Universal and species-specific bacterial ‘fungiphiles’ in the mycospheres of different basidiomycetous fungi

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Summary

In previous work, several bacterial groups that show a response to fruiting bodies (the mycosphere) of the ectomycorrhizal fungus Laccaria proxima were identified. We here extend this work to a broader range of fungal fruiting bodies sampled at two occasions. PCR-DGGE analyses showed clear effects of the mycosphere of diverse fungi on the total bacterial and Pseudomonas communities in comparison with those in the corresponding bulk soil. The diversities of the Pseudomonas communities increased dramatically in most of the mycospheres tested, which contrasted with a decrease of the diversity of the total bacterial communities in these habitats. The data also indicated the existence of universal (i.e. Pseudomonas poae, P. lini, P. umsongensis, P. corrugata, P. antarctica and Rahnella aquatilis) as well as specific (i.e. P. viridiflava and candidatus Xiphinema tobacter americani) fungiphiles, defined as bacteria adapted to the mycospheres of, respectively, three or more or just one fungal species. The selection of such fungiphiles was shown to be strongly related to their capacities to use particular carbonaceous compounds, as evidenced using principal components analyses of BIOLOG-based substrate utilization tests. The differentiating compounds, i.e. L-arabinose, L-leucine, m-inositol, m-arabitol, D-mannitol and D-trehalose, were tentatively linked to compounds known to occur in mycosphere exudates.

Introduction

Both the bacterial and fungal communities in soil play important roles in soil functioning, for instance, in key steps of mineralization processes. Both groups of organisms are thus important for the growth and development of plants (crops and trees), and also for the maintenance of soil structure (Poole et al., 2001; Johansson et al., 2004; de Boer et al., 2005; Frey-Klett et al., 2007; Uroz et al., 2007). Organisms within the two groups (bacteria and fungi) often share the same microhabitat in soil. It is likely that, given their presumed long history of coexistence in the same habitat, particular organisms in both groups may have evolved a more or less complex interaction with each other. The existence of such interactive mechanisms has already been suggested in the literature (Garbaye, 1994), but the first robust evidence of direct (mechanistic) bacterial–fungal interactions in soil was only found in the last few years (Soler-Rivas et al., 1999; Bertaux et al., 2003; Bianciotto et al., 2004; Jargeat et al., 2004; Toljander et al., 2006; Frey-Klett et al., 2007; Partida-Martinez et al., 2007). These hallmark findings open up a completely novel area of investigation in our quest to the understanding of the bacterial–fungal interactive complex in the living soil, allowing the discovery of the intricate mechanisms of interaction. For instance, the use, by a rice-pathogenic fungus, of a bacterially produced toxin in its invasion of the host plant has been revealing in that it showed the harnessing of a bacterial trait by a fungal ‘host’ in its ecophysiology (Partida-Martinez and Hertweck, 2005). Moreover, mechanisms involved in parasitic bacterial–host interactions, like type III secretion systems (TTSS), have been implicated in the selective process of particular bacteria, including pseudomonads, in the mycosphere of Laccaria proxima (Warmink and van Elsas, 2008). However, definite proof for the involvement of TTSS in the functional interaction with the fungal host has not yet been obtained.

In theory, soil bacteria that possess mechanisms which allow them to interact with fungi may obtain a fitness advantage when present in the vicinity of a fungal host. They may thus have greater ecological success and be found in higher numbers in the respective mycosphere. Such an enhancement of the densities of particular bacterial groups in the mycosphere, as compared with those in the corresponding bulk soil, offers the possibility of detection of a bacterial response to an emerging mycosphere, by using cultivation-dependent or cultivation-independent methods (Frey et al., 1997; Timonen et al., 1998; Bending et al., 2002; Warmink and van Elsas, 2008). From such studies, we now know that a considerable diversity of bacterial types has potential to be selected in different mycospheres. Thus, members of the
genera Agrobacterium, Arthrobacter, Aquamonas, Bacillus, Burkholderia, Chryseobacterium, Janthinobacterium, Mesorhizobium, Streptomyces, Sphingomonas, Paenibacillus, Pseudomonas, Rahnella, Rhodococcus and Variovorax have all been found to be selected in mycospheres of various fungi (Johansson et al., 2004; de Boer et al., 2005; Izumi et al., 2006; Boersma et al., 2008; Warmink and van Elsas, 2008). Obviously, such bacteria may take profit from their fungal hosts in a nutritional or ‘homing’ context and they may have evolved strategies to be ecologically successful with their fungal host. What we do not quite understand is to what extent the selection of particular bacterial species in the mycosphere is a robust deterministic event, or whether stochastic (chance) factors also play a role. If stochasticity is predominant, effects of environmental factors such as soil structure and composition, pH, temperature, moisture content and other organisms in the soil may become more prominent.

