Mechanisms of migration of Paraburkholderia terrae BS001 in the mycosphere

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Chapter 5

Influence of soil pH and moisture on the co-migration of *Paraburkholderia terrae* BS001 with *Lyophyllum* sp. strain Karsten in soil microcosms

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Abstract

The movement of bacterial cells along with fungal hyphae in soil (the mycosphere) has been reported in several previous studies. However, how local soil conditions affect bacterial motility in the mycosphere has not been extensively studied. Here, we investigated the influence of two soil parameters, pH and soil moisture content, on the survival, and migration, of *Paraburkholderia terrae* BS001 in the mycosphere of *Lyophyllum* sp. strain Karsten in microcosms containing a loamy sand soil obtained from Buinen (B soil). The data showed that low soil pH strongly restricted bacterial survival in the mycosphere. Remarkably, bacterial movement along the hyphal networks took place in both the “forward” and the “backward” direction in the B soil. The backward movement was weakly correlated with the amount of fungal tissue formed in the old mycelial network. Progressively lower soil pH reduced the bacterial populations that had moved, both in the forward and backward direction. The initial soil moisture content, in the range 12-17% (corresponding to 42-60% of water holding capacity), also significantly affected the bacterial population sizes along the fungal hyphae. Overall, the fungal hyphae were found to increase soil pH (under conditions of acidity), which possibly exerted protective effects on the bacterial cells.
5.1 Introduction

Soil pH and soil moisture levels are key parameters that affect soil biological activities. For instance, soil pH has been found to affect microbial community structures and microbial growth (Rousk et al., 2009; Shen et al., 2013; Zhalnina et al., 2015), steps of the nitrogen cycle (Cheng et al., 2013; Che et al., 2015), and the bioavailability and toxicity of heavy metals (Houben et al., 2013; Rees et al., 2014; Fernandez-Calvino and Baath, 2016). Also, soil moisture levels are often important determinants of bacterial activities (Stark and Firestone, 1995; Brockett et al., 2012; Liu et al., 2017), as well as community structures (Brokett et al., 2012; Shen et al., 2013). Effects on nutrient diffusion and intracellular water potential were invoked as drivers (Stark and Firestone, 1995).

Bacterial migration along with mycelium-forming organisms in soil has been extensively studied in recent years. *Pseudomonas putida* PpG7 was reported to be able to disperse along with the oomycete *Pythium ultimum* in soil, reaching sites in soil that were contaminated with phenanthrene (Wick et al., 2007). In another study, it was found that the saprotrophic fungus *Lyophyllum* sp. strain Karsten mediates the movement of *Paraburkholderia terrae* BS001 (Warmink and van Elsas, 2009) as well as several other *Paraburkholderia* strains (Nazir et al., 2012) in soil. In this context, we recently affirmed that *P. terrae* BS001 critically relies on flagellum-driven swimming motility for its dispersal along with fungal hyphae (Yang et al., 2017). In contrast, the presence of a type three secretion system (T3SS) or of type four pili (T4P) was not critical to bacterial co-migration, as these systems merely enhanced the flagellar-driven dispersion along fungal hyphae (Yang et al., 2016; 2017).

Bacterial (flagellar) motility along surfaces is affected by key local conditions. First, the hydration status of the surface is an essential driver. Thus, wet environments, in which sufficiently thick water films occur on local surfaces, were found to facilitate bacterial movement (Dechesne et al., 2010; Worrich et al., 2016). Second, the pH at surface microsites may be crucial. As flagellar motility has been reported to be driven by the proton-motive or sodium-motive force (Blair, 2003; Minamino and Imada, 2015), bacterial translocation may be spurred (in semi-solid agar) by pH decreases,
as shown in recent reports (Maurer et al., 2005; Yang et al., 2017). On another notice, factors that drive chemotaxis (e.g. particular fungal exudates) were also found to modulate bacterial motility (Furuno et al., 2010; Haq, 2016), with an abundant local nutrient supply suppressing such movement (Banitz et al., 2012).

