Migratory Response of Soil Bacteria to Lyophyllum sp. Strain Karsten in Soil Microcosms

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In this study, the selection of bacteria on the basis of their migration via fungal hyphae in soil was investigated in microcosm experiments containing Lyophyllum sp. strain Karsten (DSM2979). One week following inoculation with a bacterial community obtained from soil, selection of a few specific bacterial types was noticed at 30 mm in the growth direction of Lyophyllum sp. strain Karsten in sterile soil. Cultivation-based analyses showed that the migration-proficient types encompassed 10 bacterial groups, as evidenced by (GTG)5 genomic fingerprinting as well as 16S rRNA gene sequencing. These were (>97% similarity) Burkholderia terrae BS001, Burkholderia sordidicola BS026, Burkholderia sediminicola BS010, and Burkholderia phasianini BS028; Dyella japonica BS013, BS018, and BS021; “Sphingoterrabacterium pocheensis” BS024; Sphingobacterium daejeonense BS025; and Ralstonia basileensis BS017. Migration as single species was subsequently found for B. terrae BS001, D. japonica BS018 and BS021, and R. basileensis BS017. Typically, migration occurred only when these organisms were introduced at the fungal growth front and only in the direction of hyphal growth. Migration proficiency showed a one-sided correlation with the presence of the hrcR gene, used as a marker for the type III secretion system (TTSS), as all single-strain migrants were equipped with this system and most non-single-strain migrators were not. The presence of the TTSS stood in contrast to the low prevalence of TTSSs within the bacterial community used as an inoculum (<3%). Microscopic examination of B. terrae BS001 in contact with Lyophyllum sp. strain Karsten hyphae revealed the development of a biofilm surrounding the hyphae. Migration-proficient bacteria interacting with Lyophyllum sp. strain Karsten may show complex behavior (biofilm formation) at the fungal tip, leading to their translocation and growth in novel microhabitats in soil.

Bacterial-fungal interactions are common in a wide variety of habitats like decaying wood, human bodies, and marine and soil environments (7, 12, 13, 15, 18). Especially in soil, interactions are likely to occur frequently, as members of both kingdoms abound in this system and depend on strategies that allow them to utilize the sparse carbonaceous nutrients that are available (6, 22, 26, 27). Interactions may be deleterious, neutral, or even beneficial for either or both of the partners. In particular, the putative beneficial effects exerted by soil fungi on associated bacteria may enhance bacterial fitness and thus provide a selective force on these (4, 5, 11, 14, 29). A range of different mechanisms is thought to play a role in the putative bacterial selection, in which particular fungus-released compounds may exert key effects in this selection (1, 10, 14, 28). In addition, changes in the structure of the local (soil) habitat effected by either of the partners (2) and/or production of antibacterial substances by the fungal partner (7, 9) may play roles.

The capacity of soil bacteria to use fungal hyphae as a means to reach and colonize novel microhabitats in soil has been proposed as a mechanism for pollutant-degrading bacteria to become efficient in polluted soil (16). However, the study addressed only bacterial migration with fungi via so-called fungal highways in non-soil systems like agar plates and glass bead systems. Clearly, such fungal highways might be used by bacteria to cross air gaps (23) during growth and movement in soil, but evidence for this is lacking. Movement of the bacterial partner was probably driven by motility of the bacterial cells in the water film surrounding the fungal hyphae. The observation of bacteria moving along fungal highways was supported by an earlier study that addressed bacterial motility via dead hyphae of an oomycete in soil (32). Together, these studies suggest that bacteria can utilize the mycosphere (here defined as the fungal hyphal network) in soil to reach and colonize novel microhabitats. However, these studies do not allow an in-depth assessment of which bacteria get selected by growing fungi and how they mechanistically make use of fungal highways.

In the current study, we assessed the putative selection of organisms from a soil bacterial community that was able to migrate in the mycosphere of Lyophyllum sp. strain Karsten, a close saprotrophic relative of the ectomycorrhizal fungus Laccaria proxima. We initially assessed the selection of particular bacterial species by L. proxima (29), which was an abundant ectomycorrhizal species with hazel trees. Thus, we developed a microcosm system composed of three compartments, which allowed the outgrowth of fungal hyphae from a nutrient source into sterile soil. Different aspects of bacterial migration along with the fungal front were studied. Based on these findings, a mechanism for bacterial migration in which biofilm formation plays a role is proposed.

MATERIALS AND METHODS

Soil. We used fresh soil sampled in Gieterveen, The Netherlands, denoted G soil (28). The G soil, of sandy texture, had a pH of 4.0 and total carbon (C) and...
ments, we added 0.5% CaCO₃ to the soil, which raised the pH to 5.0. For several experiments, the total nitrogen (N) contents of 2.8% and 0.8%, respectively. For some experiments, the soil was sterilized twice at intervals of 3 days by autoclaving at 115°C for 45 min. Following each autoclaving step, a 60-min exposure to sterile air was used to release any volatile toxic compounds produced. The soil was grown overnight in 3 ml of R2A medium (Becton, Dickson and Company, Sparks, MD) at 23°C, with shaking. The cells were spun down for 5 min at 4,000 × g, washed, and resuspended in 1 ml of 0.85% NaCl; this was used directly for inoculation of soil in the migration experiments.

FIG. 1. Microcosm model based on a three-compartment petri dish.