This study examined the bacterial communities in the dense hyphal network directly underneath the feet (mycosphere) of a wide range of mushrooms of basidiomycetous fungi, encompassing ectomycorrhizal, plant-pathogenic and saprotrophic fungal species, in comparison with those in bulk soil from the same location. This way, the influence of the various fungi on the putative selective process exerted by mushroom feet on soil bacteria could be determined at a minimum of confounding factors. A special focus was placed on members of the easily cultivable genus Pseudomonas as these were thought to play an important ecological role in the mycosphere (Berg et al., 2005; Frey-Klett et al., 2007) as well as the rhizosphere, for example, in protection against plant-pathogenic fungi and bacteria (Catara, 2007; Leveau and Preston, 2008). A selection of bacteria apparently specific for particular fungi (hereafter denoted ‘specific fungiphiles’) as well as bacteria that were selected in the mycosphere of a broader range (three or more) of fungal species (universal fungiphiles) was further studied in respect of the putative mechanism involved in the association.

Results

Identification of mushroom-forming fungi via molecular analysis of fruiting bodies

Fungal DNA was successfully obtained from 25 fruiting bodies sampled in 2006 (16) and 2007 (9) at the same location in Noordlaren, the Netherlands, and subjected to sequence analyses of the 18S ribosomal RNA gene, internally transcribed spacer (ITS) region and the 23S ribosomal RNA gene. Comparison of the about 800 bp sequences obtained with those of the GenBank database identified all fruiting bodies to genus/species level (Table 1). We used as criteria the levels of > 99% similarity for species and > 95% for genus (Landeweert et al., 2003). Eight of the thus identified fungi belonged to the following known ectomycorrhizal fungi: Lactarius hepticus, Russula ochroleuca, Russula exalbicans, Russula sp. (2×), Laccaria ochropurpurea, Laccaria amethystea and Scleroderma citrina (all type species except for Russula sp.). Six other fruiting bodies sampled belonged to, respectively, Mycena galopus, Mycena sp. (2×), Gymnopilus penetrans, Rhodocollybia sp. and Tubaria furfuracea. As far as is known, these organisms are all saprotrophic fungi in soil. The remaining two fruiting bodies belonged to Armillaria tabescens and Phallus sp., which are both known as potential plant pathogens. The fruiting bodies sampled in 2007 for three fungi in triplicate

<table>
<thead>
<tr>
<th>Related to</th>
<th>Similarity (%)</th>
<th>Accession No. of closed hit</th>
<th>Year</th>
<th>Presumed ecological role</th>
</tr>
</thead>
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<tr>
<td>Mycena galopus</td>
<td>99</td>
<td>AY805614.1</td>
<td>2006</td>
<td>Saprotrophic</td>
</tr>
<tr>
<td>Lactarius hepticus</td>
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<td>2006</td>
<td>Ectomycorrhizal</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>AY281002.1</td>
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</tr>
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</tr>
<tr>
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</tr>
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<td>2006</td>
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</tr>
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</tr>
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<td>AY654886.1</td>
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</tr>
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<tr>
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<td>2006</td>
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</tr>
<tr>
<td>Armillaria tabescens</td>
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<td>DQ851572</td>
<td>2006</td>
<td>Plant-pathogenic</td>
</tr>
<tr>
<td>Scleroderma citrina</td>
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<td>AF026621</td>
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<td>Ectomycorrhizal</td>
</tr>
<tr>
<td>Phallus sp.</td>
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<td>AYT771601.1</td>
<td>2006</td>
<td>Plant-pathogenic/ectomycorrhizal</td>
</tr>
<tr>
<td>Lactarius hepticus</td>
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<td>2007</td>
<td>Ectomycorrhizal</td>
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<tr>
<td>Amanita sp.</td>
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<td>AJ889919</td>
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<tr>
<td>Scleroderma citrina</td>
<td>99</td>
<td>AF026621</td>
<td>2007</td>
<td>Ectomycorrhizal</td>
</tr>
</tbody>
</table>
were identified as belonging to, respectively, the ectomycorrhizal fungi *L. hepaticus*, *Amanita citrina* and *S. citrina*. Per species, all triplicates concurred, i.e. showed identical identifications.

**Bacterial community analyses of 16 mushrooms**

The DGGE profiles generated from all 16 mycosphere (underneath the mushroom) and corresponding bulk soil samples in 2006 visually showed clear differences between all mycosphere samples on the one hand and all corresponding bulk soil samples on the other hand (Fig. 1). Thus, we assumed the occurrence of substantial selection for particular bacterial types in all sampled mycosphere soil habitats in comparison with the corresponding bulk soil (Fig. 1). Subsequent cluster analyses of the profiles using the unweighted pair group method with mathematical averages (UPGMA) indeed revealed the distribution of all among just two major clusters, one encompassing all mycosphere samples and the other one all bulk soil samples (Fig. 2A). The similarity between the two clusters was 57.3%. All bulk soil samples were internally highly similar (>90%), which confirmed the homogeneity of the microbial communities within this habitat. In contrast, analysis of the mycosphere samples did not show any clear clustering along fungal taxonomy and/or a relation with the ecological function of the fungal host, defined as presumptively ectomycorrhizal, saprotrophic or plant-pathogenic.

Analysis of the diversities of the dominant bacteria detectable by bacterial PCR-DGGE via determination of the Shannon indices based on all profiles showed a significant (*t*-test, *P* < 0.05) decrease of the diversity level in the mycosphere soils (2.7 ± 0.1) in comparison with that in the corresponding bulk soils (3.1 ± 0.1).