Most of the aforementioned studies were conducted under laboratory conditions, using artificial media. Indeed, how soil pH and moisture content influence the degree of bacterial motility along mycelial networks has not been well elucidated yet. In this study, we hypothesized that these factors critically influence bacterial flagellar movement along with fungal hyphae through soil. In particular, we reasoned that lower soil pH might spur proton-motive force based flagellar movement. In contrast, soil pH might also act as a factor that limits bacterial survival or fitness. Therefore, we surmised that a critical balance exists between (positive) soil pH effects on proton-motive-force-driven motility and those (negative) on bacterial fitness. We thus investigated the potential relationship of soil pH with bacterial cell motility in the mycosphere using a well-established three-compartment Petri dish soil microcosm (Warmink and van Elsas, 2009; Yang et al., 2016; 2017).

5.2 Materials and methods

5.2.1 Strains and cultural conditions

*P. terrae* BS001 wild-type and mutant strains were used in this study. All strains were cultured at 28 °C in LB broth (Sigma-Aldrich Co., USA), with shaking, or on R2A agar (Difco, USA). The wild-type strain was isolated from the mycosphere (Warmink and van Elsas, 2008). Three mutant strains were derived from it. These were: *P. terrae* BS001\ΔsctD [mutant in the type three secretion system (T3SS), Yang et al., 2016], *P. terrae* BS001\ΔpilN [mutant in type four pili (T4P), Yang et al., 2017] and *P. terrae* BS001\ΔfliF [mutant in the flagellar system, Yang et al., 2017]. The fungal host used in this study was *Lyophyllum sp.* strain Karsten (DSM2979). It was grown on oat flake agar (OFA, 30 g/L oat flake, 15 g/L agar, Warmink and van Elsas, 2009) at 28 °C. Once every four weeks, the fungal strain was transferred to fresh OFA for maintenance.
5.2.2 Soil microcosms

For all experiments, soil from Buinen, the Netherlands, was used (denoted B soil). The B soil was characterized as a loamy sand soil, with pH 5.3 and organic matter content of 3.8% (Nazir et al., 2012). For some experiments, the soil was adjusted to pH 4.6, pH 4.2 and pH 3.8 by adding different amounts of 0.2 mol/L H$_2$SO$_4$. Then, it was autoclaved (121 °C, 27 min) for three times, with intermittent incubation at room temperature. The maintenance of the different pH values was confirmed following the last autoclaving before incubation. Soil microcosms were then prepared aseptically, in three-compartment Petri dishes according to the previous study (Warmink and van Elsas, 2009; Yang et al., 2016; 2017). Briefly, one of the compartments was filled with OFA and the other two with sterilized B soil [adjust soil moisture to 12% or 17%, corresponding to 42% or 60% of water holding capacity (WHC)]. Then, OFA plugs containing fungal growth were placed in the OFA compartment, and the system was incubated. Following incubation, with fungal growth reaching up to 20 mm (or 30 mm) into the soil part (28 °C), bacterial cells were introduced at the tip of fungal growth, or at the middle of the fungal hyphae. In one experimental set (set 1), when the tip of fungal growth reached 20 mm into the soil, the bacterial cells were introduced at the tip. In the other experimental set (set 2), when the tip of fungal growth reached 30 mm away from the barrier, the bacterial cells were introduced 20 mm away from the barrier in the hyphae (10 mm away from the tip of fungal growth). The wild-type and mutant strains were introduced separately, and systems were incubated at 28 °C. At set times, soil samples were recovered from introduction, backward and forward sites (4, 7 and 15 days after bacterial inoculation). The samples were suspended in water, shaken intensely (1 min, three times, with 30 s intervals), serially diluted and spread on R2A agar plates. Following incubation of the plates, colonies were enumerated and CFU numbers per gram soil were calculated. For each experiment, 3 replicates were used.

To assess the *P. terrae* BS001 population dynamics in the B soil, fresh washed suspensions of all strains (wild-type, BS001ΔsctD, BS001ΔpilN and BS001ΔfliF) were introduced respectively into the microcosm without fungal hyphae, and population densities in the soil microcosm were monitored over time.
5.2.3 Measurement of length of fungal hyphae in soil microcosms

The density of fungal hyphae in soil was measured according to the method described by Shen et al. (Shen et al., 2016). Briefly, the soil was sampled at backward section and forward section at 1 cm by 2 cm. Then, soil samples were homogenized in solution. And the aqueous extracted fungal hyphae were examined under microscopy. The fungal hyphal density was measured first time when the fungal hyphal tip reached 2 cm away from the barrier (day 0), and 4, 7, 15 days after that.

