The occurrence and ecological role of plasmids in bacterial mycosphere dwellers
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The putative effect of IncP-1β plasmid pHB44 on the iron uptake capacity of *Burkholderia terrae* BS001 and its potential transfer in soil microcosms

Miaozhi Zhang, Pu Yang, Jan Dirk van Elsas
Abstract

*Burkholderia terrae* BS001 is a well-described mycosphere dweller. In the interaction between this bacterium and its fungal host in soil, competition for Fe might be a key process. In this study, we address the capacity of the broad-host-range IncP-1β plasmid pH44, originally isolated in *Variovorax paradoxus* HB44, to enhance or modulate the Fe uptake capacity of *B. terrae* BS001 when confronted with mycelia of *Lyophyllum* sp. strain Karsten in soil microcosms. The data revealed that, in most cases, the effect of plasmid pH44 on the fitness of its host was negative. However, a strong and remarkable nullifying effect was found at the tips of the soil-exploring *L.* sp. strain Karsten mycelium, indicating that the metabolic burden imposed by plasmid pH44 to BS001 was offset under iron-limited conditions. Moreover, plasmid pH44 was transferred from *B. terrae* BS001 to *V. paradoxus* BS64 in the mycosphere. Strikingly, we found that successful plasmid transfer occurred more frequently in iron-limited than in ‘plentiful-iron’ habitats, indicating a role of plasmid pH44 in Fe acquisition as the selective force that spurred the appearance of transconjugants. Furthermore, the findings highlight the mycosphere as a selective arena in which horizontal gene transfers across the bacterial inhabitants spurs adaptive processes.
Effect of pH44 on B. terrae BS001

Introduction

Bacterial-fungal interactions are common in soil, as the two organismal groups often share the same microhabitat in this system. It can thus be posited that interactions, of different nature, have evolved between members of the two groups (de Boer et al., 2005). The organisms may cooperate, compete, antagonize or be neutral with each other, and the precise nature of the interactions may vary in time and space. The mycosphere is the microhabitat surrounding the fungal hyphae in soil, which is one of the hot spots where fungal-bacterial interactions mainly take place (Warmink and van Elsas, 2008). In this microhabitat, fungal-released compounds can provide benefits to the associated bacteria and thus establish a selective force acting on particular members of that bacterial community (Frey-Klett, 2007; Nazir et al., 2010). On the other hand, the two partners may eventually compete for limiting compounds such as available iron.

Warmink and van Elsas (2008) provided evidence that the Laccaria proxima mycosphere selects particular bacterial groups from soil in the field. Specifically, the population sizes of the culturable bacterial communities were increased in the mycosphere compared to those in the corresponding bulk soil. The selective effect was shown to be strongly related to the utilization of particular carbonaceous compounds by certain bacterial groups (Warmink et al., 2009). Boersma (2010) later revealed that glycerol is a main carbon source driving the selection of Variovorax paradoxus strain HB44 in the mycosphere of Laccaria proxima. Moreover, Warmink and van Elsas (2009) noticed that the mycosphere dweller Burkholderia terrae BS001 could migrate along with the fungal hyphae of the related fungus Lyophyllum sp. strain Karsten in soil microcosms, indicating that this organism thus may be able to co-exploit the novel microhabitats that are unlocked by the fungal partner in the soil.

Horizontal gene transfer (HGT) is a main driving force that allows bacteria to adapt and evolve in fast-changing environments (van Elsas et al., 2003). In the mycosphere, carbonaceous compounds released from the fungal hyphae stimulate the growth of bacteria that are locally present, thus activating organism-to-organism contacts that potentially include conjugational gene transfers. Therefore, the mycosphere is proposed to constitute a ‘gene transfer arena’, in which a variety of genes, including locally-adaptive ones, are exchanged across the local microbial communities (Zhang et al., 2014a). Plasmids constitute main vehicles of HGT, and broad-host-range ones can spread across divergent bacterial hosts. In a
Effect of pHB44 on *B. terrae* BS001

recent study, we provide evidence that the relative abundance of IncP-1β plasmids is enhanced in the mycosphere of several fungi in a forest soil, as compared to that in the corresponding bulk soil (Zhang et al., 2014b). Also, exogenous isolation data revealed the frequencies of mobilization of tracer plasmids to be significantly elevated with microbial communities derived from different mycospheres compared with those from corresponding bulk soil (Zhang et al., 2014b). This indicates that certain - highly transfer-proficient - plasmid groups are selected in the mycosphere, possibly playing an important role in the adaptation of their bacterial hosts. Indeed, we later showed that plasmid pHB44 could significantly enhance the fitness of *V. paradoxus* strain BS64 in soil microcosms containing *L. sp.* strain Karsten in which Fe was likely limiting, suggesting it might encode proteins involved in iron capturing or uptake (Zhang et al., 2014c).

