	Firmicutes, *Bacillaceae* (*Bacillus niacini, Bacillus bataviensis*)	Soil bacterium Felske *et al*. (1999)
PhT 15	*Eint1b*	*Cladonia coccifera*	*Bacillus* sp. (AY167860 and other 17 sequences)	99.6 (687)	Firmicutes, *Bacillaceae Bacillus mycoides, Bacillus cereus, Bacillus weihenstephanensis*)	–
	EestL1					
PhT 16	*GestL2*	*Cladonia pyxidata* (from Plankogel)	*Staphylococcus epidermidis* (AY030342 and other 31 sequences)	90 (600)	Firmicutes, *Staphylococcaceae* (*Staphylococcus epidermidis)*	–
PhT 17	*KestL1*	*Hypogymnia physodes* (on bark)	Uncultured bacterium clone X20 (DQ083105) *Bacillus niacini* (AB021194)	99.5 (778)	Firmicutes, *Bacillaceae* (*Bacillus niacini*)	Soil bacterium Goto *et al*. (2000)
PhT 18	*Kint1N*	*Hypogymnia physodes* (on bark)	*Micromonospora* sp. N0162 (AY221500)	99.3 (730)	*Actinobacteria Micromonosporaceae* (*Micromonospora matsumotoense*)	Isolated from coastal sediments Zhao *et al*. (2004)
PhT 19	*Kint2N*	*Hypogymnia physodes* (on bark)	*Micromonospora* sp. 40010 (AY295799)	96.8 (619)	*Actinobacteria Micromonosporaceae* (*Micromonospora olivasterospora*, *Micromonospora auratinigra*)	Herbicides-producing bacterium
PhT 20	*KestL1N*	*Hypogymnia physodes* (on bark)	*Streptomyces* sp. strain A46R62 (Y10842)	99.8 (813)	*Actinobacteria Streptomycetaceae* (*Streptomyces sanglieri, Streptomyces yanii*)	–
PhT 21	*KestL2*	*Hypogymnia physodes* (on bark)	Agricultural soil bacterium isolate SI-14 (AJ252581)	100 (804)	*Actinobacteria*, *Cellulomonadaceae* (*Cellulomonas xylanilytica*)	Lukow (1999)
PhT 22	*GestV1*	*Cladonia pyxidata* (from Plankogel)	Uncultured earthworm cast bacterium clone c220 (AY154578)	99.8 (410)	*Actinobacteria*, *Microbacteriaceae* (*Frigoribacterium* genomovar 2 and 3, *Curtobacterium herbarum*)	uncultured bacteria associated with the intestine of the earthworm *Lumbricus rubellus* Singleton *et al*. (2003)
PhT 23	*Gint1*	*Cladonia pyxidata* (from Plankogel)	Uncultured earthworm cast bacterium clone c220 (AY154578)	100 (720)	*Actinobacteria*, *Microbacteriaceae* (*Frigoribacterium* genomovar 2 and 3, *Curtobacterium herbarum*)	uncultured bacteria associated with the intestine of the earthworm *Lumbricus rubellus* Singleton *et al*. (2003)

Table 2. Continued.

Phylotype	Isolate	Host lichen	Most closely related sequence (Genbank accession number)	16S rRNA gene sequence similarity in % (no. of nucleotides)	Taxonomical position of isolate (most related species)	Notes/reference of most related sequence
PhT 24	*Gint1N*	*Cladonia pyxidata* (from Plankogel)	*Streptosporangium* sp. 319E04 *Streptosporangium* sp. 319B09	99 (511)	*Actinobacteria*, *Streptosporangiaceae* (*Streptosporangium vulgare*)	–
PhT 25	*Gest13N*	*Cladonia pyxidata* (from Plankogel)	nss	nss	*Alphaproteobacteria*	–

*int: bacteria isolated from the internal thallus of the lichen; est: bacteria isolated form the external surface of the lichen.
†Underlined isolates were used for the identification of the phylotype by sequencing the 16S rRNA.
‡Statistically not significant.