**TABLE 1. Bacterial strains used in this study**

<table>
<thead>
<tr>
<th>Strain³</th>
<th>Related organism</th>
<th>Genetic similarity (%)*</th>
<th>Migrator*</th>
<th>TTSS⁺</th>
<th>Antagonistic effect of:</th>
<th>Motility*</th>
</tr>
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<tbody>
<tr>
<td>BS001</td>
<td>Burkholderia terrae BS110</td>
<td>100</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BS010</td>
<td>Burkholderia xedimincola HU2-65W (EU035613)</td>
<td>98</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BS013</td>
<td>Dyella japonica RB28 (DQ984127)</td>
<td>99</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BS016</td>
<td>Dyella japonica RB28 (DQ984127)</td>
<td>99</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BS017</td>
<td>Ratstonia basilensis ER121 (AF312021)</td>
<td>98</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BS018</td>
<td>Dyella japonica RB28 (DQ984127)</td>
<td>99</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BS021</td>
<td>Dyella japonica RB28 (DQ984127)</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BS024</td>
<td>Sphingobacterium pocheensis 0032 (AB267718)</td>
<td>98</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>BS025</td>
<td>Sphingobacterium daejeonense JP10 (AB267717)</td>
<td>98</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>BS026</td>
<td>Burkholderia sordidicola SNU 020123 (AF512827)</td>
<td>97</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BS028</td>
<td>Burkholderia phenaszium HG 14 (AY154375)</td>
<td>100</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>BS043*</td>
<td>Mycobacterium hodleri</td>
<td>NA</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BS047*</td>
<td>Variovorax paradoxus</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BS053*</td>
<td>Pseudomonas poae</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BS055*</td>
<td>Mycobacterium piscium</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BS057*</td>
<td>Mycobacterium anthracenicum</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BS066*</td>
<td>Arthrobacter ramosus</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BS086*</td>
<td>Aquamonas fontana</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BS101*</td>
<td>Mesorhizobium loti</td>
<td>NA</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BS109*</td>
<td>Pseudobacillus polymyxa</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BS110*</td>
<td>Burkholderia terrae</td>
<td>NA</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BS126*</td>
<td>Chryseobacterium aurantiacum</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BS181*</td>
<td>Chryseobacterium jostellii</td>
<td>NA</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

³ Strains marked with an asterisk are isolates from the mycosphere of *L. proxa*ma (29).

NA, not applicable.

⁺Migr, capable of single-strain migration via fungal hyphae; –, not a single-strain migrator. Migration of strain BS010 was not possible because of its strong antagonistic properties against the fungal host.

As described previously (29).

+, present; –, absent. Strain BS010 has strong antagonistic properties against the fungal host, which precludes the fungus from having an antagonistic effect on the bacteria’s growth.

+, possibility of active motility of the bacterium; –, nonmotile.

total nitrogen (N) contents of 2.8% and 0.8%, respectively. For some experiments, we added 0.5% CaCO₃ to the soil, which raised the pH to 5.0. For several experiments, the soil was sterilized twice at intervals of 3 days by autoclaving at 115°C for 45 min. Following each autoclaving step, a 60-min exposure to sterile air was used to release any volatile toxic compounds produced. The soil was sterile, as evidenced by dilution plating soil suspensions prepared in 0.85% NaCl on R2A agar. Inoculation in migration experiments involved the use of a microcosm system designed to consist of three-compartment petri dishes (Greiner Bio-one, Frickenhausen, Germany). Two compartments (Fig. 1) were filled each with 9 g of moist, fresh oat flake plates for maintenance.

To prepare soil bacterial suspensions for inoculation, freshly sampled G soil was first homogenized and then added to sterile 0.85% NaCl (saline) in a 1:10 proportion (0.5 g of soil/5 ml of saline). The resulting suspensions were homogenized by vigorous mixing on a vortex shaker (at full speed three times for 1 min). Following this treatment, soil particles were allowed to settle for 1 min on the bench, after which 30 µl of the supernatant, containing roughly 10⁵ cells as estimated by direct microscopy, was used for inoculation in migration experiments.

To prepare suspensions of cultured bacterial strains (Table 1) for inoculation, bacteria were grown overnight in 3 ml of R2A medium (Becton, Dickson and Company, Sparks, MD) at 25°C, with shaking. The cells were spun down for 5 min at 4,000 × g, washed, and resuspended in 1 ml of 0.85% NaCl; this was repeated two times. The final cell suspensions were diluted to an OD₆₀₀ of 0.05 (containing an estimated 10⁷ cells/ml, using dilution plating on R2A agar). In total, 50 µl of this bacterial suspension (thus containing roughly 5 × 10⁶ cells) was used directly for inoculation of soil in the migration experiments.

**Growth and maintenance of used fungi.** The (basidiomycetous) fungi used in this study, i.e., *Lyophyllum sp.* strain Karsten (DSM2979), *Hebeloma cylindrosporum* Romagnesi (provided by I. van Aarle), *Paecilomyces lilacinus*, and *Laccaria bicolor* (strain S238N) (the last two provided by P. Frey-Klett) were grown on oat flake agar plates, prepared with 30 g of oat flake (local pet shop) and 15 g of agar (Duchefa, Haarlem, The Netherlands), filled with water to 1 liter, and sterilized at 121°C for 21 min. Once every 4 weeks, all fungal strains were transferred to fresh oat flake plates for maintenance.

**Microcosm and migration experiments.** A microcosm system was designed that consisted of three-compartment petri dishes (Greiner Bio-one, Frickenhausen, Germany). Two compartments (Fig. 1) were filled each with 9 g of moist,
sterilized G soil (moisture content of 17%, corresponding to 69% of water holding capacity, bulk density [wt/wt/vol] of about 1.3), yielding layers of 4 mm. The third compartment was filled with oat flake agar. The physical barriers between the oat flake and the two soil compartments prevented compounds from the oat flake from reaching the soil compartments. The barriers were overcome by the fungal hyphae, and hence outgrowth of a fungus from the nutrient-rich oat flake environment into the soil was achieved. The system was inoculated with the different basidiomycetous fungi on the oat flake medium and incubated at 28°C, thereby allowing the colonization of the oat flake plus about 1 to 4 mm of the sterile soil (prior to introduction of bacterial inocula).

