Interactions of Burkholderia terrae with soil fungi

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university of groningen

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Mechanisms, gene expression patterns and ecological behavior of *Burkholderia terrae* BS001 during its interaction with *Lyophyllum* sp. strain Karsten and *Trichoderma asperellum* 302 in soil

PhD thesis

to obtain the degree of PhD at the
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by

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I dedicate this book to my parents

Contents

Chapter 1

1 General introduction and thesis outline

Irshad Ul Haq

Soil, the heterogeneity of C sources and bacterial lifestyles

The very existence of microorganisms has a long-standing influence on life on Earth, as they are involved in many of the life support functions of ecosystems. A vast majority of the microorganisms resides in the living soil, which can be characterized as an intricate 'motor system' that sustains the form of life as we know it on earth. In reality, the living soil is an extremely complex environment, with a number of "players" such as bacteria, fungi, protozoa, nematodes, plants and small animals that have interactions of different kinds (Standing and Killham 2007). The nature of the interactions among these living soil organisms is strongly affected by the physical and chemical properties of the soil that determine the nature of the soil habitats. However, the activities of microorganisms in soil also bring about continuous changes in these habitats and so local conditions are commonly highly dynamic (Standing and Killham 2007).

Bacteria show a range of different lifestyles in soil (Van Elsas et al. 2007). In eco-evolutionarily terms, bacterial populations in soil — as they are subjected to changing surrounding conditions — will show adaptation to these — provided they are endowed with the capacity to be selected (selectability) — over evolutionary time. Such adaptations to soil environment may be characterized by an accumulation of capacities that enable a bacterium to deal with fluctuating environmental conditions, in particular with respect to the often low supply of carbon and energy sources (Konstantinidis and Tiedje 2004). The genomes of such bulk-soil-adapted bacteria are often large, and that may be overloaded with a diversity of substrate uptake and utilization systems. Thus, the occupation of niches in the soil by different organisms is probably directed by their capacities of efficient utilization of the different resources in soil. In theoretical (1:1) competitive situations, this precludes the co-existence of different organisms in the shared (or semi-shared) niches (Van Elsas et al. 2007).

The soil system, despite being carbon (C) scarce in an overall sense, possesses microhabitats that support enhanced organismal activities and are termed 'hotspots'. Such soil 'hotspots' are nutritionally privileged, as they can host a number of microorganisms that can co-exist on the basis of diverse resources and thus thrive. Soil under the influence of plant roots (the rhizosphere) is one of these 'hotspots', as ca. 10 to 40% of the plant photosynthates are deposited there, in the form of organic substrates and plant mucilage (De Boer et al. 2005; Sørensen and Sessitsch 2007). The rhizosphere therefore attracts a vast majority of microorganisms, including bacteria and fungi, to reside in the plant-root proximity. Whereas the rhizosphere has been relatively well studied, other activity hotspots in soil have received less attention. One such hotspot, i.e. the soil zone under the direct influence of fungi, has been coined the 'mycosphere' (Warmink and van Elsas 2008). In this microhabitat in soil, potentially 'selected' microorganisms dwell and interact 'tightly' with each other. The presence and activity of fungal hyphae as the 'drivers' of the local activity in this hotspot is thought to be dependent on the provision of colonizable surfaces (the fungal highway; Kohlmeier et al. 2005) next to nutritive compounds (carbon and energy nutrition).

Soil bacteria undergo an enhancement of activity, and potentially of interactions, when they dwell in niches in the rhizosphere, the mycosphere or the mycorrhizosphere (defined as a site influenced by combination of plant roots and fungal hyphae). In these niches, bacteria may reveal particular paths of adaptation that relate to their prior interactions in the hotspots or even in bulk soil. Disentangling these interactions and understanding the underlying mechanisms that have been responsible for such ecological and evolutionary success represents a great challenge to current-day microbiology research. In the research, the power of genomics and transcriptomics is to be unleashed on natural as well as model systems, thus allowing to interrogate these with respect to the eco-evolutionary questions on the microbial populations that are addressed.

The *Burkholderia terrae* — fungal interactome as a paradigm of soil bacterial-fungal interactions

The genus *Burkholderia* was described as early as 1942 by Walter Burkholder, and in its first description encompassed bacterial pathogens of carnation and onion (Burkholder 1942). In a second step, Yabuuchi et al. (1992) proposed a revamping of the genus *Burkholderia*, establishing a seven-species genus. After these initial efforts, the number of species belonging to the genus has increased enormously, as — (according to www.bacterio.net) — in May 2016 it was reported to contain 100 species that were obtained from a wide range of different ecosystems.

Burkholderia terrae (ter'rae. L. gen. n. *terrae* of the earth) is a Gram-negative, motile, slightly curved rod, which for the first time was isolated from forest soil near the Korea Advanced Institute of Science and Technology (KAIST), Daejeon, South Korea. The first isolate was named KMY02^T (Yang et al. 2006). Strain KMY02^T was described as an aerobic heterotrophic bacterium, which was able to grow on R2A medium in a temperature range of 25–30°C (mesophile). Nitrogen fixation is a known trait of strain KMY02^T while it does not reduce nitrate. It was reported to utilize a wide range of C compounds (Yang et al. 2006).

Another *B. terrae* strain, denoted BS001, was isolated as an occupant of the mycosphere of *Laccaria proxima* in the Netherlands. Moreover, it was found to be a co-migrant based on its capacity of movement along the hyphae of *Lyophyllum* sp. strain Karsten in controlled soil microcosm experiments (Warmink and van Elsas 2008, Warmink and van Elsas 2009). The migratory behaviour of *B. terrae* BS001 was posited as a 'trademark' phenomenon as it underlies the very concept of viable niche exploration, bearing on the Darwinian struggle for fitness and existence. This conceptualization was further strengthened by the finding of the ability of strain BS001 to form biofilms on the surfaces of *Lyophyllum* sp. strain Karsten during the co-cultivation of the two partners on water agar (Warmink and van Elsas 2009). The higher abundance of the BS001 population on young hyphal tips as opposed to older tips further corroborated the fact that migration along the growing hyphae was mediated by bacterial motility. Such motility presumably took place in water films around the fungal hyphae (Warmink and van Elsas

2009). Another intriguing property of *B. terrae* BS001 lies in its ability to 'support' non-migrant bacterial species to hitchhike along the growing fungal hyphae in soil settings (Warmink et al. 2011). Based on such capacities, strain BS001 was selected for further studies in order to ascertain its potential and success as a 'generic' mycosphere dweller. *B. terrae* BS001 was later found to induce the release of C compounds — such as glycerol — during its interaction with *Lyophyllum* sp. strain Karsten in liquid microcosms (Nazir et al. 2013). Such interactions also led to the suppression of primordium formation in *Lyophyllum* sp. strain Karsten, a phenomenon that was postulated to have important ecophysiological implications. A striking feature of *B. terrae* BS001 was its ability to associate with a number of fungal species, which highlights its successful colonization of the 'mycosphere' as well as other potential soil microhabitats (Nazir et al. 2014). As a potential pay-back to the fungi, strain BS001 was reported to have a role in the protection of fungi against antagonists in the soil (Nazir et al. 2014).

The interaction of *B. terrae* BS001 with fungi could be tracked back to an ectomycorrhizal fungus that occurs at Dutch hazel trees. A *Laccaria proxima* (phylum Basidiomycota) was thus identified. This fungus is commonly found in forests with acidic soil where it forms symbiotic relationships with the forest hazel trees (Warmink and van Elsas 2009, O'Reilly 2011). It also goes by the synonyms *Laccaria laccata* var. *proxima*, *Clitocybe proxima* and *Laccaria proximella* (O'Reilly 2011). The cap of the *L. proxima* mushroom is 2 to 8 cm in diameter; it is convex in the initial stages and becomes flat-topped at maturity (O'Reilly 2011). The younger gills are deep and pinkish-lilac which are interspersed with shorter gills. The stem of *L. proxima* ranges from six to 10 mm in diameter and is six to 12 cm tall (O'Reilly 2011). *Lyophyllum* sp. strain Karsten was reported to be a close saprotrophic relative of the ectomycorrhizal *L. proxima* (Warmink and van Elsas 2009).

Other systems involving *Burkholderia* species

The interactive lifestyle of *Burkholderia* species has been observed in other host organisms, involving various species. First, interactions of different *Burkholderia* species with plants, in particular in the rhizosphere, but extending to galls on leaves, have been well described (Salles et al. 2004, Salles et al. 2006, Weisskopf et al. 2011, Carlier and Eberl 2012, Paungfoo-Lonhienne et al. 2014, Kost et al. 2014, Stopnisek et al. 2014). Moreover, *Burkholderia* species have also been found in association with fungi, albeit from a perspective different from the above. For instance, fierce competition between *Burkholderia* species and soil fungi has been described. This includes the competitive behaviour towards different fungi, i.e. *Alternaria* sp., *Fusarium* sp., *Rhizoctonia* sp., and *Aspergillus* sp. (Groenhagen et al. 2013, Palumbo et al. 2007, Li et al. 2007, Quan et al. 2006). Such competitive interactions between *Burkholderia* (different species) and distinct fungi is apparently either antibiotic-or volatile-mediated (Groenhagen et al. 2013).

Some *Burkholderia* species have also been reported to reside in confined (secure) environments, i.e. within other organisms. One example is that of *B. rhizoxinica* – an endosymbiont of the rice pathogenic fungus *Rhizopus microsporus* (Partida-Martinez and

Hertweck 2007). The interaction incites the bacterial production of rhizoxin, which *Rhizopus* (Schmitt et al. 2008) uses to cause rice blight. Others have reported that *Burkholderia* specie can have roles in the weathering of minerals in the mycorrhizosphere of *Scleroderma citrium* (Lepleux et al. 2012), an activity that has lasting benefits for fungi and plants during their growth (Uroz et al. 2007, Uroz et al. 2009).

Motivation of this study

From the foregoing, it is apparent that the genus *Burkholderia* encompasses a highly diverse group of organisms with widely divergent life styles. In a living soil, *Burkholderia* species are likely confronted with temporally and spatially fluctuating conditions, and the prior knowledge on the interactivity of *B. terrae* with soil fungi represents a token of a highly specific evolutionary path.

Thus, I here took organism from this genus as my study object. In effect, I established a model system representing mycosphere- and soil-isolated *B. terrae* strains, *Lyophyllum* sp. strain Karsten and *Trichoderma asperellum* 302.

Outstanding questions with respect to the *B. terrae* – fungal interactions

Although bacterial motility and the type 3 secretion system (T3SS) have implicitly been mentioned in previous studies of our model bacterial-fungal interaction (Warmink and van Elsas 2008, Warmink and van Elsas 2009), the definite proof for the involvement of such systems was lacking at the start of this study. Moreover, a suite of *B. terrae* strains was found to be fungal-interactive (Nazir et al. 2012), with a single strain (BS001) interacting positively with diverse fungal hosts (Nazir et al. 2014). However, no robust mechanistic information on these interactions was available at the start of this study. I thus asked specific questions about the *B. terrae* – fungal interactions, as follows:

1. What types of genetic systems are carried and potentially used by fungal-interactive B. terrae strains?

This first and foremost question about the interactivity of *B. terrae* BS001 with a wide range of fungi (Nazir et al. 2014) inspired us to first look at the genetic potential of strain BS001. Taking into account all previous studies, our questions focused on the genetic systems that might be involved and have broad roles in the interaction with soil fungi. In particular, what makes strain BS001 such a successful dweller of the mycosphere and beyond? Do the protein secretion systems — in particular the T3SS — play vital roles in the interaction? Do we see any horizontal genetic acquirements over the trackable evolutionary life history of strain BS001 and would these guarantee its successful adaptation to the fungal-defined niches and other potential hosts? And, with reference to the findings of Nazir et al. (2013), does *B. terrae* BS001 harbor any identifiable genetic potential to reap benefits from the ecological conditions offered by the fungus in terms of released glycerol or other compounds?

2. What type of transcriptional responses are revealed by B. terrae BS001 during its interaction with Lyophyllum sp. strain Karsten?

Addressing questions regarding the presence or absence of genetic systems that presumably play roles in the interactivity of strain BS001 with different soil fungi needs a follow-up by more focused attention to the transcriptional responses of such systems. Therefore, to obtain a clear picture of the state of events during the interaction of *B. terrae* BS001 with *Lyophyllum* sp. strain Karsten, I interrogated the genes and operons that might be responsive to the presence of the fungus during a confrontation experiment. To put that in an ecological perspective, the transcriptional responses were assessed in a soil-mimicking system at different time points and physical distances between the two partners. I specifically asked the questions: what are the transcriptional responses at the early stages of the interaction when the two partners are physically apart? Do such responses change when physical interactions take place? Is this interactive process dynamic or does it follow a certain trend? What are the effects of the medium used on the outcome of the interaction?

3. Which chemical parameters provide the cues triggering a response?

Interactions between bacteria and fungi in any system would not be feasible without chemical cross-talk between the two partners. To obtain insights into which chemical cues take part in such communication and signalling, I asked the following question: Does chemotaxis of the bacterial cell population take place during the interaction between *B. terrae* BS001 and two selected soil fungi, i.e. *Lyophyllum* sp. strain Karsten and *T. asperellum* 302? The second line of investigation was to explore the putative chemical cues that are released by the two fungal strains and perceived by a cohort of *B. terrae* strains, including the type strain 17804^{T}. The potential role of oxalic acid/oxalate in the interaction of bacteria and fungi was then addressed by asking whether it is present in the system and acts as a signalling molecule or a potential carbon source. Are the chemical cues and carbon sources released by the two fungi similar or different?

4. What type of physical interactions may take place?

Earlier observations about the formation of a biofilm by *B. terrae* BS001 around the hyphae of *Lyophyllum* sp. strain Karsten by Warmink and van Elsas (2009) and Nazir et al. (2014) led us to ask the question: what are the potential anchoring sites on the fungal cell wall that strain BS001 attaches to? In collaboration with Prof. Barreto-Bergter's group (UFRJ, Brazil), I placed an emphasis on the potential of fungal cell envelope ceramide monohexosides (CMH) to serve as such anchors.

Summary

I placed a focus on: (1) the ability of *B. terrae* strains to reap the ecological benefits offered by soil fungi under carbon- and energy-limiting conditions, (2) the role of chemotaxis and metabolic signalling in the establishment of interactions with *Lyophyl-*

lum sp. strain Karsten and *T. asperellum* 302, (3) the genetic potential of *B. terrae* BS001 and (4) the gene expression patterns of strain BS001 during the onset and establishment of its interaction with *Lyophyllum* sp. strain Karsten. Using these model organisms (*B. terrae* strains, *Lyophyllum* sp. strain Karsten, and *T. asperellum* 302), this thesis took two approaches (1) *explorative* — using comparative genomics and transcriptomics, and (2) *hypothesis-driven* — assessing chemotaxis, fungal exudation, the role of oxalic acid/oxalate and the intricate physical associations between *B. terrae* cells and the target fungi. On the basis of the broad fungal interactivity of *B. terrae* strain BS001, I here posit that the findings reported in this thesis have significant impacts in advancing our fundamental understanding of the mechanisms underpinning bacterial–fungal interactions even in a broader eco-evolutionary context.

Objectives of this thesis

The objectives of this thesis are to dissect the complexity of the interactions between *B. terrae* and its target fungi in soil, with emphasis on the bacterial behaviour. Thus, I hypothesized that *B. terrae* is a soil bacterium that has part of its lifestyle in the bulk soil and that has 'learned' to take advantage of the benefits offered by the hyphae of diverse fungi should these appear in its vicinity expanding its niche. It is important to notice that soil bacteria, even if they are equipped with flagellar and other motility systems (i.e. twitching or gliding motility), normally cannot explore large distances due to a generic lack of connectivity of the water-linked pores in soil. Taking advantage of classical microbiological approaches and more advanced 'omics' tools, I here aimed at advancing our understanding of the *B. terrae* BS001 – *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 interactions in soil or soil-mimicking systems.

General research questions:

1. What are the genome size and composition of *B. terrae* BS001 and to what extent do the genes and operons underpin the interactivity of BS001 with diverse fungi in soil?
2. What genes and operons of *B. terrae* BS001 are transcribed and thus important for its survival and interaction with *Lyophyllum* sp. strain Karsten at different time points during the interaction in soil?
3. How do partners in the bacterial-fungal interaction sense the presence of each other? And, are there any communication channels between the two partners?
4. What potential anchoring sites do fungi possess on their cell walls that fungal-interactive *Burkholderia* species attach to?
5. To what extent are fungal-interactive soil *Burkholderia* species attracted towards fungal-released compounds?
6. What is the role of oxalic acid/oxalate in the interaction between *Burkholderia* species and soil fungi?

Hypotheses:

1. A myriad of genetic systems is present on the genome of *B. terrae* BS001, enabling it to be ecologically flexible and successfully interact with fungi. The ample genetic potential of strain BS001 is necessary for niche adaptation/differentiation during changing local environmental conditions.
2. The interaction between *B. terrae* BS001 and *Lyophyllum* sp. strain Karsten is complex and depends on local conditions, the physical distance between the two partners and the available resources.
3. Ecological tradeoffs (growth and tolerance) are at the basis of the interactions between *B. terrae* BS001 and *Lyophyllum* sp. strain Karsten, with the local niche conditions (availability of nutrients) determining the outcome of such interactions.
4. Specific fungal cell surface structures constitute an anchorage site for *B. terrae* enabling the organism to 'hang on' to these during the physical bacterial-fungal interaction.
5. Fungi release different compounds that serve as signalling molecules as well as potential C sources for *B. terrae* BS001 and related strains.

Outline of the thesis

This thesis sequentially addresses the questions and hypotheses discussed in the foregoing in **chapters 2** through **6**. A flow chart of the thesis questions is given in Fig. 1.1.

In order to understand the nature of the interactions between bacteria and fungi in detail, a solid background discussion regarding previous work carried out in this area is provided in **chapter 2**. This chapter provides an elaborate review of the literature related to bacterial-fungal interactions, and highlights that such interactions are complex in nature, pinpointing a number of genetic systems — from a bacterial point of view — that may drive the bacterial-fungal interactions in soil. The information gathered in **chapter 2** led to the hypothesis that the exceptionally large genome of *B. terrae* BS001 possesses ample genetic information — of complex nature — that is necessary for its successful life in soil and the interaction with other soil organisms such as fungi.

Chapter 3 analyzes the *B. terrae* BS001 genome, focusing on the genetic systems that are potentially involved in the interaction of strain BS001 with soil fungi. A comparative genomics approach was followed and questions about the commonality and differences of strain BS001 and other *Burkholderia* species are posed. This chapter also highlights some conspicuous horizontal gene transfer events that presumably occurred in the evolutionary life history of strain BS001. Furthermore, **chapter 3** provides a glimpse of the metabolic potential of *B. terrae* BS001 and addresses questions concerning the importance of membrane transporters (key to perceiving the surrounding environment) as well as metabolic systems that make part of the genome?

Chapter 4 addresses questions about the activity of particular genes and operons of *B. terrae* BS001 during its interaction with *Lyophyllum* sp. strain Karsten. These questions are answered with the perspective of the two partner organisms dwelling in a nutritionally-poor environment (posing stressful conditions to them) taking into account the

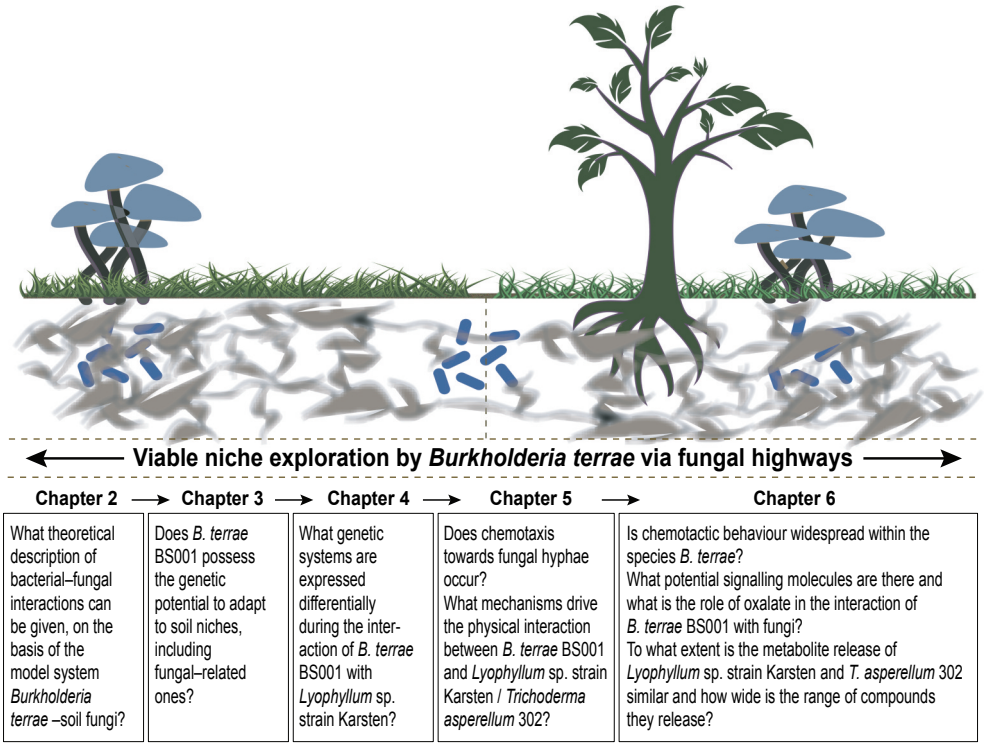

Figure 1.1 A schematic flow chart of thesis questions. Graphic resources for this figure were obtained from www.freepik.com, available as free vectors and used after modifications.

physical distance between *B. terrae* BS001 and *Lyophyllum* sp. strain Karsten, at different time points. Using RNA-based high-throughput sequencing, the transcriptome of strain BS001 is thus analyzed over time. A suite of differentially expressed genetic systems are highlighted (**chapter 4**), pointing at the involvement of bacterial motility (chemotaxis), stress relief, secondary metabolites production and specific metabolism, during the interaction of strain BS001 with *Lyophyllum* sp. strain Karsten.

