The ecological success of Burkholderia terrae BS001 and related strains in the mycosphere

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 12-01-2020
The capacity of *Burkholderia terrae* BS001 to associate with soil fungi is broad and provides protection against antifungal agents

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In preparation for publication.
Abstract
Several soil bacteria have been reported to obtain benefits from soil fungi, ranging from the provision of nutrients to the ability to access novel territories in soil. Here, we investigated the capacity of the soil bacterium *Burkholderia terrae* BS001 to comigrate with growing hyphae of a range of soil fungi, including *Lyophyllum sp.* strain Karsten, *Trichoderma asperellum*, *Rhizoctonia solani*, *Coniochaeta ligniaria*, *Phallus impudicus*, *Phanerochaete velutina* and *Fusarium* spp. Bacterial migration was found with most of these fungi, in a sometimes retarded manner. Migration further occurred mainly along the presumably hydrophilic hyphae, whereas hydrophobic ones did not allow migration. Another key finding was the ecological benefit that *B. terrae* BS001 provides to the soil fungi, *i.e.* protection from antifungal agents. In particular, *L. sp.* strain Karsten was protected from the fungal-antagonistic *Pseudomonas fluorescens* strain CHA0 as well as the fungicide cycloheximide. Protection against cycloheximide provided by *B. terrae* BS001 extended to the other fungi. Evidence was obtained for the contention that the protection was due to a combination of biofilm-produced polysaccharides and a raise in local pH. Both factors were attributed to the presence of *B. terrae* BS001 at the fungal surface. In the light of the complete dataset, we propose that the *B. terrae* BS001-*Lyophyllum sp.* strain Karsten interactions are denominated mutualistic rather than commensalistic.
Introduction

Bacteria and fungi often live together in the same microhabitat in many natural environments (Johansson et al., 2004). In some of these cohabitations, the two partners might have developed strategies to influence each other, making their mutual interaction successful (Partida-Martinez and Hertweck, 2005; Partida-Martinez et al., 2007 a, b). Soil fungi, as a result of their extendable mycelial structures, are able to cross the air-filled gaps in natural soil (De Boer et al., 2005). They also facilitate the movement of bacteria along these hyphal networks (Kohlmeier et al., 2005; Warmink and van Elsas, 2009; Warmink et al., 2011). While growing through soil, fungi may also create hospitable niches for the bacterial communities in their microhabitat (Nazir et al., 2010). Out of the many bacteria living in soil microhabitats in the vicinity of extending hyphae, some have been shown to be very successful in colonizing the newly available fungal hot spots (Warmink and van Elsas, 2009).

The genus *Burkholderia* can be found in different soils, exhibiting a range of ecological phenotypes. Thus, pathogenic, plant-growth-promoting (PGP) and fungal-interactive bacteria are encompassed in this genus (Warmink and van Elsas, 2008; Suárez-Moreno et al., 2012). In particular, specific members of the genus have been shown to be key protagonists of fungal-associated bacterial groups (Partida-Martinez et al., 2007; Warmink and van Elsas, 2008, 2009; Warmink et al., 2011; Nazir et al., 2012). Warmink and colleagues (2008) found *Burkholderia* in the soil underneath the mushroom foot of *Laccaria proxima* (denoted the mycosphere). Subsequently, they found that *Burkholderia* types are enriched in soil (mycosphere) that is colonized by the saprotrophic fungus *Lyophyllum sp.* strain Karsten (Warmink and van Elsas, 2009). Partida and colleagues discovered that a particular member of the genus *Burkholderia*, *B. rhizoxinica*, is living inside the ascomycete *Rhizopus microsporus* (Partida-Martinez et al., 2007). Interestingly, the phytotoxins that are supposedly secreted by this fungus were actually produced by the endomycotic *B. rhizoxinica* (Partida-Martinez and Hertweck, 2005; Partida-Martinez et al., 2007). The interaction between *B. rhizoxinica* and *Rhizopus microsporus* was shown to be...
very close. First, the fungus could not sporulate in the absence of the bacterial
partner (Partida-Martinez et al., 2007) and, second, the bacterium utilized its type
three secretion system (T3SS) to actively interact with the fungus (Lackner et al.,
2009). Recently, we found that the newly isolated *Burkholderia terrae* strain BS001
is highly interactive with the soil fungus *L. proxima* (Warmink and van Elsas, 2008)
as well as *L. sp.* strain Karsten (Warmink and van Elsas, 2009; Warmink et al.,
2011). Among the few bacteria able to move along growing fungal hyphae of *L. sp.*
strain Karsten, *B. terrae* BS001 stood out as a prominent one. Strain BS001 not only
migrated along the mycelial network of *L. sp.* strain Karsten but also helped other
bacteria, like *Dyella japonica* BS003, to comigrate (Warmink et al., 2011). Very
recently, we discovered that this migration capacity is not restricted to only one
species but is spread across several related species of the genus *Burkholderia* (Nazir
et al., 2012). Interestingly, the capability of each *Burkholderia* species to migrate
along growing fungal hyphae was different in different soils (Nazir et al., 2012).