By means of a pipette, washed bacterial suspensions (50 μl) were evenly distributed in one 3-mm-wide streak in the sterile soil in each soil compartment directly adjacent to the front of the growing hyphae. After incubation at 23°C, soil samples (100 mg) were taken at different time points and sites within the soil compartments by removing 4-mm-diameter cores from the soil. Samples were taken of the soil both in and against the hyphal growth direction in relation to the inoculation location. Samples (triplicates; two from each microcosm) were then used for further analyses by dilution plating and DNA extraction (see below).

Analysis of total bacterial communities by molecular means. DNA was extracted from G soil and microcosm samples with a PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. DNA of sufficient purity, size (~20 kb), and amount (20 μg/g of soil) was obtained for further analyses. These DNA extracts were used for further analyses via PCR-denaturing gradient gel electrophoresis (DGGE) to analyze the bacterial communities using primers 2 and 3 based on the 16S rRNA gene, as described previously (19).

Analysis of the culturable bacterial community. Bacterial cell suspensions from microcosm samples and fresh G soil were prepared as described above and used for dilution plating on R2A agar (Becton, Dickinson and Company, Sparks, MD). The dilution plates were incubated at 23°C for 2 weeks and regularly monitored for colony development. Enumeration of the CFU was done after 2 weeks.

From the dilution plates prepared from the microcosm samples as well as fresh G soil, randomly picked colonies were streaked to purity, resulting in 84 isolates from the microcosm and 176 isolates from G soil. All isolates were grown overnight in (3 ml) R2A medium, after which they were resuspended in 17% (vol/vol) glycerol and stored at –80°C.

Genomic fingerprinting of the bacterial isolates. To group the isolates and discern replicates, all were subjected to genomic fingerprinting by colony PCR targeting repetitive elements in the genome using primer (GTG), (20). Clustering of the patterns generated for all isolates was achieved using the unweighted pair group method with mathematical averages with the program Gelcompar II (Applied Maths, Sint-Martens-Latem, Belgium). Representative members (three per group, or just one if the group contained one to two isolates) of each group were used for further analyses.

Identification and properties of selected isolates. Representatives of each (GTG), group were subjected to identification by (partial) 16S rRNA gene sequencing as described previously (29). Furthermore, an indication for the presence of a type III secretion system (TTSS) was obtained by PCR-based detection of the hrcR gene as described previously (29). Metabolic tests using Biolog (GN2) assays (Biolog Inc., Hayward, CA) were performed on all representatives of the (GTG), groups according to the manufacturer's protocol. Intrinsically flagellar motility was tested using microscopic analyses of overnight cultures. Antagonism against Lyophyllum sp. strain Karsten was tested in a dual culture assay according to Berg et al. (4a) on oat flake medium. The inhibition zones were observed after incubation at 23°C for 7 days.

RESULTS

Design of a microcosm system for the assessment of bacterial migration with fungal hyphae. The microcosm made out of a three-compartment petri dish was used to establish a base compartment (the oat flake compartment) that would support growth of fungal mycelium in addition to two adjacent compartments containing G soil as the experimental matrix. The system allowed above-ground contact between the base and soil compartments and careful control of temperature and soil moisture. The compartments could be sampled using microsamplers without disturbing the experiment. The experiment thus allowed time courses to be performed and was easily replicable.

The basidiomycetous fungi Lyophyllum sp. strain Karsten (DSM2979), H. cylindrosporum Romagnesi, P. involutus, and L. bicolor (8238N) were tested for their performance in the microcosm experiments. All five attempts to obtain migratory growth of Lyophyllum sp. strain Karsten hyphal through non-sterile G soil failed, whereas the fungus grew readily through sterilized G soil. Similar results were obtained with H. cylindrosporum Romagnesi, P. involutus, and L. bicolor in nonsterile soil. This lack of extension through nonsterile soil was probably caused by suppression by the indigenous microbial community. It led us to perform all subsequent work in sterilized microcosms. Lyophyllum sp. strain Karsten was selected as the preferred fungus as the other fungal strains grew poorly. Lyophyllum sp. strain Karsten showed high movement of the hyphal front in the sterile soil (5 mm per day), forming a dense hyphal network in the soil. Lyophyllum sp. strain Karsten was also preferred as it is a close saprotrophic relative of L. proxima, the ectomycorrhizal fungus studied in our previous work (29). It thus supported previous assessments of L. proxima-associated bacteria in field G soil, in which strong selection of particular bacteria was shown (29) (Table 1).

Migration of soil bacteria via growing fungal hyphae. Bacterial cell suspensions prepared from fresh G soil in 2005 were used in the initial migration selection experiment through sterilized G soil, whereas the experiment was repeated in 2008 with another, fresh G soil sample to determine the reproducibility of bacterial selection under circumstances identical to those in 2005. The fungal hyphae did serve as catalysts of the movement and growth of particular bacteria through the soil as in the fungal-containing plates; the CFU counts at 30-mm distance were, on average, log 7.2 ± 0.5 CFU/g of dry soil in the first experiment and log 7.2 ± 0.5 CFU/g of dry soil in the second one. Control samples taken from the soil compartments that had been inoculated with a bacterial suspension but had not been colonized with Lyophyllum sp. strain Karsten at a 30-mm distance from the inoculation site never showed any CFU on R2A plates (detection limit, 100 CFU per g of soil). In addition, the soil bacterial suspension added to the sterile G soil did not detectably hamper hyphal extension through the soil, as found by a comparison between bacterium-inoculated and uninoculated systems, presumably as a result of the lack of strong fungistasis in such a colonizing community.