Several typical bands (Fig. 1; S1 to S11) found in the mycosphere-derived profiles were not present in corresponding bulk soil samples. A few selected conspicuous bands, namely S1, S2, S5 and S6, were presumptively identified by analysing the sequences of those clones in the clone library that yielded amplicons that co-migrated with these bands in DGGE analyses (Table 2). This analysis revealed that the two abundant bands, S1 and S2, were likely related (99% similarity) to the sequence of *Rahnella aquatilis* strain tAp10 isolated from the fungus *Suillus flavidus* (Izumi et al., 2006), and also related, at a lower similarity level (94%), to the *R. aquatilis* type strain DSM 4594 (RDP type strain database). The RDP database analysis further showed that the sequence of band S5 was related to that of *Pseudomonas antarctica* CMS 35 (94% similarity) and that of band S6 to *candidatus* Xiphinema americani. However, the latter affiliation showed low similarity (84% similarity), but considerable similarity (98%) with an as-yet-unidentified bacterium from a deciduous forest in south-west China (GenBank Accession No. AY963344).

**Comparative analysis of bacterial communities with selected mushroom samples in two years**

Comparison of the bacterial profiles obtained from the fruiting bodies of two fungi that were encountered in both sampling years (2006 and 2007), i.e. *L. hepaticus* and *S. citrina*, showed a clear clustering of all mycosphere samples (triplicates together) in one cluster and all bulk soil samples in another one. Sampling year did not greatly...
affect this clustering (Fig. 3). The profiles generated from the mycospheres of the three same fungal hosts clustered together in a larger cluster consisting of all mycosphere-derived profiles. The similarity between the triplicates per fungal species was mainly caused by the emergence of few bands that appeared to be specific for each fungal species. A number of mycosphere-unique bands was found in the profiles of both years, next to a number of different ones. This indicates the selection of similar bacteria at the same fungal species in both years.

Molecular and cultivation-based analysis of Pseudomonas mycosphere communities

PCR-DGGE analyses of the Pseudomonas communities based on the gacA gene (Costa et al., 2007) were used to assess the distribution of Pseudomonas types in the 2006 mycosphere and corresponding bulk soil samples (Fig. 4). All mycosphere samples yielded semi-complex DGGE profiles, which contained 4–14 distinct bands. On the other hand, corresponding bulk soil samples were extremely simple, mostly revealing only one strong band and a second faint band. In six bulk soil samples, no PCR products were ever obtained, indicating a Pseudomonas density in these samples below the detection limit of the PCR-DGGE system. The Shannon diversity indices showed significant increases ($t$-test; $P < 0.05$) in the mycosphere (2.0 ± 0.6) in comparison with those in the bulk soil samples (1.1 ± 0.4). Cluster analysis of the DGGE profiles showed the emergence of two broad profile groups, one containing only mycosphere-derived profiles and the other one all bulk soil-derived profiles. The mycosphere-derived samples M8, M12 and M15 clustered in the latter group (Fig. 2B).
To identify key pseudomonads in the mycosphere, we used cultivation-based analyses as an addition to the direct molecular assessments. Thus, the 119 *Pseudomonas* isolates obtained from the mycospheres of *L. hepaticus*, *A. citrina* and *S. citrina*, when subjected to GTG-5 profiling and grouping, fell into 18 groups. Identification of representatives of all 18 groups via 16S ribosomal RNA gene sequencing revealed the presence of, in total, nine different *Pseudomonas* species, as defined by similarity to type strains in the RDP database (Table 3). Thus, dominant species found in the mycospheres examined were affiliated with *Pseudomonas lini* (group P04; 40 isolates, from all replicate samples of the three mushroom species), *P. poae* (groups P01, P02, P03 and P05; 29 isolates, all triplicates of *A. citrina* and *S. citrina*) and *P. plecoglossicida* (groups P11, P13, P15 and P16; 18 isolates, all triplicates of *S. citrina*). Other strains that were found in the mycospheres, in lower numbers (Table 3), were related to *Pseudomonas rhodesiae* (P17, P18), *P. corrugata* (P09, P14), *P. frederiksbergensis* (P08, P12), *P. viridiflava* (P10), *P. umsongensis* (P07) and *P. cedrina* (P06).

Dominant culturable *Pseudomonas* species obtained from the different mycospheres as well *P. poae* BS053 (formerly *P. fluorescens* BS053), which was previously found to dominate in the mycosphere of *L. proxima* (Warmink and van Elsas, 2008), were used for the identification of bands in the *gacA*-based community profiles. Members of six of the 18 GTG-5 groups (this study) could thus be presumptively identified in the *gacA*-based *Pseudomonas* mycosphere community profiles (Table 3). These were: *P. poae* P03, *P. lini* P04, *P. cedrina* P06, *P. umsongensis* P07, *P. viridiflava* P10 and *P. corrugata* P14. Organisms akin to BS053 (Warmink and van Elsas, 2008) were also revealed in mycospheres M4 (*Mycena* sp.) and M6 (*Rhodollybia* sp.) respectively. On the other hand, the dominant band present in all bulk soil and most mycosphere profiles (Fig. 4, arrow BP1) was identified, via sequencing of the *gacA* gene fragment, as related to *P. fluorescens* PFO-1 (88% *gacA* sequence similarity).