In the current study, we transferred plasmid pHB44 to the mycosphere migrator *Burkholderia terrae* BS001, in order to assess whether it confers a similar fitness-enhancing trait to strain BS001 in the mycosphere, a presumed iron-limited habitat. We further examined whether the presence of this plasmid would affect the migration capacity of strain BS001 along the hyphae of the reference fungus *Lyophyllum sp.* strain Karsten. Besides, we tested the population dynamics of *Burkholderia terrae* BS001 (pHB44) and *Variovorax paradoxus* BS64 in a joint inoculation experiment, addressing the population dynamics of the two organisms, as well as the potential transfer, and selection, of plasmid pHB44 under different conditions of iron availability.

**Materials and methods**

**Bacterial strains, plasmid and growth conditions**

The bacterial strains and plasmids used in this study are listed in Table 1. *Burkholderia terrae* BS001 and *Variovorax paradoxus* HB44 (isolated in 2004, Warmink and van Elsas, 2008) and BS64 (a plasmid-less derivative [cured], Zhang et al., 2014) were grown either in R2A or LB broth (tryptone 10 g, yeast extract 5 g, NaCl 5 g, distilled water 1 L; pH 7.2) at 28 °C for 24 h. Streptomycin-(Sm³) or rifampicin-resistant (Rp³) mutants of strain BS001 were selected, next to a rifampicin-resistant (Rp³) mutant of strain BS64. The three selected mutants showed growth rates that were similar (P>0.05) to those of their parental strains. The mutants were stored at -80°C in 20% glycerol. The *Escherichia coli* strains used
for mating experiments were grown overnight at 37 °C in LB broth containing the appropriate antibiotics (Table 1). Agar (1.75%) was added to the media when necessary. Antibiotics were added as appropriate and their concentrations can be found in Table 1.

**Table 1. Strains and plasmids used**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Resistance</th>
<th>Plasmid / Inc group</th>
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<tr>
<td><em>Burkholderia terrae</em></td>
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<td>BS001</td>
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<td>BS001 Sm&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Sm&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>BS001 Rp&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Rp&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>BS001 Sm&lt;sup&gt;f&lt;/sup&gt; (pHB44; pSUP104)</td>
<td>Sm&lt;sup&gt;f&lt;/sup&gt; Tc&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>pHB44 / IncP-1&lt;sup&gt;β&lt;/sup&gt;; pSUP104 / IncQ</td>
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<tr>
<td>BS001 Sm&lt;sup&gt;f&lt;/sup&gt; (pHB44)</td>
<td>Sm&lt;sup&gt;f&lt;/sup&gt;</td>
<td>pHB44 / IncP-1&lt;sup&gt;β&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Variovarax paradoxus</em></td>
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<td>BS64</td>
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<tr>
<td>BS64 Rp&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Rp&lt;sup&gt;f&lt;/sup&gt;</td>
<td>-</td>
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<tr>
<td><em>Escherichia coli</em></td>
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<tr>
<td>CSH52 (pSUP104)</td>
<td>Tc&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>pSUP104 / IncQ</td>
</tr>
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</table>

**Transfer of plasmid pHB44 to *B. terrae* BS001 (Sm<sup>f</sup>)**

To allow an assessment of the effects of plasmid pHB44 on the ecology of *B. terrae* BS001, it was first transferred to *B. terrae* BS001 Sm<sup>f</sup> by using a triparental mating (Mela et al., 2008). In this system, *V. paradoxus* HB44 (pHB44) served as the plasmid donor, *E.coli* CSH52 (pSUP104) as the tracer and *B. terrae* BS001 Sm<sup>f</sup> as the recipient strain. Mobilizable plasmid pSUP104 carried chloramphenicol and tetracycline resistance determinants, and so these two antibiotics, in conjunction with streptomycin, served to preselect *B. terrae* BS001 Sm<sup>f</sup> transconjugants. Putative transconjugants were then checked to confirm the presence of plasmid pSUP104 in first place, followed by a check for the presence of plasmid pHB44. For this, PCR replicon typing (Gotz et al., 1996) as well as plasmid extractions (Birnboim and Doly, 1979) were used. Transconjugants carrying plasmid pHB44 were selected, streaked to purity, cultured and stored at -80°C with 20% glycerol.

**Curing of plasmid pSUP104 from *B. terrae* BS001 (pHB44; pSUP104)**

To eliminate plasmid pSUP104 from strain BS001 Sm<sup>f</sup> (pHB44; pSUP104), a temperature elevation strategy (33°C) was applied to serial batch cultures in LB broth of this organism. After each growth step, 50 colonies were picked to check for the putative loss of
Effect of pHB44 on *B. terrae* BS001

plasmid pSUP104 by checking for the loss of the pSUP104 antibiotic resistances. This was followed by confirmatory repB-based colony PCR. In addition, PCR targeting the trfA2 gene was performed on presumptive pSUP104-negative clones to confirm maintenance of plasmid pHB44. Finally, selected (potentially ‘cured’) clones were subjected to plasmid isolation to confirm the curing of pSUP104, yielding *B. terrae* BS001 Sm<sup>r</sup> (pHB44).