Analysis of community migrators. A suite of 84 isolates was randomly picked from plates (10° dilution) prepared from the 30-mm site and streaked to purity. (GTG),-based fingerprinting analysis of these strains, collectively denoted as community migrators, showed their distribution among 10 distinct groups, denoted groups I through X (Table 2). Groups I to IV encompassed most (86%) of the strains. Phylogenetic analysis of representatives of these groups (three isolates per group) showed that the group I, III, and IV organisms were affiliated with Dyella japonica strain RB28 (>98% similarity; 60% of all 84 migrating isolates), whereas the isolates belonging to group II were affiliated with Burkholderia terrae BS110 (100% similarity; 26.5% of all 84 migrating isolates). The other migrators (in total, 13.5% of the total) fell into six different species, as follows: group V, Burkholderia sordidicola SNU 0201230 (98% similarity); group VI, Burkholderia sediminicola HU2-65W
(100% similarity); group VII, “Sphingoterrabacterium pocheensis” (100% similarity); group VIII, Ralstonia basileensis ER121 (100% similarity); group IX, Sphingobacterium daejeonensis JP10 (96% similarity), and group X, Burkholderia phenazineum HG14 (100% similarity). The repeat experiment performed in 2008 with a fresh G soil bacterial suspension again showed selection of group II, B. terrae, as well as group III, D. japonica, strains based on (GTG)$_3$ fingerprint patterns, indicating a highly consistent selection mechanism for the same bacteria. The most abundant group (I) from 2005 was, however, not found in 2008 (Table 2).

(a) The experiment was repeated with a bacterial community isolated in 2008 from the same soil plot. +, presence of strain.

### Table 2. Community migrating strains isolated in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Related organism (accession no.)</th>
<th>Similarity (%)</th>
<th>(GTG)$_3$ group</th>
<th>No. of isolates</th>
<th>% of total bacterial population</th>
<th>Selection confirmed in 2008 soil sample$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS021</td>
<td>D. japonica RB28 (DQ984127)</td>
<td>99</td>
<td>I</td>
<td>27</td>
<td>32.5</td>
<td>+</td>
</tr>
<tr>
<td>BS001</td>
<td>B. terrae BS110</td>
<td>100</td>
<td>II</td>
<td>22</td>
<td>26.5</td>
<td>+</td>
</tr>
<tr>
<td>BS018</td>
<td>D. japonica RB28 (DQ984127)</td>
<td>99</td>
<td>III</td>
<td>13</td>
<td>15.7</td>
<td>+</td>
</tr>
<tr>
<td>BS013</td>
<td>D. japonica RB28 (DQ984127)</td>
<td>99</td>
<td>IV</td>
<td>10</td>
<td>12</td>
<td>+</td>
</tr>
<tr>
<td>BS026</td>
<td>B. sordidicola SNU 020123 (AF512827)</td>
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<td>V</td>
<td>5</td>
<td>8.4</td>
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</tr>
<tr>
<td>BS010</td>
<td>B. sediminicola HU2-65W (EU035613)</td>
<td>100</td>
<td>VI</td>
<td>2</td>
<td>2.4</td>
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<tr>
<td>BS024</td>
<td>S. pocheensis Gso0032 (AB267718)</td>
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<td>VII</td>
<td>2</td>
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<td>BS017</td>
<td>R. basilensis ER121 (AF312021)</td>
<td>100</td>
<td>VIII</td>
<td>1</td>
<td>1.2</td>
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</tr>
<tr>
<td>BS025</td>
<td>S. daejeonense JP10 (AB267717)</td>
<td>96</td>
<td>IX</td>
<td>1</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>BS028</td>
<td>B. phenazineum HG14 (AY154375)</td>
<td>100</td>
<td>X</td>
<td>1</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>

The PCR-DGGE profiles obtained from the hyphal growth contained five major bands. These profiles also showed particular bands emerging over time (Fig. 2B). Some bands that emerged in the PCR-DGGE profiles obtained from the inoculation location were also found as emerging ones in these profiles, i.e., bands B1, B2, B3, B4, and B7 (Fig. 2). However, some bands found at the inoculation spot were missing from the fungal front profiles, i.e., bands B5, B6, and B8.

Technical difficulties did not allow the direct sequencing of bands. Hence, we presumptively identified specific selected bands by comparing the comigration of amplicons obtained from the organisms described above, i.e., B. terrae BS001 and D. japonica BS021, with the bands in the direct PCR-DGGE profiles. By this analysis, bands B1, B3, and B4 in combination with the fungal hyphal front profiles, i.e., bands B5, B6, and B8.

**Single-strain migration.** Given the fact that the bacterial migration with the fungal hyphal front might have been due to either an intrinsic property of the organism itself or to a potential helper effect exerted by other migrating bacteria, representatives of all 10 community migrator groups were subjected to single-strain migration experiments performed in quadruplicate with *Lyophyllum* sp. strain Karsten in the same setup used above. Of the 10 groups, 6 could not migrate as single strains with *Lyophyllum* sp. strain Karsten, although control experiments showed good survival of all bacterial strains at the inoculation spot. These presumable obligatory community-dependent migrators were *B. phenazineum* BS028 (group X), *B. sediminicola* BS026 (group V), *D. japonica* BS013 (group IV), *S. daejeonensis* BS025 (group IX), and *S. pocheensis* BS024 (group VII). On the other hand, representatives of the remaining four groups showed a clear capacity to comigrate as a single population with growing *Lyophyllum* sp. strain Karsten hyphae. These were *D. japonica* BS021 (group I), *D. japonica* BS018 (group III), *B. terrae* BS001 (group II), and *R. basileensis* BS017 (group VIII) (Table 1).