![Fig. 3. Cluster analyses (UPGMA) via Gelcompar (Applied Maths, Sint-Martens-Latem, Belgium) of both mycosphere and bulk soil samples from 2006 and 2007 of *Lactarius hepaticus* and *Scleroderma citrina.*](image)

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Fig. 4. DGGE profiles of the *Pseudomonas* community based on the *gacA* gene. M01 to M16 are mycosphere samples from different basideomycete fungi; B01 to B16 are corresponding bulk soil samples. PCR-DGGE fragments were identified by comparing this profiles with isolated *Pseudomonas*. M01 to M16 and B01 to B16 are samples from mycosphere and bulk soil respectively (see Table 1). Marker (M) consists of 16S ribosomal RNA gene fragments of (from top to bottom): *Listeria innocua*, *Enterobacter cloacae*, *Mesorhizobium* sp., *Burkholderia cepacia* and *Arthrobacter* sp.

Table 3. Bands identified in DGGE profiles based on *Pseudomonas gacA* gene.

<table>
<thead>
<tr>
<th>Group</th>
<th>Affiliated with</th>
<th>Type strain</th>
<th>Similarity with type strain (%)</th>
<th>No. (isolates)</th>
<th>Origin of isolates</th>
<th>Detected in DGGE mycosphere profile³</th>
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</thead>
<tbody>
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<td>P01</td>
<td><em>Pseudomonas poae</em></td>
<td>DSM 14936</td>
<td>99.0</td>
<td>3</td>
<td>Amanita citrana</td>
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</tr>
<tr>
<td>P02</td>
<td><em>P. poae</em></td>
<td>DSM 14936</td>
<td>98.9</td>
<td>21</td>
<td>A. citrana, Sclerotera citrina</td>
<td></td>
</tr>
<tr>
<td>P03</td>
<td><em>P. poae</em></td>
<td>DSM 14936</td>
<td>98.3</td>
<td>3</td>
<td>A. citrana</td>
<td>M01, M02, M03³</td>
</tr>
<tr>
<td>P04</td>
<td><em>P. lini</em></td>
<td>CFPB 5737</td>
<td>99.9</td>
<td>40</td>
<td>Lactarius hepaticus, A. citrana M05, M09, M11, M14</td>
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<tr>
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<td>2</td>
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<td></td>
</tr>
<tr>
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<td>1</td>
<td>L. hepaticus</td>
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<tr>
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<td>Ps 3-10</td>
<td>99.8</td>
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<td>9</td>
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<td>M01, M02, M13</td>
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<td>S. citrina</td>
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<td>2</td>
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<td><em>P. poae</em>⁵</td>
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<td>99.9</td>
<td>9</td>
<td>L. proxima (Gieterveen)</td>
<td>M04, M06</td>
</tr>
</tbody>
</table>

a. M followed by number indicates the profile (see Fig. 1).

b. Also found in *S. citrina* isolated in 2007.

c. Renamed from former *P. fluorescens* due to a new highest match in the RDP database with *P. poae* DSM 14936 (Behrendt et al., 2003). Groups from GTG-5 profiles identified by 16S ribosomal RNA gene sequencing.
revealed the occurrence of similar bands across different mycosphere-derived profiles, which were completely absent from corresponding bulk soil-derived profiles (Tables 2 and 3). This indicated the selection of similar types in the mycospheres of different fungi. Specifically, five bands, i.e. S1, S2, S3, S4 and S5 (Fig. 1), in the total bacterial and another four, i.e. P03, P04, P07 and P14 (Fig. 4), in the *Pseudomonas* community profiles dominated in minimally three different mycospheres, whereas they were completely absent from, or weakly present in, the corresponding bulk soils. These bands were postulated to reflect putatively universal fungiphiles (Tables 2 and 3). For total bacteria, at least six bands, i.e. S6, S7, S8, S9, S10 and S11, were specific for just one fungal species, whereas these were completely absent from bulk soil (next to the other mycosphere) profiles (Fig. 2). The organisms reflected in these bands can be regarded as potential species-specific fungiphiles, as they were only found with one fungal type.

The bacterial as well as *Pseudomonas* community profiles from 2007 showed a similar selection of both specific and universal fungiphiles. The universal fungiphile *R. aquatilis* found in 2006 (bands S1 and S2) was also found in the 2007 profiles (Table 2), whereas *P. poae* P03 and *P. umsongensis* P07 from the *Pseudomonas* community were also found in both the 2006 and 2007 profiles (Table 3).

On the other hand, the bulk soil profiles showed the occurrence of bands that were shared across at least eight samples. Of the total of 32 bands, 19 were never found in any mycosphere. The latter bands may thus reflect organisms that become relatively less prevalent in the mycosphere, here denoted as ‘fungiphobes’.

The dominance of particular bacterial types in the mycosphere-derived DGGE profiles (Fig. 1) was correlated to the type of specificity of these bacteria for fungal species (i.e. universal or species-specific). When the relative peak areas (reflecting bacterial dominance) were examined (Fig. 5), a strong trend was shown, albeit not significant (*t*-test, *P* > 0.05). Specifically, the universal fungiphile bands, i.e. S1, S2, S3, S4 and S5, were consistently found at relatively low abundances (< 3.9% of total peak surface), whereas the species-specific fungiphile bands, i.e. S6, S7, S10 and S11, were stronger (4–12% of total peak surfaces). An increase of specificity was thus found to coincide with an increase of relative dominance within the DGGE profiles (Fig. 5).