**Soil microcosms - preparation**

Soil (top 10 cm) was freshly sampled from a field in Gieterveen, the Netherlands (denoted G soil), mixed to homogenize, and used through all the experiments. The soil was then sterilized at least two times at intervals of 3 days by autoclaving at 115°C for 45 min. The G soil, characterized as a loamy sand, had a pH of about 4.8, and contained total carbon (C) and total nitrogen (N) contents of 2.8% and 0.8%, respectively. Prior to the experiments, it was amended with 0.5% CaCO<sub>3</sub> to establish a pH of 6.8. The pH-6.8 G soil was further manipulated to contain different iron levels, i.e. (1) native Fe level, (2) additional Fe (as FeCl<sub>3</sub>) at 5 µM FeCl<sub>3</sub> over the water content of the soil (75% of WHC is 15% total water content, and (3) additional Fe at 50 µM FeCl<sub>3</sub>. This yielded ‘native-Fe’, ‘intermediate-Fe’ and ‘high-Fe’ soils. Soil microcosms were then prepared and inoculated with *Lyophyllum sp.* strain Karsten as described (Warmink and van Elsas, 2009). In short, three-compartment Petri dishes were used, of which two compartments were filled with sterile G soil at bulk density 1.2 and 60-65% of water-holding capacity (WHC), and the other compartment was filled with oat flake agar (OFA), consisting of 30 g oat flake and 15 g agar in 1 L of water (Warmink and van Elsas, 2008). Following inoculation of the OFA compartment with *L.* sp. strain Karsten, the Petri dishes were sealed with parafilm to stabilize the soil moisture contents, after which they were incubated at 28 °C for 5 days until the fungal front had entered around 5-8 mm into both soil compartments.

**Population dynamics of *B. terrae* BS001 Rp<sup>r</sup> and BS001 Sm<sup>r</sup> (pHB44) - Separate inoculation**

Cells from overnight cultures of *B. terrae* BS001 Rp<sup>r</sup> and BS001 Sm<sup>r</sup> (pHB44) were washed and introduced into the native-Fe, intermediate-Fe and high-Fe G soils at the fungal front, establishing population densities around 10<sup>6</sup> cells g<sup>-1</sup> dry soil at about 75% of WHC. Control plates did not receive fungal inoculum. Following sealing, the microcosms were incubated at 28 °C. Over time, triplicate soil samples (4-mm dia core) were taken from the inoculation and migration sites (fungal hyphal front, Warmink and van Elsas 2009); the
samplings were respectively at days 0, 1, 4, 7 and 14. Dilution plating was performed and, following incubation of the plates, the population dynamics of the bacterial inoculants was followed by CFU counts.

**Population dynamics of B. terrae BS001 Rp\(^{r}\) and BS001 Sm\(^{r}\) (pHB44) - Joint inoculation**

In order to investigate the competitive behavior of strains BS001 Rp\(^{r}\) and BS001 Sm\(^{r}\) (pHB44) under different iron concentrations as well as the effect of plasmid pHB44 on the migratory capacity along the L. sp. strain Karsten hyphae, joint inocula of *B. terrae* BS001Rp\(^{r}\) and BS001 Sm\(^{r}\) (pHB44), at about 10\(^6\) cells g\(^{-1}\) soil, were introduced to the soil microcosms. Control plates did not receive fungal inoculum. At days 0, 1, 4, 7 and 14, triplicate microcosms were sampled at both the inoculation and migration sites, and CFU counts were obtained from rifampicin-containing agar plates for strain BS001 Rp\(^{r}\) and from streptomycin-containing plates for strain BS001 Sm\(^{r}\) (pHB44). To detect putative plasmid transfer, colonies from the rifampicin-containing plates (n=20 per replicate system) were checked for the presence/absence of plasmid pHB44 using trfA-based colony PCR.

**Dynamics of V. paradoxus BS64 Rp\(^{r}\) and B. terrae BS001 Sm\(^{r}\) (pHB44) and potential plasmid transfer**

Washed cells from fresh cultures of *B. terrae* BS001 Sm\(^{r}\) (pHB44; pSUP104) and *V. paradoxus* BS64 (Rp\(^{r}\)), at about 10\(^8\) cells g\(^{-1}\) soil, were introduced into the native-Fe, intermediate-Fe and high-Fe G soils in stripes at the mycelial growth front. Control plates did not receive fungal inoculum. Over time, triplicate soil samples were taken at the inoculation and migration sites and processed for dilution plating; samplings were at days 1, 4 and 7. Rifampicin- and streptomycin/chloramphenicol-containing agar plates were used to select strains BS64 Rp\(^{r}\) and BS001 Sm\(^{r}\) (pHB44; pSUP104), respectively. Rifampicin/chloramphenicol-containing plates were used to select putative transconjugants. To detect the presence of plasmid pHB44 in these, colonies from the latter medium (n=20 per replicate system) were checked by using trfA-based colony PCR and >90% of the tested transconjugants were trfA-positive, confirming the co-transfer of pHB44 with the tracer plasmid pSUP104. Transfer frequencies were calculated as the ratio of the total number of transconjugants to the total number of recipients. To assess the potential occurrence of matings during the plating procedure, soil with recently introduced mixtures of washed BS64 Rp\(^{r}\) and BS001 Sm\(^{r}\) (pHB44; pSUP104) cells (10 min) were subjected to dilution plating on the double-selective medium. No transconjugants were detected as a result of plate matings.
Effect of pHB44 on *B. terrae* BS001

To recover transconjugants from soil, soil with native-Fe, intermediate-Fe and high-Fe was sampled and amended with LB broth supplemented with rifampicin and chloramphenicol at the ratio of 1:100. Following incubation at 24°C for 0, 4, 7 and 24 h, soil was dilution-plated on Rp+Cm-containing plates to screen transconjugants. Control soil without the fungus was processed the same way to examine the recovery of transconjugants.