The *D. japonica* group IV was closely related to the *D. japonica* groups I and III on the basis of its similar 16S rRNA gene sequence and also revealed some similarities in the (GTG)$_3$ patterns (data not shown). Remarkably, strains of this group did not show any migration via the fungal hyphae.
whereas group I and III strains revealed avid bacterial migration.

Properties of selected bacteria. (i) TTSSs. For all strains, a clear correlation was found between the capacity to comigrate with *Lyophyllum* sp. strain Karsten as a single strain through soil and the presence of the *hrcR* gene (an indicative marker for the presence of a TTSS) as all four single-strain migrators possessed the *hrcR* gene, whereas four of the other six community migrators (except *B. sordidicola* BS026 and *B. phenazine* BS028) did not possess the *hrcR* gene (Table 1). We also assessed the comigration capacity of 12 organisms (Table 1) obtained from the mycosphere of *L. proxima* (related to *Lyophyllum*) in natural G soil (29). Of these, only the *hrcR*-positive *B. terrae* BS110 (Table 1) did not possess the *hrcR* gene (Table 1). We also assessed the comigration capacity of 12 organisms (Table 1) obtained from the mycosphere of *L. proxima* (related to *Lyophyllum*) in natural G soil (29). Of these, only the *hrcR*-positive *B. terrae* BS110 (Table 1) did not possess the *hrcR* gene (Table 1). We also assessed the comigration capacity of 12 organisms (Table 1) obtained from the mycosphere of *L. proxima* (related to *Lyophyllum*) in natural G soil (29). Of these, only the *hrcR*-positive *B. terrae* BS110 (Table 1) did not possess the *hrcR* gene (Table 1).

(ii) Intrinsic flagellar motility. All community migratory strains as well as strains obtained from the *L. proxima* mycosphere were microscopically analyzed in wet mounts for intrinsic (flagellar) motility. With the exception of *Chryseobacterium piscium* BS055 (obtained from the *L. proxima* mycosphere), all strains were positive as they showed active motility that was not caused by Brownian motion (Table 1).

(iii) Antagonism. The putative antagonistic properties of all community migrator (and *L. proxima* isolated) strains against *Lyophyllum* sp. strain Karsten were then tested in a dual-culture assay. Growth of *Lyophyllum* sp. strain Karsten was inhibited by only six strains, i.e., *B. sediminicola* BS010, *Pseudomonas poae* BS053, *Chryseobacterium piscium* BS055, *Aquamonas fontana* BS086, *Chryseobacterium aurantiacum* BS126, and *Chryseobacterium joosteii* BS181. All other bacterial strains did not have an influence on the development and growth of *Lyophyllum*. Interestingly, none of the strains except *B. sediminicola* BS010 showed antagonistic properties in microcosm experiments (data not shown).

On the other hand, *Lyophyllum* sp. strain Karsten present in the microcosm limited the survival of bacterial strains *Arthrobacter ramosus* BS066, *Mycobacterium hodleri* BS043, *Paenibacillus polymyxa* BS109 and *C. joosteii* BS126 (all *L. proxima* isolates). These bacterial species were not found at the inoculation spot (detection limit, <200 CFU/g of soil) after a 1-week incubation following the introduction at an inoculum density of 10^5 CFU/g of dry soil (Table 1) although these bacterial strains showed survival in the corresponding soil without the fungus.

Metabolic tests using Biolog. Metabolic profiles were obtained via Biolog substrate utilization tests and cross-compared using principal components analyses (PCA). A strong clustering of (group II) *B. terrae* (strain BS001) and (group VI) *B. sediminicola* (BS010) with strains denoted as universal “fungiphiles” in previous work (27) was found (Fig. 3). This clustering was primarily based on the utilization of 15 of 18 com-

FIG. 2. PCR-DGGE analyses of development of the bacterial community at the inoculation (A) as well as hyphal front (B) in time. Markers for 16S rRNA gene fragments of (from top to bottom): *Listeria innocua*, *Enterobacter cloaceae*, *Mesorhizobium* sp., *Burkholderia cepacia*, and *Arthrobacter* sp. B1 through B8 represent key bands in the profiles that were (partially) presumptively identified (see the text).
pounds, which have previously been postulated to be often present in fungal exudates (28), in the Biolog assay (14, 21, 24, 25, 31). The compounds utilized by B. terrae (BS001) were L-arabinose, D-arabitol, α-D-glucose, M-inositol, D-mannitol, D-trehalose, citric acid, D-alanine, L-aspartic acid, L-glutamic acid, L-phenylalanine, L-proline, L-threonine, and glycerol. In the PCA analyses, the three D. japonica strains, i.e., BS003, BS013 and BS021, clustered together, but they formed a cluster separate from the typical universal fungiphile cluster established earlier (Fig. 3). For these D. japonica strains, the utilization of such compounds was limited to D-trehalose, L-aspartic acid, L-glutamic acid, L-leucine, L-phenylalanine, L-proline and L-threonine. The other community migrators, i.e., R. basilensis (BS017), B. sordidicola (BS026), and B. phena-zinium (BS028), did not show clear clustering and were found between the fungiphile groups and the bulk soil isolates based on metabolic profiles.

The migratory direction of B. terrae and D. japonica in soil microcosms. We selected two strains, i.e., D. japonica (BS021; group I) and B. terrae (BS001; group II), representing the abundant single-strain migrators, for further studies, as these were likely most strongly selected by the Lyophyllum sp. strain Karsten hyphae. Both B. terrae BS001 and D. japonica BS021 were introduced as single strains (inoculum density of about 10⁵ CFU/g of dry soil) at the hyphal growth front, and their migration either with or against the growth direction of the Lyophyllum sp. strain Karsten hyphae was determined.