5.2.4 Statistical analysis of the data

All data obtained were subjected to analysis of variance (ANOVA). Also, unpaired two-tailed t-tests were performed in cases two treatments were compared. Differences of the means were considered to be significant at \( P<0.05 \). Also, all the data were analyzed by Classification and Regression Tree (CART).

5.3 Results

In a previous paper, we showed that migration of \( P. \ terrae \) BS001 with soil-exploring hyphae of \( Lyophyllum \) sp. strain Karsten in an acid (pH 4.1-4.5) soil, denoted G, was only detectable in the fungal growth direction (Warmink and van Elsas, 2009). Moreover, cells that did not live in the vicinity of the fungal hyphal network in soil were found to progressively lose viability. Here, we extend this work, using another soil (a loamy sand soil taken from an agricultural field denoted B, with native pH of 5.3), in microcosms. To understand the contribution of the flagellar, the T3SS and the T4P systems to the survival and movement of strain BS001, we included, next to the wild-type, the respective mutant strains of these systems (denoted BS001\( \Delta fliF \), BS001\( \Delta sctD \) and BS001\( \Delta pilN \), see Yang et al., 2016; 2017) in all experiments. The inclusion of the mutants was justified based on previous indications that pH affects the expression and/or function of the T3SS (Rappl et al., 2003; Jitprasutwit et al., 2010; Rogge and Thune, 2011) and T4P/twitching motility (Bakkali, 2013; Kurre et al., 2013), whereas the
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flagellum is essential for strain BS001 migration in the mycosphere (Yang et al., 2017). The cells were inoculated at the tip (experiment set 1) or middle (experiment set 2) of the fungal growth in the soil compartment (introduction site, see above 5.2.2). Following incubation of the systems, these were sampled at two sites away from the introduction site, coined the “forward” and “backward” migration sites.

5.3.1 Population dynamics of *P. terrae* BS001 in bulk B soil and in the mycosphere

The data showed the cell population sizes to decrease progressively with time, irrespective of strain type in B soil in the absent of fungal hyphae. The populations reached the detection limit, of 24 CFU/g dry soil, at day 7 in native soil (Figure S5.1). Moreover, introduced cell lost their cultural ability within 2 days in the soil at pH 4.6 (data not shown) and within 1 day in the soil at lower pH (pH 4.2 and 3.8, data not shown). Thus, remarkably, the B soil did not support the survival of BS001, and progressive lowering of pH induced faster decline rates. In contrast, in the presence of fungal hyphae, all bacterial strains survived and, on occasion, even grew at the introduction sites (Figure 5.1).

A full factorial ANOVA performed on all data on the population dynamics at the introduction sites revealed that soil moisture (F=1.073, *P* > 0.1) and strain type (F=0.276, *P* > 0.1) did not significantly influence bacterial survival in soil (Table S5.1). In contrast, (initial) soil pH was shown to be a strong determinant of bacterial survival (F=1063.807, *P* < 0.0001), whereas introduction site (tip versus middle of fungal hyphae) was the second important factor (F=79.437, *P* < 0.0001). Time also drove the abundance of CFUs at the introduction sites (F=16.968, *P* < 0.0001). The relative effects of the different factors were also analyzed by CART and are presented in Figure S5.2 and Table S5.1. For more details in survival of all the bacterial strains in mycosphere, please see Figure S5.3-S5.6.

A closer look at the effect of initial soil pH revealed strain BS001 to survive and even grow in the mycospheres in the pH-5.3 and pH-4.6 soils (Figure 5.1). In contrast, its dynamics in the mycosphere in the pH-4.2 soil was erratic, with cell abundances decreasing at days 4 and 7 and increasing later (day 15, Figure 5.1). Moreover, the population dynamics in the
mycosphere at pH-3.8 soil clearly depended on introduction site (Figure 5.1), with poor survival already after four days (tip introduction, Figure 5.1a, 5.1c) versus survival until day 15 (middle introduction, Figure 5.1b, 5.1d), even though cell abundances decreased, with one exception, strongly and significantly ($P<0.05$) over 15 days (Figure 5.1b, 5.1d).