**Statistical analysis of the data**

All experiments were performed in triplicate per treatment. Several experiments were repeated in time. At each time point, the data obtained were log-transformed, after which average values and standard deviations were determined. The differences between treatments were tested for significance with One-Way ANOVA (p < 0.05), in the data analysis function of Microsoft Excel. Bars in graphs and the number between brackets behind means of values represent the standard error of the mean (SE).

**Results**

*Introduction of plasmid pHB44 into B. terrae BS001*

To perform experiments on the effect of plasmid pHB44 on *B. terrae* BS001, we first transferred this plasmid from *V. paradoxus* HB44 to *B. terrae* strain BS001 Sm, with the help of the IncQ tracer plasmid pSUP104 (present in *Escherichia coli* CSH52) in a triparental mating. The mating yielded presumptive transconjugant colonies at a rate of $10^{-7}$ per recipient. A suite of 20 putative transconjugants was then screened by repB-based PCR to examine the presence of pSUP104 in these; all clones gave positive signals. Subsequently, the presence of plasmid pHB44 was checked in ten of the 20 selected pSUP104-positive clones by trfA2-based PCR, and positive signals were found in five clones. These five positive clones were plasmid-extracted, after which PCRs were again run, in addition to electrophoretic gel detection of covalently closed circular (ccc) bands. All five picked transconjugant clones were positive for both the trfA2 and repB PCRs and showed, next to a band representing the chromosome, two bands with sizes 60 kb and 9.5 kb (data not shown).

Then, plasmid pSUP104 was removed by sequential batch culturing of two selected clones of strain BS001 Sm (pHB44; pSUP104). Five 1:1000 transfers were used, and growth took place at elevated temperature (34°C). This procedure, after transfer 5, yielded colonies which had lost plasmid pSUP104, as evidenced by the absence of the chloramphenicol and
Effect of pHB44 on *B. terrae* BS001

tetracycline resistances. We confirmed the nature of five randomly–picked ones by their consistently negative *rep*B-based PCR but positive *trf*A2-based PCR, in addition to the presence of the typical ~60-kb band and the absence of the smaller (~9.5 kb) band in gel analyses of plasmid extracts. Two of the five clones were streaked to purity, grown and stored in 20% glycerol in a -80 °C freezer.

An additional experiment examined the growth rate of *B. terrae* BS001 Sm′ (pHB44) in comparison with that of BS001 Rp′. This experiment revealed slightly different growth rates between the two strains, with a fitness disadvantage for the plasmid-carrying strain. However, growth of both strains was still comparable and the rate differences were not significant (p>0.05). Collectively, these data warranted the use of these strains in further experiments on the potential fitness-enhancing properties of plasmid pHB44 in the mycosphere.

Population dynamics of *B. terrae* strains BS001 Rp′ and BS001 Sm′ (pHB44) in soil with *L. sp.* strain Karsten at different Fe levels – Separate inoculations

To investigate the effect of plasmid pHB44 on *B. terrae* strain BS001 in G soil with the fungus at different iron levels, the behavior of strains BS001 Rp′ and BS001 Sm′ (pHB44) at the inoculation site in the soil microcosm (with progressively ageing fungal tissue) was determined. Fig.1 provides the details of the experiment. In the native-Fe systems, plasmidless strain BS001 Rp′ showed progressive growth, with population densities varying from ~10^6 to 5x10^7 (d 4) and, finally, around 10^8 CFU per g dry soil (d 14). Strain BS001 Sm′ (pHB44) showed a similar trend of growth during the time course, increasing from ~10^6 to 5x10^7 CFU per g dry soil (d 14). However, it revealed lower CFU counts than did plasmidless strain BS001 Rp′ at all time points, be it that only at day 7 the difference was significant (P<0.05) (Fig. 1A). In the intermediate-Fe soil, the population dynamics of strains BS001 Rp′ and BS001 Sm′ (pHB44) was similar. Both populations started from ~10^6 cells per g dry soil and reached maximum densities, of ~10^8 CFU per g dry soil at day 14. At days 7 and 14, strain BS001 Rp′ again showed slightly higher cell densities than plasmid-bearing strain BS001, but the differences were not significant (P>0.05) (Fig. 1B). In the high-Fe soil, the behavior of strains BS001 Rp′ and BS001 Sm′ (pHB44) was also similar, with growth from about 10^6 to around 10^8 CFU per g dry soil (d 14). No significant differences between the population sizes of the two strains were observed at any time point (P>0.05) (Fig. 1C).
Effect of pHB44 on *B. terrae* BS001

**Fig. 1** Effect of plasmid pHB44 on the dynamics of *B. terrae* BS001 Sm\(^r\) in the mycosphere of *Lyophyllum* sp. strain Karsten in G soil microcosms. Separate inoculations. A. Native-Fe soil, B. Intermediate-Fe soil, C. High-Fe soil (as indicated in Materials and Methods).