**Properties of fungiphilic *Pseudomonas* spp.**

Metabolic profiling. For several selected *Pseudomonas* fungiphiles, the utilization of a range of substrates was determined via BIOLOG-based substrate utilization assays and tests for oxalate and chitin utilization. The metabolic patterns obtained were compared with the patterns of selected pseudomonads that had been obtained from bulk soil, i.e. strains B1, B2, B3, B4, B5 and B6 (R. Costa, unpublished). Principal components analysis (PCA) clustering of the substrate utilization profiles showed a clear separation of all bulk soil pseudomonads in comparison with those obtained from the mycosphere (Fig. 6). Compounds that could be utilized by the majority...
of the fungiphiles (> 75%) were: L-arabinose, m-inositol, maltose, L-rhamnose, D-sorbitol, D-glucuronic acid, succinamic acid, glucuronamide, L-alaninamine, L-leucine and L-ornithine. These compounds could generally not be utilized by any of the bulk soil isolates, resulting in a clear separation of the organisms into two groups in the PCA. L-arabinose, L-leucine and m-inositol are components that often occur in fungal exudates (Sun et al., 1999). The substrates α-D-glucose, D-mannitol, D-alanine, L-aspartic acid, L-glutamic acid and L-proline were utilized by isolates from both mycosphere and bulk soils. These substrates can also make part of fungal exudates (Sun et al., 1999; Rangel-Castro et al., 2002). None of the tested Pseudomonas isolates was able to use chitin or oxalate as energy or carbon sources.

Interestingly, the BIOLOG substrate utilization analysis showed that all cultured universal fungiphiles (P. poae P03, P. lini P04, P. umsongensis P07 and P. corrugata P14) formed a narrow cluster in the midst of the large cluster that encompassed all mycosphere-derived pseudomonads (Fig. 6). This indicates that these organisms utilize a core set of specific substrates responsible for the clustering of all universal fungiphiles. These substances were D-arabitol, D-mannitol, D-trehalose, formic acid and L-leucine, which are all known to occur in exudates of different fungal species (F.G.H. Boersma, S.P. Moolenaar, R. Otten, J.A. Warmink, and J.D. van Elsas, submitted; Sun et al., 1999; Rangel-Castro et al., 2002). In total, nine other compounds (maltose, L-rhamnose, D-sorbitol, D-glucuronic acid, p-hydroxy-phenylacetic acid, succinamic acid, glucuronamide, L-alaninamine and L-ornithine) were also identified as being universal fungiphile substrates. However, we lack knowledge as to the putative release of these compounds by fungi in soil.

**Type III secretion.** Tests for the presence of the hrcR gene (marker for TTSS) gave positive results for three Pseudomonas groups, namely groups P01, P02 and P03 (all closely related to P. poae; Table 3). These Pseudomonas groups represented about 25% of all isolated pseudomonads and thus formed an important part of this bacterial population in different mycospheres. On the basis of both 16S ribosomal RNA gene sequence analyses and BIOLOG assays, Pseudomonas groups P01 and P02 were highly similar to P. poae strain BS053, which is the Pseudomonas type that has previously been shown to dominate in the mycosphere of L. proxima (Warmink and van Elsas, 2008).

**Discussion**

The selection of soil bacteria by conditions that are locally established by fungi has been known for a long time (Mosse, 1962). Whereas in most previous studies, the mycorrhizosphere was the habitat studied, in this study a special focus was placed on the sphere surrounding the hyphae under fruiting bodies of basidiomycetous fungi, here denoted as the mycosphere. The microbiota in mycosphere soil presumably plays an important role in the carbon and nutrient cycling between plants and trees and the living soil environment. Selection of diverse bacterial species has been observed previously with different fungi under different environmental conditions (Johansson et al., 2004; de Boer et al., 2005; Boersma et al., 2008; Warmink and van Elsas, 2008). Reaching firm conclusions about the generality of specific adaptations of soil bacteria to particular fungal species in soil has so far not been possible, as (a)biotic conditions in the soil environment constitute confounding factors that affect this apparent adaptation process (Standing and Killham, 2007). The existence of bacterial species adapted to competitive life in the mycosphere of different fungal species can thus be argued, but this existence has never been robustly proven (Andrade et al., 1997; de Boer et al., 2005).
In this study, we sampled the mycospheres of a range of fungal species that appeared in the same forest area in Noordlaren, the Netherlands, in two consecutive years. Total bacterial and *Pseudomonas*-specific community analyses were performed on these mycosphere and corresponding bulk soil samples to gain insight into the putative effects the different fungal species exert on the soil bacterial communities, including pseudomonads. A special focus was then placed on the impact of compounds presumably present in fungal exudates in the selection of, primarily, the pseudomonads.