The ITS 16S–23S profiles showed one to seven different bands for each isolate and we were generally able to unambiguously recognize the bacteria with the same profile. The isolates with unclear differences in ITS 16S–23S profiles (i.e. isolates Aint1 and Bint1, Table 2) were assigned to phylotype based on Amplified Ribosomal DNA Restriction Analysis (ARDRA) performed with three restriction endonucleases (*Alu*I, *Taq*I and *Hinf*I). Phylotypes 9 and 12 were represented in external parts of three different lichen species, respectively (phylotype 9 in three different genera, phylotype 12 in three species of *Cladonia*). Both phylotype 1 and phylotype 8 were found in two different *Cladonia* species. *Cladonia digitata* and *Cladonia rangiferina* contained phylotype 1 internally, and phylotype 8 was found inside *Cladonia pyxidata* and externally in *C. rangiferina*. Phylotype 15 comprised isolates from both the surface and the inside of *Cladonia coccifera*. All other phylotypes are represented by a single isolate (Table 2).

Identification of strains and phylogenetic analysis

The 16S ribosomal gene of one isolate representing each of the phylotypes was partially sequenced. Identification by BLASTn searches for significant sequence similarity revealed that seven phylotypes (nine isolates) represent the genus *Paenibacillus* (Firmicutes), four phylotypes (eight isolates) belong to the genus *Burkholderia* (*Betaproteobacteria*), and three phylotypes (four isolates) can be assigned to the genus *Bacillus* (Firmicutes). Phylotype 12 (three isolates) is related to *Luteibactor rhizovicina* (*Gammaproteobacteria*), whereas phylotypes 22 and 23 (two isolates) belonged to the *Microbacteriaceae* (*Actinobacteria*); however, an unambiguous identification at genus level was not possible. The other phylotypes were identified as *Micromonospora* sp. (two phylotypes, two isolates), *Streptomyces* sp. (one phylotype, one isolate), *Streptosporangium* sp. (one phylotype, one isolate), *Cellulomonas* sp. (one phylotype, one isolate), *Staphylococcus* sp. (one phylotype, one isolate) and *Inquilinus limosus* (one phylotype, one isolate). Phylotype 25, containing one isolate belonging to the *Alphaproteobacteria*, was not identified at the genus level (Table 2). The phylogenetic analysis of the 16S ribosomal gene sequences resulted in a highly resolved Neighbour-Joining tree with moderate to high bootstrap support values for most clades (Fig. 1). A monophyletic clade of closely related phylotypes (CLU1) can be clearly identified in the *Paenibacillus* branch. The clade contains the phylotypes 1, 2, 4, 6 and 7, which are closely related to the species *P. pabuli*, and *P. amylolyticus*. CLU1 includes seven of the nine isolates identified as *Paenibacillus* sp., isolated on TY medium from both the internal thallus and the external surface of four lichen species. Phylotype 3 (from *C. coccifera*) and phylotype 5

(from *Pseudevernia furfuracea*) represent two further *Paenibacillus* species isolated from the internal parts of their host thalli (Table 2). A second cluster (CLU2) includes *Burkholderia* strains, including the phylotypes 8 and 9. These are closely related to the species *Burkholderia glathei* and *B. sordidicola*. CLU2 includes six of the eight isolates identified as *Burkholderia* sp., isolated on TY (phylotype 8) and N-free medium (phylotype 9) from the external surface of five lichen samples (from four different species, one of them from distant sampling sites). A third cluster (CLU3) contained phylotypes 22 and 23 in the *Actinobacteria* branch; both of these phylotypes were formed by a single isolate identified as *Micromonospora* sp., related to *Micromonospora auratinigra* (PhT 23) and *Micromonospora matsumotoense* (PhT 22) and both were isolated from the internal thallus of *Cladonia pyxidata*. A fourth cluster (CLU4), including the phylotypes 14 and 17, encompasses the *Bacillus* branch. These phylotypes are closely related to the species *Bacillus bataviensis* and *Bacillus niacini*, and were isolated from the surface of *C. coccifera* and *Hypogymnia physodes*, respectively. Distinct from this group are two other phylotypes from *Cladonia* species: phylotype 15 (related to *Bacillus* sp. according to Genbank information) and phylotype 16 (related to *Staphylococcus epidermidis*). Phylotype 12 from three *Cladonia* species was identified as *Luteibactor rhizovicina*, which was recently described from the rhizosphere of *Hordeum vulgare* (Johansen *et al.*, 2005). All others phylotypes were isolated from the external surfaces of lichens, and grouped with other strains found on lichens.