Thus, irrespective of the Fe level of the soil, the population dynamics of strains BS001 Rp\(^r\) and BS001 Sm\(^r\) (pHB44) at the fungus was largely similar, with a tendency of the plasmid-containing strain to have an (ephemeral) ecological disadvantage. This suggested that, in the soil system with the fungus, plasmid pHB44 indeed confers a metabolic burden to the host *B. terrae* BS001, and is unable to provide a significant fitness advantage to this host bacterium. In the control soils (deprived of the fungus), both strains BS001 Rp\(^r\) and BS001 Sm\(^r\) (pHB44) grew out, from the added ~10\(^6\) to about 10\(^8\) CFU per g dry soil over...
Experimental time, with no significant differences (P>0.05) in the population sizes of the two strains at any time point. The dynamics of both strains was also not affected by the iron content of the soil (data not shown).

The effect of plasmid pH44 on the migration of strain BS001 in soil with L. sp. strain Karsten at different Fe levels – Separate inoculations

To detect whether plasmid pH44 exerts an effect on the migration proficiency and competitiveness of B. terrae strain BS001 at the L. sp. strain Karsten migration front, we analyzed the population densities of strain BS001 Sm(f) (pHB44) in comparison to BS001 Rp(f) at the migration spots, as described (Warmink and van Elsas, 2009). First, soils without L. sp. strain Karsten never showed the presence of strain BS001 Rp(f) or BS001 Sm(f) (pHB44) cells at the (virtual) migration spots, confirming that bacterial migration was due to co-migration with the soil-exploring fungal hyphae. Then, in the fungus-containing native-Fe soil, at all sampling days (1, 4 and 7), the dynamics of both strains was statistically similar, although strain BS001 Rp(f) showed somewhat higher cell densities than did strain BS001 Sm(f) (pHB44) (p>0.05) (Fig. 2). Thus, no significant ecological disadvantage of plasmid carriage was noted in this situation. Moreover, in the intermediate-Fe and high-Fe soils, the population sizes of strains BS001 Rp(f) and BS001 Sm(f) (pHB44) also showed no significant differences at days 1 or 4 (P>0.05) (Fig. 2A,B). However, at day 7, the plasmid-less strain BS001 Rp(f) was found to strongly outcompete strain BS001 Sm(f) (pHB44), with highly significant differences (P<0.001) (Fig. 2C). This indicated that conditions of plentiful Fe in the soil nullified the ‘compensating’ effect on host population size of the plasmid observed in the soil without added Fe.

The collective data thus indicated that, after 7 d, the bacterial-fungal competition for Fe in the native soil at the migration front may have been fierce, resulting in the offsetting of the metabolic burden conferred by plasmid pH44 on B. terrae BS001 Sm(f) in the native-Fe soil (which was seen in intermediate-Fe and high-Fe soils).
Effect of pHBA on B. terrae BS001

Fig. 2 Effect of plasmid pHBA on the dynamics of B. terrae BS001 Smr at migration spots in native-Fe, intermediate-Fe and high-Fe soil microcosms in the mycosphere of Lyophyllum sp. strain Karsten. Separate inoculations. A. day 1, B. day 4, C. day 7. Similar letters above bars indicate similar statistical classes (P>0.05), letters different between bars indicate significant differences (P<0.05).

Population dynamics of B. terrae strains BS001 Rpr and BS001 Smr (pHB44) in soil with L. sp. strain Karsten at different Fe levels – Joint inoculations
To more closely examine the competition for Fe\textsuperscript{3+} between plasmid-less and plasmid-containing \textit{B. terrae} BS001 strains in soil containing \textit{L. sp.} strain Karsten, the behavior of these strains was assessed upon joint introduction (1:1) into microcosms containing G soil with different levels of Fe. The occurrence of large-scale plasmid transfer – which would thwart the experiment - was ruled out by data from \textit{trfA2}-based colony PCR (colonies picked from putative recipient-selective plates) which showed that the level of initially plasmid-less BS001 Rp\textsuperscript{r} cells that had received plasmid pHB44 \textit{in situ} was consistently below 10\% of the CFU counts (see Materials and methods).

\textbf{Fig. 3} Population dynamics of \textit{B. terrae} BS001 Sm\textsuperscript{r} (pHB44) versus BS001 Rp\textsuperscript{r} in G soil microcosms in the mycosphere of \textit{Lyophyllum} sp. strain Karsten. Joint inoculations. A. Native-Fe soil, B. Intermediate-Fe soil, C. High-Fe soil.
Effect of pHB44 on *B. terrae* BS001