Here, we extend the previous work on *B. terrae* BS001 comigration with
extending hyphal networks, to answer the following fundamental research questions:

1. Is *B. terrae* BS001 able to comigrate with hyphae of different fungi and
is the migration efficiency similar across these different fungi,  
2. Which hyphal characteristic (hydrophobicity versus hydrophilicity) is favorable to
*B. terrae* BS001 migration, and
3. Is there an effect of *B. terrae* BS001 on the growth and/or
survival of its fungal partner in soil in the face of adverse conditions?

**Materials and methods**

**Growth and maintenance of microorganisms**

The fungal strains used in this study, i.e. *Lyophyllum sp.* strain Karsten (DSM2979),
*Rhizoctonia solani, Trichoderma asperellum* 302, *Coniochaeta ligniaria, Phallus
impudicus, Phanerochaete velutina and Fusarium* spp. were routinely grown on oat
flake agar (OFA) plates, prepared with 30 g of oat flake (local shop) and 15 g of agar
(Duchefa, Haarlem, The Netherlands) in milliQ water to 1 litre, and sterilized at
121°C for 21 min. Once every 4 weeks, the fungal cultures were transferred to fresh OFA plates for maintenance.

The bacterial strains used in this study (B. terrae BS001, P. tolaasii BS295, Chryseobacterium aurantiacum, C. joostei and Pseudomonas fluorescens CHA0) were maintained as frozen cultures in 20% glycerol (-80 °C). Working stocks were maintained on R2A agar plates (Becton, Dickinson and Company, Sparks, MD), normally at room temperature, which were streaked onto new plates every week to maintain cell viability. After each 3rd transfer, bacterial cultures were re-established from the original -80 °C stock.

Microcosm experiment

Preparation of bacterial inocula

To prepare suspensions of cultured strains for inoculation, bacteria were grown overnight in 5 ml of LB medium (pH 7.0; Sigma-Aldrich, Haarlem NL) at 23 °C, with shaking. The cells were spun down for 5 min at 5,000 x g, washed, and resuspended in 1 ml of sterile saline (0.85 % w/vol NaCl). This procedure was repeated twice. The final cell suspensions were then diluted to an OD660 of 0.05 (containing an estimated 10^7 cells ml^-1, as evidenced using dilution plating on R2A agar). In total, 50 µl of this bacterial suspension was used directly for inoculation of soil in the migration experiments.