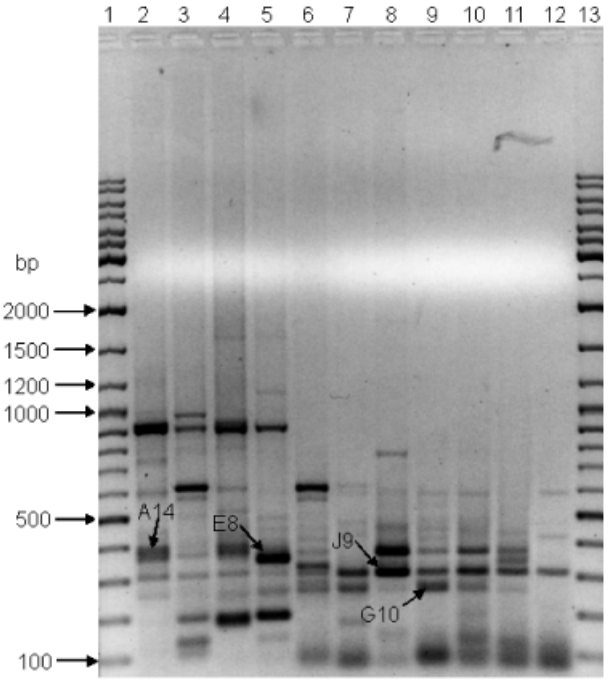

Fig. 2. Bacterial internal transcribed spacer profiles obtained by analysis of total DNA extracts from surface-sterilized thalli of 11 lichen samples (2.5% agarose gel, ethidium bromide stained). Excised and sequenced bands are indicated (see text for details). Lanes 1 and 13: DNA marker; lanes 2–12: lichen samples in the following order: A, B, D, E, R, H, J, G, K, C and S.

Cultivation-independent analysis

As the DNA extracted from the 11 lichen samples by the CTAB/chloroform-isoamyl alcohol method could not directly be visualized on an ethidium bromide-stained agarose gel, we arbitrarily used 5 μL of crude extract as template for a first PCR reaction to amplify the 16S ribosomal gene and the ITS 16S–23S with conserved primers for Eubacteria. The PCR products were hardly visible on an agarose gel and we therefore used 10 μL of the PCR product as template for a nested PCR to amplify the ITS 16S–23S. The multiple PCR products were clearly visible as distinct bands of variable intensity (Fig. 2). We repeated this approach several times and with different visualization methods to confirm the consistency of the results. The profiles – considering size and fluorescence intensity – were compared to assess the variation in banding patterns between the different communities. A matrix was constructed and used for the calculation of the ecological indices of Shannon-Wiener (diversity index) and Simpson (dominance index). The values of the Shannon-Wiener index ranged between 0.90 for the bacterial community of *C. coccifera* (lichen sample 'E') and 2.28 for the bacterial community of *C. digitata* (lichen sample 'A'). The average values, considering all 11 lichen-associated bacterial communities, were 1.71 ± 0.38 for the Shannon-Wiener diversity index and 0.23 ± 0.097 for the Simpson dominance index. The comparison showed a high specificity of profiles from the different samples (including unique bands). On the other hand, several bands are shared among different profiles, indicating the occurrence of similar bacteria in different lichens, and from different geographical regions. Four bands were excised and sequenced (Fig. 2). BLAST searches indicated the presence of additional bacteria that were not detected by culture methods. A14 showed the same identity values in the BLAST search with *Nitrosomonas* and *Nitrosospira* ssp., which are ammonia-oxidizing *Betaproteobacteria*. Band E8 did not match with any ITS sequence in Genbank and represents a hitherto unknown bacterial type. Band J9 fits well with *Propionibacterium* ssp., an obligate anaerobic *Actinobacterium*, and G10 was identified as *Bacillus subtilis*, which has previously been found in lichens (Henkel & Plotnikova, 1973).