First, in soil without *L.* sp. strain Karsten, both strains BS001 Rp\(^{f}\) and BS001 Sm\(^{f}\) (pHB44) showed similar trends, increasing their population sizes from \(\sim 10^6\) to about \(10^8\) CFU per g dry soil over time. No significant difference in the population densities of the two strains was detected at any time point, irrespective of the Fe content of the soil (data not shown). In the fungus-containing systems, strains BS001 Rp\(^{f}\) and BS001 Sm\(^{f}\) (pHB44) also grew out, revealing a population dynamics that was very akin to that in the separate-inoculation experiment (Fig. 3). In the native-Fe soil, both strains BS001 Rp\(^{f}\) and BS001 Sm\(^{f}\) (pHB44) increased their population densities in the mycosphere gradually over time, from \(\sim 10^6\) to about \(10^7\)-\(5\times 10^7\) CFU per g dry soil. From day 4 onwards, strain BS001 Rp\(^{f}\) showed a consistently higher population density than strain BS001 Sm\(^{f}\) (pHB44), but the difference was only significant at day 14 (\(P<0.05\)) (Fig. 3A). In intermediate-Fe soil, both strains grew out from \(\sim 10^6\) to \(5\times 10^7\) CFU per g dry soil after 7 days. At one occasion (day 14), plasmid-less strain BS001 Rp\(^{f}\) displayed a significantly raised CFU count as compared to strain BS001 Sm\(^{f}\) (pHB44) (\(P<0.05\)) (Fig. 3B). In high-Fe soil, the dynamics of both strains demonstrated a similar trend, with significant differences only being observed at day 14 (\(P<0.05\)) (Fig. 3C). Thus, in an overall sense, plasmid pHB44 revealed a trend to confer an energetic burden onto its host *B. terrae* BS001, since at all time points strain BS001 Rp\(^{f}\) outperformed strain BS001 Sm\(^{f}\) (pHB44), albeit slightly.

The effect of plasmid pHB44 on the migration of *B. terrae* BS001 in soil with *L.* sp. strain Karsten at different Fe levels – Joint inoculations

To assess the effect of plasmid pHB44 on the migration behavior of strain BS001, we compared data from the fungal ‘migration spots’ in the mixed inoculum experiment. First, in the soil without the growing fungus, no bacteria were detected in the (imaginary) migration spots. Then, to validate our data, we checked for the occurrence of plasmid transfer in the putative recipients by *trfA2*-based colony PCR. This analysis revealed no evidence for such transfers being rampant (frequency < 5% per recipient), and hence the population densities of strain BS001 obtained were not biased by high plasmid transfer frequencies. In the native-Fe soil mycelial fronts, strains BS001 Rp\(^{f}\) and BS001 Sm\(^{f}\) (pHB44) were detected at about \(5\times 10^7\) CFU per g dry soil at day 1. After this, strain BS001 Rp\(^{f}\) maintained a density of about \(10^8\) CFU per g dry soil, whereas the population density of BS001 Sm\(^{f}\) (pHB44) declined slightly to \(4\times 10^7\) CFU per g dry soil at day 7. However, there was no significant difference between the population densities of the two strains (\(P>0.05\)) (Fig. 4). The behavior in the soil microcosms amended with Fe was different. At the mycelial fronts of the intermediate-Fe and
Effect of pHB44 on *B. terrae* BS001

High-Fe soils, both strains were also present at 5x10^7 CFU per g dry soil initially. Strain BS001 Rp', with an initial decline to ~10^7 CFU per g dry soil at day 4, recovered to 3x10^8 (intermediate-Fe) and 5x10^7 (high-Fe) CFU per g dry soil at the end of the experiment. However, the population densities of strain BS001 Sm' (pHB44) decreased strongly at both levels of added Fe, to 1x10^6 (intermediate-Fe) and 5x10^5 (high-Fe) CFU per g dry soil. Strain BS001 Rp' thus showed significantly higher cell densities than did strain BS001 Sm' (pHB44) at day 7 (P<0.05) (Fig. 4C). Thus, the data - as from day 7 - suggested that the more iron was present in the soil, the more plasmid pHB44 conferred an ecological disadvantage to its host.
Effect of pHB44 on B. terrae BS001

Fig. 4 Population dynamics of B. terrae BS001 Sm\textsuperscript{r} (pHB44) versus BS001 Rp\textsuperscript{r} at migration spots in native-Fe, intermediate-Fe and high-Fe G soil microcosms in the mycosphere of Lyophyllum sp. strain Karsten. Joint inoculations. A. day 1, B. day 4, C. day 7. Similar letters above bars indicate similar statistical classes (P>0.05), letters different between bars indicate significant differences (P<0.05).

Plasmid transfer from B. terrae BS001 Sm\textsuperscript{r} (pHB44; pSUP104) to V. paradoxus BS64 Rp\textsuperscript{r} in soil with L. sp. strain Karsten at different Fe levels