The soil bacterial communities were highly homogeneous across samples, as confirmed by the similar DGGE profiles in different bulk soil samples (Fig. 1). This rather homogeneous background thus allowed to address questions in respect of the influence of the flush of activity in emerging fungal hyphae, occurring at nascent mycorrhizal rooms, on the bacterial communities that are locally present. Clearly, the homogeneity of the bacterial communities did extend across time, as we obtained relatively similar profiles when bulk soil samples from two years were compared side by side.

The decrease of the diversity of the dominant bacteria in the mycosphere as compared with that in the bulk soil indicates the selection of (few) bacterial types that presumably dispose of ecophysiological strategies which allow to benefit from the fungal habitat. Bacterial species that lack such ability either remained unaffected or were even negatively influenced by the fungus, thus loosing the competition in the mycosphere with the presumably better-adapted bacterial species. Indeed, we found the total bacterial diversity to decrease in the mycosphere as opposed to the bulk soil, thus confirming the hypothesis of selection of a subset of the soil bacterioflora by the mycosphere. In a somewhat counterintuitive way, this hypothesis was also supported by the increase in the diversity of the *Pseudomonas* communities in the mycosphere in the face of the poor diversity as well as abundance of these communities in the corresponding bulk soil. On the basis of cultivation-based assessments, particular pseudomonads have been known for their ability to thrive within the mycosphere of fungi for some time now (Danell *et al.*, 1993; Varese *et al.*, 1996; Brule *et al.*, 2001; Mansfeld-Giese *et al.*, 2002; de Boer *et al.*, 2005; Frey-Klett *et al.*, 2005). For some types, the relative importance may indeed be reliably estimated from enumerations using the culturable fractions. However, for others the relative importance may be overestimated, as these types are often very easily cultured. This is in contrast to the total soil bacterial community, of which only up to 5% of the bacterial species can be cultured. This is known as the great plate anomaly (Staley and Konopka, 1985). In this study, we bypassed the problem posed by the great plate anomaly by using DNA isolated from soil in combination with a *Pseudomonas*-specific gacA-based PCR-DGGE system (Costa *et al.*, 2007).

The increase of *Pseudomonas* diversity in the mycosphere provided indications for adaptive life strategies within particular members of this community, allowing these to benefit from the mycosphere. These adaptive strategies can be manifested as a fast response to the novel hyphae emerging in the soil. The capacity to give a rapid growth response to such emerging ecological opportunities is a common trait for the known pseudomonads, which are typically regarded as r-strategists. Also, the physical conditions provided by the hyphal network in soil can offer selection for colonization and so biofilm formation might be favoured (Hogan and Kolter, 2002). Attachment and migration in soil using the fungal network may yet form another selective asset. In ecological terms, beneficial effects of *Pseudomonas* spp. can also be supposed, as several *Pseudomonas* species have been identified as mycorrhiza helper bacteria (Garbaye, 1994; Frey-Klett *et al.*, 2007). *Pseudomonas* spp. also play an important role in the rhizosphere as several pseudomonads were identified as plant growth promoting bacteria as well as biological control agents against plant-pathogenic fungi (Johansson *et al.*, 2004; Catara, 2007).

We postulate that the soil bacteria that are able to thrive in the mycosphere have the capacity to give a rapid ecophysiological response to emerging fungal hyphae; they may do so in a broad or specific way, giving rise to either universal or species-specific fungiphilic compounds. Hence, such fungiphiles may take profit from properties that are shared across different fungi or are specific per species. Compounds released by the fungal tissue are the obvious candidate selective factors (Timonen *et al.*, 1998). Strong evidence for a role of particular compounds of fungal exudates in the selection of bacteria was provided using BIOLOG substrate utilization assays. In particular, the universal fungiphiles *P. poae* P03, *P. lini* P04, *P. unsson- gensis* P07 and *P. corrugata* P14 were well fitted to utilize the compounds L-arabinose, L-leucine, m-inositol, D-arabitol, D-mannitol and D-trehalose. As such compounds may abound in fungal exudates, this presumably makes these organisms capable of colonization of, and persistence in, different mycospheres. The use of bulk soil isolates in this comparison from a chemically and structurally similar, but locationally different, soil was necessary as no *Pseudomonas* isolate could be obtained from bulk soil from the same sampling location. This phenomenon was shown before by Timonen and colleagues (1998) and the low pH (3.1) of the forest bulk soil is hypothesized to play a role in this; survival in this soil may depend on fungal development, as further worked out in F.G.H. Boersma, S.P. Moolenaar, R. Otten, J.A. Warmink, and J.D. van Elsas (submitted). In contrast, the dominance of species-specific fungiphiles in the...
respective DGGE profiles can be ascribed to their strong adaptation to the particular mycospheres. The putative ability of these organisms to utilize particular substrates exuded only by their specific fungal host would provide support for this contention.