The comparison of the profiles showed relative similarity values of 14–74% (Fig. 3). According to the ITS fingerprints, the differences of the bacterial community seem to be rather high among the sampling sites, and there is no clear congruence with the relationships of the hosting lichen.

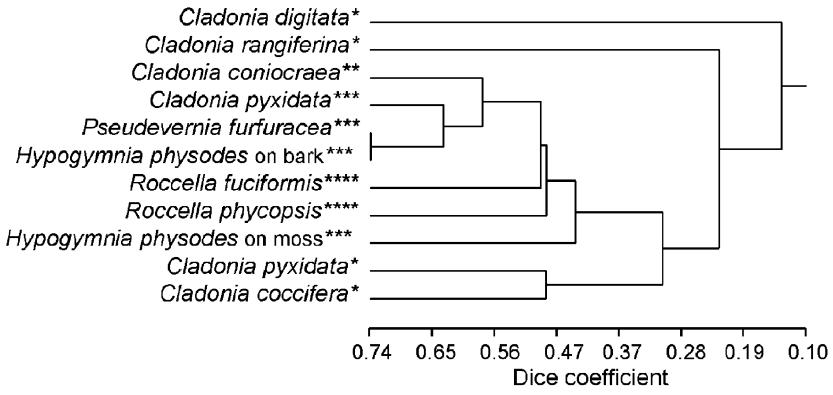

Fig. 3. UPGMA-tree of the lichen-associated bacterial communities based on the amplified ribosomal intergenic spacer profiles (ITS 16S–23S), as obtained by using bacterial primers ITSF/ITSReub and separated by both agarose and acrylamide gel electrophoresis (* from Rabenwald, ** from Botanical Garden of Graz, *** from Plankogel, **** from St. Malo).

The greatest diversity was found among the *Cladonia* samples, whereas the bacterial communities associated with different lichens sampled in Plankogel, St. Malo and the Botanical Garden of Graz formed a cluster with similarity values of 44%. The bacterial communities from two distantly collected *C. pyxidata* are diverse, whereas the bacterial communities associated with lichens from Rabenwald did not form a unique cluster, owing to the low similarity between the bacterial communities of both *C. digitata* and *C. rangiferina* and the other communities.

Discussion

Although lichen symbioses generally comprise two obligate partners with clear functional roles, i.e. the photobionts (either green algae or cyanobacteria, or sometimes both) and the mycobionts, additional fungi and bacteria can occur as optional symbionts in lichen thalli. In this contribution we used DNA sequence data to investigate the composition of the bacterial communities associated with lichens from temperate habitats and to assess the phylogenetic position of all detected culturable strains. The characterization of the culturable bacterial community was carried out in a two-step approach. First, with the ribosomal intergenic spacer analysis we assessed the genetic variation of strains cultivated from lichens, and second, by the analysis of rRNA gene sequences of a representative of each strain group, we assigned most bacteria to their respective genera and placed the discovered strains in a phylogenetic framework. However, as we generally purified only distinct phenotypes from each lichen sample, we may still have missed other genetically different strains with the same morphology. Nonetheless, we found 25 different phylotypes representing discrete bacterial taxa. The strains were assigned to different genera, of which three belong to Firmicutes, four are found among the *Actinobacteria* and three among the *Proteobacteria*. The association of *Paenibacillus* and *Burkholderia* with lichens seems to be common, irrespective of relationship and habitats of the host. Their presence is not surprising, as these genera are known also from associations with diverse other fungi. For example, *Burkholderia* was isolated from Basidiomycota, from Glomeromycota, and from Zygomycota (Bianciotto *et al*., 2000; Lim *et al*., 2003; Partida-Martinez & Hertweck, 2005), and *Paenibacillus* is found in Basidiomycota and in Glomeromycota (Budi *et al*., 1999; Poole *et al*., 2001; Bertaux *et al*., 2003). Several genetically distinct strains within these genera are associated with lichens. We also detected representatives of actinomycetes, which have previously been found in other studies (Zook, 1983; Gonzáles *et al*., 2005). However, we did not detect bacteria of the genus *Azotobacter*, repeatedly mentioned in the literature and isolated from other lichens (reviewed in Lenova & Blum, 1983), nor did we find other genera previously isolated from lichens, such as *Clostridium*, *Bejerinckia* or *Pseudomonas*. We assume that the composition of the bacterial communities in lichens is affected by diverse biotic and abiotic factors, which include the phylogenetic position of the lichens, but also the geographic origin, the substrate, the microhabitat conditions, and the pattern of fungal secondary metabolites.