Inoculation site - As plasmid pHB44 has previously been shown to be able to confer a fitness advantage to its host V. paradoxus BS64 in an iron-limited soil environment (Zhang et al., 2014c), we investigated whether plasmid transfer from B. terrae BS001 to V. paradoxus BS64 would occur in the mycosphere, and whether transconjugants of the latter strain would be selected by iron deficiencies. The results first revealed that, irrespective of the Fe level, plasmid pHB44/pSUP104 transfer from strain BS001 Sm\textsuperscript{r} to BS64 Rp\textsuperscript{r} in soil without L. sp. strain Karsten was undetectable over experimental time (day 1 to 7), thus indicating that such transfers might only occur to a significant extent in soil hot spots such as the mycosphere (data not shown). Indeed, plasmid transfer was detected in soil containing growing mycelium of L. sp. strain Karsten, as specified in the following (Fig. 5A). In native-Fe soil, the transfer frequency was about 2.5x10\textsuperscript{-4} per recipient at day 1, dropping to 4.7x10\textsuperscript{-5} after 4 d and maintaining this level till day 7. In intermediate-Fe soil, the plasmid transfer frequency was 3x10\textsuperscript{-4} (d 1), decreasing to 1.5x10\textsuperscript{-5} after 7 days. In high-Fe soil, pHB44 was transferred to strain BS64 Rp\textsuperscript{r} at 3.6x10\textsuperscript{-4} per recipient at day 1, dropping to 1.6x10\textsuperscript{-5} per recipient at day 4 and 1.1x10\textsuperscript{-5} at day 7. In all cases, the plasmid transfer frequencies showed a trend to being reduced over time, which may relate to the ageing of the fungal mycelium in the inoculation spots. Remarkably, with the increasing amount of iron in the soil, the frequency of successful transfer of plasmid pHB44 tended to decrease over time (Fig. 5A), with a significant difference between the native-Fe and high-Fe soils at d 4 and d 7 (P<0.05). Possibly, under plentiful iron, the selective force for plasmid acquisition and maintenance in the mycosphere was weaker, thus the rate of successful transfer was lower.

At d 7, absolute levels of transconjugants were low (1.5~2.0 log CFU/g dry soil) and similar across different Fe levels (Fig. 5B). To test whether transconjugants would show a response in the soil to a selective growth condition, we added LB supplemented with plasmid pSUP104-selective antibiotics to the soil and determined the population densities of the transconjugants after 7 h. Upon growth in soil under selection, divergent responses were observed: (a) at native-Fe, a significant raise in transconjugant level was seen (P<0.05); (b) at
Effect of pHB44 on B. terrae BS001

intermediate-Fe, level remained similar (no growth/ no death); (c) at high-Fe, the number of transconjugants decreased significantly (P<0.05) (Fig. 5B). This clearly showed the divergent effects of plasmid pHB44 carriage by strain BS64 as related to Fe level in soil, i.e. growth-enhancing at low Fe vs growth-limiting at high Fe. Control soil (without fungus) was processed the same way, and no transconjugants were detected after 7 h, nor after 24 h of incubation.

![Graph A](image)

**Fig. 5A** Transfer frequencies of plasmid pHB44 from B. terrae BS001 Smr to V. paradoxus BS64 Rp in G soil microcosm colonized by *Lyophyllum* sp. strain Karsten at different iron levels (native-Fe, intermediate-Fe and high-Fe). Log-transformed frequencies of transfer are shown.

*B. terrae* BS001 (pHB44; pSUP104) was used, and pSUP104 was tracked using as markers chloramphenicol and tetracycline; presence of pHB44 was confirmed by *trfA2*-based colony PCR.

**Fig. 5B** *in situ* growth of transconjugants under soil condition with native-Fe, intermediate-Fe and high-Fe.

* significantly higher than all other values (P<0.05); 
# significantly lower than all other values (P<0.05).
Effect of pHB44 on B. terrae BS001

Migration site – at the migration site, we never found any transconjugants at d 1, 4 or 7, irrespective of the Fe level in the soil. Moreover, V. paradoxus BS64 CFU were not found, and hence we concluded strain BS64 was not migrating with strain BS001 along the fungal hyphae in soil.

Discussion

Soil is often ‘limited’ with respect to providing easily-available nutrient sources to its microbial occupants, the reason being the ‘locking’ of such nutrients in microhabitats that are unreachable to much of the soil bacteriota (van Elsas et al., 2007). The mycosphere constitutes a so-called ‘hot spot for enhanced activity’ in soil, in which nutrients may become increasingly accessible and thus bacterial activities can be enhanced (Warmink and van Elsas, 2008). However, microbial activities in the mycosphere occur in various ways, and one may assume that growth runs into limitations by ‘bottleneck’ compounds. Next to carbon, such a bottleneck compound may well be iron. Recently, the mycosphere has also been pinpointed as a prime arena for horizontal gene transfer (Zhang et al., 2014a). In this habitat, locally-selected genes are presumed to be transferred between bacterial populations, thus stimulating the fitness of the latter and accelerating evolution in soil. Plasmids are prime vehicles that spur such gene transfers. In particular, IncP-1β plasmids may perform such roles, as they are more prevalent in the mycosphere than in the corresponding bulk soil (Zhang et al., 2014b). The IncP-1β plasmid pHB44, originally found in V. paradoxus HB44 (isolated from the mycosphere of L. proxima), has been shown to enhance the fitness of another V. paradoxus strain, BS64, in soil microcosms containing L. sp. Strain Karsten under iron limitation (Zhang et al., 2014c).