Microcosm experiments – bacterial migration through soil with growing fungal hyphae and protection against antifungal agents

The microcosm system used in this study consisted of three-compartment petri dishes (Greiner Bio one, Frickenhausen, Germany), as described elsewhere (Warmink and van Elsas, 2009). Briefly, two of the compartments were filled with pre-sterilized (autoclaved) test G (Gieterveen) soil (moisture contents corresponding to 60% of water holding capacity, bulk density of about 1.3 (g/cm³), establishing a layer of approximately 8 mm depth. The third compartment was filled with OFA and served as a nutrient source for the fungus. The physical barriers between the oat
flake and two soil compartments prevented compounds from the OFA compartment to reach the soil compartments. The barriers were overcome, though, by the fungal hyphae from the OFA compartment, and hence outgrowth of a fungus from the nutrient-rich OFA environment into the soil was achieved. The system was inoculated with the respective fungi, *i.e.* *Lyophyllum sp.* strain Karsten, *Rhizoctonia solani*, *T. asperellum* 302, *Coniochaeta ligniaria*, *Phallus impudicus*, *Phanerochaete velutina*, *Fusarium oxysporum* or *F. oxysporum lini* on the OFA medium and incubated at 28 °C, thereby allowing the colonization of the OFA compartment plus about 1-2 mm of the soil (prior to introduction of bacterial inocula).

Using a pipette, washed suspensions (50 µl) of cells of the inoculant bacterium, *e.g.* strain BS001, were placed evenly in one 3-mm-wide streak in the soil compartment directly adjacent to the front of the growing fungal hyphae. Control treatments consisted of the addition of BS001 cells in a similar streak to microcosms without fungal mycelium, and of sterile water added to fungal-plus microcosms in a similar streak.

The experiments served two purposes, *i.e.* analyses of (1) the comigration behaviour of strain BS001 and (2) the putative protective effect of the presence of *Burkholderia terrae* BS001 on the fungal partner. For the comigration experiments, the systems were incubated and sampled after 9, 12, 15 and 18 days of bacterial inoculation. For the protection experiments, a fungal antagonist (see Table 2), or the antifungal compound cycloheximide, were applied in a similar fashion, about 15 mm away from the growing fungal front. Replicate systems were then incubated at 23 °C, taking care that their moisture contents remained at the initial level.

**Bacterial population dynamics in the microcosms**

About 12-14 days following bacterial inoculation, samples (about 100 mg) were punched out (using a 4-mm dia auger) at different sites within the soil compartments (corresponding with inoculation of helper and antagonist). Replicate samples were then used for further analyses by dilution plating onto different media (R2A, Gould’s S1; Gould et al., 1985 and/or PCAT; Salles et al., 2006) followed by incubation of the plates and CFU counting after incubation.
OFA plate inhibition experiments

The protective effect of *B. terrae* BS001 for *Lyophyllum sp.* strain Karsten and other fungi (see results) against the antagonistic pressure exerted by the antifungal compound cycloheximide was tested. Different concentrations of cycloheximide, *i.e.* 0, 6, 12.5, 25, 50, 75 and 100 µg/ml, were applied in the OFA. After solidification of the OF agar, plates were inoculated as follows below. To OFA plates without cycloheximide, *P. fluorescens* strain CHAO was introduced as a fungal antagonist away from the spot where fungal and protective bacteria were co-inoculated. To cycloheximide-containing plates, bacterial and fungal cells (about $10^6$ per ml) were placed either in monocultures or in combination, and the growth of fungal mycelium over the plate was monitored over time, *i.e.* 7, 14, 21 and 28 days. The fungal migration ‘progress’ was expressed as mm per unit time. To monitor the bacterial presence, the systems were sampled by removal of an agar plug from the OFA medium with fungal colonization and vortex-mixing it for 15 min in 1 ml sterile saline. Afterwards, the suspensions were serially diluted and appropriate dilutions plated onto R2A to subsequently enumerate the cells after 2-4 days of incubation at 23 °C.

Data analysis

All experiments were performed in triplicate per treatment and the data are presented as geometric averages. Some of the experiments were repeated in time (see Results). At each time point, the CFU data were log-transformed, after which average values and standard deviations were calculated. Comparisons were made by statistical tests (t-test, ANOVA) using the SPSS package (SPSS, IBM, Statistics 1.8 for Windows), and data are reported as significant at $P<0.05$.