Migration of both inoculant strains was shown to occur at the hyphal front of Lyophyllum sp. strain Karsten but only in the direction of hyphal growth (Table 3). Migration in the opposite direction was never observed, whereas the presence of fungal hyphae did not hamper bacterial outgrowth on plates.

![FIG. 3. PCA of the metabolic profiles obtained with the Biolog assay. BSXXX codes (where X is a number 0 to 9) represent strains that were obtained in this study; P01 to P18 and B1 to B8 were obtained in a previous study (28) and represent Pseudomonas isolates from mycospheres and bulk soil isolates, respectively.](http://aem.asm.org/)

<table>
<thead>
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<th>TABLE 3. Summary of migrational behavior of B. terrae BS001 and D. japonica BS021 with Lyophyllum sp. strain Karsten hyphae through sterile G soil</th>
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<td><strong>Migrational direction</strong></td>
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<td>B. terrae BS001</td>
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*Data show bacterial densities after 7 days at six sampling spots. The microcosms were inoculated with 10⁵ CFU per g soil at the inoculation site. Controls revealed no migratory activity in soil without fungal hyphae. Plus, ≥10⁵ CFU/g of soil; minus, <200 CFU/g of soil.*
Distribution and growth rate of *B. terrae* and *D. japonica* cells on fungal hyphae. To assess the distribution of inoculant cells established on growing *Lyophyllum* sp. strain Karsten hyphae in the microcosm, $2 \times 10^6$ (*B. terrae* BS001) and $7 \times 10^6$ (*D. japonica* BS021) cells were introduced at the hyphal front, yielding after 3 h (locally) about log 5.3 CFU of *B. terrae* BS001 per g of dry soil and log 5.8 CFU of *D. japonica* BS021 per g of dry soil. After 1 week, the bacterial densities were determined at three different spots in the soil compartments, i.e., the introduction site as well as 15 and 30 mm (hyphal growing front) in the fungal growth direction. Bacterial CFU were never found from the 15- and 30-mm sites on plates prepared from control soil compartments (without the presence of the fungus).

For the *Lyophyllum* sp. strain Karsten systems that had received *B. terrae* BS001 cells, very similar strain BS001 CFU counts of approximately log 8.6 ± 0.15 per g of dry soil were found at all three sampling sites, and no significant differences ($t$ test, $P > 0.05$) were observed between the CFU counts found at these locations. Also, *D. japonica* BS021 was detected at log 8.2 ± 0.19 CFU/g of soil, again showing no significant differences between the three locations in the soil compartments (Fig. 4). The *B. terrae* BS001 CFU counts were higher ($t$ test, $P < 0.05$) than those of *D. japonica* BS021 under the same conditions (Fig. 4). Thus, for both strains, growth resulting in an estimated >1,000-fold population increase was observed in the total soil compartment in comparison to the cell density that had originally been introduced into this compartment. Specifically, this indicated that at least nine cell divisions of the introduced bacteria had taken place within a week.

**Bacterial growth and rescue from low pH by *Lyophyllum* sp. strain Karsten.** The influence of *Lyophyllum* sp. strain Karsten hyphae on the putative growth of *B. terrae* BS001 in G soil was monitored over a period of 9 days using CaCO$_3$-amended (pH 5.0) as well as unamended (pH 4.0) soil. Both the inoculation site (initially at the hyphal front) and the moving hyphal front were sampled during this time period in addition to controls without the fungus. In the control samples, a strong decrease of *B. terrae* BS001 CFU numbers from about $10^5$ to below the detection limit (200 CFU/g of dry soil) was noticed after day 2. This strong decrease in CFU numbers in the absence of *Lyophyllum* sp. strain Karsten hyphae was overcome, and growth of *B. terrae* BS001 was even observed when the soil pH had been raised from 4.0 to 5.0 (data not shown). Thus, in soil that contained growing *Lyophyllum* sp. strain Karsten hyphae, strong increases in the *B. terrae* BS001 CFU numbers were observed from the initial ± log 5 CFU/g of dry soil to a maximum of about log 7.3 CFU/g of dry soil. The latter value was supposed to roughly represent the maximum carrying capacity for *B. terrae* BS001 in the specific *Lyophyllum* sp. strain Karsten mycosphere system (Fig. 5). At the growing hyphal front, the level was reached after 3 days and did not change significantly afterwards, indicating a maximal level of bacterial density at the fungal hyphae. Starting with an inoculum of $10^5$ cells per gram of soil, the inoculation site reached this maximum level at day 5, thus indicating that the bacterial growth rate was limited on these (older) hyphae.
Microscopic analysis of bacterial *Lyophyllum* sp. strain Karsten interactions. Microscopic analyses of *Lyophyllum* sp. strain Karsten hyphae growing on water agar showed two morphological hyphal types, i.e., (i) a “normal” type, mainly found directly on the agar layer, and (ii) an aberrant type, identified as aerial hyphae growing into the air and not contacting the agar. The diameter of the aerial hyphal type (1.5 μm) was about half that of the normal hyphal type (3 μm) and showed hydrophobic properties, as evidenced by observing the behavior of small droplets of water added onto the fungal mat. These aerial hyphae were also observed on top of the G soil that was colonized by the fungus. The normal hyphae were probably present between the soil particles; however, microscopic analyses of these were impossible, given the fact that the hyphal structure in soil is destroyed during sample preparation.

Microscopic analyses of the migration of *B. terrae* (BS001) via *Lyophyllum* sp. strain Karsten hyphae on water agar showed the existence of a biofilm formed by bacterial cells on the growing hyphae (Fig. 6). The migration followed a similar pattern as in soil microcosms in that, as determined by plating...