Our direct analysis of bacterial diversity by PCR approaches using total DNA extracts of surface-sterilized material (using H_2O_2 to degrade superficial DNAs) is a first step towards assessing the entire bacterial diversity within lichen thalli. The results revealed variation among all samples analysed, especially in the mostly soil- and moss-inhabiting representatives of *Cladonia*. Some shared bands suggest the presence of similar strains in different lichens, which agrees with the data from the cultured fraction. Sequence analysis of selected bands revealed additional bacterial strains and further analysis of cloned PCR products is the focus of ongoing studies.

A clear statement cannot be made about the ecological role of lichen-inhabiting bacteria. It is, nevertheless, interesting that many strains are capable of growing readily on N-free media. In the case of *Paenibacillus*, this agrees with

published data about strain-specific N-fixing capacities in this genus. Eight strains are known in this genus as nitrogen fixers (Rodriguez Coelho *et al.*, 2003). All our lichen-associated strains of *Paenibacillus* grow readily on N-free medium, but their closest relative according to rRNA gene sequence data, *P. pabuli*, is not known as a nitrogen-fixer. Our data would be in accordance with a nitrogen-fixing role of lichen-associated bacterial strains, but further experiments are needed to assess whether fixed nitrogen is available in significant amounts to the symbionts. There is no clear evidence for a defensive role (Zook, 1983), but as antifungal properties are known from bacteria, e.g. from strains in *Burkholderia* (Opelt & Berg, 2004), this hypothesis should be investigated further. It also remains to be studied whether bacteria use extracellular compounds produced by lichens for their growth and to what extent they are involved in the degradation of lichens (e.g. a chitinolytic activity is known from *Paenibacillus* and *Streptomyces*). As bacteria are commonly observed in decaying lichens and especially in the soil interface (e.g. Asta *et al.*, 2001), it is also possible that bacteria could benefit from phenolic substances leaching from lichens (Stark & Hyvärinen, 2003). On the other hand, it might also be argued that lichen-associated bacteria could have a helper effect for the establishment of fungal-algal symbioses, similar to mycorrhizal helper bacteria (Garbaye, 1994). So far, lichen-associated bacteria seem to be present on the surface of thalli or in intercellular spaces (e.g. De los Ríos *et al.*, 2005); their endosymbiotic occurrence is not known. As this was found in diverse non-lichenized fungi, Ascomycota (Barbieri *et al.*, 2000), Basidiomycota (Bertaux *et al.*, 2003, 2005), Glomeromycota (Bianciotto *et al.*, 2000), and Zygomycota (Partida-Martinez & Hertweck, 2005), further investigations might also test whether endosymbioses with bacteria also occur in lichens. The ubiquitous presence of bacteria in lichens will have to be considered in studies of certain paralogs of functional genes in lichen mycobionts, such as MSAS type I polyketide synthases (Kroken *et al.*, 2003), which may occur both in fungi and bacteria. This is also underlined by the recent finding of an abundance of these genes in lichen-associated actinomycetes (Gonzáles *et al.*, 2005). Moreover, the occurrence of *Burkholderia* or *Propionibacterium* raises the question whether lichens might also act as a reservoir for opportunistic pathogens, as has been shown for rhizosphere-associated bacteria (Berg *et al.*, 2005).