In **chapter 5**, further questions regarding the chemotaxis of *B. terrae* towards two selected soil fungi, *Lyophyllum* sp. strain Karsten and *T. asperellum* 302, and the involvement of potential fungal cell envelope anchorage sites during the physical interactions with the two counterparts, are answered. By providing different carbon sources in a minimal medium, the chemotactic behavior of *B. terrae* BS001, towards *Lyophyllum* sp. strain Karsten and *T. asperellum* 302, was investigated. I further shifted the focus towards the physical interaction of *B. terrae* BS001 and the aforementioned fungi, and in collaboration with Prof. Barreto-Bergter's group (UFRJ, Brazil), elucidated the biochemistry of the presumed anchorage sites on the fungal cell surface and the binding capacity of *B. terrae* BS001.

Chapter 6 presents a detailed analyses of the compounds exuded by two selected fungi (*Lyophyllum* sp. strain Karsten and *T. asperellum* 302). This chapter addresses four main research questions: (1) Do fungal-interactive *B. terrae* strains show chemotactic behaviour towards oxalic acid/oxalate in different concentrations? (2) Does a similar chemotactic response occur towards fungal exudates? (3) Do both *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 release oxalic acid/oxalate, next to other compounds? (4) Does oxalic acid/oxalate act as a carbon source for *B. terrae* BS001? **Chapter 6** focuses on the conundrum of whether *B. terrae* BS001 perceives oxalic acid/oxalate as a signaling molecule and/or as a carbon source, in dependency of the concentration.

Chapter 7 presents a synthesis of all observations gathered and analyzed during the course of this study. The results and observations obtained are presented from the broader perspective of bacterial-fungal interactions in soil. Future perspectives and new research lines in the field of bacterial-fungal interactions in general, and fungal-interactive *Burkholderia* strains in particular, are discussed.

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Chapter 2

2 The interactions of bacteria with fungi in soil: emerging concepts

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Abstract

In this chapter, we review the existing literature on bacterial–fungal interactions in soil, exploring the role fungi may play for soil bacteria as providers of hospitable niches. A focus is placed on the mycosphere, i.e., the narrow zone of influence of fungal hyphae on the external soil milieu, in which hypha-associated bacterial cells dwell. Evidence is brought forward for the contention that the hyphae of both mycorrhizal and saprotrophic fungi serve as providers of ecological opportunities in a grossly carbon limited soil, as a result of their release of carbonaceous compounds next to the provision of a colonizable surface. Soil bacteria of particular nature are postulated to have adapted to such selection pressures, evolving to the extent that they acquired capabilities that allow them to thrive in the novel habitat created by the emerging fungal hyphae. The mechanisms involved in the interactions and the modes of genetic adaptation of the mycosphere dwellers are discussed, with an emphasis on one key mycosphere-adapted bacterium, *Burkholderia terrae* BS001. In this discussion, we interrogate the positive interactions between soil fungi and bacteria, and refrain from considering negative interactions.

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Introduction and the importance of microhabitats in the living soil

The living soil provides a natural habitat to diverse communities of organisms, which play important roles in the decomposition of soil organic matter as well as in the cycling of plant nutrients (Coleman et al. 2004, Wardle et al. 2004). These processes represent key ecosystem services with massive impacts on global ecology. The living soil has thus been mentioned as a hotspot in soil science, given its complex nature and the role it plays in global biogeochemical cycling processes (Van Elsas et al. 2007).

The heterogeneous nature of the living soil governs the vast diversity of the soil microbiota (Standing and Killham 2007). Soil is biologically very rich and diverse, as compared to other natural habitats (Dance 2008). The prokaryotic abundance, which is largely bacterial (Torsvik et al. 2002), can be as high as 4.8×10^{9} to 2.1×10^{10} cells cm^{-3}, with, in some cases, up to 8,800 different species genomes, depending on the soil type. Next to bacteria, other organisms such as archaea, fungi, protozoans, and nematodes are abundant in soil (Nazir et al. 2010). Key factors in the soil, such as nutrient status, soil type, pH, moisture, and plant species and age, direct the activity, diversity, and community composition of soil organisms (Graystone et al. 1998). Among the soil organisms, in particular bacteria and fungi are important, as they are by far most numerous and also because of their essential roles in the functioning of soil (De Boer et al. 2005, Frey-Klett et al. 2007, Johansson et al. 2004, Poole et al. 2001, Uroz et al. 2007).

How is the microbiota distributed in soil? We know that soil consists of different microenvironments, also called "microhabitats". For instance, depending on local influences, we can distinguish the rhizosphere (zone of influence of plant roots), mycosphere (zone of influence of fungal hyphae; Warmink and van Elsas 2008), mycorrhizosphere (zone of influence of roots and hyphae), next to the residuesphere (zone determined by decaying organic matter) and bulk soil. A particular form of the mycosphere occurs at the dense fungal hyphae in soil immediately under fungal fruiting bodies (Warmink and van Elsas 2008), often of mycorrhizal fungi. In this mycosphere, bacterial growth is stimulated, as photosynthates from plants become available via the hyphal network of the fungi, in the form of exudates or lysed and sloughed-off cells. Such compounds will then be used by soil bacteria for their growth and clonal expansion in the mycosphere. The bacterial abundances in the mycosphere have been shown to be higher than the bulk soil (Warmink and van Elsas 2008), indicating a selective effect of fungi on soil bacteria. Although we understand the mycosphere effect in simple terms (i.e. as provider of carbon), we know very little of the underlying bacterial–fungal interactions and hence the fungal–soil interface needs to be explored comprehensively.

In addition, the mycorrhizosphere is defined as the microhabitat in soil where plant roots are surrounded by fungal hyphae and the two living entities affect their surroundings (Rambelli 1973). Both the plant roots and the associated fungal networks are drivers of the microbial communities in the mycorrhizosphere (Nazir et al. 2010). The mycorrhizosphere is often quite persistent along the plant's life span, as mycorrhizal associations are vital for many plants (Frey-Klett et al. 2007). Therefore, mycorrhizal

fungi have a great influence on mycorrhizosphere-dwelling bacterial communities and vice versa (Johansson et al. 2004).

In contrast to the mycorrhizosphere, the mycosphere may be rather transient, as it is affected by the growth, survival, aging, and death of the fungal hyphae that built the structure. The conditions in this microhabitat are shaped, in different ways, by fungi as well as their associated bacteria. Generally speaking, the ecological effects exerted by both the mycorrhizosphere and mycosphere on inhabitant soil bacteria could be positive, neutral, or detrimental, depending on how the local conditions are affected by the fungi (Nazir et al. 2010). In this review, we focus on the positive effects of soil fungi on bacteria, as great progress has been made in recent years. However, we recognize the important developments made in studies on negative interactions. For instance, it has recently been shown that the polyyne-like collimomycins produced by *Collimonas fungivorans* Ter331, inhibited the growth of *Aspergillus niger*, indicating detrimental association (Fritsche et al. 2014).

Bacterial–fungal interactions in soil

Prevalent bacterial communities associated with soil fungi

Bacterial communities associated with soil fungi, including saprotrophic and mycorrhizal ones, have been explored according to microbiological (cultivation-based) as well as molecular (DNA-based) methods (Table 2.1). What makes bacterial species so apt to occupy niches in soil that are influenced by fungi (mycosphere; transient) or both fungi and plants (mycorrhizosphere)? Is there a selection of bacterial types by fungi or is the association process totally random? Irrespective of the type of microhabitat, it appears logical that bacteria are selected by fungi that are locally present. Here, we review some earlier work that at least highlights those bacterial genera that are associated with fungi. In early work, several bacterial taxa, i.e. members of the genera *Burkholderia*, *Pseudomonas*, and *Bacillus*, were found to be associated with soil fungi (De Boer et al. 2005). Among these, *Burkholderia* and *Pseudomonas* types may be the most abundant colonizers of fungi in soil, in terms of relative abundances and species composition (Frey-Klett et al. 2005, Rangel-Castro et al. 2002, Timonen and Hurek 2006, Warmink and van Elsas 2009). However, rather specific bacterial types stand out by their capacity to migrate with growing hyphae of *Lyophyllum* sp. strain Karsten, namely *Burkholderia terrae*, *Dyella japonica*, and *Ralstonia basilensis* (Nazir et al. 2012, Warmink et al. 2011, Warmink and van Elsas 2009). The finding of such bacterial occupants of fungal surfaces is very likely not coincidental, as was shown by Warmink et al. (2009).

Moreover, Boersma et al. (2009) described the selection of *Sphingomonadaceae* family members and in particular, *Variovorax* types by the ectomycorrhizal (EM) fungi *Laccaria proxima* and *Russula exalbicans*. In other work on oak forest soil, numerous bacterial species were isolated from the *Scleroderma citrinum* mycorrhizosphere. These belonged to the genera *Burkholderia*, *Collimonas*, *Pseudomonas*, and *Sphingomonas* (Uroz et al. 2007). Moreover, particular streptomycetes were found to be associated with EM fungi,

which can act as modulators of symbiosis with the plant (Schrey and Tarkka 2008). Also, bacterial populations in the mycosphere (Fig. 2.1) of *L. proxima* encompassed, among others, members of *Pseudomonas fluorescens*, *Chryseobacterium piscium* and *Mycobacterium* sp.. Concerning the mycorrhizosphere, EM fungi like *Paxillus involutus* and *Suillus bovinis* have been shown to host diverse bacterial communities, indicating the existence of different "territories" in their mycorrhizospheres (Nurmiaho-Lassila et al. 1997). Thus, bacterial community structures in such mycorrhizospheres are thought to rely more on the type of fungus than on the host plant (Roesti et al. 2005, Singh et al. 2008). Moreover, soil pH influences the bacterial assemblages in the mycosphere (Nazir et al. 2010), which were found to be spatially organized. On the basis of all of these data, we conclude that the bacterial community structure in the mycorrhizosphere is determined by both the fungal host and the bacterial types that are locally present, and so is bacteria–mycorrhiza specific. Moreover, we here would like to postulate that deterministic processes most likely lie at the basis of most of the microbial community structures seen in association with soil fungi.

Interactome of soil fungi and their associated bacteria

Interactions between bacteria and fungi in soil are important, as the nutritional dynamics they confer, strongly affects soil health/quality. Nutritional interactions imply that

Table 2.1 Different bacterial genera associated with fungi in soil.

Main genera	Fungal host(s)	References
Burkholderia, *Rahnella*, *Chryseobacterium*, *Dyella* and *Pseudomonas*	*Laccaria proxima*, *Russula exalbicans*, *Lactarius hepaticus*, *Laccaria ochropurpurea* and *Scleroderma citrina*	Warmink & van Elsas (2008) Warmink & van Elsas (2009)
Chondromyces, *Cellvibrio*, *Lysobacter*, *Flexibacter* and *Pseudomonas*	*Glomus geosporum* and *Glomus constrictum*	Roesti et al. (2005)
Streptomyces	*Amanita muscaria*	Schery et al. (2007)
Collimonas, *Sphingomonas*, *Burkholderia* and *Pseudomonas*	*Scleroderma citrinum*	Uroz et al. (2007)
Bacillus, *Pseudomonas*, *Paenibacillus* and *Arthrobacter*	*Glomus* sp. MUCL 43205 and *Glomus intraradices* MUCL 43194	Toljander et al. (2006)
Pseudomonas	*Gigaspora rosea*	Gamalero et al. (2008)
Bacterium-like objects (BLOs), *Candidatus Glomeribacter gigasporarum*	*Glomus catedonius*, *Gigaspora margarita*, *Scutellospora persica*, *Scutellospora castanea* and Glomaceae	MacDonald & Chandler (1981) Scannerini & Bonfante (1991) Bianciotto et al. (1996) Bianciotto et al. (2003)
Burkholderia	*Gigaspora decipiens*	Levy et al. (2003)
Collimonas	*Chaetomium globosum*, *Fusarium culmorum*, *Mucor hiemalis* and *Aspergillus niger*	De Boer et al. (2005) Fritsche et al. (2014)

either bacteria feed on fungi or their released products (De Boer et al. 2005, Leveau and Preston 2008) or, vice versa, fungi feed on bacteria or their released products (Hildebrandt et al. 2002, Hildebrandt et al. 2006). Bacteria can obtain their nutrients from fungi by (1) living as endosymbionts inside the fungal host, (2) feeding on dying or dead fungal tissue, or (3) taking profit of compounds exuded by the fungi. The latter is likely to be a very prominent mechanism, and it may imply commensalism or true mutualism (Nazir 2012). In this context, it has been reported that *Burkholderia* sp. of a particular type can colonize and penetrate senescing spores of the mycorrhizal fungus *Gigaspora decipiens*. They may also attach to fungal hyphae following germination, as was found using green fluorescent protein-tagged bacteria (Levy et al. 2003). Similarly, members of the genera *Bacillus*, *Pseudomonas*, *Paenibacillus*, and *Arthrobacter* were found to attach to living or non-living *Glomus* sp. hyphae, suggesting that attachment of bacteria to hyphae is controlled by fungal vitality and is species-specific (Toljander et al. 2006). Moreover, several bacterial types, i.e. *Chondromyces*, *Cellvibrio*, *Lysobacter*, *Flexibacter*, and *Pseudomonas*, were shown to feed on the outer hyaline spore layer of arbuscular mycorrhizal (AM) fungi (Roesti et al. 2005). The underlying mechanisms determining this bacterial feeding on fungi are poorly understood. On the positive side, two mutant strains of *P. fluorescens* CHA0 showed enhanced capacities to synthesize extracellular polysaccharides as compared to the wild type, enabling them to adhere to the surface of AM fungi. Thus, these bacteria apparently acquired an enhanced capacity to occupy the 'fungal' niche. This finding emphasized the importance of outer cell surface structures for successful attachment of bacteria to fungal surfaces (Bianciotto et al. 2001).

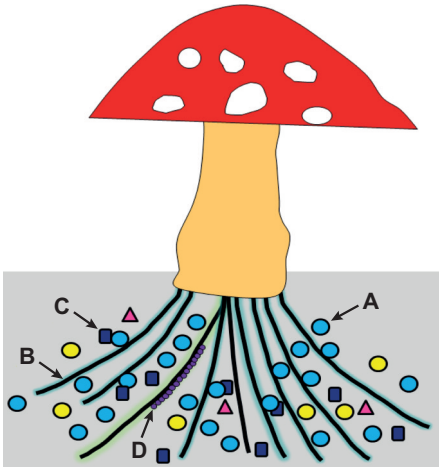

Figure 2.1 The microbial "loop" in the mycosphere. Different bacteria show different abundances in the mycosphere. Some bacteria can attach to fungal cells (A) and stimulate the production of fungal exudates (B). They can also mediate the adhesion of other bacteria (C). Moreover, some of them can form a biofilm along the fungal hyphae (D).

Akin to attachment to plant roots, lectins secreted by truffles allow binding of cells of *Rhizobium* sp.. Thus lectins constitute a molecular determinant of rhizobial attachment (Cerigini et al. 2008). Other associations of bacteria with soil fungi have also been found. For instance, *in situ* hybridization revealed the occurrence of a cryptic bacterium, belonging to *Paenibacillus* sp., in the culture pool of *Laccaria bicolor* S238N (Bertaux et al. 2003). The organism was found in fungal mats and fruiting bodies and was able to grow with living and dead fungal cells. However, it was rarely found inside fungal hyphae (Bertaux et al. 2005). Interestingly, *Paenibacillus validus* has been shown to support the growth and spore formation of *Glomus intraradices* independently of the plant species with which this fungus associated (Hildebrandt et al. 2002). The probable release of raffinose and an unidentified trisaccharide made the organism efficient in sustaining fungal growth until new germinating spores emerged (Hildebrandt et al. 2006). Thus, this AM fungus could develop and complete its life cycle, possibly at the expense of bacteria and independently of the plant host.

Mycorrhization helper bacteria and interactions

Particular bacteria in soil can assist mycorrhizal fungi in the establishment of a mycorrhizal association with the plant. This led to the concept of mycorrhization helper bacteria (MHB), as first described and confirmed by Duponnois and Garbaye (1991). MHB are currently a well investigated group of bacteria (Frey-Klett et al. 2007). Importantly, they can exert a key role in symbiotic associations that are under stress due to drought (Vivas et al. 2003b) or heavy metals like cadmium (Cd (II)) (Kozdroj et al. 2007), zinc (Zn) (Vivas et al. 2006), and lead (Pb) (Vivas et al. 2003a).

Mechanisms involved in the MH effect include the production of factors that stimulate the growth of mycelia, fungal spore germination, increased root colonization and reduction of stress by detoxification of substances that are antagonistic. For instance, 1-aminocyclopropane-1-carboxylate deaminase producing *Pseudomonas putida* UW4 (blocks ethylene release by acting on an ethylene precursor) was found to stimulate the association of the AM fungus *Gigaspora rosea* with cucumber. However, mutant *P. putida* UW4 lacking ACC-deaminase activity could not block ethylene synthesis and, as a result, was less capable of stimulating mycorrhization (Gamalero et al. 2008).

MHB can also incite changes in gene expression of mycorrhizal fungi. Thus, *P. fluorescens* BBc6R8 was shown to stimulate the growth of *L. bicolor* S238N and change its gene expression, activating genes involved in transcription regulation, recognition and synthesis of primary metabolism proteins (Deveau et al. 2007). Hyphal growth and association with the host plant by the EM fungus *Amanita muscaria* was stimulated by *Streptomyces* sp. AcH 505. Morphological changes in the actin cap of the hyphae in the presence of bacteria were observed through immunofluorescence microscopy (Schrey et al. 2007). From these observations, we may infer that, in the establishment of bacterial–fungal interactions, diffusible molecules are important, second to the physical contact between partners. Recently, volatile organic compounds (VOCs) were also pinpointed as key agents affecting the communication and interactions among the soil biota (Tarkka and

Piechulla 2007). Thus, bacterial VOCs may affect soil fungi (including mycorrhiza) as well as soil bacteria. These bioactive molecules are considered to be very important drivers of symbioses. To fully understand the mechanisms underlying the interactions of MHB and fungi, identification of the bioactive molecules and their mode of action needs to be done.

Endobacteria and their interactions with mycorrhizal fungi

Already in early work, so-called bacterium-like objects (BLOs) were found inside the hyphae of endomycorrhizal fungi (MacDonald and Chandler 1981, Scannerini and Bonfante 1991). Interestingly, in later work *Burkholderia*-related bacteria were found to live inside different members of the *Gigasporaceae* based on 16S rRNA gene analysis (Bianciotto et al. 1996). The key representative BLO, which presumably has conquered a key ecological role in the host fungus goes by a new suggestive name, *Candidatus Glomeribacter gigasporarum*. So far, this organism could not be cultured (Bianciotto et al. 2003). However, confocal and electron microscopy coupled with molecular tools confirmed that the organism is a bacterium, possibly on its way to become a true endosymbiont or even further down the road to turning into an organelle (Bianciotto et al. 1996).

Sequence of events in bacterial–fungal interactions, taking the B. terrae BS001–Lyophyllum sp. strain Karsten interaction as the model

In our own work, we have taken the interaction between the mycosphere dweller *B. terrae* BS001 and the reference fungus *Lyophyllum* sp. strain Karsten as the basis for the emerging theories and concepts about the fungal–bacterial interactome. This, as several key features of this interaction have been, or are being, unveiled in our recent work. On the basis of the observations so far, we surmise that the process of bacterial–fungal interactions in soil is either truly dependent or relatively independent of cell-to-cell contact between the partners in the association, resulting in different and divergent processes of interaction. This is further explored hereunder (Fig. 2.2).

Cell-to-cell contact-independent interaction

In this interaction, there is no dependency on physical contact between the interacting partners (Fig. 2.2A). The perception of one partner by the other one is presumed to come about as a result of secreted or volatilized signaling molecules. We discern the following steps:

Secretion

Sender cells (of either partner) release signalling molecules to the external milieu. These molecules could be quorum-sensing molecules, antibiotic-like substances, metabolites, as well as VOCs. VOCs may confer an advantage, as they extend the “reach” of interaction (Garbeva et al. 2014).

Capture

Recipient cells first sense and then utilize or process the signal molecules in the cell. In this step, there is contact between the signal and the recipient cell, which starts up a reaction to this signal by modulating its signal response system.

Response

The recipient cells fully respond to the signal molecules, altering their gene expression patterns, and resulting in diverse effects such as organic compound releases (Nazir et al. 2013), or growth inhibition or stimulation.

This first set of steps may then result in an interaction which keeps cells distant from each other ("platonic" interaction) or which incites cell-to-cell approximation ("intimate" interaction) much like described below.