**Figure 5.1** Survival of *P. terrae* BS001 wild-type in mycosphere. (a) Sampled from the mycosphere with 60% WHC and tip introduction; (b) sampled from the mycosphere with 60% WHC and middle introduction; (c) sampled from the mycosphere with 42% WHC and tip introduction; (d) sampled from the mycosphere with 42% WHC and middle introduction


### 5.3.2 Effects of the presence of fungal hyphae on soil pH

In the absence of fungal hyphae, the pH of the soil in the microcosms did not change over the time of the experiment ($P>0.05$, Figure S5.7). In contrast, soil pH was raised, in all cases, in the presence of fungal hyphae, both at the introduction and migration sites ($P<0.05$, Figure 5.2). For instance, the native-pH soil revealed a pH shifting from initially $5.25\pm0.05$ (bulk soil) and $5.41\pm0.09$ (experiment set 1, introduction site) to $5.95\pm0.02$ at day 15 ($P<0.01$, Figure 5.2a), or from $5.50\pm0.03$ (introduction site, experiment set 2) to $5.98\pm0.02$ ($P<0.001$, Figure 5.2e). Similar results of progressively increasing soil pHs were found for the other soil microcosms with pH set at 4.6, 4.2 and 3.8 (Figure 5.2b-d, f-h). In these, soil pH increases from $4.56\pm0.01$ to $5.29\pm0.10$, from $4.15\pm0.01$ to $4.75\pm0.05$ and from $3.83\pm0.03$ to $4.34\pm0.01$ were found, respectively.

### 5.3.3 Migration of P. terrae BS001 in the B soil mycosphere

In the native-pH soil microcosm (pH-5.3), movement of the flagellar mutant (BS001ΔfliF) along with the growing *Lyophyllum* sp. strain Karsten hyphae was never detected. This confirmed the key relevance of a functional flagellar apparatus for migration. In subsequent work, we thus used this mutant as the negative control of migration. In contrast, all flagellum-positive strains (wild-type strain, BS001ΔsctD and BS001ΔpilN) were found to translocate in the canonical growth direction (forward) of the developing hyphae, reaching similar elevated population densities at the hyphal fronts (Figure 5.3c). Very surprisingly, consistent migration of these strains in the backward direction (against the direction of fungal growth) was also noticed in these systems (Figure 5.3a).

Starting from the premise that soil pH will affect the forward/backward migration with fungal hyphae (next to affecting bacterial fitness), we then tested the strain BS001 migrational behavior with *Lyophyllum* sp. strain Karsten hyphae in the B soil at different pH values (at both 42% and 60% of WHC). The overall ANOVA of the CFU counts (Figure S5.3-S5.5) showed, for all three strains, an absence of differences between the CFU numbers of
Figure 5.2 pH changes along with fungal growth. Top row, mimic the condition that bacterial cells were inoculated at the tip of fungal growth; bottom row, mimic the condition that bacterial cells were inoculated at the middle of the fungal growth; (a), (e) native soil (pH 5.3); (b), (f) soil at pH 4.6; (c), (g) soil at pH 4.2; (d), (h) soil at pH 3.8.
the three different strains at the forward versus backward migration sites (F=1.632, P>0.05). Thus the \( \Delta scTD \) or \( \Delta pilN \) mutations did not affect single-strain migrational behavior as compared to that of the wild-type strain (F=0.543, P>0.05). The ANOVA further affirmed that soil pH was the main effector of the bacterial population densities resulting from migration in the mycosphere (F=2179.117, P<0.0001). Other drivers of these densities were time (F=155.202, P<0.0001), soil moisture content (F=24.764, P<0.0001) and introduction site (F=14.589, P<0.001). In contrast, sampling site (forward/backward) was unimportant. Similarly, CART analysis revealed (Figure S5.2b, Table S5.1) soil pH to drive the first split and time the second. Figure 5.3 shows only the data of wild-type strain. For more details in migration of all the bacterial strains (wild-type and three mutant strains) in mycosphere, please see Figure S5.3-S5.5.

The analyses of the data further revealed that bacterial cells reached the migration sites (forward or backward) in the pH-4.6 soil (Figure 5.3) later than in the native-pH soil (Figure 5.3). Moreover, the BS001 population densities at the migration sites were greatly restricted in the soil at pH-4.2 and pH-3.8 (Figure 5.3). In the pH-4.2 soil, poor migration was noted in some of the cases (Figure 5.3a, 5.3b, 5.3d, 5.3f), whereas a population resulting from migration was not found in the pH-3.8 soil.