Acknowledgements

We thank Barbara Stelzl-Marx (Graz) for help with the translation of publications in Russian. Joël Boustie (Rennes) is acknowledged for providing the *Roccella* samples. This work was partially supported by Agenzia Regionale per la Protezione dell'Ambiente (ARPA-Sicilia).

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.

Atlas RM & Bartha R (1993) *Microbial Ecology Fundamentals and Applications*. pp. 140–145. The Benjamin/Cummings Publishing Company, Redwood City, CA.

Asta J, Orry F, Toutain F, Souchier B & Villemin G (2001) Micromorphological and ultrastructural investigations of the lichen-soil interface. *Soil Biol Biochem* **33**: 323–337.

Barbieri E, Potenza L, Rossi I, Sisti D, Giomaro G, Rossetti S, Beimfohr C & Stocchi V (2000) Phylogenetic characterisation and in situ detection of a *Cytophaga-Flexibacter-Bacteroides* phylogroup bacterium in *Tuber borchii* Vittad. ectomycorrhizal mycelium. *Appl Environ Microbiol* **66**: 5035–5042.

Bassam BJ, Caetano-Anolles G & Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal Biochem* **196**: 80–83.

Berg G, Eberl L & Hartmann A (2005) The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Environmental Microbiology* **7**: 1673–1685.

Bertaux J, Schmid M, Chemidlin Prevost-Boure N, Churin JL, Hartmann A, Garbaye J & Frey-Klett P (2003) *In situ* identification of intracellular bacteria related to *Paenibacillus* spp. in the mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N. *Appl Environ Microbiol* **69**: 4243–4248.

Bertaux J, Schmid M, Hutzler P, Hartmann A, Garbaye J & Frey-Klett P (2005) Occurrence and distribution of endobacteria in the plant-associated mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N. *Environ Microbiol* **7**: 1786–1795.

Bianciotto V, Lumini E, Lanfranco L, Minerdi D, Bonfante P & Perotto S (2000) Detection and identification of bacterial endosymbionts in arbuscular mycorrhizal fungi belonging to Gigasporaceae. *Appl Environ Microbiol* **66**: 4503–4509.

Budi SW, van Tuinen D, Martinotti G & Gianinazzi S (1999) Isolation from the *Sorghum bicolor* mycorrhizosphere of a bacterium compatible with arbuscular mycorrhiza development and antagonistic towards soilborne fungal pathogens. *Appl Environ Microbiol* **65**: 5148–5150.

Cardinale M, Brusetti L, Quatrini P, Borin S, Puglia AM, Rizzi A, Zanardini E, Sorlini C, Corselli C & Daffonchio D (2004) Comparison of different primer sets for the Automated Ribosomal Intergenic Spacer Analysis (ARISA) of Complex Bacterial Communities. *Appl Environ Microbiol* **70**: 6147–6156.

Cubero OF, Crespo A, Fatehi J & Bridge PD (1999) DNA Extraction and PCR amplification method suitable for fresh, herbarium stored and lichenized fungi. *Plant Syst Evol* **216**: 243–249.

De los Ríos A, Sancho LG, Grube M, Wierzchos J & Ascaso C (2005) Endolithic growth of two *Lecidea* lichens in granite from continental Antarctica detected by molecular and microscopy techniques. *New Phytol* **165**: 181–190.

Felsenstein J (2004) *PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author*. Department of Genome Sciences, University of Washington, Seattle, WA.

Felsenstein J & Churchill GA (1996) A hidden Markov model approach to variation among sites in rate of evolution. *Mol Biol Evol* **13**: 93–104.

Felske A, Wolterink A, van Lis R, de Vos WM & Akkermans AD (1999) Searching for predominant soil bacteria: 16S rDNA cloning versus strain cultivation. *FEMS Microbiol Ecol* **30**: 137–145.

Garbaye J (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. *New Phytol* **128**: 197–210.

Gargas A, De Priest PT, Grube M & Tehler A (1995) Multiple origins of lichen symbioses in fungi suggested by SSU rDNA phylogeny. *Science* **268**: 1492–1495.