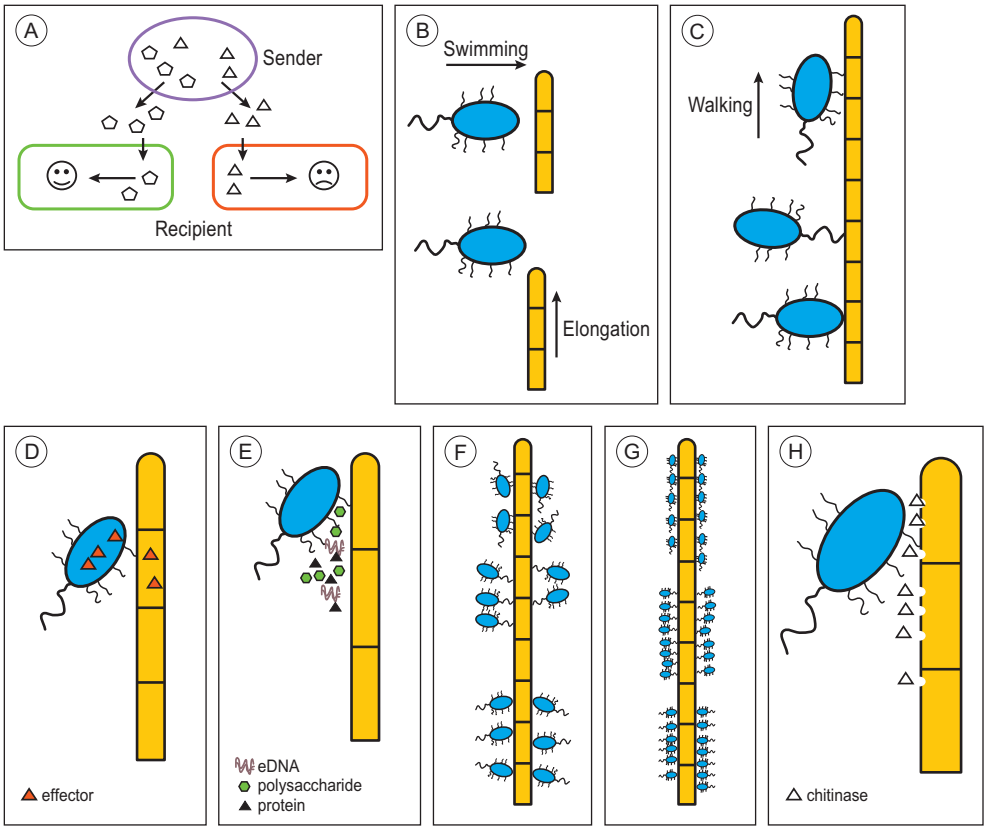

Figure 2.2 The process of bacterial–fungal interactions. (A) Cell-to-cell contact independent interaction. (B–H) Cell-to cell contact-dependent interaction; (B) approximation; (C) recognition and attachment; (D) effector injection; (E) EPS alteration; (F) bacterial growth; (G) biofilm formation; (H) cell wall degradation.

Cell-to-cell contact-dependent interaction

In this interaction, the perception of an interaction partner is presumed to depend on direct cell-to-cell (physical) interaction. Hence, physical contact between the interacting partners has to establish first, before any other step is undertaken. The following steps can be discerned:

Approximation

Bacterial cells swim close to fungal hyphae or fungal hyphae extend to sites close to the bacterial cells. Following either or both of these steps, the partners get in touch with each other (Fig. 2.2B). The approximation step may be random/stochastic or it may be "directed," i.e. either or both of the partners sense a signal produced by the other one (akin to the above mechanism).

Recognition

Cells of the two partners in close proximity directly recognize signal molecules at the cell wall of their interacting partner. For bacteria like *B. terrae* BS001, diverse cellular appendages may be involved, whereas for fungi glycolipids, glycoproteins, or even chitin may be essential for recognition (Fig. 2.2C).

Attachment

Bacterial cells attach to fungal hyphae, possibly in two steps, i.e. a reversible (loose) interaction first, followed by an irreversible (strong) one later. They may also adhere to the fungal cell wall on the basis of shared hydrophobicity/hydrophilicity levels, according to the "like-dissolves-like" principle. Additionally, bacteria start to "walk" along fungal hyphae by i.e. twitching motility (Fig. 2.2C).

Effector injection

Bacteria can modulate fungal cell physiology by injection of effector proteins (Fig. 2.2D).

Extracellular polymeric substance alteration

When bacteria attach to fungal surfaces, they may change the composition of their extracellular polymeric substance (EPS) to facilitate subsequent processes such as intermittent adherence and (twitching) motility. Additionally, fungi may form water films to promote bacterial motility (Fig. 2.2E).

Bacterial growth

Bacteria may grow at the surface of fungi (Fig. 2.2F), possibly at the expense of fungal-released compounds.

Biofilm formation

Bacteria can form biofilms around fungal hyphae. EPS production and possible alteration may play a crucial role in this step (Fig. 2.2G).

Cell wall degradation

Bacteria may start to secrete enzymes that degrade fungal cell walls such as chitinases (Fig. 2.2H).

Selected mechanisms involved in bacterial fitness in fungal-affected microhabitats

As proposed in the foregoing, several bacterial mechanisms may act in bacterial–fungal interactions in soil (Table 2.2). In the light of the likely complexity of the interaction of soil bacteria with emerging fungal hyphal tissues in the soil, we by no means would like to suggest that the selected mechanisms are exhaustive. However, there is emerging evidence for the involvement of these mechanisms in some of the steps in bacterial–fungal interactions, and therefore these are briefly discussed in the following text.

Secretion systems

In order to productively infect fungal hosts, bacteria may excrete effector proteins into the host cytoplasm or into the milieu. Seven different types of secretion systems, numbered I through VII, have been identified in bacteria (Beeckman and Vanrompay 2010). We here discuss just the systems that are deemed to be most important in bacterial–fungal interactions, i.e., the type three and type four secretion systems (T3SS; T4SS).

Type three secretion system

Among the secretion systems, the T3SSs can facilitate the export of particular virulence proteins from Gram-negative bacteria into eukaryotic cells (Arnold et al. 2010). The T3SS is a complex protein secretion system that is composed of up to 25 proteins (Beeckman and Vanrompay 2010). It is based in the bacterial inner membrane, spans this as well as the outer membrane, reaching out to the exterior on one side and the bacterial cytoplasm on the other. It may be involved in antagonistic interactions, as revealed by the fact that *P. aeruginosa* needs a T3SS to kill *Acanthamoeba castellanii* (Abd et al. 2008). Additionally, the biocontrol activity of *P. fluorescens* against *Pythium ultimum* on cucumber was strongly reduced in the *hrcV* deficient mutant strain (*hrcV* is a key gene of the T3SS) that was used (Rezzonico et al. 2005). Moreover, *Burkholderia rhizoxinica* uses a T3SS in its interaction with its fungal host to establish a stable symbiosis (Lackner et al. 2011). Finally, the *B. terrae* BS001 strain, an excellent colonizer of fungal hyphae, was shown to contain a T3SS (Warmink and van Elsas 2008) and there is circumstantial evidence that it may play a role in the interaction.

The effector molecules that are potentially introduced into fungal cells may have essential roles, and, as we understand very little, we are in need of tools to detect them. Several methods have been developed to predict these effectors in genome sequences (Arnold et al. 2010). However, actual proof of effector activity should come from experiments. Moreover, in some cases, flagella and pili were indicated to play essential roles in effector molecule transport (He et al. 2012, O'Boyle et al. 2013).

From work with non-fungal eukaryotic hosts, we know that expression of T3SS genes is influenced by environmental conditions, including Ca^{2+}, contact with host cells, metabolic stress, DNA damage, Cu^{2+} and osmolarity (Yahr and Wolfgang 2006). Moreover, quorum sensing, such as via the *Pseudomonas* quinolone signal (PQS), may be involved (Singh et al. 2010). Such chemical signals may be primordial, as *cis*-2-dodecanoic acid played a vital role in T3SS expression as well as biofilm formation in *P. aeruginosa* (Deng et al. 2013). Possibly, they regulate the expression of T3SS by modulating the SOS and/or QS systems (Mellies et al. 2007, Singh et al. 2010). Additionally, the production of PQS was inhibited by farnesol, which is found in many organisms, including *Candida albicans* (Singh et al. 2010). As a consequence, the expression of T3SS can be regulated by fungi through farnesol, next to PQS. We here conclude that, although there is accumulating information about the complex regulation of the T3SS across systems, we still lack substantial specific data on this regulation in bacterial–fungal interactions.

Type four secretion system

It has been reported that type four secretion systems (T4SSs) can act as secretors of virulence proteins to host cells (Voth et al. 2012). However, T4SSs are traditionally known to be involved in conjugation, allowing the transfer of conjugative plasmids into target cells. Whereas such gene transfer has been known for quite some time for bacterial–bacterial interactions, particular bacteria have also been found to be able to introduce plasmids into fungi. The most prominent organism involved was *Agrobacterium*, which was shown to transfer its Ti plasmid into the soil fungi *Aspergillus awamori* (Gouka et al. 1999), *Agaricus bisporus* (Chen et al. 2000) and *L. bicolor* (Kemppainen and Pardo 2011), albeit under laboratory conditions. These observations reveal one key point, and that is that the T4SS allows cytoplasmic bridges between bacterial and fungal cells to be formed. With this possibility as the basis, we can have an open view for what the genomes of

Table 2.2 Candidate systems involved in *B. terrae* BS001–*Lyophyllum* sp. strain Karsten interaction.

Systems	Role	References
Type three secretion system (T3SS)	Attachment, effector protein injection	Warmink & van Elsas (2008)
Type four secretion system (T4SS)	DNA secretion, biofilm formation (extracellular DNA)	Haq et al. (2014)
Flagella	Attachment, swimming	Haq et al. (2014)
Type 4 pili	Attachment, swarming	Nazir (2012)
Chitinase	Fungal cell wall degradation	Nazir (2012)
Glycerol uptake system	Glycerol uptake and growth promotion	Haq et al. (2014)
Glucose-rich exopolysaccharide biosynthesis (Pel)	Biofilm formation	Haq et al. (2014)
Poly-beta-1,6-N-acetyl-D-glucosamine (PGA) biosynthesis	Biofilm formation	Haq et al. (2014)

mycosphere-dwelling bacteria offer us. For instance, the T4SS harbored by the genome of the mycosphere-competent *B. terrae* BS001, which resides on a genomic island of 72.4 kb, might be involved in conjugation (Haq et al. 2014), across bacteria in the mycosphere, or even from the bacterial host into the fungus. However, we do not know if this system can also transfer effector molecules into its fungal host. Given the possibility that genomic islands, such as the one discussed here, may play important roles in bacterial–fungal interactions, it is vital that more emphasis is placed on studying these.

Pili and flagella

Pili of different types, as well as flagella, are appendages that occur at the outer surface of the cells of many bacterial species. Among these, type IV pili (T4P) are key factors involved in the attachment to host cells. Such attachment allows twitching motility and subsequent virulence (Lemkul and Bevan 2011). The T4P is about 3–4 m long and adhesion of T4P to a surface is normally mediated by its tip (Bakkali 2013). Host glycolipids and glycoproteins are the principal anchoring points for pilus mediated attachment (Lo et al. 2013). It has further been indicated that T4P may be associated with biofilm formation (Heijstra et al. 2009). In the absence of fungal cells, a non–flagellated mutant *Pseudomonas* strain exhibited a higher level of fitness than the wild-type strain (Pion et al. 2013). However, the opposite situation was observed in the presence of fungal mycelia, thus indicating that the presence of flagella offers an advantage for bacteria when occurring at fungal surfaces (Pion et al. 2013). Flagellin, the structural unit of the flagellum, may act as an adhesive factor for binding to the surface (Tran et al. 2011). Flagella can also be involved in biofilm formation and cell-to-cell interactions and play crucial roles in the adaptation of bacteria to non-water saturated environments (Pion et al. 2013). Supporting this view was the finding that swimming and/or swarming motility of bacteria is crucial for bacterial movement along fungal mycelia (Kohlmeier et al. 2005). Moreover, our own group also reported that motility was, in all cases, correlated with the ability of mycosphere bacteria to comigrate with the reference fungus *Lyophyllum* sp. strain Karsten (Nazir et al. 2012). Bacterial cells can use flagella to swim to sites close to a surface through hydrodynamic interactions. When they attach to the surface, they can "walk" on the latter by twitching motility using their T4P (Conrad 2012). Fungi can form liquid water films around their hyphae, which support the bacterial motility. However, a mutant strain of *P. aeruginosa* PAO1, which lacked both pili and flagella, was not able to swim or twitch, yet showed swarming motility on swarming motility plates. Also, flagella-mutant strains could not swarm on such a plate, whereas pilus-mutant strains showed increased spreading ability (Murray and Kazmierczak 2008). Similar results were obtained in a study on *P. aeruginosa* strain PAK. The rate of association of a mutant *P. aeruginosa* strain PAK (lacking pili) with a surface was similar to that of the wild-type strain and was fourfold that of a *pilU* mutant strain (twitching motility negative and surface pili overexpression). Thus, pili may not be required for binding and hyperpilation may hinder binding to a surface in *P. aeruginosa* strain PAK (Tran et al. 2011). So, this is still a controversial issue. Swarming ability is further affected by the

carbon and glucose sources that are available (Murray and Kazmierczak 2008). In certain strains, the pilus may act as a lectin, mediating bacterial adherence to the host (O'Boyle et al. 2013). The *B. terrae* BS001 genome was found to carry gene clusters that encode flagella and pili biosynthesis. It is likely that these systems are also required in the *B. terrae* BS001–*Lyophyllum* sp. strain Karsten interaction.

Chitinase

Chitin is an insoluble linear β-1,4-linked polymer of N-acetylglucosamine (GlcNAc) and it is an essential structural component of fungal cell walls. Chitinases are hydrolytic enzymes that catalyze the degradation of chitin (Ubhayasekera and Karlsson 2012). They occur as free proteins in open environments or as surface-bound protein (Jagmann et al. 2012). Hjort and coworkers identified a novel chitinase, Chi18H8, from disease-suppressive soil cropped with cabbage, and the enzyme showed antifungal activity (Hjort et al. 2014). Similarly, studying a metagenomic library produced in fosmids from a chitin-treated soil, Cretoiu and colleagues in our group recently found a gene for a family-18 ChiA protein (fosmid clone 53), which expressed chitobiosidase activity in *Escherichia coli* (Cretoiu et al. 2015). Moreover, the abundance of fungal plant pathogens was shown to decrease in chitin-amended soil (Cretoiu et al. 2013), whereas chitinase activity was enhanced in this treatment (Kielak et al. 2013). Additionally, a recombinant strain of *Burkholderia vietnamiensis*, containing a new chitinase gene in its chromosome, decreased the growth of various fungi by 15–21% when compared to the wild-type strain (Zhang et al. 2012). Thus, chitinase, when expressed in bacterial associates of soil fungi, may play a crucial role (controlling or parasitizing the fungal host) in bacterial–fungal interactions. Additionally, *N*-acetylglucosamine, the product of the action of chitinase on chitin, can induce the release of antifungal volatiles by *Collimonas pratensis* and *C. fungivorans*, further inhibiting fungal growth (Garbeva et al. 2014). A range of soil fungi and bacteria can produce chitinases. In fact, fungal cell wall chitinases have essential roles during growth, morphogenesis, and sporulation (Adams 2004). It was suggested that chitinase orthologs can evolve under different selective constraints following horizontal gene transfer (HGT) between bacteria and fungi (Ubhayasekera and Karlsson 2012). In previous work in our lab, it was found that the expression level of the *B. terrae* BS001 *chiA* gene increased significantly in liquid microcosms with fungal mats in the so-called compartment "E," which is closely linked to the *Lyophyllum* sp. strain Karsten surface, as compared to compartment "B" (Nazir 2012). These data indicate that the *chiA* gene product may be involved in the inhibition of hyphal growth, potentially related to mushroom formation.

Biofilm formation genes

Bacteria such as *B. terrae* BS001 can form biofilms along growing fungal hyphae (Warmink and van Elsas 2009), as was shown with the reference fungi *Lyophyllum* sp. strain Karsten, as well as with *Trichoderma harzianum*. Studies on other systems confirmed that EPSs, which consist of polysaccharides, proteins, nucleic acids, and lipids can

facilitate biofilm formation, surface adhesion and biofilm stability (Flemming and Wingender 2010). EPSs may also mediate fungal–bacterial interactions (Toljander et al. 2006). Particularly, hydrophobic EPSs were correlated with attachment of the biofilm layers to surfaces (Ras et al. 2013). Among the different compounds that make part of the biofilm matrix, extracellular DNA (eDNA) has attracted a lot of attention. In *Bacillus cereus*, eDNA–RNA complex was present in a biofilm population, but it was absent from a planktonic population. Mutant strains that could not release eDNA lost the capability to attach and form a biofilm (Vilain et al. 2009). Similarly, almost no biofilm was detected in the presence of the enzyme exonuclease I, which specifically degrades single-stranded DNA (Zweig et al. 2013). Remarkably, Novotny and coworkers found that eDNA and nucleic acid-binding protein were essential for biofilm stability in *Burkholderia cenocepacia* (Novotny et al. 2013). Another study showed that eDNA plays a primary role in initial bacterial attachment, but had little effect on mature biofilm (Tang et al. 2013). So, eDNA can play diverse roles in distinct strains and we need more information to elucidate its role in biofilm formation and stability. eDNA also acted as a layer that protects against the action of actinomycin D, a DNA-interacting antibiotic agent (Vilain et al. 2009). In some cases, eDNA was similar to chromosomal DNA, as it was derived from a lysed subpopulation of the bacteria or from membrane vesicles (Allesen-Holm et al. 2006, Renelli et al. 2004). Remarkably, the involvement of T4SS in the secretion of eDNA has recently been suggested (Zweig et al. 2013), which is consistent with the recent finding in our lab that plasmids of the IncP-1β type were able to strengthen the biofilms formed by the mycosphere dweller *Variovorax paradoxus* (Zhang et al. 2015b). Extracellular polysaccharides (exopolysaccharides) are also important, as in *Myxococcus xanthus* the attachment of eDNA to surfaces was mediated by these compounds (Hu et al. 2012). Exopolysaccharides are abundant in fungi and may thus play vital roles in the biofilm formation of bacteria around fungal mycelia.

With respect to bacterial adhesion and induction of activities, a particular case is formed by chitin. It was shown that *Francisella novicida* can form biofilms on a chitin surface (crab shell pieces). Moreover, chitinase was essential for biofilm formation by this bacterium. So, activation of a specific biofilm formation program in a bacterium on a chitin surface may result in the degradation of chitin, spurring bacterial growth and survival. In addition, adhesion of the bacterium to the substrate was mediated by the so-called 'Sec' translocon (Margolis et al. 2010). In other cases, T4P (Frischkorn et al. 2013) or cellulose (Brandl et al. 2011) were required for biofilm formation by bacteria on chitin. Bacteria that use different chitin degradation mechanisms might also coexist by formation of a mixed-species biofilm (Jagmann et al. 2012). As fungal cell walls are composed of chitin, such chitins may constitute true anchoring points for the attachment of bacteria to fungal mycelia. This may result in directed gene expression, including biofilm formation.

Indeed, the fungal-interactive bacterium *B. terrae* BS001 was shown to contain genes for two systems involved in biofilm formation, i.e. the poly-beta-1,6-*N*-acetyl-D-glucosamine (PGA) as well as glucose-rich exopolysaccharide (Pel) biosynthetic systems Table 2.2 (Haq et al. 2014). As mentioned, *B. terrae* BS001 also contains a T4SS, which may

secrete eDNA into the milieu and mediate biofilm formation. Moreover, chitin, a component of the *Lyophyllum* sp. strain Karsten cell wall, could be used as an anchoring surface inciting biofilm formation.

Fungal-released compounds in bacterial–fungal interactions

As indicated in the foregoing, the utilization of fungal-released compounds, in particular low-molecular-weight carbonaceous compounds, is a major driver of the life of those bacteria that associate with fungal hyphae. The release of trehalose and polyols such as mannitol by EM fungi is certainly of influence on the local selection of bacteria (Danell et al. 1993, Frey et al. 1997, Rangel-Castro et al. 2002a, Rangel-Castro et al. 2002b). For instance, the use of fungal-released trehalose by fungal-associated *Pseudomonas* sp. is considered to be an important element of bacterial growth in the mycosphere of the EM fungus *Cantharellus cibarius* (Danell et al. 1993). Particular bacteria associated with fungi in soil are thus selected preferentially over others. Moreover, *V. paradoxus*-like bacteria (for example strain HB44) from the mycosphere of *L. proxima* were shown to be able to grow on compounds released by *Lyophyllum* sp. strain Karsten, particularly glycerol. The study also reported the release of other compounds, such as acetic acid and formic acid, by the fungus (Boersma et al. 2010). More recently, it was found that *Lyophyllum* sp. strain Karsten releases glycerol-rich exudates due to a stimulatory effect exerted by *B. terrae* BS001, which is also an avid glycerol consumer. This phenomenon may be of great significance for the fitness of strain BS001 in the mycosphere (Nazir et al. 2013). The selection of particular bacterial groups in the mycosphere and other fungal-influenced habitats in soil has thus been, at least partially, attributed to fungal-exuded carbonaceous compounds. Interestingly, we recently found that the genome of *B. terrae* BS001 possesses a set of genes that encode membrane-bound glycerol uptake (GUP) transporters, next to genes responsible for glycerol metabolism (Haq et al. 2014). The *gup* gene was located in a region of genome plasticity, which led to the hypothesis that it might have been acquired through HGT, allowing survival value in the mycosphere.

Genomics of the interactome of *B. terrae* BS001 and *Lyophyllum* sp. Strain Karsten

To better understand the ecophysiology of *B. terrae* BS001 interacting with the fungus *Lyophyllum* sp. Karsten, we here review recent findings from analyses of the *B. terrae* BS001 genome. The exceptionally large size of this genome (~11.5 Mb) suggests that a patchwork of diverse genetic systems drives the interaction of this organism with its environment, including fungal counterparts. Indeed, strain BS001 harbors genetic systems involved in flagellar biosynthesis, chemotaxis and biofilm formation, next to the T3SS (Haq et al. 2014). This is consistent with the occurrence of physical interactions between the two entities of the interactome. However, the exact nature of this physicality can only be understood when mutational analyses of selected gene regions of strain BS001 is carried out.