FIG. 5. Influence of the fungus *Lyophyllum* sp. strain Karsten on growth of strain BS001 in time at two sites (inoculation spot and fungal growth front) in the microcosm. The detection limit for this experiment was 200 CFU/g of dry soil. Values below this limit are shown as zero. Control, no fungal host; Fungus (I), system with fungus, inoculation spot; Fungus (F), system with fungus, fungal growth front.

FIG. 6. Microscopic analyses of fungal hyphae with biofilm formation. (A) Inoculated with BS001. (B) Not inoculated. The arrow indicates biofilm aerial hyphae.
and microscopic analyses, bacterial cells were always found on the extending hyphal tips, even at distances about 50 mm from the initial introduction spot. Controls without *Lyophyllum* sp. strain Karsten did not show any migration of *B. terrae* BS001 point inoculated on water agar. Biofilm formation was shown to occur on the thicker (normal) *Lyophyllum* sp. strain Karsten hyphae as the aerial hyphae remained free of any visual bacterial colonization (Fig. 6).

**DISCUSSION**

Selection of particular soil bacteria by hyphae (forming the mycosphere) of soil fungi can encompass a range of mechanisms. For instance, the ability of bacteria to utilize particular substances secreted by the fungal host (10, 14, 28) can play a role as can bacterial activity to capture a carbon source directly from fungal hyphae via local cell wall degradation (8). Although not extensively studied in this paper, selective pressure by the fungus on the soil bacterial community can also be mediated by the provision of antibiotic substances in the mycosphere (7). Kohlmeier et al. (16) showed the migration of bacteria on fungal hyphae via intrinsic motility, and they propose the use of the so-called fungal highways to introduce pollutant-degrading bacteria into contaminated soil systems. We hypothesized that migration via hyphal structures in the soil can be used by bacteria to reach and colonize new sites in soil and thus be successful in the colonization of such newly emerging niches in soil. We thus set up experiments in sterile soil microcosms in order to study the selection, migration, and interaction of bacteria in the mycosphere of the soil fungus *Lyophyllum* sp. strain Karsten (DSM2979).

The microcosm systems were designed to contain sterilized soil as this opens opportunities to investigate bacterium-fungus interactions in bipartite or otherwise simple communities without interference from the complexity of the native soil inhabitants. Indeed, colonization of unsterilized G soil by the fungus used, *Lyophyllum* sp. strain Karsten, was not observed, and this may be attributed to the grossly fungistatic character of the native G soil, possibly linked to suppression of incoming fungal hyphae by the native microbiota that may have broad antagonistic properties against invading organisms (17).

The experiments in which soil bacterial communities were interrogated for their capacity to comigrate with *Lyophyllum* sp. strain Karsten through G soil revealed a strong reduction of migratory bacterial diversity, both at the culturable as well as at the total bacterial community levels. This indicates that a strong selective force was exerted on the bacterial community for migratory fitness (Table 1 and Fig. 2). The selection was clearly exerted by conditions provided by the growing fungus *Lyophyllum* sp. strain Karsten, as in the relevant nonfungal controls no bacterial migration or growth was ever observed. The selection was quite reproducible, since in an experiment executed with a G soil microbial community sampled 3 years later, two identical bacterial species were found in abundance after migration, i.e., *B. terrae* group II and *D. japonica* group IV types. The rise and fall of particular bacterial phylotypes over time, as seen in the PCR-DGGE profiles in this experiment, indicated the occurrence of competition between the different types, resulting in shifting balances between these over time. Hence, a glimpse of shifting fitness maxima across time, encompassing several bacterial species in the mycosphere, was given. In these fluctuating communities, *B. terrae* BS001 and *D. japonica* BS013 likely play important roles, given the consistency with which they were detected.

Migration of bacterial cells with *Lyophyllum* sp. strain Karsten hyphae through soil was not a commonality for all bacteria found to migrate in the initial selection experiments. In fact, an important part (25%) of the organisms that could migrate as part of the community could not do so when tested as single strains. The property to migrate as a single strain was thus found in only 4 of the 10 bacterial groups, i.e., *D. japonica* BS021 (group I), *B. terrae* BS001 (group II), *D. japonica* BS013 (group III), and *R. basilensis* BS017 (group VIII) (Table 1). Strikingly, these were the groups that contained most individuals (75%) and, hence, were most strongly (migration and growth) selected. By implication, the other, less abundant migrators (denoted community migrators) may have profited from the single-strain migrators by means of a postulated migration helper effect. This migration helper effect is defined by the possibility that community migrators benefit from single-strain migrators and are able to migrate, as outlined elsewhere (Warmink et al., unpublished data).

The capacity to actively migrate—and grow—with growing fungal hyphae through soil may confer a specific fitness asset that has emerged in particular soil bacteria, allowing them to avidly respond to a growing mycosphere and find their preferred ecological niche in it. This asset may actually be complex and involve capacities to adhere to and grow and migrate in the mycosphere. Novel ecological niches are presumably constantly created at growing hyphal tips, and bacteria that possess the migrational fitness trait can be highly competitive versus the other bacteria that are locally present. The colonization efficiency of bacteria with this presumed migrational fitness trait was, however, not similar between strains. This is shown (Fig. 4) for *B. terrae* BS001, which under similar circumstances was more successful and reached higher cell numbers than *D. japonica* BS021 in G soil microcosms.