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

Goto K, Omura T, Hara Y & Sadaie Y (2000) Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. *J Gen Appl Microbiol* **46**: 1–8.

Henkel PA & Yuzhakova LA (1936) Nitrogen-fixing bacteria in lichens. *Izv Biol Inst Permsk Gos Univ* **10**: 9–10.

Henkel PA & Plotnikova TT (1973) Nitrogen-fixing bacteria in lichens. *Izv Akad Nauk SSR, Ser Biol* **1973**: 807–813.

Heyrman J & Swings J (2001) 16S rDNA Sequence analysis of bacterial isolates from biodeteriorated mural paintings in the Servilia Tomb (Necropolis of Carmona, Seville, Spain). *Syst Appl Microbiol* **24**: 417–422.

Hunter PR & Gaston MA (1988) Numeric index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol* **26**: 2465–2466.

Ingólfsdóttir K, Bloomfield SF & Hylands PJ (1985) In vitro evaluation of the antimicrobial activity of lichen metabolites as potential preservatives. *Antimicrob Agents Chemother* **28**: 289–292.

Iskina RY (1938) On nitrogen fixing bacteria in lichens. *Isv Biol Inst Permsk* **11**: 133–139.

Johansen JE, Binnerup SJ, Kroer N & Mølbak L (2005) *Luteibacter rhizovicinus* gen. nov., sp. nov., a yellow-pigmented gamma-proteobacterium isolated from the rhizosphere of barley (*Hordeum vulgare* L.). *Int J Syst Evol Microbiol* **55**: 2285–2291.

Krasil'nikov NA (1949) Does *Azotobacter* occur in lichens? *Microbiologiya* **18**: 3–6.

Kroken S, Glass NL, Taylor JW, Yoder OC & Turgeon BG (2003) Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. *Proc Natl Acad Sci USA* **100**: 15670–15675.

Lagacé L, Pitre M, Jacques M & Roy D (2004) Identification of the bacterial community of maple sap by using amplified ribosomal DNA (rDNA) restriction analysis and rDNA sequencing. *Appl Environ Microbiol* **70**: 2052–2060.

Lenova LI & Blum O (1983) To the question on the third component of lichens. *Bot J* **68**: 21–28.

Lim YW, Baik KS, Han SK, Kim SB & Bae KS (2003) *Burkholderia sordidicola* sp. nov., isolated from the white-rot fungus *Phanerochaete sordida*. *Int J Syst Evol Microbiol* **53**: 1631–1636.

Lukow T (1999) *Thesis. Max-Planck-Institut fuer terrestrische Mikrobiologie*. Philipps-Universität Marburg, Marburg, Germany.

Lutzoni F, Pagel M & Reeb V (2001) Major fungal lineages are derived from lichen symbiotic ancestors. *Nature* **411**: 937–940.

Nogales B, Moore ERB, Llobet-Brossa E, Rossello-Mora R, Amann R & Timmis KN (2001) Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. *Appl Environ Microbiol* **67**: 1874–1884.

Ntougias S, Zervakis GI, Kavroulakis N, Ehaliotis C & Papadopoulou KK (2004) Bacterial diversity in spent mushroom compost assessed by amplified rDNA restriction analysis and sequencing of cultivated isolates. *Syst Appl Microbiol* **27**: 746–754.

Opelt K & Berg G (2004) Diversity and antagonistic potential of bacteria associated with bryophytes from nutrient-poor habitatas of the Baltic Sea Coast. *Appl Environ Microbiol* **70**: 6569–6579.

Page RD (1996) Treeview: an application to display phylogenetic trees on personal computers. *Appl Biosci* **12**: 357–358.

Panosyan AK & Nikogosyan VG (1966) The presence of *Azotobacter* in lichens. *Akad Nauk Armian SSR, Biol Zhurn Armen* **19**: 3–11.

Partida-Martinez LP & Hertweck C (2005) Pathogenic fungus harbours endosymbiotic bacteria for toxin production. *Nature* **437**: 884–888.

Petrini O, Hake U & Dreyfuss MM (1990) An analysis of fungal communities isolated from fruticose lichens. *Mycologia* **82**: 444–451.