Standard deviations are shown between brackets in the text and/or as error bars in the figures.
Results

Growth of test fungi through the soil microcosms

All fungi, i.e. *Lyophyllum* sp. strain Karsten, *Rhizoctonia solani*, *Trichoderma asperellum* 302, *Coniochaeta ligniaria*, *Phallus impudicus*, *Phanerochaete velutina*, *Fusarium oxysporum* and *F. oxysporum lini* grew from the OFA compartment into and through the soil in the section ahead. The growth pattern through the G soil microcosms was different for the different fungi. *T. asperellum* 302 exhibited the fastest growth i.e. 4-5 mm/d while *P. velutina* was slowest i.e. 0.8 mm/d. Other fungi, i.e. *L. sp.* strain Karsten, *C. ligniaria*, *R. solani* and *Fusarium* spp. showed intermediate growth rates, i.e. 1.6, 1.14, 1.28 and 1.5 mm/d respectively. *P. impudicus* had difficulties to grow in this soil matrix. Noticeably, *Fusarium* spp. entered into the soil compartments, grew for about 20 mm and did not depict any visualisable aerial hyphae. Furthermore, for none of the test fungi was there any noticeable inhibition of growth by the presence of *B. terrae* BS001.

*B. terrae* BS001 migration with hyphal networks of different soil fungi

*B. terrae* BS001 has been reported to migrate along *Lyophyllum* sp. strain Karsten hyphae through a range of soils (Nazir et al. 2012). It was used as a positive control and reference in this study. Much like the migration along *L. sp.* strain Karsten hyphae through G soil (establishing population densities of >10^8 CFU/g of dry soil), *B. terrae* BS001 was able to migrate along with developing hyphae of the phytopathogenic fungus *Rhizoctonia solani* AG3, establishing slightly lower CFU numbers, i.e. 10^7 CFU/g of soil. Moreover, strain BS001 was found to migrate with the plant-growth-promoting biocontrol fungus *Trichoderma asperellum* 302, establishing CFU numbers of around 10^6 per g dry G soil. Migration with growing mycelium of the fungi *Coniochaeta ligniaria*, *Fusarium oxysporum*, and *Fusarium oxysporum* pv *lini* was noted, however the quantification of the bacterial load at the fungal growth fronts needs to be substantiated. At the same instance, we were unable to detect any migration of *B. terrae* BS001 along growing hyphae of *Phallus impudicus* and *Phanerochaete velutina* through the tested G soil.
We conclude that *Burkholderia terrae* strain BS001 appears to have a remarkable capacity, *i.e.* it is able to associate and migrate with moving hyphal fronts of many fungi through soil.

**Hydrophilicity of fungal hyphae supports migration of *B. terrae* strain BS001 along with extending hyphae**

We first analyzed the physico-chemical characteristics of hyphae of the fungus *L. sp.* strain Karsten by contact angle measurements. The contact angles of *L. sp.* hyphae when growing in dry conditions (such as in dry air) were about 128°, indicating a strongly hydrophobic environment. In contrast, the angle was about 80° when hyphae had been in contact with water (such as in soil with water films in soil pores), indicating a hydrophilic environment. Furthermore, the surface charge of these fungal hyphae tended to be negative, as the zeta potential was measured as -1.09. In a similar fashion, we determined the hydrophobicity/hydrophilicity of hyphae of *R. solani* AG3, and found the contact angle values to be about 120° and about 75°, respectively.