Our analyses further unveiled the presence of a repertoire of membrane bound transporters in strain BS001 that may be involved in capturing nutrients from the fungal counterpart. This includes carbohydrates, amino acids, fatty acids, glycerol, and cell wall detritus. In addition, the genome of strain BS001 was found to carry a plethora of genomic islands, which together, constituted 16.48% of the total genome size. This included the aforementioned integrated 70.4 kb plasmid-like stretch containing a complete T4SS next to some other plasmid-typical genes (Haq et al. 2014). *B. terrae* BS001, in its life in soil, may have faced situations or conditions, in which the acquisition of certain genetic traits allowed it to survive under the highly demanding soil conditions. It is likely that survival and adaptation to the mycosphere of different soil fungi is one determining facet of its lifestyle in the soil.

Mutational analysis to understand bacterial–fungal interactions in soil

In order to further promote our understanding of the exact role of particular genetic systems in bacterial–fungal interactions, mutational analysis followed by ecological experiments needs to be applied. Rezzonico and colleagues inactivated the *hrcV* gene in *P. fluorescens* KD, which encodes the T3SS inner membrane channel protein. This resulted in the biocontrol activity of *P. fluorescens* KD against *P. ultimum* on cucumber being strongly reduced (Rezzonico et al. 2005). In addition, T3SS-defective *B. rhizoxinica* cannot establish a stable symbiosis with its fungal host *Rhizopus microsporus* (Lackner et al. 2011), whereas the wild-type strain can, showing that the T3SS has a definite function in the symbiosis. Also, site-specific mutagenesis was used in analyzing the role of bacterial flagella. Thus a flagellar mutant (Δ*fliM*) of bacterial strain *P. putida* KT2440 showed reduced dispersal on *Morchella crassipes* hyphae (Pion et al. 2013). Moreover, following RT-PCR analysis, gene inactivation experiments confirmed that the polyketide synthase gene *orsA*, which is in the genome of *Aspergillus nidulans* and involved in secondary metabolite biosynthesis, is induced by physical *Streptomyces hygroscopicus–A. nidulans* interactions (Schroeckh et al. 2009).

In general, site-specific mutagenesis is key to identifying the genes that are involved in particular processes in bacterial–fungal interactions. However, it is limited in that it needs a predefined list of candidate genetic systems that are potentially involved in the interactions. The alternative, random insertion mutagenesis, is a time-consuming method used to investigate the ecological mechanisms, such as those involved in bacterial–fungal interactions. Although tedious, this method enables us to get broader insights into the plethora of systems that play a role in bacterial–fungal interactions. Heijstra and colleagues made a random transposon mutant library of *Acidovorax temperans* CB2; they screened for biofilm-deficient strains in 2,500 mutants and found that inactivation of the *pilB* gene resulted in the absence of T4P, as well as the ability of biofilm formation (Heijstra et al. 2009).

In conclusion, both random and site-specific mutagenesis methods followed by ecological experiments provide us with strategies that allow to pinpoint the mechanisms

involved in bacterial–fungal interactions. Both methods are thus extremely helpful in fostering our understanding of bacterial–fungal interactions, however, the process is critically dependent on the availability of sound methods for screening the effects of the mutations, i.e. the phenotypes.

Horizontal gene transfer and adaptability of bacteria in the mycosphere

HGT is the main driving force for bacteria in that it enables rapid evolution and adaptation to local conditions. HGT is carried out by three different mechanisms: transformation, transduction and conjugation. Of these, conjugation is considered to be an essential mechanism of bacterial adaptation in soil (Thomas and Nielsen 2005). Mobile genetic elements such as plasmids, transposons and integrative and conjugative elements are the vehicles of HGT (Frost et al. 2005). Whereas some horizontal gene acquisitions may be neutral or deleterious, others may confer selective fitness in the mycosphere. Examples may be resistance to fungal-produced antibiotics, degradation of fungal-released "recalcitrant" compounds, virulence and symbiosis/association factors. In particular, conjugative plasmids play important roles for bacteria in their adaptation to local challenges which may range from local toxicity (antibiotics), nutrient scarcity or local opportunities (Heuer and Smalla 2012, Zhang et al. 2014). There is evidence that plasmid transfers are stimulated in the mycosphere (Zhang et al. 2015a). In these, essential nutrients may increase the metabolic activity of bacteria, stimulating plasmid transfer. Van Elsas et al. (2003) already indicated the importance of the enhanced nutrient availability in soil hot spots for HGT processes. What plasmid types might we encounter in the mycosphere? We recently obtained evidence for the presence of two plasmids of the IncP-1β group, denoted pHB44 and pBS64, in cultured *V. paradoxus* types that dominated the mycosphere of *L. proxima* (Zhang et al. 2015b). Moreover, there is additional evidence (obtained from triparental exogenous isolations) for the presence of IncP-1β plasmids in mycosphere-dwelling bacterial communities (Zhang et al. 2015a). Specifically, we isolated several IncP-1β plasmids from the mycosphere of the EM fungi *Inocybe* and *Clitocybe* sp., next to PromA group plasmids (Van der Auwera et al. 2009) from mycosphere bacterial communities (Zhang et al. 2015a).There is emerging evidence that plasmids of the latter group might play roles in the mycosphere. PromA stands for (highly) promiscuous, i.e. it constitutes a novel broad host range plasmid group. The first piece of evidence for this contention was the finding of plasmid pIPO2 from the rhizosphere of wheat (Van Elsas et al. 1998). Later, another very similar plasmid, pTER331, was found inside a *C. fungivorans* strain isolated from Marram grass (*Ammophila arenaria*) [in which there was the likely presence of a fungus]. The finding of this plasmid inside only one in 22 strains of the same species indicated it might have incidental fitness value for the mycosphere-associated *Collimonas* population. As indicated in the foregoing, another piece of evidence is provided by the recent isolation, by triparental exogenous isolation, of PromA-like plasmids from the mycosphere of the EM fungus *Inocybe* sp. (Zhang et al. 2015a). The 'action' of plasmids in the mycosphere depicts this habitat as a true 'arena' of

genome plasticity, as recently suggested (Zhang et al. 2014). Indeed, plasmids from the IncP-1β and PromA groups are key to our considerations about bacterial adaptability in the mycosphere and will certainly be the target of further studies as to their potential ecological relevance.

What type of ecological support could be provided by plasmids? Plasmid accessory genes are known to often greatly contribute to the phenotypic traits of their hosts. In particular, plasmids that have roles in symbioses, i.e. those that allow rhizobia to interact with host plants, are important. Yet, as argued in the foregoing, other plasmids, referred to as cryptic ones, i.e. those that do not show an easily recognizable phenotype, may also have their 'raison d'être' in the mycosphere. Concerning the two plasmid types found in the mycosphere (IncP-1β and PromA), in the IncP-1β plasmids the accessory genes were found to always insert into the backbone at the same sites. The insertion usually occurs either between the *tra* and *trb* region or between the *trfA* gene of the replication region and the origin of vegetative replication *oriV*. It is obvious that these insertions take place at sites which would not influence the basic functioning of the plasmid (Dennis 2005, Schlüter et al. 2007) and is due to targeted insertion and selection for plasmid stability (Sota et al. 2007). Very recently, Norberg et al. (2011) demonstrated that homologous recombination is a prominent feature of IncP-1 plasmid backbone evolution. Such plasmids apparently confer adaptive value to a variety of local conditions, at small temporal or spatial scales (Slater et al. 2010). For instance, a response to sub-inhibitory concentrations of antibiotics may enhance antibiotic resistance genes (Beaber et al. 2004); this process might have allowed soil bacteria to explore new habitats (Aminov 2009). Also, the abundance of heavy metal resistance genes in plasmids of soil bacteria may be correlated with local pollution (Smalla and Heuer 2006, Smit et al. 1998), being IncP-1 plasmids of importance in adaptation to mercury (Smalla et al. 2006). On the other hand, plasmids may confer an energy burden to the host; if the cost of carrying a plasmid is not balanced by fitness enhancement, plasmids will not persist (Ochman and Davalos 2006). We here posit that the mycosphere environment shows periodic challenges/selective force, and so there are benefits to be gained by bacterial populations from plasmids. Transfer, recombination, occasional selection of plasmid-carrying strains and/or co-selection are prime mechanisms that play roles (Heuer and Smalla 2012).

The two IncP-1β group plasmids of *V. paradoxus* isolated from the mycosphere of *L. proxima* (Zhang et al. 2015b) increased the production of biofilms in their host, an important asset of successful mycosphere colonizers (Warmink et al. 2009). Moreover, an iron-uptake system assisted the host endowed with this plasmid in the mycosphere, an effect that was only detectable in the case the iron levels were low. In contrast, in the soil with additional Fe, the plasmid-carrying strain revealed poorer survival than its plasmidless counterpart, revealing the energy burden conferred by the plasmid upon the host cell. The features carried by both plasmids had most likely recombined into these following a HGT.

Conclusions and outlook

The living soil, being a rich environment in terms of biodiversity, confers a unique suite of microhabitats, in which organisms from different realms of life (prokaryotes and eukaryotes) live, either independently or in close association with one another. The microhabitat provided by soil fungi allows soil bacteria to deal with unique resources provided by the fungal partner that would not be available in the bare soil. Thus, deterministic bacterial–fungal associations are successfully established, as the availability of nutrients and the capabilities to access them are guaranteed. It is well possible that long-lasting bacterial–fungal associations are based on a "give and take" policy, ensuring that mutual benefits are warranted for both partners of the association. Here, we highlighted the mycosphere as a highly specific habitat of the soil and discussed how mycosphere-inhabiting organisms may adapt to the respective niches offered by the soil fungi, in addition to considerations as to what factors are primordial in their adaptation. In particular, the mechanisms of interactions of bacterial mycosphere associates with their fungal host are discussed. We emphasized the role of carbonaceous compounds as major drivers of the interactions, next to the importance of the processes leading to the physical association between the two partners (i.e. sensing, migration, attachment, growth and biofilm formation). Moreover, the putative key roles of 'parasitic' phenomena, such as mycophagy, with a role for chitinase and secretion of T3SS effectors, is highlighted. One clear take-home message obtained from the available evidence so far is that the reality of the interactome in the mycosphere is remarkably complex, as there is an interplay of several mechanisms in both the bacterial and the fungal partner that act at the same time in this habitat. Concerning the bacterial partner, the role of plasmids and of HGT in the adaptation to the mycosphere is discussed. Different plasmids are likely to effectively play a role in the adaptation of bacteria to the niches offered by the fungal partner, enabling them to acquire important genetic traits, that give them an edge over those bacteria that lack such traits. Overall, we are currently seeing only a glimpse of the interactions that take place in this crosspoint of two soil constituents of major relevance to soil function, i.e. soil bacteria and soil fungi. There is a growing need to explore this nascent field, which lays at the crossroads of soil microbiology and ecology from different perspectives using state-of-the-art techniques such as metagenomics, transcriptomics and metabolomics. These approaches will, by and large, enable us to better understand bacterial–fungal interactions and the genes and genetic systems that are at the forefront in establishing such interactions.

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Chapter 3

3 The genome of the fungal-interactive soil bacterium *Burkholderia terrae* BS001—a plethora of outstanding interactive capabilities unveiled

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Abstract

Burkholderia terrae strain BS001, obtained as an inhabitant of the mycosphere of Laccaria proxima (a close relative of *Lyophyllum* sp. strain Karsten), actively interacts with *Lyophyllum* sp. strain Karsten. We here summarize the remarkable ecological behavior of *B. terrae* BS001 in the mycosphere and add key data to this. Moreover, we extensively analyze the approximately 11.5-Mb five-replicon genome of *B. terrae* BS001 and highlight its remarkable features. Seventy-nine regions of genomic plasticity (RGP), that is, 16.48% of the total genome size, were found. One 70.42-kb RGP, RGP76, revealed a typical conjugal element structure, including a full type 4 secretion system. Comparative analyses across 24 related *Burkholderia* genomes revealed that 95.66% of the total BS001 genome belongs to the variable part, whereas the remaining 4.34% constitutes the core genome. Genes for biofilm formation and several secretion systems, under which a type 3 secretion system (T3SS), were found, which is consistent with the hypothesis that T3SSs play a role in the interaction with *Lyophyllum* sp. strain Karsten. The high number of predicted metabolic pathways and membrane transporters suggested that strain BS001 can take up and utilize a range of sugars, amino acids and organic acids. In particular, a unique glycerol uptake system was found. The BS001 genome further contains genetic systems for the degradation of complex organic compounds. Moreover, gene clusters encoding nonribosomal peptide synthetases (NRPS) and hybrid polyketide synthases/NRPS were found, highlighting the potential role of secondary metabolites in the ecology of strain BS001. The patchwork of genetic features observed in the genome is consistent with the notion that 1) horizontal gene transfer is a main driver of *B. terrae* BS001 adaptation and 2) the organism is very flexible in its ecological behavior in soil.

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Introduction

As a result of the spatial separation in soil between bacterial cells and substrate, as well as the general recalcitrancy of substrate to degradation, soil bacteria most of the time perceive conditions of nutrient scarcity in their habitat and occur in a state of dormancy. However, there are so-called hotspots in soil in which soil bacteria are activated. One such hotspot for bacterial activity is the mycosphere, that is, the narrow zone of influence around the fungal hyphae in soil.

Evolutionary and genome-shaping events in soil bacteria (mutations and horizontal gene transfer and selective processes) are thought to mostly take place in high-activity microenvironments, and hence a thorough investigation of the genomic diversity of organisms dwelling in the mycosphere is warranted. On the basis of two different experimental setups, *Burkholderia terrae* like organisms were previously found to be key associates with fungal hyphae in soil (Warmink and van Elsas 2009). Indeed, since this initial discovery, a fascinating interaction between the mycosphere-isolated *B. terrae* strain BS001 and *Lyophyllum* sp. strain Karsten has been unveiled (Warmink and van Elsas 2009, Nazir et al. 2012a).

The draft genome sequence of strain BS001 was recently announced (Nazir et al. 2012b), but no detail on key genetic systems and on how the genomic information determines the ecophysiological behavior of the organism was provided. From the previous work, circumstantial ecologically based evidence was obtained for the contention that the presence of type 3 secretion system (T3SS), motility and biofilm formation traits as well as glycerol uptake and metabolism genes might offer selective advantages to the organism in the mycosphere. However, a tight linkage of the ecological data with genome features of strain BS001 has been lacking so far, in spite of the fact that this linkage is needed to build hypotheses that are testable in further experiments.

Thus, in this study, we provide an in-depth analysis of the genome of *B. terrae* BS001, focusing on the plethora of genetic systems that potentially drive the interactions between *B. terrae* BS001 and the soil fungi it associates with. As the reference fungus, *Lyophyllum* sp. strain Karsten was used. To achieve our aim of assessing the genome in a broad sense and also addressing the uniqueness of particular features for strain BS001,we then performed comparative analyses of selected salient features of the genome of *B. terrae* BS001 with those of other (related) *Burkholderia* strains. From the data, we make inferences about the implications for the behavior of strain BS001 in soil, in an attempt to narrow the gap between (selected) genotype and phenotype.

Materials and Methods

Genome analysis and annotation

The genome of *B. terrae* BS001 was submitted to MicroScope platform that is hosted at Genoscope, for analysis. The genome data was integrated to PkGDB database of MicroScope and we have used the gene locus tags from the same database in this study. The

genome sequencing data had been submitted to NCBI under accession number PRJNA157903/AKAU00000000.

Pan–core genome computation

The pan–core genome analysis was computed based on single-linkage clustering algorithm in the software package siLix (SIngle LInkage Clustering of Sequences), hosted by MicroScope. The algorithm implemented in the siLix is based on a principle where a sequence A if considered homologous to sequence B and sequence B is homologous to C, then all the sequences (A, B, and C) are clustered in a same family, irrespective of the similarity between sequences A and C (Miele et al. 2011). Therefore if a gene X is homologous to gene Y, they are clustered together and if Y is a homolog of Z, then all three of them are grouped into the same MicroScope gene family (MICFAM).

Synteny calculations

We employed MicroScope platform to compute synteny of *B. terrae* BS001 using data from PkGDB and NCBI RefSeq databases. Genes that satisfied BLASTP alignment threshold (35% sequence identity over 80% length of the smallest protein) or bidirectional best hit criteria, were defined as putative orthologs. Based on these relationships, synteny groups or syntons among other bacterial genomes were searched. The approach allows chromosomal rearrangements including insertions, deletions, and inversions. Gap parameter was set to five genes, representing maximum number of consecutive genes not part of synteny group.

Bioinformatics and comparative genome analysis

For comparative analysis of genomes, their metabolic profile prediction and pan–core genome analysis and genomic islands predictions, we used MicroScope platform of the Genoscope (http://www.genoscope.cns.fr/agc/microscope/home/index.php, last accessed June 26, 2014). The RGP finder was used to detect more than 5-kb synteny breaks across a query genome and closely related (comparator) ones, followed by screening of the identified region for HGT features, such as the presence of mobility genes, tRNA hotspots and deviation of the G+C% from the main value. It then employed "SIGI-HMM" (based on the use of Hidden Markov Models, using codon usage to characterize the origin of genes) to measure codon usage aberrations (Waack et al. 2006) and "Alien-Hunter," which is an "Interpolated Variable Order Motif" that uses variable-order motif distributions (2–8) to exploit compositional biases (Vernikos and Parkhill 2006).

Membrane transporters prediction and annotation were performed using TransAPP (Transporter Automatic Annotation Pipeline) of TransportDB (www.membranetransport.org, last accessed June 26, 2014). Potential type 3 effectors were predicted using www.effectors.org (last accessed June 26, 2014) with parameters (classification: [standard set] cutoff: [0.9999; selective]), whereas signal peptides were predicted using online server SignalP 4.1 (Petersen et al. 2011). Antibiotics and secondary metabolites analysis shell (antiSMASH) (Blin et al. 2013) online server was used for prediction and analysis of secondary metabolites.

Metabolic pathways comparison

The metabolic profiles of *B. terrae* BS001 and other genomes in this study were analyzed using MicroCyc based on MetaCyc (Caspi et al. 2010) of the MicroScope platform, as a reference pathway database. For each genome, comparative analysis of metabolic pathways was accomplished using MicroScope platform (https://www.genoscope.cns.fr/agc/microscope/metabolism, last accessed June 26, 2014). Each pathway has its own completion value (completion value =the number of enzymatic reactions in a particular pathway in a given organism divided by the total number of enzymatic reactions in the same pathway in MetaCyc). Hierarchical cluster analysis of the MicroCyc predicted metabolic pathways for *B. terrae* BS001 was performed using MeV tool integrated in the MicroScope, taking result comparisons as input and using pathway completion values. The analysis was performed taking into consideration other *Burkholderia* and a number of additional bacterial genomes integrated in the PkGDB database.

Phylogenetic analysis

Phylogenetic trees were made with MEGA 6 (Tamura et al. 2013). Poorly aligned regions and gaps were manually eliminated. Maximum-likelihood method based on the JTT matrix based model (Jones et al. 1992) was used to infer the evolutionary history for the T3SS, T4SS, ACC-deaminase, and tyrosinase genes. For modeling of evolutionary rate differences among sites, discrete Gamma distribution was used (4 categories [+G, parameter =3.8674]). For network analysis of membrane transporters, amino acid sequences of random ORFs (ATP binding component of ABC and MFS transporters) were aligned using ClustalW in MEGA. The matrix was then analyzed in SplitsTree4 software using WAG model with Gamma 4.0 and ProteinMLdist (characters).

Results and Discussion

Genome properties

We first briefly describe the overall genome characteristics, before considering particular genetic systems that might relate to the ecological behavior of *B. terrae* BS001. The genome has an estimated size of about 11.5 Mb, and was found to consist of five replicons with a G+C content of 61.8% (Nazir et al. 2012b). The genome of *B. terrae* BS001 is extremely large compared with the genomes of other *Burkholderia* species, that is, *B. rhizoxinica* HKI454 (3.75 Mb) (Lackner et al. 2011b), *B. phytofirmans* PsJN (8.21 Mb) (Mitter et al. 2013), and *B. phymatum* STM815 (8.68 Mb). The total number of predicted coding sequences (CDSs) was 12,047, compared with 4,146 in the genome of *B. rhizoxinica* HKI454, 8,043 in that of *B. phytofirmans* PsJN, and 8,434 in that of *B. phymatum* STM815 (Table 3.1). A relatively high number of CDSs, amounting to 38% of the genome, was predicted to encode proteins of unknown function.

The giant genome size and dense gene coverage suggest that strain BS001 disposes of a suite of lifestyle choices in the soil that are selected as a result of the conditions that may reign locally in the microhabitat it occupies in soil. In previous studies, we found a

(one-sided) correlation between fungal interactivity in soil bacteria and the presence of 1) motility traits and 2) a T3SS. In the following section, we highlight the ecological features of *B. terrae* BS001 in soil and explore the genome for the presence of these as well as other characteristics that may correlate with the (fungal-interactive and otherwise) lifestyle in soil.

Ecological features that presumably drive the genome structure of B. terrae BS001

Salient ecological features of *B. terrae* BS001 in soil and the mycosphere are summarized in supplementary Table S3.1. *B. terrae* was found to be a key inhabitant of the mycosphere of *Laccaria proxima* in the field, whereas *B. terrae* BS001 was an avid migrator with growing *Lyophyllum* sp. strain Karsten mycelium. The rapid migration along the mycelial growth front endowed strain BS001 with the remarkable capacity to successfully reach novel (remote) microhabitats in the soil (Warmink and van Elsas 2009). Moreover, strain BS001 revealed a migration helper effect, as it stimulated the comigration of the nonmigrator *Dyella japonica* BS003 along with the fungal hyphae (Warmink et al. 2011). The organism further showed avid biofilm formation around hyphae of the fungal host. The extracellular polysaccharides constituting the biofilm are thought to act like a shield against antifungal agents (Warmink and van Elsas 2009), allowing protection of the fungal host. *B. terrae* BS001 was further found to be able to comigrate with a range of other selected soil fungi through soil, allowing the notion that it is a "generalist" migrator. The fungal-interactive capacity was suggested to involve bacterial motility as well as the activity of a T3SS (Warmink and van Elsas 2009). Moreover, considering resource utilization, *B. terrae* BS001—much like *Variovorax paradoxus* HB44—was found to grow on *Lyophyllum* sp. strain Karsten released glycerol (tested in liquid microcosms). The organism was even able to trigger the release of glycerol from host cells by an as-yet-unknown mechanism (Boersma et al. 2010, Nazir et al. 2013). Furthermore, it grew avidly on glycerol as the sole carbon source (data not shown). In this sense, *B. terrae* BS001 is likely to be an excellent competitor in soil in competitive situations where carbon sources such as glycerol become available. Finally, *B. terrae* BS001 was indicated to affect the physiology of *Lyophyllum* sp. strain Karsten, including the induction of a secretome that contained high levels of glycerol, next to the inhibition of primordium formation by fungal mycelial mats in the microcosms (Nazir et al. 2013).