Burkholderia terrae BS001 has been isolated as a prime responder to the fruiting bodies of Laccaria proxima and its genome has revealed the presence of a plethora of fungal-interactive systems (Haq et al., 2014). To better understand the role of plasmid pHB44 as a putative enhancer of the iron uptake capacities of mycosphere dwellers, in the current study this plasmid was moved into Burkholderia terrae BS001. To further explore the putative selective force exerted by soil Fe levels on plasmid transfer and maintenance in the mycosphere, we also monitored the transfer of plasmid pHB44 between B. terrae BS001 and V. paradoxus BS64.
The collective results obtained by us indicated that plasmid pHB44 has two possible effects on its (novel) host *B. terrae* BS001, which is related to the iron content of the soil (being ‘native’ [limiting], intermediate or plentiful). On the one hand, plasmid pHB44 was observed to confer an ecological disadvantage, decreasing the fitness of its host *B. terrae* BS001, mostly under conditions of plentiful supply of Fe. On the other hand, the plasmid energetic ‘burden’ imposed on the host was nullified under conditions of (presumed) Fe scarcity, such as in the native soil with the bacterium accompanying the fungal exploration of the soil habitat. Haq et al. (2014) provided evidence that the 11.5 Mb five-replicon genome of *B. terrae* BS001 already contains potential iron-uptake systems. Thus, the native Fe capturing system of strain BS001 may allow for sufficient influx of Fe under most conditions in the mycosphere, allowing the plasmid to be ‘superfluous’ and thus imposing a true burden on the cellular energy reserves of the host. However, in highly competitive situations (possibly linked to Fe levels being extremely limited) the presence of plasmid pHB44 might be of assistance to the host, as shown by us for the situation at the fungal hyphal fronts at d 7 (Fig. 2 and 4). Thus plasmid-containing strain BS001 Sm<sup>T</sup> (pHB44) might be expected to grow slower than the plasmid-less strain BS001 Rp<sup>T</sup> in cases of non-limiting iron, but such an effect is obscured in situations where iron is limiting. In the ‘native-iron’ mycosphere soil habitats, plasmid pHB44 carriage may thus have conferred a relative advantage, as it may assist strain BS001 to, possibly more efficiently, capture iron from the environment. This putative Fe-rescuing activity might be at the basis of the ‘recovery’ of BS001 Sm<sup>T</sup> (pHB44) cells, coming up to the level of BS001 Rp<sup>T</sup> in the ‘native-Fe’ soil at the fungal migration spots after 7 days.

Remarkably, transfer of plasmid pHB44 from the *B. terrae* BS001 into the *V. paradoxus* BS64 derivatives was found to occur at detectable levels in the L. sp. strain Karsten mycosphere soil, whereas any evidence for such transfers in bulk soil was lacking. This finding is consistent with the notion that the mycosphere constitutes an arena in soil where genetic interconnections are stimulated (Zhang et al., 2014a). Previous work, in which exogenous plasmid isolations were used, also revealed plasmid transfer frequencies to be significantly raised with mycosphere communities as compared with systems with bulk soil ones (Zhang et al., 2014b). We posit that the mycosphere offers conditions of enhanced carbon supply as well as cell-to-cell contact possibilities, stimulating locally grown bacteria and thus potentially enhancing conjugational gene transfers. The data also pinpointed pHB44 as a plasmid that can transfer across diverse species in the mycosphere habitat. Broad-host-range transfer and replication is indeed a major characteristic of IncP-1β plasmids, however
the degree to which successful plasmid transfer and spread across natural bacterial communities occur naturally is as yet unknown.

The effect of the soil Fe level on successful plasmid transfers was striking. Under plentiful iron, the rate of selection of plasmid pHB44 bearing cells might have been lower than under conditions of Fe scarcity, pointing to a role of a plasmid-borne Fe capturing system. Thus, such a system was a strong driver of bacterial plasmid acquisition and maintenance, being detectable only under Fe-limited conditions. The fact that the difference in transfer frequency in the mycosphere between the native- and iron-amended soil became stronger at day 7 than at day 1 further demonstrated that, with the ageing of fungal hyphae, the environmental conditions become harsher, spurring successful plasmid transfer and/or maintenance.

The differential in situ outgrowth of transconjugants - with significantly higher levels in the native-Fe soil than in the intermediate- and high-Fe soils – confirmed the role of Fe level as an in situ selector of plasmid pHB44. Moreover, the growth of fungal hyphae plays an important role, akin to a two-sided sword, for the local bacterial communities, as it, on the one hand, can enhance the ecological opportunities for fungal associates, and, on the other hand, stimulate adaptive plasmid transfers for these due to the creation of highly selective (iron-limited) conditions (Zhang et al., 2014a).

This study showed - for the first time – that a plasmid such as pHB44, when present in a new host, i.e. B. terrae BS001, can offer particular benefits to this novel host under particular conditions in the mycosphere (Fe limitation). However, under other conditions, plasmid pHB44 clearly imposes a metabolic burden on the BS001 cells. Another remarkable finding was that successful transfer of plasmid pHB44 to a taxonomically remote host did occur in the mycosphere, and more frequently so in native-Fe than in plentiful-Fe habitats. This finding highlighted both the propensity of the mycosphere as a gene transfer arena and the intricacies of the selective forces in this habitat modulating the carriage of selective agents (such as plasmid pHB44) that exert two divergent ecological forces on their hosts.

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References


Effect of pHB44 on *B. terrae* BS001