Time as a driver of the BS001 population dynamics in the mycosphere was particularly relevant for the pH-4.6 (Figure 5.3) and pH-4.2 (Figure 5.3) soils. Clearly, in pH-4.6 soil, the population sizes were positively related to time under these conditions. Thus, whereas bacteria were detected early-on in the native-pH mycosphere (day 4, Figure 5.3), they were only detected later in both low-pH mycospheres (day 7 to day 15 in the pH-4.6 and 4.2 mycospheres, Figure 5.3).

Additionally, soil moisture level affected bacterial migration in particular cases. In the native-pH (5.3) soils with soil moisture at 42% of WHC, migration was significantly (P<0.05) retarded as compared to 60% WHC systems (tip introduction; Figure 5.3a, 5.3e). In the pH-4.6 soils, the forward sites were reached earlier in soil with moisture level at 60% of WHC than in that with moisture at 42% WHC [day 4 (Figure 5.3c, 5.3d) vs day 15 (Figure 5.3g, 5.3h)].
Figure 5.3 Migration of *P. terrae* BS001 wild-type in mycosphere. Left column, tip introduction, right column, middle introduction. (a), (b) sampled at backward site in the soil microcosm with 60% WHC; (c), (d) sampled at forward site in the soil microcosm with 60% WHC; (e), (f) sampled at backward site in the soil microcosm with 42% WHC; (g), (h) sampled at forward site in the microcosm with 42% WHC.
5.3.4 Dynamics of development of hyphal density in soil (forward and backward)

Importantly, in native-pH soil (pH-5.3), the hyphal density in the backward direction increased from 8.39±3.53 (m/g dry soil) at day 0 to 26.52±9.01 (m/g dry soil) at day 4, after which it progressively decreased (Figure 5.4a). This indicated the formation of novel fungal tissue over time in the older hyphal parts. However, in soils with progressively-lower pH (4.6, 4.2 and 3.8), the hyphal densities were already high at the early phases (day 0) of the experiment (50.69±22.49 m/g dry soil at pH 4.6, 28.27±7.99 m/g dry soil at pH 4.2 and 26.65±21.87 m/g dry soil at pH 3.8). These densities decreased progressively at the later sampling times ($P > 0.05$, Figure 5.4a). In contrast, at the forward site, hyphal densities increased from day 0 to day 7 in native-pH as well as pH-3.8 soil (Figure 5.4b). In the pH-4.6 and pH-4.2 soils, the respective hyphal densities increased until day 4, after which they fluctuated slightly (Figure 5.4b).

5.3.5 Hyphal density in soil, pH alteration and bacterial movement

As described above, the presence of fungal hyphae was essential for soil pH alteration. Moreover, it spurred bacterial survival as well as movement in soil. An overall analysis revealed no positive or negative relationships between the soil hyphal density values and soil pH changes (pH at specific time points vs initial pH values, Figure S5.8). Remarkably, when the “backward site” bacterial CFU data (at pH values 4.6 and 4.2) were compared with the (previous) fungal biomass measurements (backward), a weak correlation was found ($R^2 = 0.21$, Figure S5.9), providing evidence for the contention that a higher density of young hyphae was consistent with enhanced bacterial motility in these low-pH soils.

5.4 Discussion

Migration of bacteria along with fungal hyphae has received increased focus in recent years, using artificial conditions or soil microcosms. On agar surfaces, *Pseudomonas putida* KT2440 was found to disperse along with the
hyphal network of *Morchella crassipes* via flagellar-driven motility (Pion et al., 2013). Migration of *Achromobacter* sp. SK1 along with hydrophilic *Fusarium oxysporum* Fo47, allowing to cross air gaps was also reported (Kohlmeier et al., 2005). In soil, translocation of *Pseudomonas putida* PpG7 along with mycelium of the oomycete *Pythium ultimum* was reported (Wick et al., 2007). In our lab, migration of *Paraburkholderia* spp. along with