Aerial (hydrophobic) hyphae of *L. sp.* strain Karsten were sampled with sterilized forceps from the soil microcosms, whereas the soil layer beneath the aerial hyphae was sampled as it contained the hydrophilic hyphae of the same fungus. Bacterial loads on the hydrophilic hyphae were significantly higher than those on the hydrophobic ones, *i.e.* 10^8 CFU per g of soil (estimated to equal 10^10 CFU per g of hyphal tissue, at hyphal occupancy of 0.01) compared to only 500 CFU per g hyphal tissue present on hydrophobic hyphae (P<0.05). Furthermore, similar observations were made with *B. terrae* BS001 migrating along with growing hyphae of *R. solani* AG3. Bacterial loads on hydrophilic and hydrophobic hyphae of *R. solani* AG3 were 5x10^7 and 900 CFU per g, respectively. These values were also significantly different (P<0.05). Moreover, the bacterial cells sampled from the hydrophobic hyphae might have resulted from some hydrophilic residues in the hydrophobic hyphal samples.
Table 1 Migration of *B. terrae* BS001 along different fungal hyphae

<table>
<thead>
<tr>
<th>Test fungi</th>
<th>Ecological feature of the fungus</th>
<th>Hyphal type</th>
<th>Migration observed (CFU/g soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lyophyllum sp.</em> strain Karsten</td>
<td>Saprotroph</td>
<td>Thread like mycelia</td>
<td>++</td>
</tr>
<tr>
<td><em>Rhizoctonia solani</em> AG3</td>
<td>Phytopathogen</td>
<td>Thread like mycelia</td>
<td>+</td>
</tr>
<tr>
<td><em>Trichoderma harzianum</em></td>
<td>Biocontrol</td>
<td>Thread like mycelia</td>
<td>+</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>Biocontrol</td>
<td>Thread like mycelia</td>
<td>++</td>
</tr>
<tr>
<td><em>F. oxysporum</em> pv. <em>lini</em></td>
<td>Phytopathogen</td>
<td>Thread like mycelia</td>
<td>+</td>
</tr>
<tr>
<td><em>Coniochaeta ligniaria</em></td>
<td>Saprotroph</td>
<td>Thread like mycelia</td>
<td>++</td>
</tr>
<tr>
<td><em>Phallus impudicus</em></td>
<td>Wood rot</td>
<td>Cords</td>
<td>-</td>
</tr>
<tr>
<td><em>Phanerochaete velatina</em></td>
<td>Wood rot</td>
<td>Cords</td>
<td>-</td>
</tr>
</tbody>
</table>

++ means > 10⁷ - 10⁸ per g soil; + means 10⁴ - 10⁷ per g soil; - <10⁴ per g soil

The protective effect of *B. terrae* strain BS001 against fungal antagonists

In a 21-day experiment in microcosms with G soil, *Lyophyllum sp.* strain Karsten alone was unable to grow over any of the antagonists, *i.e.* *Pseudomonas fluorescens*
Comigration and protection effect of *B. terrae* BS001

CHA0, *P. tolaasii* BS295, *Chryseobacterium aurantiacum* and *C. joostei* that were initially applied at 15 mm distance of the mycelial front. Over the experimental time, the fungus could grow up to the zone in which each of the antagonists had been inoculated, where it stopped its growth (Fig. 1). The visualizable fungal biomass density (till the point where it stopped) was higher in the antagonist-treated microcosms than in the controls without antagonists. Strikingly, the fungus was able to grow over the zone where antagonists had been introduced in all cases when *B. terrae* BS001 had been introduced at the inoculation site at the young mycelial front (Fig. 1). Hence, the distances over which the mycelial fronts migrated in the presence of the antagonists were significantly higher in the presence of *B. terrae* BS001 than in its absence (*P*<0.05). Similar findings were obtained when the mycelial front encountered cycloheximide (25 µg / g) in the soil. No such difference was noted in control experiments in which the fungus was grown over non-antagonist plates in the presence or absence of *B. terrae* strain BS001 (*P*>0.05). The results of these experiments are summarized in Table 2.