Table 3.1 Comparison and general features of *Burkholderia* genomes.

Bacterial specie	*B. terrae* BS001	*B. rhizoxinica* HKI454	*B. phytofirmans* PsJN	*B. phymatum* STM815
Genome size (Mb)	11.5	3.75	8.21	8.68
GC content (%)	61.8	60.7	62.2	62.3
Number of CDSs	12,047	4,146	8,043	8,434
tRNAs	51	59	63	62
rRNA operons	4	9	18	16

Core and pan genome analyses

We used the "MicroScope" platform (Vallenet et al. 2013) to determine the genetic landscape of the *B. terrae* BS001 genome, taking as references the available (MicroScope) genomes of 23 other *Burkholderia* species, that is, *B. phymatum* STM815, *B. phytofirmans* PsJN, *B. rhizoxinica* HKI454, *B. glumae* (strains; AU6208, LMG 2196, BGR1, and 3252-8), *Burkholderia* sp. (strains; CCGE1002, CCGE1003, and TJ149), *B. lata* 383, *B. kuruiensis* M130, *B. thailandensis* E264, *B. mallei* ATCC 23344, *B. pseudomallei* K96243, *B. cenocepacia* AU 1054, *B. phenoliruptrix* BR3459a, *B. ambifaria* AMMD, *B. gladioli* (strains; 3848s-5 and BSR3), *B. vietnamiensis* G4, *B. multivorans* ATCC 17616, and *B. xenovorans* LB400. First, *B. terrae* BS001 had the largest genome of all (11.5 Mb compared with 9.7 Mb for *B. xenovorans* LB400), indicating a large accessory gene part. The pan genome across these genomes is defined as the sum of the core genome (genes present in all twenty four organisms), the variable genomes (genes found in some organisms while being absent from the others) and the strain-specific genes (Medini et al. 2005), whereas the core genome is the collective number of shared genes between all genomes. The analysis revealed a total of 180,124 CDSs to be present in the pan genome of the 24

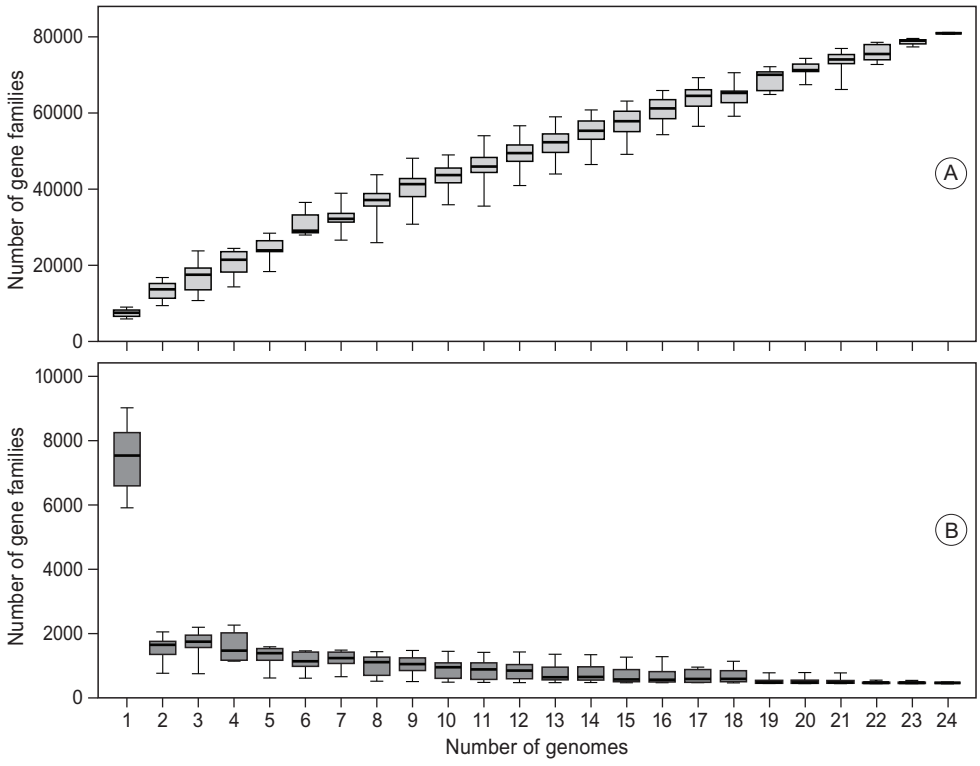

Figure 3.1 Core–pan genome size evolution. (A) Pan genome: Pan genome size is directly proportional to the number of genomes. (B) Core genome: Core genome size is inversely proportional to the number of genomes.

genomes, which were divided over 81,027 MicroScope gene families (MICFAM; see Materials and Methods section). The core genome across all *Burkholderia* species comprised 472 gene families; however, the exact number of core CDSs was different across the compared genomes, as a result of some core CDSs having more than one copies. Thus, strain BS001 was found to have a core of 523 CDSs as several core genes had paralogs. The 523 CDSs core genome of strain BS001 encompassed only 4.34% of its total genome. The remaining 95.66% (11,522 CDSs) thus constituted the variable or accessory part. Of these, there were 6,099 (50.63%) strain-specific CDSs in strain BS001 whereas the remainder (~5,000) were volatile, meaning that they were present across a limited number of genomes (Supplementary Table S3.2). Clearly, as found by others, we also found that the sizes of the core and pan genomes depended strongly on the number of genomes analyzed, resulting in shrinking core and expanding pan genomes with depth of genome sampling (Fig. 3.1). Also, across the *Burkholderia* strains that were analyzed, the pan genome should be considered to be open, as, with every new genome sequenced, several hundreds of novel genes are found (data not shown). Our analysis further revealed that the core genome across all 24 *Burkholderia* genomes consisted of CDSs encoding key cellular functions such as DNA recombination, replication, metabolism, transcription, translation, glycolysis, amino acid activation (tRNAs), chaperoning, RNA modification, transcription regulation, DNA repair, fatty acid biosynthesis, peptidoglycan biosynthesis, posttranslational modification, and cell division. Overall, the level of homology (translated amino acid sequences) of the genome of *B. terrae* strain BS001 with these genomes was between 55% and 60%.

Phylogeny and synteny groups

We first constructed a maximum-likelihood tree on the basis of seven concatenated genes for representative *Burkholderia* species from groups A (plant/soil related, non-pathogens) and B (pathogens) (Estrada-de los Santos et al. 2013) to locate the position of strain BS001. Indeed, strain BS001 belonged to group A, being very closely related to *B. phymatum* STM815 (Fig. 3.2). In this analysis, we further found that *B. rhizoxinica* HKI454 could not be related to any of the other *Burkholderia* groups, as also observed by Estrada-de los Santos et al. (2013). We then performed an analysis of synteny between the strain BS001 genome versus selected available genomes in the NCBI (National Center for Biotechnology Information) RefSeq (reference sequence) database. The analyses (Fig. 3.3) revealed that the *B. phymatum* STM815 genome had highest CDSs synteny to the BS001 genome, that is, 76.78% (Fig. 3.2), followed by *B. graminis* C4D1M, *B. phytofirmans* PsJN, and *B. kururiensis* M130 (69.47%, 67.97%, and 64.79%, respectively). These four *Burkholderia* species are members of *Burkholderia* group A (Estrada-de los Santos et al. 2013) to which *B. terrae* BS001 also belongs (Nazir et al. 2012a). A trend of decreasing % CDSs synteny was observed as we moved from *Burkholderia* group A members to those of group B, that is, *B. cenocepacia* AU 1056 (58.66%), *B. pseudomallei* K96243 (58.1%), *B. mallei* ATCC 23344 (55.49%), and *B. vietnamiensis* G4 (51.34%) (Fig. 3.3). The levels of synteny of the outgroups, that is, *Ralstonia*, *Cupriavidus*, and

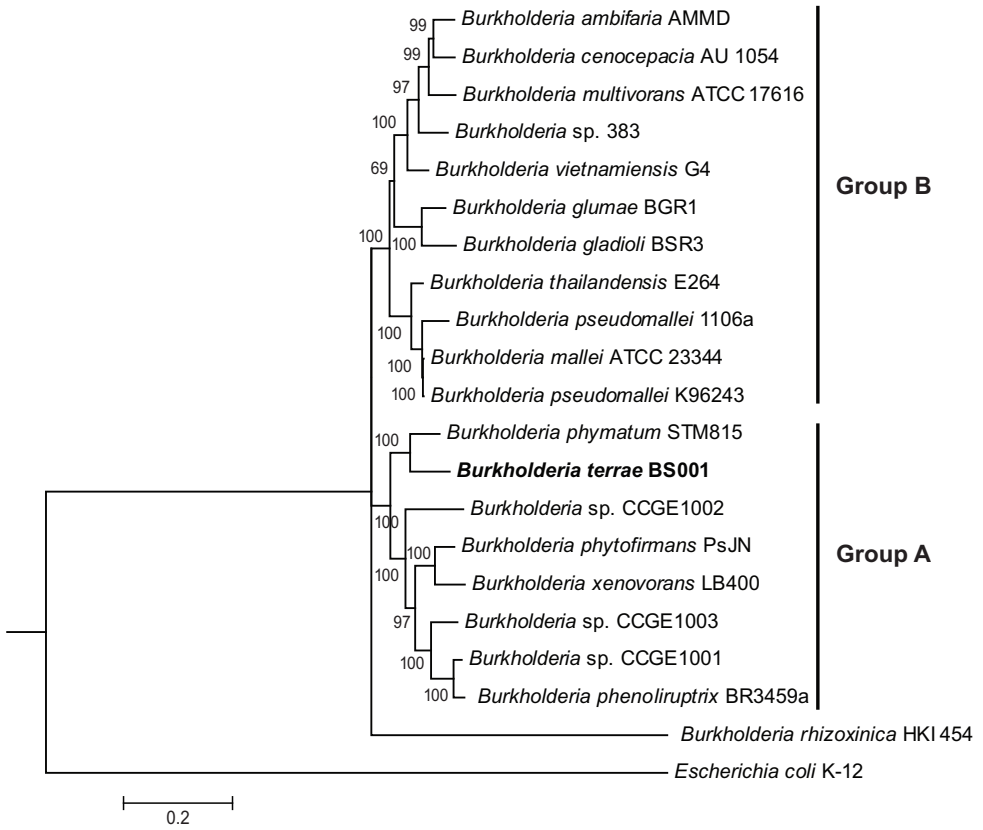

Figure 3.2 A maximum-likelihood tree illustrating the relationship of selected *Burkholderia* species. The tree is based on alignment of seven concatenated core genes (*aroE*, *dnaE*, *groeL*, *gyrB*, *mutL*, *recA*, and *rpoB*) and was generated using MEGA. *Burkholderia terrae* BS001 falls within group A and is closely related to *B. phymatum* STM815. Bootstrap values (more than 50%) are shown at each node. Group A and group B are defined by Estrada-de los Santos et al. 2013.

Escherichia, with the genome of the strain BS001, were much lower. These findings suggest that the conservation of synteny was highest among members of the group A *Burkholderia* and it gradually decreased as the phylogenetic distance increased.

Secretion systems and effector proteins

Secretion systems such as the T3SS may be important for bacteria when these interact with hosts such as fungi (Lackner et al. 2011a). We investigated the genome of *B. terrae* BS001 for the presence of (protein) secretion systems, which have a potential role in the interaction with *Lyophyllum* sp. strain Karsten. Basically, we found all known protein secretion systems, that is, the type 1 secretion system (T1SS) through to the type 6 secretion system (T6SS), in the genome of *B. terrae* BS001. The T1SS is normally composed of three constituents, including ATP-binding cassette (ABC) transporters, membrane

fusion proteins, and outer membrane proteins (Delepelaire 2004, Holland et al. 2005). The BS001 genome harbored ABC transporters and related proteins that are part of the T1SS (discussed later in detail).

The type 2 secretion system (T2SS) (constituted of 9–11 genes), which is used to translocate a wide range of proteins, was present as three gene clusters, numbered 1 (AKAUv1_257005–AKAUv1_2570061), 2 (AKAUv1_2570149–AKAUv1_2570159), and 3 (AKAUv1_790112–AKAUv1_790123) across the genome. The latter (11-genes) cluster was completely syntenous and highly homologous (>80% amino acid identity [AAI]) to a similar system in *B. phymatum* STM815. The BS001 genome also revealed the presence of two copies of a subtype of the T2SS, the Tad (tight adherence) macromolecular transport system. *Tad* genes encode a machinery that is required for the assembly of adhesive fimbrial low-molecular-weight protein (Flp) pili. The machinery is associated with a so-called widespread colonization island (Tomich et al. 2007). The Flp pili play crucial roles in the formation of biofilms, colonization, and pathogenesis across several bacterial genera (Tomich et al. 2007). The first 14-genes *tad* cluster (AKAUv1_990039– AKAUv1_990052) was highly syntenous and homologous to similar systems in *B. phytofirmans* PsJN (~60–70% AAI) and *B. phymatum* STM815 (~70–75% AAI). The second, 13-gene, *tad* copy (AKAUv1_550027–AKAUv1_550039), located at a different position, also had

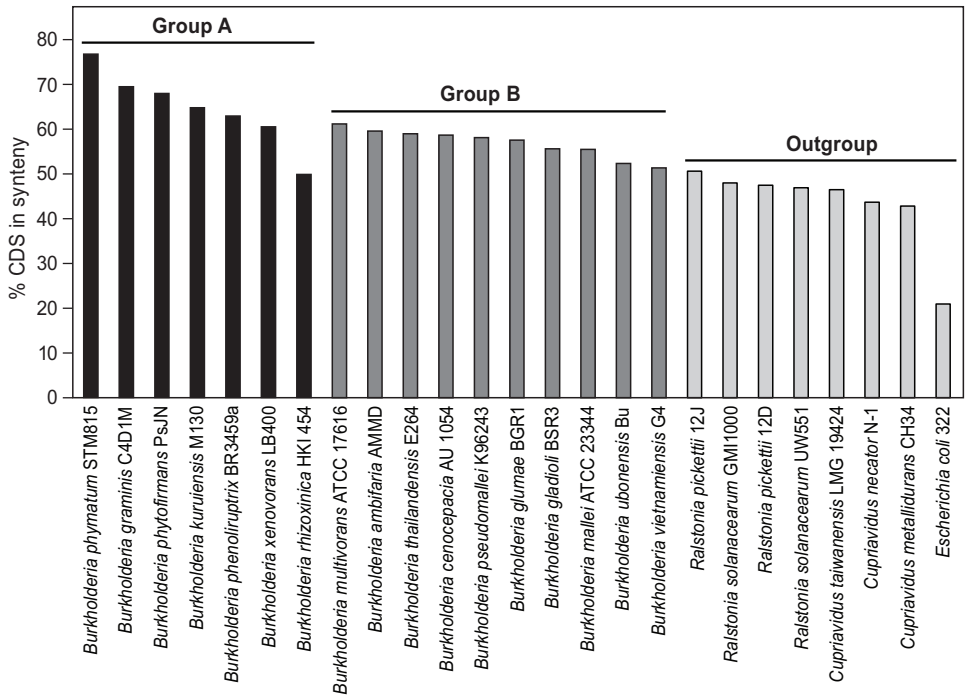

Figure 3.3 Synteny groups. Syntenous CDSs (%) of *Burkholderia* species from group A and group B, compared with *B. terrae* BS001, are shown. *Ralstonia*, *Cupriavidus*, and *Escherichia coli* species are included as outgroups.

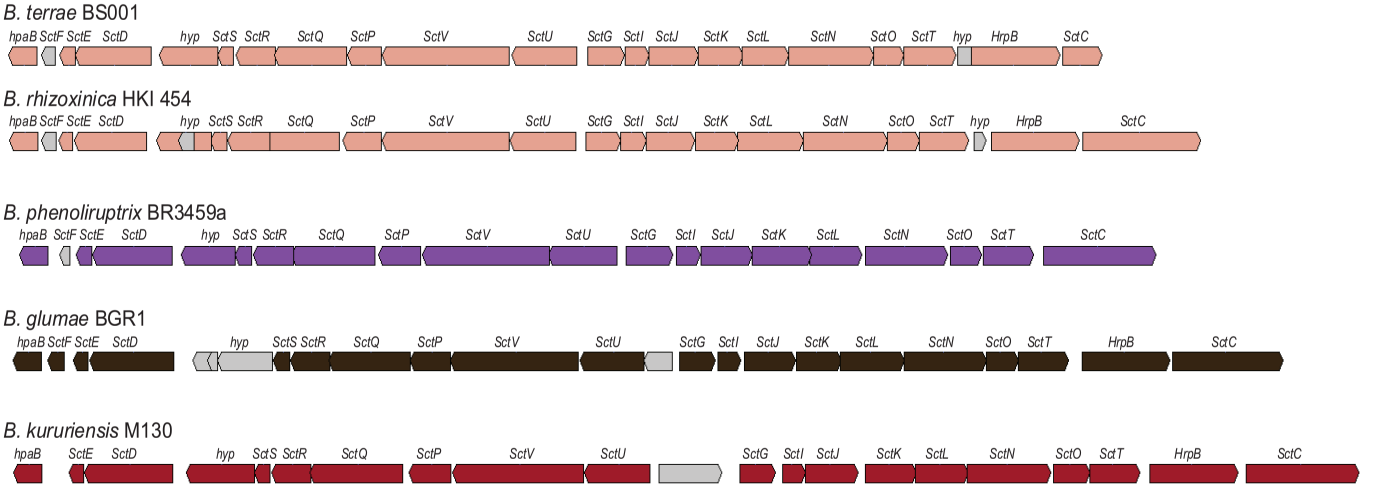

Figure 3.4 Type 3 secretion system (T3SS). Genetic organization of the T3SS gene cluster found in *Burkholderia terrae* BS001 compared with other *Burkholderia* species including *B. rhizoxinica* HKI454, *B. phenoliruptrix* BR3459a, *B. kururiensis* M130 and *B. glumae* BGR1.

highest synteny with a second system in *B. phymatum* STM815 (>70% AAI) and *B. phytofirmans* PsJN (>55% AAI).

The T3SS has a crucial role in the virulence of several plant and human pathogens (Tseng et al. 2009). It may also be involved in bacterial–fungal interactions, in particular aiding bacteria in their migratory response to extending fungal hyphae in the mycosphere (Warmink and van Elsas 2009). Indeed, the few bacterial types that were able to migrate along with the hyphal front of *Lyophyllum* sp. strain Karsten in soil microcosms were all positive for the presence of the T3SS (Warmink and van Elsas 2009). The T3SS makes membrane-bound complexes that can secrete effector proteins into eukaryotic host cells. The *B. terrae* BS001 genome possesses one T3SS gene cluster, containing 22 canonical T3SS genes (AKAUv1_540178–AKAUv1_540199). Out of these, nine were found to be highly conserved, making up the proposed core apparatus (*SctSRQVUJNTC*), however without a canonical gene for the "needle." Interestingly, the system was found to be highly syntenous and homologous (60–65% AAI) to the T3SS of the fungal-interac-

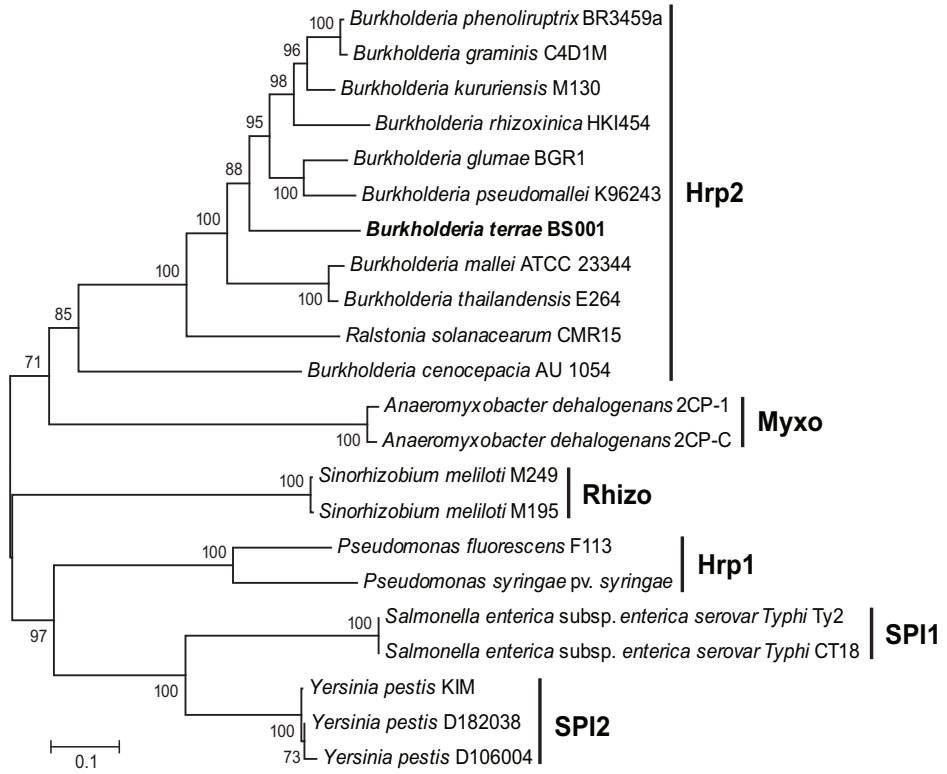

Figure 3.5 A maximum-likelihood phylogenetic tree. The evolutionary relationship and position of the T3SS of *Burkholderia terrae* BS001 was inferred from concatenated amino acid sequences of eight conserved T3SS genes (*SctS*, *SctR*, *SctQ*, *SctV*, *SctU*, *SctJ*, *SctN*, and *SctT*). The T3SS of *B. terrae* BS001 belongs to the Hrp2 family of T3SS. Hrp2, Myxo, Rhizo, Hrp1, SPI1, and SPI2 are families within T3SSs (Abby and Rocha 2012).

tive strain *B. rhizoxinica* HKI454 as well as that of *B. glumae* BGR1 (Fig. 3.4). Remarkably, a CDS with unknown function (AKAUv1_540179) was found, of which the location and length compelled us to argue that it encodes a needle protein. Based on PSIPRED predicted α-helix structures, we assume that this CDS is a functional homolog of *SctF* (*B. rhizoxinica* HKI454) or its *Ralstonia* counterpart *HrpY* (Lackner et al. 2011a). To understand the evolutionary position of the T3SS of strain BS001, we constructed a phylogenetic tree (Fig. 3.5) using eight concatenated core genes (*SctS*, *SctR*, *SctQ*, *SctV*, *SctU*, *SctJ*, *SctN*, and *SctT*) that were conserved across the tested genomes. The analysis supports the conclusion that the T3SS of *B. terrae* BS001 belongs to the so-called Hrp2 family, to which most of the *Burkholderia* and *Ralstonia* T3SSs belong (Abby and Rocha 2012). The T3SSs of *B. terrae* BS001 and *B. rhizoxinica* HKI454 might fulfill similar roles. Both strains interact actively with fungal hosts and there is evidence that T3SSs play a role in the interaction in both cases (Lackner et al. 2011a, Warmink and van Elsas 2008).