The most abundant universal fungiphile, reflected in bands S1 and S2 (see bacterial PCR-DGGE), was probably similar (99%) to R. aquatilis tAp10 (Izumi et al., 2006) and to the RDP type strain R. aquatilis DSM 4594 (94%). The former organism has recently been found by Izumi and colleagues (2006) in association with the ectomycorrhizal fungus S. variegatus. Based on their conclusions, this Rahnella strain was tightly associated with S. variegatus as they found this bacterium after surface sterilization of the hyphae. It can also play a role in mineral weathering and nitrogen fixation (Berge et al., 1991; Kim et al., 1998). Such properties are highly beneficial for mycorrhizal life, in which exchange of minerals with the soil and the plant partner is essential. Based on this, a role for R. aquatilis as a mycorrhiza helper bacterium might even be argued (Garbaye, 1994; Frey-Klett et al., 2007), but additional work will be required to prove this contention. Together with our findings, the data may indicate that members of R. aquatilis are universal and successful fungiphiles. The relatively high similarity between R. aquatilis and our fungiphile in comparison with the DSM 4594 (Brenner et al., 1998) type strain (isolated from water) can be attributed to a selection of this R. aquatilis type by the mycosphere rather than an aquatic habitat.

The dominant Pseudomonas groups P01, P02 and P03 were highly similar to P. poae BS053 (Warmink and van Elsas, 2008), a strain isolated from L. proxima at a different location. This indicates an important role for this P. poae type in the mycosphere of at least four different mycorrhizal fungi. Similar Pseudomonas types (based on sequence similarity) were also found within the hyphae of the ectomycorrhizal fungi S. variegatus and Russula paludosa in Scotland (Izumi et al., 2006). This defines members of the P. poae clade as key fungiphilic types, independent of location and soil. We here indicate their successful life in the mycospheres of different ectomycorrhizal fungi, in addition to their clear selection in the L. proxima mycosphere (Warmink and van Elsas, 2008).

Experimental procedures

Sampling location and soil

All samples (mushrooms plus mineral soil underneath) were taken in a forest area containing deciduous and coniferous trees (Quercus robur and Larix × marschilinsii Coaz) near Noordlaren in the Netherlands over two years (2006 and 2007). The Noordlaren (N) soil was a sandy soil type with a pH-H2O (Weaver et al., 1994) of 3.1 ± 0.1, determined over six samples taken in both years throughout the forest soil. The organic matter content in the mineral layer just below the litter layer was 3.0%.

The mycospheres (mushroom feet) of, in total, 16 different basidiomycetous fungi, were sampled in autumn 2006. In addition, three selected basidiomycetous fungi were sampled in triplicate, in autumn 2007. That is, per fungal species three fruiting bodies surrounding the same tree were obtained. All samples were obtained by digging out intact mushrooms, taking care that the underlying soil (5 cm depth, without litter) was included. Bulk soil samples were also taken to the same depth (with an auger, after removal of a shallow litter layer) in close proximity (30 cm) to the fruiting bodies, taking care that, in this case, no intense hyphal network was sampled. Samples were taken to the laboratory and used immediately for the analyses. In the laboratory, the shallow zones in the soil directly underneath the fruiting bodies of the fungi were severed, yielding approximately 0.2 g of soil. In addition, a homogenized sample of corresponding bulk soil, from which stones and roots were removed, was also included. These samples were used for direct soil DNA isolation as well as for the isolation of fluorescent pseudomonads, as described (Warmink and van Elsas, 2008).

Identification of fungal and bacterial species by sequence analyses

The collected mushrooms as well as selected bacterial isolates were subjected to identification via analysis of their small subunit ribosomal RNA gene sequences, as described (Warmink and van Elsas, 2008). All new sequences generated in this study were deposited in GENBANK under Accession Numbers EU863627–EU863644 and EU924771–EU924789.

Total bacterial and Pseudomonas-specific community analyses via PCR-DGGE fingerprinting

Extraction and purification of DNA from the soil samples, as well as amplification via PCR with primers F968-GC and 1378R (Nübel et al., 1996; Heuer and Smalla, 1997; Bruns and van Elsas, 2008), were performed as described previously (Warmink and van Elsas, 2008), whereas samples from 2006 and 2007 were compared according to Muyzer and colleagues (1993). To assess the Pseudomonas communities, a specific nested gacA-based PCR-DGGE was performed, as described (Costa et al., 2007). DGGE analyses were performed on a PhorU2 system (Ingény International, Goes, the Netherlands) according to the manufacturer’s protocol, using a gradient (Muyzer et al., 1993) of 40–70% denaturants (urea/formamide) (100% denaturants is 8 M urea plus 40% formamide), a buffer temperature of 60°C and 110 V for 18 h. Following electrophoresis, the polyacrylamide gels were stained using silver staining (Heuer et al., 2001), after which they were digitized for further analyses with Gelcompar.