**Table 2** Protection provided by *B. terrae* BS001 to *L. sp.* strain Karsten against fungal antagonists in G soil

<table>
<thead>
<tr>
<th></th>
<th>Cycloheximide</th>
<th><em>C. aurantiacum</em> BS126</th>
<th><em>C. joostei</em> BS181</th>
<th><em>P. tolaasii</em> BS295</th>
<th><em>P. fluorescens</em> CHAO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungus alone</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fungus+ BS001</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*C: Chryseobacterium; P: Pseudomonas; - : no fungal growth over the antagonists; + : fungal growth, similar to control, passing over the antagonists.*
**Bacterial population dynamics in soil microcosms with L. sp. strain Karsten** - The protective *B. terrae* BS001 was introduced into the microcosms at about $5 \times 10^6$ cells per g of soil. Expectedly, it migrated with *L. sp.* strain Karsten, increasing its numbers up to carrying capacity of the system. Interestingly, the fungal antagonists *P. fluorescens* CHA0, *P. tolaasii* BS295, *C. aurantiacum* and *C. joostei* persisted at the inoculum level, *i.e.* $10^6$ CFU per g dry soil over experimental time. In the light of these data, we surmised that an active antagonist population was present, in the face of which the growing hyphae of *L. sp.* strain Karsten required a protective effect by *B. terrae* BS001 to allow progression.

**The protective effect of *B. terrae* strain BS001 against cycloheximide**

A range of cycloheximide concentrations (0, 6, 12.5, 25, 50, 75 and 100 µg/ml) in OFA plates was evaluated for inhibition of *L. sp.* strain Karsten growth. The control OFA plate, without cycloheximide, showed a fungal growth rate of about 8.5 mm/day. The presence of cycloheximide, in all tested concentrations, was clearly harmful to fungal development. Specifically, 6 and 12.5 µg/ml already inhibited fungal growth, as this was reduced to 5.5 and 4.0 mm/day, respectively. Levels of 25 and 50 µg per ml further reduced the fungal growth rates to 2.5 and 1.5 mm/day. The presence of 75 and 100 µg of cycloheximide in the OFA yielded no apparent fungal growth, not even three months following fungal inoculation (Fig. 2). Thus, expectedly, the progressively increasing cycloheximide concentrations linearly increased the level of inhibition that the fungus experienced (measured by fungal growth rate in mm/day).

Very interestingly, the presence of *B. terrae* BS001 (at about $10^6$ - $10^7$ CFU initially) at the *L. sp.* strain Karsten hyphal plug reduced the inhibitory effect of cycloheximide on hyphal outgrowth. At any of the applied cycloheximide concentrations, the time to completely cover the OFA plate was significantly reduced in the presence of strain BS001 compared to the situation without bacterial inoculant. As above, the fungal growth rates were 5.5, 4.0, 2.5 and 1.5 mm/day with 6, 12.5, 25 and 50 µg/ml cycloheximide in the absence of strain BS001 while these
were respectively 7.0, 6.5, 3.5 and 1.7 mm/day in the presence of B. terrae BS001 (Fig. 2). Furthermore, the highest cycloheximide levels, i.e. 75 and 100 µg/ml, were initially deleterious to L. sp. strain Karsten, even in the presence of the bacterium. However, in the presence of BS001 the fungus started growth after 21 days and bacterial presence had a large and significant positive effect on fungal growth rate afterwards, being 0.7 and 0.3 mm/day. Such growth was absent even after 12-13 weeks of fungal inoculation in the absence of bacterial inoculum (Fig. 2).