The correlation between the presumptive presence of an active TTSS and the capacity to migrate as a single strain with hyphae of *Lyophyllum* sp. strain Karsten was striking. TTSSs may be involved in the active attachment of bacteria, coupled or not to an effect on the fungal cell wall to the extent that fungal cells are weakened, biochemical pathways are changed, and enhanced nutrients may become available in a way beneficial for the bacteria. Such mechanisms may have played a role in the observed migrational fitness. The hypothesis of active involvement of a TTSS is corroborated by the finding of the TTSS-negative *D. japonica* BS013 (group IV), which was based on (GTG) fingerprinting and 16S rRNA gene sequence similar to the single-strain migrator *D. japonica* BS021 (group I) but did not show the ability to migrate as a single strain (Table 1). In a previous study (28), we observed the selection of TTSS-containing bacteria under the influence of *L. proxima*, the ectomycorrhizal fungus related to *Lyophyllum* sp. strain Karsten, although a specific function of the TTSS was not indicated. Interestingly, one TTSS-positive species obtained from that study, *B. terrae* BS110, was similar to one of the most frequently selected organisms, the group II *B. terrae* BS001 in the current microcosm studies. Selection by migration may thus have played a role in the selective process that
took place at the fruiting bodies of *L. proxima* in natural G soil (28). In this previous study (28), the prevalence of TTSSs in bulk soil communities (used for inoculation in this study) was found to be low (<3% of the culturable bacterial community). The low prevalence of TTSSs, together with the finding that all single-migrating strains are TTSS positive, provided evidence supporting a role of the TTSS in bacterial migration. Assessment of the behavior of a TTSS mutant versus the wild-type would provide much more compelling evidence for this contention. However, our attempts to produce such a mutant via integration of a selectable marker into the TTSS gene cluster were, unfortunately, not successful (data not shown).

Migration of bacteria via fungal hyphae has been shown previously (16). The authors showed the occurrence of migration via intrinsic bacterial motility through continuous liquid films surrounding the fungal hyphae. Our results indicate a second mechanism for migration via the fungal hyphae. We could not find any proof for bacterial migration via intrinsic motility in the water film surrounding fungal hyphae as the only factor for migration in our soil microcosm. In 11 non-single migrants, motility and also survival in mycosphere soil were observed (Table 1). Based on “the intrinsic motility migration hypothesis,” these strains should be able to migrate. The lack of migration of all strains in the direction opposite to the *Lyophyllum* sp. strain Karsten hyphal growth direction also indicates a more complex mechanism in the migratory process than just the use of intrinsic motility for migration in soil via hyphal-bound water films.

We propose that bacterial biofilm formation, as observed in the cocultures of *B. terrae* BS001 and *Lyophyllum* sp. strain Karsten on water agar (Fig. 6), plays a key role in the migratory behavior of the fungus-responsive bacteria. The single-strain migrator bacteria can possibly move with the fungus by growing, as well as by using (swarming) motility, in association with the biologically active and extending hyphal tip (3) and in this way produce a biofilm, which stays behind on old hyphae but also moves/extends with newly emerging hyphae. This hypothesis was supported by the finding of a rather even abundance of bacterial cells on young and older hyphae (Fig. 7). This hypothesis was supported by the finding of a rather even abundance of bacterial cells on young and older hyphae. This hypothesis was supported by the finding of a rather even abundance of bacterial cells on young and older hyphae (Fig. 7).

Moreover, intrinsic flagellar motility may be involved in the bacterial colonization of hyphal tips before attachment, given the fact that all bacteria with migration capacity showed flagellar motility (Table 1). Concerning hyphal differentiation, aerial hyphae of *Lyophyllum* sp. strain Karsten did not show biofilm formation with *B. terrae* BS001, as evidenced by microscopy. Aerial hyphae are known to produce hydrophobins in their walls and in this way gain hydrophobicity in order to escape from wet surfaces (30). It is known that most bacteria do not attach well to hydrophobic surfaces (16).

The formation of bacterial biofilms in contact with fungal hyphae has been observed previously (11, 13). However, our study indicates a relationship between the formation of bacterial agglomerates (here denoted as biofilms) at hyphal tips and the concomitant introduction of bacterial cells to novel niches by growing hyphal tips in soil. This is a novel finding that opens doors for direct studies on bacterium-fungus interactions and biofilm formation in soil, possibly extending into the natural soil environment.

The substrate utilization assays, in particular of the single-strain migrator *B. terrae* BS001 (Fig. 3), indicated an important role for particular compounds (some of them implied as components of fungal exudates) in the functioning of this strain in the mycosphere. Fifteen of 18 compounds that have been found in fungal exudates were utilized by strain BS001. However, our assumptions as to the release of such carbon sources by *Lyophyllum* sp. strain Karsten are based on knowledge of such releases from other (related) fungi (28). The utilization of this wide array of compounds supports our hypothesis of a passive or even active (using TTSS) capture of resources from *Lyophyllum* sp. strain Karsten by *B. terrae* (BS001). The clustering of this organism within the PCA-determined universal fungiphile cluster (Fig. 3) may indicate that it is probably successful in other mycospheres as well. On the other hand, the *D. japonica* isolates BS003, BS013, and BS021 did not cluster within the fungiphile cluster, in spite of the fact that these showed utilization of seven potentially fungus-released compounds. The issue of allocation of particular strains to fungus–interactive groups clearly requires further study; however, utilization of these seven compounds may be pinpointed as important in the selection of these strains.

In conclusion, a very restricted subset of a soil bacterial community was found to be able to comigrate as single strains with hyphae of *Lyophyllum* sp. strain Karsten through sterile G soil and presumably utilized a complex array of characteristics allowing this migration, which may have included a TTSS. This complex array of mechanisms is possibly not widespread in the bacterial community of bulk G soil, as members of only three bacterial species from among the total complexity, i.e., *B. terrae*, *D. japonica*, and *R. basiennis*, were found to possess this property, resulting in positive selection. These were detected after inoculation with the whole bacterial community and showed single-strain migration. For one strain, *B. terrae* BS001, indications were found for biofilm formation in the mycosphere and for the utilization of particular fungus-released compounds as potential driving forces behind successful comigration with *Lyophyllum* sp. strain Karsten hyphae.

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