**Figure 5.4** Fungal hyphal density in the soil. (a) backward section; (b) forward section.
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growing fungal hyphae in soil has been reported in several papers (Warmink and van Elsas, 2009; Nazir et al., 2012; 2014). In another work, it was found that the soil fungus *Trichoderma* sp. even mediated the movement of eight bacterial types over an air gap between two agar surfaces (Bravo et al., 2013). However, although we now understand that particular bacteria in soil make use of the networks provided by soil fungi, how soil pH and moisture affect the translocation of such bacteria along these hyphae has not been elucidated yet. Clearly, the bacterial population size measurements made by us, as CFU’s per g soil, per site, were the net result of bacterial migration, survival, growth and death. We therefore first address the impact of soil pH, soil moisture and the presence of fungal hyphae on bacterial population densities.

5.4.1 Bacterial survival is impaired in B bulk soil and fostered by the presence of fungal hyphae

In the current study, all BS001 strains tested revealed impaired survival in the pre-sterilized B soil without fungal mycelium. This rather surprising observation indicated that the sterilization process (three-fold autoclaving) incited conditions in the soil that may be, in an unknown manner, restrictive to incoming populations. This may include carbon limitation, irrespective of the carbon released by the sterilization process. Bacterial survival in soil is determined by factors such as soil textural type, soil nutrient availability, soil moisture, soil pH, soil pore size distribution, and the presence of toxic chemicals (van Veen et al., 1997; Inglis and Sagripanti, 2006). Clearly, the presence of *Lyophyllum* sp. strain Karsten protected strain BS001 from potentially hostile conditions in the B soil, much like previously found for the G soil. Although we lack evidence for this, it is possible that the fungus, by providing nutrients such as glycerol and oxalate (Nazir et al., 2013; Haq, 2016), furnished energy to the inoculant cells which subsequently were able to better establish and survive in the system.

5.4.2 Bacterial migration with fungal hyphae detected in the “backward” direction

In a previous study in our laboratory, movement of strain BS001 against the fungal growth direction (backward) could never be detected in the low-pH
Gieterveen soil (G soil, pH 4.1-4.5, Warmink and van Elsas, 2009; Nazir et al., 2012; 2014). Hence, we surmised that the presence of young, actively-growing fungal mycelium is a prerequisite for strain BS001 movement along with the hyphae. Here, to our surprise, we found that all flagellated forms of *P. terrae* BS001 were able to migrate (in addition to the growth direction) in the non-growth direction of *Lyophyllum* sp. strain Karsten in the B soil, whereas the non-flagellated mutant was not. This occurred at (initial) soil pH values as low as 4.2. Previous data have shown that flagellar motility is isotropic on general agar media (Yang et al., 2017) but tropic in the presence of fungal hyphae (Haq et al., 2016). We thus surmised that migration in the mycosphere in soil microcosms was, in this case, either uncoupled from the hypothesized fungal tip tropism (Warmink and van Elsas, 2009), or that the development of new mycelia in soil continued in the backward direction. Supportive of the latter is the finding that fungal density increased at the backward site from day 0 to day 4 in the pH-5.3 soil (Figure 5.4a), allowing a degree of tip tropism. Moreover, our trend analysis revealed a correlation (albeit weak) between backward migration and fungal hyphal density. Consistent with findings by Haq et al, the migration might be mediated by chemotaxis towards glycerol or oxalate (Furuno et al., 2010; Haq et al., 2016). Alternatively, the movement in the “backward” direction might have had another directional driver, including old/senescent hyphae leaking nutrients. In previous work by others, movement at old fungal hyphae has been reported for *Achromobacter* sp. SK1 in its interaction with *Fusarium oxysporum* Fo47 (Kohlmeier et al., 2005). In an ecological sense, the capability to move to both directions along fungal hyphae extends the suite of colonizable niches in a fungal network in soil, potentially enhancing the overall bacterial population fitness.