Poole EJ, Bendino GD, Whipps JM & Read DJ (2001) Bacteria associated with *Pinus sylvestris-Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation in vitro. *New Phytol* **151**: 743–751.

Prillinger H, Kraepelin G, Lopandic K, Schweigkofler W, Molnar O, Weigang F & Dreyfuss MM (1997) New species of *Fellomyces* isolated from epiphytic lichen species. *Syst Appl Microbiol* **20**: 572–584.

Quatrini P, Scaglione G, Cardinale M, Caradonna F & Puglia AM (2002) *Bradyrhizobium* sp. nodulating the Mediterranean shrub Spanish broom (*Spartium junceum* L.). *J Appl Microbiol* **92**: 13–21.

Rodriguez Coelho MR, von der Weid I, Zahner V & Seldin L (2003) Characterization of nitrogen-fixing *Paenibacillus* species by polymerase chain reaction-restriction fragment length polymorphism analysis of part of genes encoding 16S rRNA and 23S rRNA and by multilocus enzyme electrophoresis. *FEMS Microbiol Lett* **222**: 243–250.

Rohlf FJ (1998) *NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, Version 2.0*. Applied Biostatistics, Inc, New York, NY.

Saitou N & Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406–425.

Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Springs Harbor Laboratory, Cold Spring Harbor, NY.

Scott GD (1956) Further investigations of some lichens for fixation of nitrogen. *New Phytol* **55**: 111–116.

Singleton DR, Hendrix PF, Coleman DC & Whitman WB (2003) Identification of uncultured bacteria tightly associated with the intestine of the earthworm *Lumbricus rubellus* (Lumbricidae; Oligochaeta). *Soil Biol Biochem* **35**: 1547–1555.

Sneath PHA & Sokal RR (1973) *Numerical Taxonomy*. W.H. Freeman, San Francisco, CA.

Stark S & Hyvärinen M (2003) Are phenolics leaching from the lichen *Cladina stellaris* sources of energy rather than allelopathic agents for soil microorganisms? *Soil Biol Biochem* **35**: 1381–1385.

Suessenguth K (1926) Zur Frage der Vergesellschaftung von Flechten mit Purpurbakterien. *Ber der Deutsch Bot Ges* **44**: 573–578.

Taylor TN, Hass H, Remy W & Kerp H (1995) The oldest fossil lichen. *Nature* **378**: 244.

Thompson JD, Higgins DG & Gibson TJ (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680.

Uetanabaro P, Wahrenburg C, Hunger W, Pukall R, Spröer C, Stackebrandt E, de Canhos VP, Claus D & Fritze D (2003) *Paenibacillus agarexedens* sp. nov., nom. rev., and *Paenibacillus agaridevorans* sp. nov. *Int J Syst Evol Microbiol* **53**: 1051–1057.

Uphof JCT (1925) Purple bacteria as symbionts of a lichen. *Science* **61**: 67.

Urzi C, Brusetti L, Salamone P, Sorlini C, Stackebrandt E & Daffonchio D (2001) Biodiversity of Geodermatophilaceae isolated from altered stones and monuments in the mediterranean basin. *Environ Microbiol* **3**: 471–479.

Venter JC, Remington K, Heidelberg JF, *et al.* (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304**: 66–74.

Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**: 697–703.

Wilson MS, Herrick JB, Jeon CO, Hinman DE & Madsen EL (2003) Horizontal transfer of *phnAc* dioxygenase genes within one of two phenotypically and genotypically distinctive naphthalene-degrading guilds from adjacent soil environments. *Appl Environ Microbiol* **69**: 2172–2181.

Zhao H, Kassama Y, Young M, Kell DB & Goodacre R (2004) Differentiation of *Micromonospora* isolates from a coastal sediment in wales on the basis of fourier transform infrared spectroscopy, 16S rRNA sequence analysis, and the amplified fragment length polymorphism technique. *Appl Environ Microbiol* **70**: 6619–6627.

Zook PD (1983) *A study of the role of bacteria in lichens*. M.A. thesis). Clark University, Worcester, MA.