The type 4 secretion system (T4SS) is responsible for secreting proteins and, most importantly, nucleic acids (coupled to proteins; nucleoproteins) across the inner and outer membranes into recipient cells or into the external milieu. It plays a key role in the transfer of plasmids or integrated chromosomal elements, next to being involved in virulence on animals (protein secretion, i.e., in *Helicobacter pylori*) or plants (plasmid transfer, i.e., in *Agrobacterium tumefaciens*) (Dale and Moran 2006). Remarkably, the genome of *B. terrae* BS001 was found to contain three T4SS gene clusters. The first one, T4SS-I (20 CDSs; AKAUv1_3130024–AKAUv1_3130044), with all canonical T4SS functions (Fig. 3.6), is present on the 70.42-kb region of genome plasticity (RGP) RGP76. The predicted *virB10* gene of T4SS-I had very high AAIs with similar genes from a *Burkholderia* sp. BT03 plasmid (94%) and the *B. phymatum* STM815 genome (71%). Furthermore, non-T4SS open reading frames (ORFs) on RGP76 were predicted to encode a DNA circulation family protein, DNA replication protein *tnpB*, transposase *tnpA*, IS5 transposase

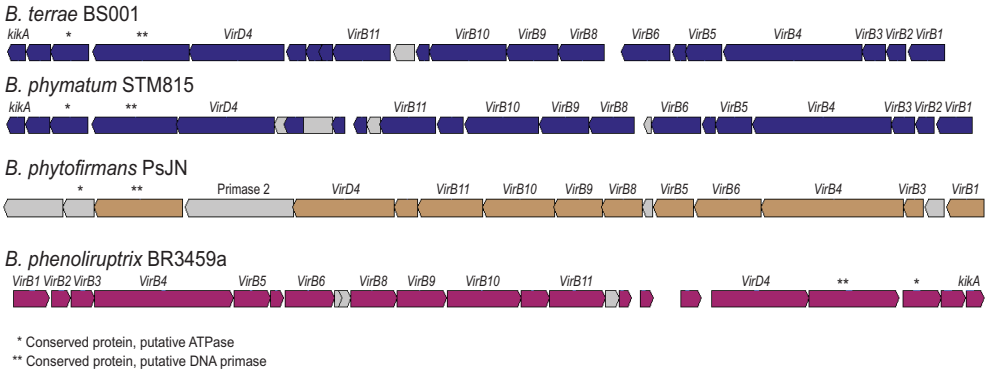

Figure 3.6 Type 4 secretion system (T4SS). The gene organization of T4SS-I (gene cluster 1) of *Burkholderia terrae* BS001 compared with the T4SSs *B. phymatum* STM815, *B. phenoliruptrix* BR3459a, and *B. phytofirmans* PsJN, is shown.

insH, next to seven integrases, including two phage family integrases. Further, at the downstream boundary of RGP76, four transposases and two integrases were localized. The T4SS was completely syntenous and highly homologous (70–75%) with a *B. phymatum* STM815 T4SS located on plasmid pBPHY02 (595,108 bp). Moreover, the regions flanking RGP76 also contained CDSs potentially involved in gene mobility, that is, ORFs similar to the typical plasmid replicon genes *parA*, *parB*, *parD*, *parE*, *mobB*, *mobC*, *repB*, *korC*, and *klcA*. RGP76 further carried several accessory genes, that is, those predicted to function as a putative xanthine dehydrogenase/carbon monoxide dehydrogenase acceptor (AKAUv1_3070015, AKAUv1_3070016) and molybdenum binding xanthine dehydrogenase (AKAUv1_3130017). The remaining accessory ORFs were predicted to encode conserved proteins of unknown functions.

A second system, T4SS-II, containing 13 genes, was found to be present elsewhere in the genome (AKAUv1_1810006–AKAUv1_1810019). This cluster had a gene composition different from the first one, as it contained the *virB8*, *virB9*, and *virB10* genes but lacked other vir genes. Instead, it contained CDSs for lytic transglycosylase (two CDSs), *pilL*, toxin coregulated pilus biosynthesis protein Q, *pulD*, *pulE*, *pulF* (T2SS), and conserved hypothetical proteins genes.

The third system, T4SS-III, (AKAUv1_1120025–AKAUv1_1120032) contained only eight T4SS related genes. T4SS-III contained *virB4*, *traG*, *traL* (relaxase) *virB6*, *trbJ*, *trbD*, *trbC*, and *trbB* genes. However, T4SS-III lacked the canonical *virB10* gene. In order to assess the origin of the former two T4SSs, we performed a phylogenetic analysis of their respective *virB10* gene amino acid sequences. The analysis showed that T4SS-I is highly similar (72% AAI) to T4SSs of both *B. phymatum* STM815 and *B. phenoliruptrix* BR3459a. The T4SS-II, being very different from T4SS-I (22% homology), was closely related (76% AAI) to a T4SS of *Burkholderia* sp. SJ98 (Supplementary Fig. S3.1).

The *B. terrae* BS001 genome further revealed the presence of four copies of predicted T6SSs, numbered T6SS-I through T6SS-IV (12–25 genes). T6SS-1 (16 genes; AKAUv1_650058–AKAUv1_650074) had highest synteny and homology (>40%) with a genomic region of *B. gladioli* BSR3. T6SS-II (19 genes; AKAUv1_1080195–AKAUv1_1080214) was highly syntenous and homologous (85–95%) with a similar cluster of *B. phymatum* STM815. T6SS-III and T6SS-IV comprised lower numbers of genes, that is, 8 (AKAUv1_1680089–AKAUv1_1680097) and 5 (AKAUv1_920001–AKAUv1_920005), respectively. T6SS-III had highest synteny with clusters in *B. phenoliruptrix* BR3459a and *B. kururiensis* M130 (both 55–60% homology). T6SS-IV, found on RGP25, was syntenous with five-genes clusters (40–50% homology) in *B. glumae* strains BGR1 and AU6208 and *Burkholderia* sp. CCGE1002 (Supplementary Fig. S3.2). The *B. terrae* BS001 genome also contained several genes for signal peptide bearing proteins (Supplementary Table S3.3) that may be exported in a sec-dependent fashion. Among these, the so-called type II secreted proteins fall in the category of lytic and toxin-related proteins (Cianciotto 2005). Furthermore, putative *ppiA* (encoding peptidyl–prolyl cis–trans isomerase A; rotamase A; AKAUv1_720068), next to genes for parvulin-type peptidyl–prolyl cis–trans isomerase (AKAUv1_1270066), and peptidyl–prolyl cis–trans isomerase (AKAUv1_1390107)

were found. The latter protein may have pathogenicity-enhancing abilities in *Legionella pneumophila* and *Xanthomonas campestris* pv. *campestris* 8004 (Fischer et al. 1992, Zang et al. 2007). It has also been reported to exist in *B. rhizoxinica* HKI454 (Lackner et al. 2011b). We also found gene *iagB* (AKAUv1_790111) and its three duplicates (45–65% AAI), which produces an invasion protein and may be involved in the invasion of eukaryotic host cells by *Shigella flexneri* and *Salmonella enterica* serovar typhi (Allaoui et al. 1993, Miras et al. 1995). Three genes predicted to encode ankyrin (AKAUv1_540128, AKAUv1_920061, AKAUv1_1270092) and tetratricopeptide (TPR) repeat containing proteins were also present in the BS001 genome. These proteins may be involved in protein–protein interactions as host interactive factors (Edqvist et al. 2006). Both ankyrin and TPR repeat proteins have also been reported in *B. rhizoxinica* HKI454 (Lackner et al. 2011b).

Putative T3SS secreted effector proteins were then searched for using www.effectors.org (last accessed June 26, 2014) (Jehl et al. 2011) (Supplementary Table S3.4), which were analyzed with respect to the putative interaction of *B. terrae* BS001 with *Lyophyllum* sp. strain Karsten. These effectors need confirmation, as we ignore their functions at this moment. Nevertheless, we found putative phytoene synthase (AKAUv1_920106), of the squalene–hopene cyclase enzyme family and may be involved in the biosynthesis of terpenoids. This is consistent with the presumption that terpenoids mediate the interactions of *B. terrae* BS001 with its host, like reported for *B. rhizoxinica* HKI454 in its interaction with the fungal host (Lackner et al. 2011b). Among the predicted effectors, we also found flagellin proteins (coined pathogen-associated molecular patterns) both in plants and in mammals (Ronald and Beutler 2010, Wei et al. 2013).

Pilus, biofilm formation, motility, and insecticidal toxin complexes

The genome of *B. terrae* BS001 further carries gene clusters predicted to encode flagellar biosynthesis, biofilm formation, motility, and (seven genes) type 4 pili. The latter pili may play a role at fungal surfaces, as, once attached to the surface, bacteria can "walk" on it by employing the action of such pili (Conrad 2012). Genes that encode such pili, that is, *pilMNQ* (AKAUv1_1170009, AKAUv1_1170010, AKAUv1_1170012), were present in a cluster. We also found pilin precursor gene *PilA* (AKAUv1_2170003), signal peptidase *PilD* (AKAUv1_2940033), NTP-binding protein *PilB* (AKAUv1_2940037), and *PilC* (AKAUv1_2940035) in the genome.

Bacterial biofilm formation is dependent on the ability to produce extracellular matrix which may be composed of polymers like poly-beta-1,6-*N*-acetyl-D-glucosamine (PGA) (Izano et al. 2007, Bossé et al. 2010). We found several ORFs predicted to be involved in PGA production, that is, *pgaA* (PGA synthesis protein; AKAUv1_1000016), *pgaB* (PGA synthesis lipoprotein; AKAUv1_1000017), and *pgaC* (PGA synthesis *N*-glycosyltransferase; AKAUv1_1000018). In addition, two other biofilm synthesis gene clusters (both representing a Pel operon homolog) were identified. The Pel operon provides a scaffold in the early stage of biofilm formation by *Pseudomonas aeruginosa* PA14, (Colvin et al. 2011). Pel is thought to encompass a glucose-rich polysaccharide polymer that is

encoded by a seven-genes operon (*pelA–F*) (Friedman and Kolter 2004). Cluster 1 comprised *pelABCDEFGA* (AKAUv1_2280066–AKAUv1_2280073), and cluster 2 *pelGFEDCBA* (AKAUv1_100003–AKAUv1_100009). Thus the Pel region may have undergone a recent duplication followed by some reshuffling, as evident from our analysis. Two genes, taken as proxies in the two systems, that is, *pelG* and *pelF*, were 61.84% and 64.69% identical, respectively, whereas *pelABCDE* had lower (43.52%, 36.33%, 47.9%, 40.08%, and 45.55%) AAI. An extensive screening further revealed the presence of ten other CDSs (i.e., 4 *epsA*, 4 *epsB*, and 2 *epsP*) in the genome that are potentially involved in the biosynthesis of other exopolysaccharide (EPS). Overall, the presence of the *pel* gene clusters reflects another capacity of *B. terrae* BS001 to form biofilms during its interaction with *Lyophyllum* sp. strain Karsten (as well as other hosts) in soil.

Moreover, the *B. terrae* BS001 genome harbors one gene cluster that encodes flagellar biogenesis proteins. The cluster revealed the presence of 46 genes (AKAUv1_120002–AKAUv1_120048) in a region that was highly syntenous with regions of the STM815 (80–90% AAI) and PsJN (75–85% AAI) genomes. Moreover, the BS001 genome contained a gene cluster comprised 11 genes (AKAUv1_790001–AKAUv1_790011) associated with bacterial chemotaxis and motility. Again, this cluster was syntenous with a similar one in strain STM815 (85–95% AAI). Elsewhere in the genome, another nine flagellar genes (*flhA*, *flhB*, 2 [*flhC*], 3 [*flhD*], *fliC*, and *fliD*) were found, next to a seven-genes chemotaxis/motility cluster (AKAUv1_920023–AKAUv1_920030).

Interestingly, the genome of *B. terrae* BS001 harbored two ORFs (AKAUv1_2130030 and AKAUv1_2130031) coding for a putative insecticidal toxin complex, as also reported to be present on the 822-kb megaplasmid pBRH01 in *B. rhizoxinica* HKI454 (Lackner et al. 2011b). We analyzed the putative horizontal mobility of such ORFs. Indeed, the two ORFs formed part of the largest genomic island, RGP53 (102,873 bp), suggesting that they may have been acquired horizontally. Thus strain BS001 might have insect pathogenicity traits, indicating its putative interaction with soil insects.

Genomic islands across the genome of B. terrae BS001

We employed "MicroScope" (Vallenet et al. 2013) to predict the presence of RGPs in the BS001 genome. The analysis predicted the existence of 79 RGPs, which were scattered across the genome (Supplementary Table S3.5). The total size of the collective RGPs was 1,896,071 bp, accounting for 16.48% of the genome. Among the RGPs, we found the aforementioned 70.42 kb plasmid-like RGP76 (AKAUv1_3070018–AKAUv1_3130074). An overview of ten important RGPs with plasmid-related and functional genes is given in supplementary Table S3.6.

Briefly, on RGP34, we identified two ORFs (AKAUv1_1280007 and AKAUv1_1280008) encoding putative addiction proteins (toxin and antitoxin). In this region, four ORFs (AKAUv1_1930029, AKAUv1_1930030, AKAUv1_1230100, and AKAUv1_1230101) encoding *HigA*, *HigB* (plasmid maintenance protein), *StbD* and *StbE* (stabilization proteins) were found just outside of the RGP. Similarly, putative plasmid segregation antitoxin *CcdA* and *CcdB* toxin proteins are encoded by ORFs AKAUv1_2410015 and

AKAUv1_2410016, both located outside plasticity regions. On the other hand RGP61 harbored two ORFs, AKAUv1_2490012 and AKAUv1_2490013, which encoded *CcdB* and *CcdA*. Downstream of these genes, the region harbored an ORF (AKAUv1_2490015) predicted to encode a protein having 39% (coverage 77%) homology with cryptic plasmid protein A from *Neisseria gonorrhoeae* NCCP11945. Interestingly, it also revealed an ORF (AKAUv1_2490048) that encoded a beta-lactamase (with 55% and 51% similarity to a similar protein in *Bradyrhizobium* SG-6C and *R. etli* CFN 42, respectively). Furthermore, we identified putative *parE* and *parD* genes (AKAUv1_2860003 and AKAUv1_2860004) in RGP71, whereas another ORF (AKAUv1_330002) encoding "prevent host death" family protein; *Phd* was present outside the RGPs. We also identified *HigB* and *HigA* (AKAUv1_360025 and AKAUv1_360026) as well as *MazE* and *MazF* addiction modules (AKAUv1_920172 and AKAUv1_920173), outside the predicted RGPs. Such toxin–antitoxin system proteins play a role in mediating stability of plasmids by imposition of an addiction mechanism to the host (Arcus et al. 2005). They may also play a role in mediating growth arrest during stress situations (Gerdes et al. 2005).

The largest RGP, RGP53, was found to span 102,873 bp (AKAUv1_2120017–AKAUv1_2140005), carrying ORFs for seven transposases and one integrase. A screen for functional genes in this RGP revealed the presence of one gene *sip* encoding a siderophore interacting protein (SIP) (AKAUv1_2130016) highly similar to a *sip* from *Burkholderia* sp. BT03 (99%). The gene had a duplicate downstream (AKAUv1_ 2130093), having (99%) similarity with sip of *Burkholderia* sp. BT03. Another ORF (AKAUv1_ 2130052) was predicted to encode invasion plasmid antigen J (*IpaJ*); it had 35% homology with *IpaJ* from *S. flexneri* 2002017. RGP53 also carried a gene for type 3 effector protein *HopJ* (AKAUv1_2130053); it was 53% homologous to similar gene from *Salinivibrio costicola*. It appears to furnish a wealth of secondary functions to its host, which may be useful in a wide range of ecological conditions. The clear mobility and divergent accessory nature of RGP53 might suggest that it is an ecological flexibility island.

Primary metabolites and metabolic pathways

We used the "MicroCyc" metabolic database (Caspi et al. 2010) to compare the metabolic potential in the genome of *B. terrae* BS001 with that of other *Burkholderia* genomes (Supplementary Table S3.7).

Based on the values computed for pathway completion, hierarchical cluster analysis was performed for the *B. terrae* BS001 genome next to 23 other genomes including next-of-kin *Burkholderia* species (Supplementary Fig. S3.3). The analysis revealed that the *Burkholderia* strains tended to group together, *B. terrae* BS001 being close to *B. kururiensis* M130 and *B. phymatum* STM815, whereas *B. phytofirmans* PsJN grouped with *B. vietnamiensis* G4 and *B. ambifaria* AMMD. Interestingly, *B. rhizoxinica* HKI454 grouped outside of the main *Burkholderia* cluster; however, this might have been compromised by its smaller genome size.

B. terrae BS001 clearly invested in diverse primary metabolisms, as evidenced from the presence of more than 1,200 putative CDSs for carbohydrate metabolism (Nazir et

al. 2012b). Specifically, we found a putative glycerol kinase, *glpK* (AKAUv1_1300029), which was preceded by a glycerol-3-phosphate dehydrogenase (*glpD*, AKAUv1_1300028).

Remarkably, in the *B. terrae* BS001 genome, we found a gene for a specific glycerol uptake family transporter, denoted GUP. This system was absent from all other *Burkholderia* genomes and it is actually typical for the yeast *Saccharomyces cerevisiae*. In this organism, active uptake of glycerol occurs mainly by *GUP* 1 and its close homolog *GUP* 2, which both encode multimembrane-spanning proteins (Holst et al. 2000). Interestingly, the gene for *GUP* (AKAUv1_1930108) was present on RGP47, showing highest AAI (49%) with *Magnetospirillum magneticum* AMB-1 (Fig. 3.7). The upstream ORF AKAUv1_1930107 encodes protein of unknown function that also shares 25% AAI with a hypothetical protein from *M. magneticum* AMB-1. The unique presence of this *GUP* gene in *B. terrae* BS001 is consistent with the hypothesis that fungal-exuded glycerol (Nazir et al. 2013), which feeds the bacterium, may be taken up through it. The presence of this system on RGP47 may indicate that the bacterium acquired and fixed it in its quest to thrive in the mycosphere at the expense of exuded glycerol.

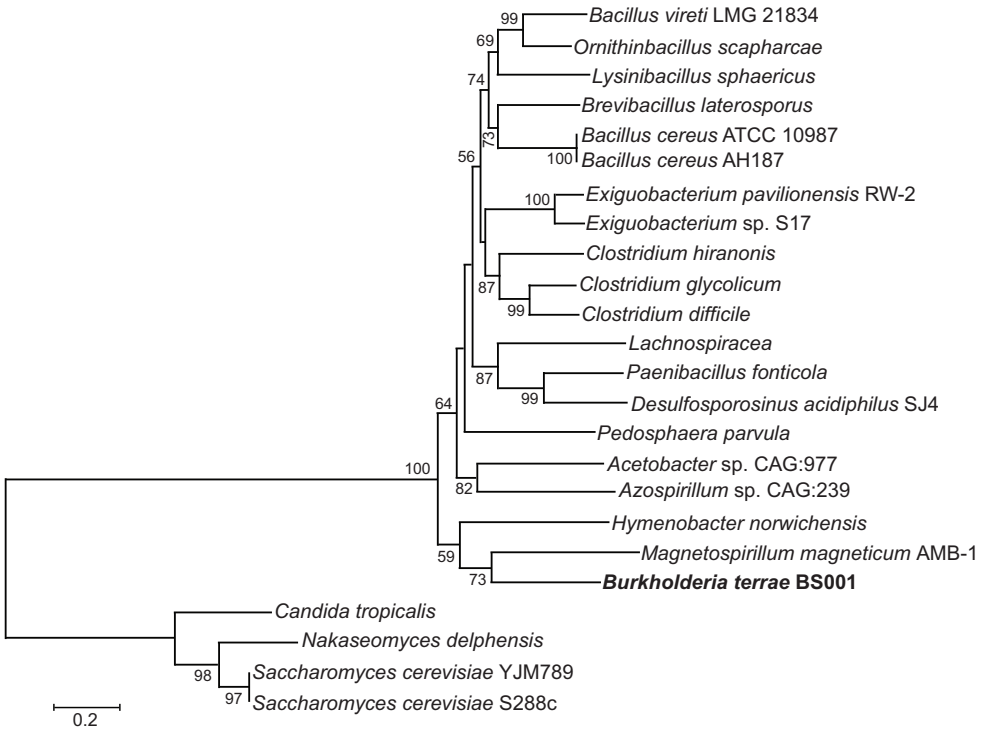

Figure 3.7 A neighbor joining tree of glycerol uptake gene (*GUP*) of *Burkholderia terrae* BS001 and close relatives. Based on the amino acid sequence of *GUP* gene, *B. terrae* BS001 *GUP* clustered with a similar gene product of *Magnetospirillum magneticum* AMB-1. *GUP* amino acid sequences of *Saccharomyces cerevisiae* strains s288c, YJM 789, *Candida tropicalis* and *Nakaseomyces delphensis* are taken as an outgroup. Bootstrap values (more than 50%) are shown at the nodes.