### 5.4.3 Role of flagella, T3SS and T4P in bacterial migration in the mycosphere

In previous work, the T3SS has been shown to be related to the attachment of BS001 to fungal hyphae (Haq et al., 2016), whereas T4P have been related to twitching motility (Yang et al., 2017). Recent work in our lab demonstrates that both systems enhance, but are not essential for, the bacterial migration along with fungal hyphae (Yang et al., 2016; 2017). In
contrast, the presence of functional flagella was found to be essential (Yang et al., 2017), which was here confirmed and extended to migration against the canonical growth direction of the fungal soil colonizer. Given that local pH can regulate the expression/function of the T3SS and T4P (Rappl et al., 2003; Jitprasutwit et al., 2010; Kurre et al., 2013), we here included the respective mutant strains to examine whether these systems would show any major effect in the soil pH range tested here. Overall, and in concordance with the previous study (Yang et al., 2016; 2017), both mutant strains showed patterns of single-strain migration in the mycosphere that were akin to those of wild-type strain BS001, indicating that the T3SS and T4P do not have major roles in the migrational/survival responses in the mycosphere. Overall, the roles of the T3SS, T4P and flagella in strain BS001 migration with fungal hyphae were consistent in the different B soils pre-set at various soil pH values.

5.4.4 The size of the translocated populations is inversely related to soil pH

The finding that the relationship between the bacterial densities at the migration sites and the soil pH was inverse to our expectations, was interesting. A first striking observation was that, for all initial soil pH values, the pH in the mycosphere increased along with fungal growth in the B soil. This was consistent with findings from an earlier study in another soil (G soil, Nazir et al., 2010), and suggested that the hyphae of *Lyophyllum* sp. strain Karsten secrete compounds into the surrounding soil that raise the pH (Nazir et al., 2010). However, in all cases mere upward soil pH shifts were noted, rather than completely changed patterns.

An observation on semi-solid agar revealed that *P. terrae* BS001 exhibits stronger swimming at lower (5.2) than at higher pH levels (6.0, 6.8 and 7.5). This was attributed to the fact that the motor proteins, that drive flagellar movement, are powered by the proton motive force (Yang et al., 2017). However, in the presence of fungal hyphae in soil, bacterial cells reached the migration sites (forward and backward) generally later at the lower than at the higher pre-set pH values. Two processes, which work in an opposite fashion in terms of pH dependency, might have driven the observed population densities, i.e. (1) general pH-driven toxicity and (2) the proton-
motive force. With respect to general pH toxicity, at pH<5.0, the proton level may have been deleterious to strain BS001 cells, thus reducing overall survival. In soil with lower pH values, BS001 cells may therefore have shown enhanced death (and overall reduced growth) rates (or even no growth in the mycosphere with pH-3.8 soil) as compared to soil at higher pH. Thus, the events that drive the cell densities at the introduction sites likely strongly influenced those found at the corresponding migration sites (Figure S5.10). Apparently, pH toxicity affected strain BS001 survival directly and, via this effect, migration along with fungal hyphae. Although at low soil pH the proton-motive force may have been high, the resulting pH toxicity may have restricted bacterial cell densities to a too large extent, thus overriding the proton motive force effect.

5.4.5 Model that explains bacterial migration patterns with fungal hyphae

To explain our observations discussed in the foregoing, we here propose a model in which the final strain BS001 population densities in soil are strongly determined by the very events that take place directly following introduction. Thus, the cells of strain BS001, upon introduction into the mycosphere, immediately perceive and respond to the local conditions. A key immediate response is the allocation of energy (obtained from the local environment in which the fungal counterpart is a major component) to physiological processes that foster local establishment, survival and possible growth. Motility may be inhibited in this case. The initial events thus may allow the inoculant population to establish, to some degree, viable adapted cell populations at the introduction site(s). As time progresses, the cell population may grow, which restricts the overall energy supply per cell, and so a cue may appear in the population that activates flagellar mediated translocation of cells along with the fungal hyphae. The final outcome would be the persistence of a population at maximal cell density at the local introduction site, along with the spread of “explorer cells” along with the fungal hyphae to novel microhabitats. This is followed by the subsequent establishment, and outgrowth, of a population at the latter. Thus, the motility of such explorer BS001 cells in the mycosphere may be seen as a behavioral response to initial crowding in the mycosphere.
5.4.6 The effect of water content on bacterial migration with fungal hyphae

Water content can also influence bacterial dynamics and motility in soil. Bradyrhizobium japonicum was found to disperse up to 7 mm in soil at high water content (80-100% of WHC) via flagellar-driven motility (Covelli et al., 2013). However, the motility of other bacteria has been shown to be restricted in bulk soil at lower soil moisture (Warmink and van Elsas, 2009; Wolf et al., 2013). In the current study, and consistent with a previous one, strain BS001 did not migrate in soil without fungal hyphae (Warmink and van Elsas, 2009). The presence of fungal hyphae can indeed bridge soil particle aggregates, with the presence of water films around these facilitating migration. Possibly, a thicker water film was present around fungal hyphae in the soil with higher water content, allowing enhanced strain BS001 swimming. Thus, soil water content also played an important role in bacterial migration.