**Does B. terrae BS001 grow on cycloheximide as a nutrient?**

B. terrae BS001 did not exhibit any growth response in minimal medium (MM) with cycloheximide as the sole C source. The bacterium was inoculated as $10^6$ cells/ml in the medium, and remained roughly at this level even after one month of incubation at 28 °C (shaking). In the control (MM) medium with added glucose (0.5%), strain BS001 showed vigorous growth after only 2 days at 28 °C. These data indicate that strain BS001 is unable to utilize cycloheximide as the sole carbon source. Hence, the protective effect against cycloheximide might lie in a mechanism (e.g. shielding as a result of a biofilm) which makes this compound unavailable to the growing fungal hyphae.
Protective effect of *B. terrae* BS001 against cycloheximide for different fungi

The growth dynamics of *Fusarium oxysporum* pv lini and *Rhizoctonia solani* AG3 under cycloheximide pressure were also evaluated in relation to the effect of *B. terrae* strain BS001. The growth of both fungi was significantly inhibited by the presence of 75 and 100 µg/ml of cycloheximide in the OFA medium, whereas lower concentrations permitted fungal outgrowth. Quantification of the growth inhibition revealed that both fungi grew at rates of 1.2 mm/day in the presence of cycloheximide, while the rates were 3 and 6.5 mm/day, respectively, in the controls devoid of cycloheximide.

The protective effect of *B. terrae* BS001 against cycloheximide pressure (75 µg/ml) was prominent for *Rhizoctonia solani*, as the fungal migration distance after 15 d was consistently 10 mm larger in the presence of strain BS001 than in its absence. The protective effect exerted by strain BS001 on *Fusarium oxysporum* pv lini against cycloheximide (75 µg/ml) was also clearly visible 1 week after inoculation. That is, the fungal front was 30 mm advanced in the presence of strain BS001 compared to the situation without strain BS001. However, the effect turned out to be ephemeral, as after 3 weeks the differences had diminished.

Bacterial biofilm formation around fungal hyphae

*B. terrae* BS001 formed biofilms around growing hyphae of many fungi that were tested in this study. Microscopic observations as well as CFU counts revealed that the whole mycelial network of *L. sp.* strain Karsten, *T. asperellum* 302 and *R. solani* was covered with bacterial cells. Figure 3 shows the colonization of *B. terrae* BS001 cell on growing hyphae of *T. asperellum* 302. We, thus, hypothesized that these biofilms may protect growing hyphae against antagonists and/or antibiotics by shielding effects exerted by the matrix of polysaccharides secreted in the *B. terrae* BS001 around the growing fungal hyphae.
Comigration and protection effect of *B. terrae* BS001

**Fig. 2** Growth rates of *Lyophyllum* sp. strain Karsten on OFA supplemented with cycloheximide.
- Light grey bars: growth of fungus alone
- Dark grey bars: growth of fungus coinoculated with *B. terrae* BS001
- Digits indicate cycloheximide concentration in medium as µg/ml.

**Fig. 3** Microscopic image of *Trichoderma asperellum* mycelium (marked as arrows) covered by the biofilm of *Burkholderia terrae* BS001 (rod like) cells.
Modulation of pH due to *B. terrae* strain BS001

An experiment was performed with *B. terrae* BS001 growing in OF liquid medium amended with cycloheximide at either zero or 100 µg/ml. The initial pH of the medium (before bacterial inoculation) was 6.5, whereas it increased to 7.5 after overnight growth of *B. terrae* BS001. This increase in pH took place due to bacterial growth irrespective of the level of cycloheximide.