Genes for glycerol degradation pathways I and V were predicted to be present in the genome of *B. terrae* BS001. In comparison with the comparator strains, glycerol degradation pathway V was only complete in *B. terrae* BS001, whereas it was incomplete in strains PsJN and STM815 while strain HKI454 completely lacked it. Pathway V encompasses two reactions, and only the *B. terrae* BS001 genome carried ORFs (AKAUv1_760319, AKAUv1_2440011, and AKAUv1_1890037) encoding enzymes catalyzing these. The first two ORFs are duplicates of each other, having 42.9% sequence identity.

Strikingly, the BS001 genome further carried ORF AKAUv1_1970009, which is predicted to encode a tyrosinase (involved in L-dopachrome biosynthesis), present on RGP49. We analyzed the potential phylogenetic history of this gene using a maximum-likelihood tree (Supplementary Fig. S3.4) with closest hits in the database. Blasting the whole NCBI database only yielded four close hits, with highest AAI (73%) with *Anaeromyxobacter dehalogenans* 2CP-1. The ORF directly upstream in BS001, AKAUv1_1970008, was predicted to encode a twin-arginine signal translocation protein (Tat). This gene also occurred (55% AAI) directly upstream in *A. dehalogenans* 2CP-1. However, the region downstream of ORF AKAUv1_1970010 was different. The presence of the *Tat* gene upstream of the tyrosinase gene is consistent with the fact that the tyrosinase may require a signal peptide containing transactivator for its export and translocation (Berks et al. 2000; Schaerlaekens et al. 2001). This commonality between the rather unique *B. terrae* system and one in *A. dehalogenans* is striking. The latter organism belongs to a very different bacterial clade (delta-Proteobacteria) than *B. terrae* BS001; however, it occurs in the same habitat (soil) and is a very motile (gliding) bacterium (Thomas et al. 2008). Moreover, its mosaic genome has been postulated to be composed of parts coming from diverse proteobacteria. Based on this analysis and the uniqueness of the commonality, we postulate that the gene for tyrosinase may have been acquired from an organism like *A. dehalogenans* 2CP-1 through horizontal gene transfer. L-dopachrome is the building block of melanin, which can protect against ultraviolet radiation (Liu et al. 2004). Moreover, melanin in the cell wall of *Aspergillus nidulans* has been correlated to resistance against digestion by chitinases, glucanases, and proteases (Kuo and Alexander 1967). Bacterial melanin has been shown to interact with double-stranded DNA and its cellular localization may inhibit cell metabolism (Geng et al. 2010). Bacterial tyrosinases may also act on particular phenolic compounds, such as chlorophenols (Marino et al. 2011) and fluorophenols (Battaini et al. 2002). The role of the unique tyrosinase found in the *B. terrae* BS001 genome is not well understood, but it may serve the organism to cope with stressful environments and detoxify fungal/plant phenolic compounds.

Furthermore, the *B. terrae* BS001 genome revealed an enormous biosynthetic potential for a wide range of amino acids, see supplementary Table S3.7, indicating its flexibility in synthesizing the essential cellular building blocks of different kinds.

Plant-Interactive traits

In the *B. terrae* BS001 genome, we further found biosynthetic potential for plant hormones, that is, genes for ethylene biosynthesis pathway III (two out of three enzymes are present) and indole acetic acid (IAA) biosynthesis IV and V, whereas IAA biosynthesis pathway VI is partially present. This may suggest that *B. terrae* BS001 spends (part of) its life occupying a niche close to plants like the rhizosphere. The presence of three copies of a 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase—*acdS*) gene (AKAUv1_150009, AKAUv1_510080, and AKAUv1_780047), denoted *acdS*-I, *acdS*-II and *acdS*-III, gives support to this hypothesis. Maximum-likelihood phylogenetic analyses showed that *acdS*-III clustered separately from the other two genes and might have been acquired through horizontal gene transfer (HGT). In contrast, *acdS*-I and *acdS*-II may be the result of a duplication very early in evolution (Supplementary Fig. S3.5).

Similarly, RGP53 was found to carry two CDSs, here denoted as p*nodW* (putative *nodW* genes) (AKAUv1_2130064 and AKAUv1_2130080) that may act as two-component response regulators, with 81% AAI to a putative two-component response regulator from *P. resinovorans* NBRC 106553. Further analyses revealed that up to 18 paralogs of p*nodW* genes were present across the genome, with similarities of 30–69%. Downstream one ORF (AKAUv1_2130067), we also found a PAS sensor protein (sensor kinase; *nodV*). This sensor kinase had seven paralogs in the genome with AAI of 35–46%. Both *nodV* and *nodW* belong to the two-component regulator family that activates the expression of other nodulation genes at the plant surface and in response to isoflavonoides (Sanjuan et al. 1992, Loh et al. 1997, Loh et al. 2002). We could not find other nodulation genes (such as *nodABCD*); however, elsewhere in the genome we found CDSs similar to *nodN*, *nodI*, *nodJ*, and *nodT*. The exact role of the two-component regulators (*nodV* and *nodW*) in strain BS001 is not known yet, but the presence of such an array of *nod* genes allows the hypothesis that *B. terrae* BS001 might also display a plant-interactive lifestyle. On the other hand nitrogen fixation genes were not present in the genome, excluding a classical nitrogen fixation interaction.

Detoxification potential

The *B. terrae* BS001 genome revealed great potential to detoxify arsenate, phenylmercuric acetate, methylglyoxal, cyanate, fluoroacetate, and superoxide radicals. The gene encoding arsenate reductase (*arsC*) (AKAUv1_1500002) is present on RGP38. Interestingly, two genes (AKAUv1_1500022 and AKAUv1_1500025) involved in cyanate degradation are located downstream on RGP38. Moreover, ORF AKAUv1_3230012, encoding aliphatic nitrilase that catalyzes a reaction in acrylonitrile degradation, was found to be harbored by RGP78. Another putative ORF, AKAUv1_2920114, was harbored by RGP73. It encodes a putative 2-nitropropane dioxygenase that is involved in alkylnitronate degradation. We also found that strain BS001 is capable of degrading salicylate, anthranilate, benzoate, and catechol. Similarly, proteins for degradation of compounds that are released during the degradation of plant lignin in soil, such as protocatechuate, ferulate and vanillate, as described for *Ralstonia solanacearum* (Genin and Boucher

2004), are represented by ORFs in the genome of *B. terrae* BS001.

The afore analyses indicate that *B. terrae* BS001 has invested considerable genetic luggage in detoxification systems, which may relate to its persistence in ecological niches that are affluent in such compounds and may explain its versatile ecological behavior.

Membrane transporter landscape

As gatekeepers to the outside, membrane transporters are important for metabolic activities. Lifestyle is thought to be a key driver of the evolution in numbers and types of transporters (Gelfand and Rodionov 2007). Transporters can be classified into three different classes based on their structure and mechanism of action, that is, energy-dependent (ATP dependent) membrane pumps, ion channels, and secondary transporters (Nagata et al. 2008). We used TransAPP (Transporter Automatic Annotation Pipeline) in the search for membrane transporters. A total of 1,338 transporter-like ORFs were found (Supplementary Table S3.8), and so the membrane transporter landscape of *B. terrae* BS001 was different from that of the other *Burkholderia* strains. *B. phymatum* STM815 had 935 transporters, *B. rhizoxinica* HKI454 267 while according to Mitter et al. (2013), *B. phytofirmans* PsJN has 1,196 membrane transporters. As the transporter database lists 997 transporters for *B. phytofirmans* PsJN, we used this figure for our analysis. Across these *Burkholderia* species, a relatively constant percentage of the total genomic space is thus dedicated to transporters, and so the larger genomes such as BS001 (11.1%), STM815 (10.8%), and PsJN (12.1%) have more transporters compared with smaller ones such as HKI454 (7.1%). Overall, the ATP-dependent systems represented the bulk of transporters (648), followed by major facilitator family (MFS) (237) membrane transporters. A detailed account of the transporters across all four *Burkholderia* genomes analyzed is given in supplementary Table S3.9. A total of 81 ATP binding component CDSs of ABC transporters as well as 55 MFS CDSs (Supplementary Table S3.10) were randomly picked and analyzed using SplitsTree4 (Huson and Bryant 2006). Thus, for both classes of transporters, a neighbor-net splits network was generated in order to depict different splits indicating potential evolutionary events. The ATP binding components of the ABC transporters revealed varying splits and radiations, indicating duplications to serve different functions in the genome (Fig. 3.8A). Transporters of branched-chain amino acids clustered together, as opposed to those involved in glycerol-3-phosphate and sugars. Similarly, another split indicated that phosphate, Zn/Mn, Fe^{3+}, and nod factor transporters clustered together and are duplicates. Thus, a range of duplication events followed by mutational drift and selection is at the basis of the current diversity of membrane transporters in strain BS001. Similarly, MFS transporters having similar roles clustered together, indicating another suite of duplication events (Fig. 3.8B). Thus, expansion of the genome of *B. terrae* BS001 may have been driven mainly by nutritional needs in the light of locally fluctuating nutrient availability, for which a diversity of transporter systems is essential. Ren and Paulsen (2005) indicated that gene duplications might be at the basis of expansion, diversification, and selection of distinct transporters families.

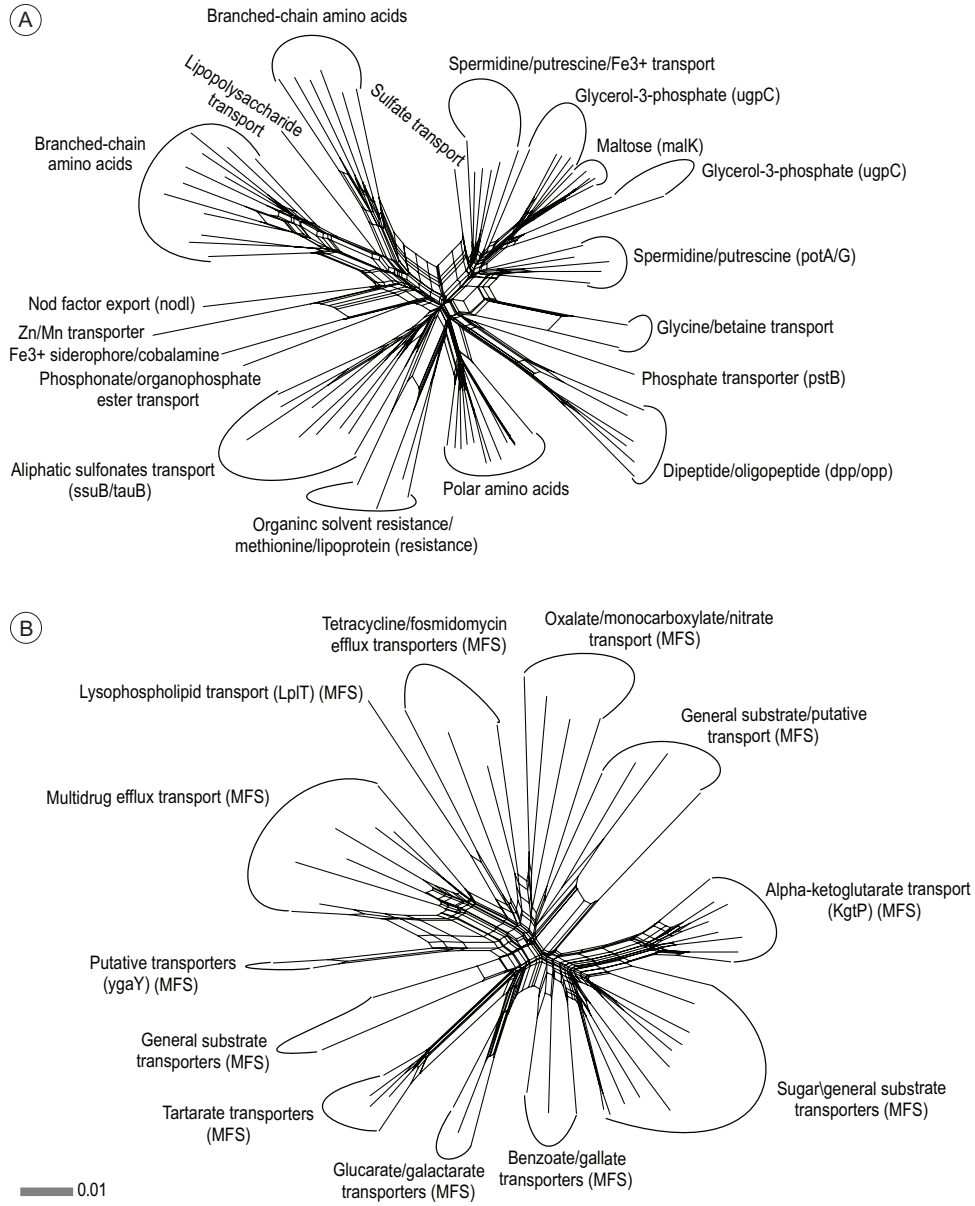

Figure 3.8 Neighbor-net splits graphs depicting the membrane transporters landscape of *B. terrae* BS001. (A) NNet splits graph inferred from alignment of 81 ATP binding component CDSs (amino acid sequences) of ABC (ATP binding cassette) transporters. (B) NNet splits graph derived from alignment of 55 MFS (major facilitator superfamily) CDSs (amino acid sequences). Alignments were manually curated and the resulting "nexus" files were analyzed using ProteinMLdist and WAG model (Gamma 4.0) within SplitsTree4 software. The splits indicate that membrane transporters (both ABC and MFS) split out and cluster by their respective functions and that gene duplications are at the basis of membrane transporters diversity. The list of CDS analyzed, is given in the supplementary Table S3.10.

Secondary metabolite biosynthetic potential

Many *Burkholderia* species exhibit a high potential for secondary metabolite production (Kang et al. 1998, Partida-Martinez and Hertweck 2005, Partida-Martinez et al. 2007, Knappe et al. 2008, Schmidt et al. 2009, Rohm et al. 2010, Seyedsayamdost et al. 2010, Tawfik et al. 2010, Mahenthiralingam et al. 2011, Franke et al. 2012). As *B. terrae* BS001 possesses an exceptionally large genome, one might predict the ample presence of novel biosynthesis gene clusters. Analyses with antiSMASH (Medema et al. 2011, Blin et al. 2013) revealed the existence of a locus (14,592 bp) that likely encodes a nonribosomal peptide synthetase (NRPS) (AKAUv1_1360004–AKAUv1_1360005) and another one (12,975 bp) coding for a hybrid polyketide synthase (PKS)/NRPS enzyme (AKAUv1_1710055, AKAUv1_1710056, and AKAUv1_1710057) (Fig. 3.9A). The NRPS locus

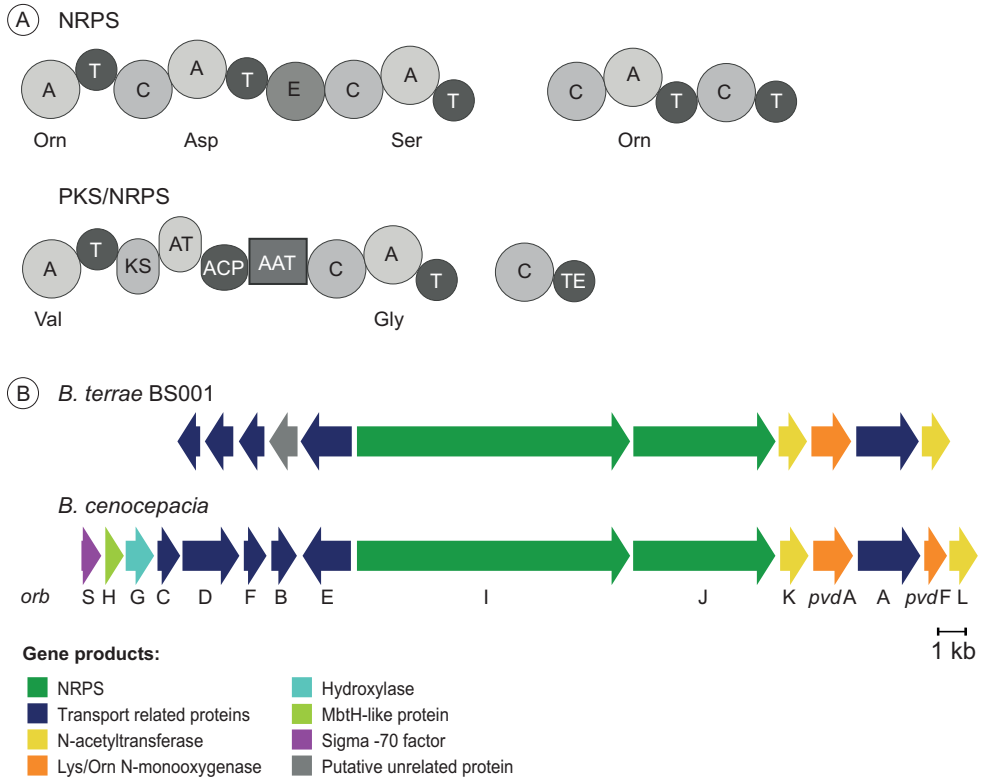

Figure 3.9 Modular organization of the NRPS (nonribosomal peptide synthetase) and the PKS (polyketide synthase)/NRPS enzymes encoded in the genome of *Burkholderia terrae* BS001. (3.9A) Predicted substrates that get activated by the A-domains, are shown. Orn (Ornithine), Asp (Aspartic acid), Ser (Serine), Val (Valine) and Gly (Glycine). A, adenylation domain; T, thiolation domain; C, condensation domain; E, epimerization domain; KS, ketosynthase domain; AT, acetyl transferase domain; ACP, acyl carrier protein; AAT, aminotransferase; TE, thioesterase. (3.9B) Comparison of the ornibactin (orb) biosynthesis gene cluster of *B. cenocepacia* and the NRPS gene cluster of *B. terrae* BS001. *orb* (S, H, G, C, D, F, B, E, I, J, K, A, and L) and *pvd* (pyoverdine) A and F, are shown.

(AKAUv1_1360004–AKAUv1_1360005) displays similarity (63% and 60%, respectively) to an NRPS locus of *B. cenocepacia* that is known to encode the siderophore ornibactin (Agnoli et al. 2006). As a mycosphere-inhabiting bacterium, *B. terrae* BS001 is postulated to depend on iron uptake systems under conditions of low iron. However, in the predicted ornibactin-like NRPS locus, four genes (*pvdF* [*N5*-hydroxyornithine transformylase], *orbS* [sigma factor], *orbH* [unknown function], *orbG* [α-ketoglutarate-dependent hydroxylases]) were missing. Moreover, several transporter genes (*orbB*, *orbC*, *orbD*, and *orbF*) involved in transport and utilization, differed from those of the ornibactin locus of *B. cenocepacia* (Fig. 3.9B).

The modular organization of another cluster—a hybrid PKS/NRPS—is shown in Fig. 3.9A. Homologs of such hybrid PKS/NRPS gene clusters have been found in other bacterial genomes by BLASTP analyses (Altschul et al. 1990), notably in *Burkholderia* sp. BT03 and *B. phymatum* STM815. However, no secondary metabolite has been described for such gene clusters yet. It would mean a great step forward if the products synthesized by these two clusters could be identified.

The BLAST search for homologs of enzymes of the ribosomally synthesized and post-translationally modified peptides (RiPPs) pathway revealed no hits, suggesting that *B. terrae* BS001 is not able to produce any such RiPPs.

Conclusion

We analyzed the genome of the fungal-interactive *B. terrae* BS001, which was recently sequenced (Nazir et al. 2012b). The genome is the largest among all hitherto available *Burkholderia* genomes, and it is equipped with a repertoire of genetic systems that encode predicted fungal- as well as plant-interactive traits. We thus identified systems potentially involved in physical interactions (biofilm formation and twitching motility) with fungi, next to protein (as well as DNA) secretion systems (in particular T3SS) that may modulate fungal host physiology and unique genes for glycerol uptake and metabolism. The genome further contains extensive RGPs (16.48% of the genome space), which carry functional genes as well as plasmid-related traits, indicating the presumed great role of horizontal gene transfer in shaping the genome. Moreover, *B. terrae* BS001 apparently invested considerable energy in primary (carbohydrate and amino acid) metabolism, membrane transporters, and even insect-interactive traits, indicating a versatile life style in soil. Such life style may have included (fungal/plant) host-interactive phases interspersed with free-living phases. Concerning the latter, the BS001 genome also contained genes that potentially encode secondary metabolite biosynthesis, which is useful for highly competitive situations that may occur in the soil upon exploration for colonization space. Overall, our analysis unveiled a plethora of ecological features in the genome that provide a firm basis for further studies based on transcriptomics of ecological situations, including interactions with (fungal) hosts.