5.5 Conclusion

In conclusion, this study confirmed that flagellated cells of P. terrae strain BS001 can move along with fungal hyphae through a loamy sand (B) soil forward to fungal growth tips. In addition, we provide evidence for the contention that movement against the fungal growth direction also takes place in the B soil mycosphere. Soil pH was found to exert a strong negative effect on bacterial survival and motility in the mycosphere, whereas soil moisture, in the range 42%-60% of WHC had weaker, yet significant, effects. The presence of fungal hyphae provided protection to the introduced BS001 populations for survival, potentially by alleviating pH stress, and a “highway” for bacterial cells to migrate. The migrational behavior was likely driven by nutrient availability. Bacterial migration in the backward direction was weakly related to recently produced fungal mycelium, but not to pH alteration in the soil.
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Influence of soil pH and moisture


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Supplementary information

**Figure S5.1** Bacterial cell abundance in native bulk soil without fungal hyphae.
Figure S5.2 CART analysis of the factors influencing cell survival (a, data at inoculation site) and migration (b, data from both migration backward and forward). The pruned tree at (a) explained 92.62% of the variance in cell survival. Of this, 61.23% was accounted for by the first split, and 12.27% by the second split. The pruned tree at (b) explained 77.38% of the variance in cell migration. Of this, 56.23% was explained by the first split, and 10.85% by the second split.
Figure S5.3 Cell abundance in the microcosm with native soil (pH 5.3). Left two columns (a, b, e, f, i, j), 17% soil moisture; right two columns (c, d, g, h, k, l), 12% water moisture; 1st and 3rd columns (a, c, e, g, i, k), introduced at the tip of fungal growth; 2nd and 4th columns (b, d, f, h, j, l), introduced at the middle of fungal growth; top row (a, b, c, d), backward migration site; middle row (e, f, g, h), introduction site; bottom row (i, j, k, l), forward migration site.
Figure S5.4 Cell abundance in the microcosm with soil at pH 4.6. Left two columns (a, b, e, f, i, j), 17% soil moisture; right two columns (c, d, g, h, k, l), 12% water moisture; 1st and 3rd columns (a, c, e, g, i, k), introduced at the tip of fungal growth; 2nd and 4th columns (b, d, f, h, j, l), introduced at the middle of fungal growth; top row (a, b, c, d), backward migration site; middle row (e, f, g, h), introduction site; bottom row (i, j, k, l), forward migration site.
**Figure S5.5** Cell abundance in the microcosm with soil at pH 4.2. Left two columns (a, b, e, f, i, j), 17% soil moisture; right two columns (c, d, g, h, k, l), 12% water moisture; 1st and 3rd columns (a, c, e, g, i, k), introduced at the tip of fungal growth; 2nd and 4th columns (b, d, f, h, j, l), introduced at the middle of fungal growth; top row (a, b, c, d), backward migration site; middle row (e, f, g, h), introduction site; bottom row (i, j, k, l), forward migration site.
Figure S5.6 Cell abundance at introduction site in the microcosm with soil at pH 3.8. (a), (b) 17% soil moisture; (c), (d) 12% soil moisture; (a), (c) introduced at fungal growth tip; (b), (d) introduced at the middle of fungal growth.
Figure S5.7 pH changes in bulk soil.
Figure S5.8 Correlation between fungal hyphal density and soil pH changes ($R^2=0.008$).
Figure S5.9 Correlation between fungal hyphal density and bacterial abundance ($R^2=0.214$).
Figure S5.10 (a) Relationship of the CFU counts at introduction site and backward site ($R^2=0.678$). (b) Relationship of the CFU counts at introduction site and forward site ($R^2=0.717$).
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</table>

\(^a\) data from introduction site.

\(^b\) data from both forward and backward migration site.