Discussion

Several members of the genus *Burkholderia* have been reported to be enriched in the mycosphere in soil (Warmink and van Elsas, 2008). These strains apparently utilize the hyphal network as a colonizable surface (Warmink and van Elsas, 2009) where they may find their ecological niche driven by nutritional and colonization demands. Another facet of bacterial association with mycelial networks is the fungal facilitation of passage of air-filled voids in the soil (De Boer et al., 2005), which allows bacteria to access new microhabitats (Nazir et al., 2010; Kohlmeier et al., 2005; Furuno et al., 2010). The phenomenon of bacterial migration along growing fungal hyphae in soil is not confined to only one *Burkholderia* species but is, in fact, widespread in this genus (Nazir et al., 2012). Interestingly, the capacity of a bacterium to migrate along with hyphae of the same fungal species in different soils is different (Nazir et al., 2012). Furthermore, at least one of the *Burkholderia* strains, denoted *B. terrae* strain BS001, was shown to not only migrate itself but to also assist other bacteria, like *Dyella japonica*, to move along the fungal highway (Warmink et al., 2011). Living in association with growing fungal hyphae, the highly interactive *B. terrae* BS001 has probably developed an optimal capacity to live off fungal-released compounds. It also has the capacity to tinker with (delay) mushroom formation by *L. sp.* strain Karsten (Nazir et al., 2012; see chapter 4). Also, during the extended delay of mushroom formation by *L. sp.* strain Karsten, strain BS001 induces the release of glycerol, a compound that it can utilize for its own growth and survival. Based on our current results, we feel confident to suggest that *B. terrae* BS001, on the basis of its capacity to migrate along with the hyphae of
a range of soil fungi, has a broad fungal-interactive ability. This broad capacity most likely resides in the physical ability of strain BS001 to deal with a fungal surface, respond and interact with it, culminating in the construction of a biofilm associated with the hyphal network. Furthermore, we found that the migration of strain BS001 was mostly with hydrophilic hyphae, as expected. Hydrophobicity being an important criterion that modulates the interactions, it becomes apparent how local (water) conditions govern the interactions between the soil bacterium B. terrae BS001 and the hyphal network. Moreover, it was very interesting to observe that B. terrae BS001 apparently not only obtains a benefit from its fungal partner but also protects it against antagonists like P. fluorescens strain CHA0 and/or antifungal compounds present in its microhabitat. In natural soils, many antagonists exist that can inhibit fungal growth and survival as a result of their production of secondary metabolites (Keel et al., 1992). To nail down the mechanisms, we did some experiments with the antifungal compound cycloheximide, which is normally produced by bacteria of the genus Streptomyces (Abou-Zeid and El-Sherbini, 1975; Dykstra and Wang, 1990; Kominek, L.A., 1975 a,b). This analysis revealed that the B. terrae strain BS001-induced protective effect might relate to shielding or inactivation of the antagonistic compound. Protection of L. sp. strain Karsten by B. terrae BS001 against antifungal agents has environmental significance. It may explain why, for example, plant disease management may fail or does not fully work in nature because the target plant-pathogenic fungi may have acquired bacterial protection much like found in our study. Furthermore, the efficiency of some biocontrol agents might be improved using the protective strategy, i.e. by coupling a biocontrol fungus such as Trichoderma asperellum 302 with protective bacteria like B. terrae BS001. We propose that the protection is provided by the bacterial biofilm that is formed around the growing fungal hyphae. The bacterium, following its growth, creates a biofilm around fungal hyphae, of which the extracellular polysaccharides diffuse out into the vicinity and deactivate or shield away the antifungal agent present. Thus, the B. terrae strain BS001 biofilm around fungal mycelia may work as a physical barrier for any available antifungal agent to target the living hyphae. This barrier could also be physicochemical, binding the antifungal
compounds in the matrix of polysaccharides. This way, the bacterium may make the antifungal compound unavailable to the growing fungus. Furthermore, the growing bacterium was able to increase the pH in its vicinity, which might cause the instability/degradability of cycloheximide (Lawes, B.C. 1961) or any other antifungal compound in this micro-environment. As a result of these two bacterial strategies, the antifungal compounds are unable to adversely target the living mycelium and consequently the fungus keeps growing.

In the light of the current data that reveal mutually-beneficial effects of the two partners in this bacterial-fungal relationship, we propose that the *B. terrae* BS001 – *L. sp.* strain Karsten interaction should be regarded as mutualistic rather than commensalistic.

**Acknowledgements**

We gratefully acknowledge the financial support to Rashid Nazir by the HEC programme of the government of Pakistan. He was further assisted by the Soil Biotechnology Foundation.

**References**


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