Clone libraries and identification of bacterial types in DGGE profiles

Clone libraries were constructed based on DNA extracted from two selected mycospheres, denoted M01 (M. galopus)
and M05 (G. penetrans). For that purpose, DNA was amplified with primers F968 and 1378R (Garbeva et al., 2004) and amplicons were treated as described previously (Warmink and van Elsas, 2008). The amplicons were ligated into the pGEM-T easy vector (Promega, Madison, WI, USA) according to the manufacturer’s protocol. The ligation products were then introduced into Escherichia coli MM294 competent cells (Syntiphium Life Sciences, Groningen, the Netherlands) by transformation according to the supplier’s protocol, after which cells were plated onto selective media. Following growth at 37°C, white colonies were randomly picked, and checked for the right insert by a PCR assay with primers SP6R and T7F based on the pGEM-T vector. The 25 μl of PCR mix consisted of 2.5 μl of PCR buffer (Roche, Basel, Switzerland), 200 μM of each deoxynucleoside triphosphate, 200 nM of each primer, 2% DMSO, 0.06 U Taq DNA polymerase (Roche, Basel, Switzerland) and 20.7 μl of H2O. The following PCR programme was used: 94°C for 3 min; 94°C for 1 min, 50°C for 1 min, 72°C for 3 min (30 cycles); 72°C for 10 min (one cycle). The PCR products (600 bp, insert + flanking regions of the pGEM-T vector) were visualized on 1% agarose gels, to determine size and quality. Clones containing expected inserts were used in a PCR with primer pair F968-GC and 1378R (Heuer et al., 2001) under the above conditions. These PCR products were used in DGGE analyses as described and fragment migration was compared with the profiles obtained from the mycosphere. Clones that had produced amplicons that co-migrated with particular bands of the mycosphere profiles were taken as indicative for those bands and used for sequence identification by (partial) 16S ribosomal RNA gene fingerprinting using primer GTG-5 (Rademaker et al., 2004) and the profiles obtained were then clustered – yielding GTG-5 groups – using UPGMA with the program Gelcompar II (Applied Maths, Sint-Martens-Latem, Belgium). One representative of each GTG-5 group was then subjected to identification by (partial) 16S ribosomal RNA gene sequencing as described (Warmink and van Elsas, 2008). The identified pseudomonads were also used in gacA-based DGGE analyses to identify bands in the Pseudomonas (gacA-based) DGGE profiles.

Isolation of culturable Pseudomonas species

Isolates belonging to the genus Pseudomonas were obtained from the (replicate) mycospheres of the three fungal species sampled in 2007 in accordance with Warmink and van Elsas (2008). Attempts to obtain Pseudomonas isolates from corresponding bulk soil were not successful [detection limit 200 colony-forming units (cfu) per gram of soil], and hence random Pseudomonas isolates from similar (unforested) soil, at 10 km distance (Haren, the Netherlands), were used (R. Costa, pers. comm.). Briefly, a Pseudomonas-specific medium (S1) (Gould et al., 1985) was used to obtain isolates by dilution plating. From plates containing colonies, ±40 colonies were randomly picked per fungal species and used for DNA isolation as described (Warmink and van Elsas, 2008). This gave a total of 119 isolates. All strains were subjected to genomic fingerprints using primer GTG-5 (Rademaker et al., 2004) and the profiles obtained were then clustered – yielding GTG-5 groups – using UPGMA with the program Gelcompar II (Applied Maths, Sint-Martens-Latem, Belgium). One representative of each GTG-5 group was then subjected to identification by (partial) 16S ribosomal RNA gene sequencing as described (Warmink and van Elsas, 2008). The identified pseudomonads were also used in gacA-based DGGE analyses to identify bands in the Pseudomonas (gacA-based) DGGE profiles.

Characterization of Pseudomonas isolates

Metabolic tests using BIOLOG (BIOLOG, Hayward, USA) were performed on representatives from each Pseudomonas group determined with GTG-5 genomic fingerprinting-based cluster analyses according to the manufacturer’s protocol. Additional tests were performed in glass tubes for the ability of these strains to utilize and grow on oxalate as the sole carbon source in M9 salts medium (Sambrook et al., 1989). The medium was prepared with M9 salts supplemented with 1% Na oxalate and was adjusted to pH 7.0 with NaOH. Following inoculation (1:100 from an overnight culture), the 3 ml of cultures in glass tubes were incubated (shaking at 235 r.p.m.) at 23°C for 1 week. Strains were scored positive if increased turbidity was observed. Controls for growth consisted of the same strains introduced into M9 salts with glucose, whereas an oxalate user obtained from soil served as the positive control for oxalate utilization. The ability to use chitin as an energy and carbon source was tested by growing selected isolates on chitin-containing plates (Li et al., 2002). Strains were scored positive if a clear halo was formed surrounding the bacterial colonies. Positive controls consisted of two selected chitinase-positive Serratia strains, Serratia plymuthica 23 (kindly provided by R. Trifonova) and Serratia sp. 2-col (kindly provided by W. de Boer). All data were analysed in the program CANOCO for Windows 4.5 (WUR, Wageningen, the Netherlands).

Statistics and bioinformatics

All statistical analyses were performed using P ≤ 0.05 as the cut-off criterion. PCR-DGGE patterns and GTG-5 genomic fingerprint patterns were evaluated and clustered using UPGMA (cosine coefficient) within the program Gelcompar II (Applied Maths, Sint-Martens-Latem, Belgium). The Shannon index of diversity, based on the number of peaks and the surface area of each peak from the PCR-DGGE profiles, was used to determine the influence of the fungal fruiting bodies on the diversity of the dominant members of the respective bacterial communities. The 16S ribosomal RNA gene sequences obtained were compared with those of type strains in the Ribosomal Database Project II (RDB database) using the option Seqmatch. Sequences obtained from the fungal ITS regions were compared with those in the GenBank database for identification. Principal components analysis clustering was performed on the substrate utilization (BIOLOG) data using CANOCO (CANOCO 4.5, Biometris, Wageningen, the Netherlands).

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References


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