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Supplementary Material

Supplementary Tables (S3.3, S3.4, S3.5, S3.7, and S3.8) are available at Genome Biology and Evolution online (http://gbe.oxfordjournals.org/content/suppl/2014/06/11/evu126.DC1) and can be downloaded in the form of Microsoft Excel files.

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Supplementary tables and figures

Table S3.1 Salient features underpinning the behavior of *B. terrae* BS001.

Bacterial strain	Ecological feature	Remarks (reference)
B. terrae BS001	Migration along fungal hyphae	Warmink & van Elsas 2009
B. terrae BS001	Migration helper effect/hitchhiking	Warmink et al. 2011
B. terrae BS001	Universal migrator with diverse fungi	Nazir 2012
B. terrae BS001	Glycerol use	This paper
B. terrae BS001	Co-migration in a range of soils	Nazir et al. 2012a
B. terrae BS001	Fungal glycerol release induction	Nazir et al. 2013
B. terrae BS001	Primordia setting inhibition	Nazir et al. 2013
B. terrae BS001	Protection against antifungal agents	Nazir 2012

Table S3.2 Pan-core genome analysis parameters and data.

MicroScope gene families parameters
80% amino acid identity 80% alignment coverage

Component	Families	Genes
Pan-genome	81027	180124
Core-genome	472	11836
Variable-genome	80555	168288

Table S3.2 Continued.

Organism	CDS	CDS (without artefact families)	Pan CDS	Core CDS	Variable CDS	Strain specific CDS	Core CDS (%)	Variable CDS (%)	Strain specific CDS (%)
Burkholderia rhizoxinica HKI 454	4146	4146	4146	479	3667	3246	11.553	88.447	78.292
Burkholderia glumae AU6208	5518	5518	5518	483	5035	1378	8.753	91.247	24.973
Burkholderia mallei ATCC 23344	5682	5681	5681	484	5197	1016	8.52	91.48	17.884
Burkholderia thailandensis E264	6071	6071	6071	487	5584	1372	8.022	91.978	22.599
Burkholderia kururiensis M130	6436	6436	6436	496	5940	3339	7.707	92.293	51.88
Burkholderia pseudomallei K96243	6527	6527	6527	489	6038	1219	7.492	92.508	18.676
Burkholderia glumae 3252-8	6671	6671	6671	483	6188	1434	7.24	92.76	21.496
Burkholderia multivorans ATCC 17616	6909	6900	6900	493	6407	1600	7.145	92.855	23.188
Burkholderia glumae LMG 2196	6760	6760	6760	479	6281	1960	7.086	92.914	28.994
Burkholderia cenocepacia AU 1054	6940	6940	6940	488	6452	1287	7.032	92.968	18.545
Burkholderia phenoliruptrix BR3459a	7355	7346	7346	513	6833	1987	6.983	93.017	27.049
Burkholderia ambifaria AMMD	7131	7129	7129	491	6638	1630	6.887	93.113	22.864
Burkholderia sp. CCGE1003	7266	7263	7263	494	6769	2312	6.802	93.198	31.833
Burkholderia glumae BGR1	7341	7331	7331	486	6845	1277	6.629	93.371	17.419
Burkholderia sp. CCGE1002	8025	8025	8025	506	7519	3649	6.305	93.695	45.47
Burkholderia phytofirmans PsJN	8043	8041	8041	500	7541	2593	6.218	93.782	32.247
Burkholderia gladioli 3848s-5	8056	8056	8056	486	7570	1597	6.033	93.967	19.824
Burkholderia lata 383	8305	8305	8305	498	7807	2370	5.996	94.004	28.537
Burkholderia phymatum STM815	8434	8426	8426	500	7926	2834	5.934	94.066	33.634
Burkholderia gladioli BSR3	8560	8555	8555	492	8063	1769	5.751	94.249	20.678
Burkholderia vietnamiensis G4	8830	8824	8824	494	8330	3587	5.598	94.402	40.65
Burkholderia sp. TJI49	9096	9091	9091	487	8604	4418	5.357	94.643	48.598
Burkholderia xenovorans LB400	10038	10037	10037	505	9532	4564	5.031	94.969	45.472
Burkholderia terrae BS001	12047	12045	12045	523	11522	6099	4.342	95.658	50.635

Table S3.6 Composition and contents of ten regions of genomic plasticity (RGPs).

RGP	Size (bp)	No. of genes	Functional genes	Accessory genes			
RGP76	70422	89		T4SS	6 transposases	9 integrases	*parA*, *parB*, *parD*, *parE*, *korC*, *repB*, *MobB*, *MobC*, *KlcA*
RGP53	102873	113	Insecticidal toxin complex, *nodV*, *nodW*, Nickel transporter, T3 effector *HopJ*, invasion plasmid antigen J, uracil degradation gene, transporters		7 transposase	1 integrase	
RGP47	46205	62	Glycerol uptake gene (*GUP*), GDP-L-fucose, GDP-mannose, peptidoglycan and colanic acid building blocks biosynthesis genes		2 transposases	2 integrases	
RGP20	48143	60	Nitrate reduction, ACC-deaminase, amino acids metabolism, glycolysis/gluconeogenesis pathways, tartarate transporter		5 transposase	1 integrase	
RGP24	31450	36	Glycogen degradation genes, TCA cycle, aerobic respiration			1 integrase	*parA*, *parB*
RGP25	46914	64	Various degradation pathways: 2-oxopentenoate degradation, gamma-hexachlorocyclohexane degradation, salicylate, vanillate, tyrosine, valine degradation	T6SS	1 transposase		
RGP38	53810	84	Arsenate detoxification, cyanate degradation (nitrogen metabolism), beta-lactamase, chloride peroxidase, efflux pumps (membrane transporters)		3 transposases		
RGP49	96211	120	Acrylonitrile degradation, glycolate/glyoxalate degradation, nitrile biocatalysis, mandelate degradation, methylglyoxal degradation, formaldehyde assimilation, melanin biosynthesis, membrane transporters		6 transposases	2 integrases	
RGP61	51149	74	Amino acid metabolism, glutamate; 4-amino butyrate degradation, caffeine degradation, fatty acid degradation, ethanol degradation, 4-hydroxybenzoaate biosynthesis, membrane transporters		3 transposases		*CcdB* (toxin), *CcdA* (anti-toxin)
RGP62	53217	69	Ketogluconate metabolism, methylglyoxal degradation, gamma-hexachlorocyclohexane degradation, polycyclic aromatic hydrocarbon degradation, aminobenzoate degradation, benzoate/fluorobenzoate degradation, ethylbenzene degradation, quinate degradation, chorismate biosynthesis, membrane transporters		1 transposase		

Table S3.9 List of all membrane transporters across four *Burkholderia* genomes.

***Burkholderia terrae* BS001**	**Number**	***Burkholderia rhizoxinica* HKI454**	**Number**	***Burkholderia phymatum* STM815**	**Number**	***Burkholderia phytofirmans* PsJN**	**Number**
ATP dependent							
ABC Family	648	ABC Family	107	ABC Family	471	ABC Family	485
ArsAB family	3	ArsAB family	0	ArsAB family	0	DNA-T family	1
DNA-T family	2	DNA-T family	1	DNA-T family	1	ArsAB family	1
F-ATPase family	15	F-ATPase family	9	F-ATPase family	14	F-ATPase family	16
IIISp family	11	IIISp family	5	IIISp family	6	IIISp family	10
IVSP family	6	IVSP family	6	IVSP family	3	IVSP family	13
MTB family	12	MTB family	4	MTB family	4	MTB family	4
P-ATPase family	11	P-ATPase family	5	P-ATPase family	8	P-ATPase family	7
Total	**708**	**Total**	**137**	**Total**	**507**	**Total**	**537**
Ion channels							
Amt family	2	Amt family	1	Amt family	2	Amt family	2
Bestrophin family	1	Bestrophin family	0	Bestrophin family	1	Bestrophin family	4
EVE1-C family	1	EVE1-C family	0	EVE1-C family	1	EVE1-C family	1
IRK-C family	1	IRK-C family	0	IRK-C family	1	IRK-C family	1
MIP family	2	MIP family	1	MIP family	2	MIP family	3
MIT family	3	MIT family	1	MIT family	2	MIT family	2
Mot/EXB family	13	Mot/EXB family	4	Mot/EXB family	8	Mot/EXB family	12
MscL family	1	MscL family	1	MscL family	1	MscL family	1
MscS family	8	MscS family	3	MscS family	7	MscS family	9
TRIC family	2	TRIC family	0	TRIC family	2	TRIC family	2
UT family	1	UT family	0	UT family	1	UT family	1
VIC family	5	VIC family	0	VIC family	2	VIC family	2
Total	**40**	**Total**	**11**	**Total**	**30**	**Total**	**40**
Outer membrane porins							
OOP family	7	OOP family	1	OOP family	7	OOP family	6
Phospotransferase system (PTS)							
GPTS	3	GPTS	2	GPTS	3	GPTS	3
SSPTS	5	SSPTS	2	SSPTS	4	SSPTS	3
Total	**8**	**Total**	**4**	**Total**	**7**	**Total**	**6**
Secondary Transporters							
2-HCT family	2	2-HCT family	4	2-HCT family	1	2-HCT family	0
AAE family	1	AAE family	0	AAE family	1	AAE family	2
ACR3 family	1	ACR3 family	0	ACR3 family	0	ACR3 family	2
AEC family	5	AEC family	0	AEC family	1	AEC family	2
AGCS family	1	AGCS family	1	AGCS family	0	AGCS family	0
AI-2E family	10	AI-2E family	3	AI-2E family	6	AI-2E family	7
APC family	14	APC family	4	APC family	15	APC family	9
ArAE family	16	ArAE family	2	ArAE family	8	ArAE family	10
ArsB family	3	ArsB family	2	ArsB family	2	ArsB family	3
AtoE family	3	AtoE family	0	AtoE family	0	AtoE family	0
BASS family	1	BASS family	0	BASS family	0	BASS family	2
BenE family	2	BenE family	1	BenE family	1	BenE family	1
CaCA family	1	CaCA family	2	CaCA family	1	CaCA family	2
CDF family	5	CDF family	1	CDF family	3	CDF family	4
CHR family	5	CHR family	1	CHR family	5	CHR family	8
CitMHS family	1	CitMHS family	1	CitMHS family	1	CitMHS family	1
CIC family	11	CIC family	2	CIC family	4	CIC family	6
CLIC family	1	CLIC family	0	CLIC family	0	CLIC family	0
CNT family	0	CNT family	0	CNT famiy	0	CNT family	1

Table S3.9 Continued.

Burkholderia terrae BS001	Number	*Burkholderia rhizoxinica* HKI454	Number	*Burkholderia phymatum* STM815	Number	*Burkholderia phytofirmans* PsJN	Number
Secondary Transporters							
CPA1 family	5	CPA1 family	2	CPA1 family	2	CPA1 family	3
CPA2 family	5	CPA2 family	3	CPA2 family	4	CPA2 family	4
DAACS family	18	DAACS family	2	DAACS family	7	DAACS family	5
DASS family	1	DASS family	1	DASS family	0	DASS family	1
DMT family	35	DMT family	11	DMT family	31	DMT family	26
ESS family	5	ESS family	0	ESS family	0	ESS family	0
FNT family	1	FNT family	0	FNT family	2	FNT family	1
GntP family	3	GntP family	1	GntP family	1	GntP family	3
GPH family	0	GPH family	0	GPH family	1	GPH family	0
HAAAP family	0	HAAAP family	0	HAAAP family	1	HAAAP family	0
GUP family	1	GUP family	0	GUP family	0	GUP family	0
KDGT family	3	KDGT family	0	KDGT family	2	KDGT family	1
KUP family	2	KUP family	1	KUP family	2	KUP family	2
LctP family	3	LctP family	0	LctP family	2	LctP family	1
LIV-E family	2	LIV-E family	1	LIV-E family	2	LIV-E family	2
LysE family	1	LysE family	1	LysE family	1	LysE family	1
MFS	237	MFS	29	MFS	155	MFS	175
MOP family	12	MOP family	7	MOP family	14	MOP family	12
MSS family	2	MSS family	0	MSS family	0	MSS family	2
NCS1 family	10	NCS1 family	0	NCS1 family	5	NCS1 family	7
NCS2 family	4	NCS2 family	1	NCS2 family	4	NCS2 family	6
NhaA family	1	NhaA family	0	NhaA family	1	NhaA family	0
NiCoT family	3	NiCoT family	0	NiCoT family	1	NiCoT family	2
NramP family	9	NramP family	1	NramP family	7	NramP family	5
Oxa-1 family	1	Oxa-1 family	1	Oxa-1 family	1	Oxa-1 family	1
PiT family	2	PiT family	1	PiT family	2	PiT family	2
PNaS family	3	PNaS family	0	PNaS family	2	PNaS family	1
POT family	1	POT family	0	POT family	1	POT family	1
RhtB family	21	RhtB family	4	RhtB family	20	RhtB family	18
RND family	33	RND family	7	RND family	20	RND family	18
SSS family	28	SSS family	5	SSS family	16	SSS family	21
SulP family	9	SulP family	0	SulP family	9	SulP family	5
Tat family	3	Tat family	3	Tat family	3	Tat family	3
TDT family	2	TDT family	0	TDT family	0	TDT family	1
TRAP-T family	8	TRAP-T family	0	TRAP-T family	2	TRAP-T family	7
TTT family	3	TTT family	0	TTT family	0	TTT family	1
VIT family	2	VIT family	0	VIT family	2	VIT family	1
NhaD family	0	NhaD family	1	NhaD family	0	NhaD family	0
Total	**562**	**Total**	**107**	**Total**	**372**	**Total**	**399**
Unclassified Transporters							
FeT family	1	FeT family	0	FeT family	2	FeT family	2
HCC family	2	HCC family	2	HCC family	2	HCC family	2
ILT family	3	ILT family	2	ILT family	2	ILT family	1
TerC family	2	TerC family	1	TerC family	3	TerC family	2
UBS1 family	1	UBS1 family	1	UBS1 family	1	UBS1 family	0
YggT family	1	YggT family	1	YggT family	1	YggT family	1
MgtE family	0	MgtE family	0	MgtE family	1	MgtE family	1
Total	**10**	**Total**	**7**	**Total**	**12**	**Total**	**9**

Table S3.10 List of ABC transporters (ATP component) and MFS transporters used for SplitsTree (neighbor-net) analysis.

Locus_tag	ABC Transporters	Locus_tag	MFS Transporters
AKAUv1_830016	Branched-chain amino acids	AKAUv1_2130084	alpha-ketoglutarate
AKAUv1_790086	Branched-chain amino acids	AKAUv1_1150001	alpha-ketoglutarate
AKAUv1_3020047	Branched-chain amino acids	AKAUv1_1770047	alpha-ketoglutarate
AKAUv1_2610041	Branched-chain amino acids	AKAUv1_460038	alpha-ketoglutarate
AKAUv1_160073	Branched-chain amino acids	AKAUv1_920209	alpha-ketoglutarate
AKAUv1_160061	Branched-chain amino acids	AKAUv1_760139	alpha-ketoglutarate
AKAUv1_1550002	Branched-chain amino acids	AKAUv1_1000002	alpha-ketoglutarate
AKAUv1_2270033	Branched-chain amino acids	AKAUv1_1390087	alpha-ketoglutarate
AKAUv1_110171	Branched-chain amino acids	AKAUv1_1010089	Sugar/general substrate
AKAUv1_1330001	Branched-chain amino acids	AKAUv1_1570009	Sugar/general substrate
AKAUv1_2560043	Branched-chain amino acids	AKAUv1_140024	Sugar/general substrate
AKAUv1_790077	Branched-chain amino acids	AKAUv1_1480018	Sugar/general substrate
AKAUv1_580056	Lipopolysaccharide transport	AKAUv1_1410023	Sugar/general substrate
AKAUv1_1330002	Branched-chain amino acids	AKAUv1_1080161	Sugar/general substrate
AKAUv1_1550003	Branched-chain amino acids	AKAUv1_1000065	Sugar/general substrate
AKAUv1_160074	Branched-chain amino acids	AKAUv1_1680017	Sugar/general substrate
AKAUv1_160062	Branched-chain amino acids	AKAUv1_2570222	Sugar/general substrate
AKAUv1_790087	Branched-chain amino acids	AKAUv1_2870098	Sugar/general substrate
AKAUv1_2840143	Branched-chain amino acids	AKAUv1_3190084	Sugar/general substrate
AKAUv1_1710005	Sulfate transporter	AKAUv1_1680073	Benzoate/gallate
AKAUv1_1230094	Spermidine/putrescine	AKAUv1_1080444	Benzoate/gallate
AKAUv1_220030	Spermidine/putrescine	AKAUv1_930055	Benzoate/gallate
AKAUv1_2440007	Spermidine/putrescine	AKAUv1_1880037	Glucarate/galactarate
AKAUv1_1080401	Spermidine/putrescine	AKAUv1_2120003	Glucarate/galactarate
AKAUv1_1080047	Fe^{3+}	AKAUv1_3140065	Glucarate/galactarate
AKAUv1_1140023	Fe^{3+}	AKAUv1_760286	Tartarate
AKAUv1_1390018	Glycerol-3-phosphate	AKAUv1_920044	Tartarate
AKAUv1_1080422	Glycerol-3-phosphate	AKAUv1_300003	Tartarate
AKAUv1_730008	Glycerol-3-phosphate	AKAUv1_1880005	Tartarate
AKAUv1_750029	Glycerol-3-phosphate	AKAUv1_1010100	Tartarate
AKAUv1_280009	Glycerol-3-phosphate	AKAUv1_2820061	General substrate
AKAUv1_760328	Maltose	AKAUv1_760279	General substrate
AKAUv1_2490059	Maltose	AKAUv1_920191	Putative/general substrate
AKAUv1_1930164	Maltose	AKAUv1_1230090	Putative/general substrate
AKAUv1_1010085	Maltose	AKAUv1_1650004	Multidrug efflux
AKAUv1_3020076	Maltose	AKAUv1_760047	Multidrug efflux
AKAUv1_2440014	Glycerol-3-phosphate	AKAUv1_760033	Multidrug efflux
AKAUv1_1660022	Glycerol-3-phosphate	AKAUv1_160026	Multidrug efflux
AKAUv1_2450102	Spermidine/putrescine	AKAUv1_1820013	Multidrug efflux
AKAUv1_110165	Spermidine/putrescine	AKAUv1_3280037	Multidrug efflux
AKAUv1_550007	Spermidine/putrescine	AKAUv1_580096	Multidrug efflux
AKAUv1_930094	Spermidine/putrescine	AKAUv1_920180	Multidrug efflux
AKAUv1_1560025	Spermidine/putrescine	AKAUv1_720061	Lysophospholipid
AKAUv1_2340009	Glycine/betaine	AKAUv1_2340014	Tetracycline/fosmidomycin efflux
AKAUv1_2450050	Glycine/betaine	AKAUv1_1420001	Tetracycline/fosmidomycin efflux
AKAUv1_2840097	Phosphate transporter	AKAUv1_930033	Tetracycline/fosmidomycin efflux
AKAUv1_120067	Dipeptide/oligopeptide	AKAUv1_1660049	Tetracycline/fosmidomycin efflux
AKAUv1_120066	Dipeptide/oligopeptide	AKAUv1_3140071	Oxalate/monocarboxylate/nitrate
AKAUv1_2870036	Dipeptide/oligopeptide	AKAUv1_2660032	Oxalate/monocarboxylate/nitrate

Table S3.10 Continued.

Locus_tag	ABC Transporters	Locus_tag	MFS Transporters
AKAUv1_2870035	Dipeptide/oligopeptide	AKAUv1_1310008	Oxalate/monocarboxylate/nitrate
AKAUv1_3200007	Dipeptide/oligopeptide	AKAUv1_1930146	Oxalate/monocarboxylate/nitrate
AKAUv1_1360049	Dipeptide/oligopeptide	AKAUv1_1360018	Putative/general substrate
AKAUv1_1230062	Polar amino acids	AKAUv1_2540040	Putative/general substrate
AKAUv1_2270066	Polar amino acids	AKAUv1_3050020	Putative/general substrate
AKAUv1_1580007	Polar amino acids	AKAUv1_790232	Putative/general substrate
AKAUv1_1010076	Polar amino acids		
AKAUv1_760115	Polar amino acids		
AKAUv1_1700052	Polar amino acids		
AKAUv1_1480036	Polar amino acids		
AKAUv1_1980017	Polar amino acids		
AKAUv1_650080	Polar amino acids		
AKAUv1_1010065	Polar amino acids		
AKAUv1_140075	Polar amino acids		
AKAUv1_2140008	Polar amino acids		
AKAUv1_220054	Organic solvent resistance		
AKAUv1_1270059	Organic solvent resistance		
AKAUv1_760125	Methionine		
AKAUv1_720008	Lipoprotein		
AKAUv1_930007	Aliphatic sulfonates		
AKAUv1_650037	Aliphatic sulfonates		
AKAUv1_1710029	Aliphatic sulfonates		
AKAUv1_1390091	Aliphatic sulfonates		
AKAUv1_1350003	Aliphatic sulfonates		
AKAUv1_110135	Aliphatic sulfonates		
AKAUv1_110015	Aliphatic sulfonates		
AKAUv1_110005	Aliphatic sulfonates		
AKAUv1_1010059	Aliphatic sulfonates		
AKAUv1_1710014	nod factor export		
AKAUv1_2330006	Mn/Zn		
AKAUv1_1970065	Fe^{3+}		
AKAUv1_2730004	Phosphonate/organophosphonate		

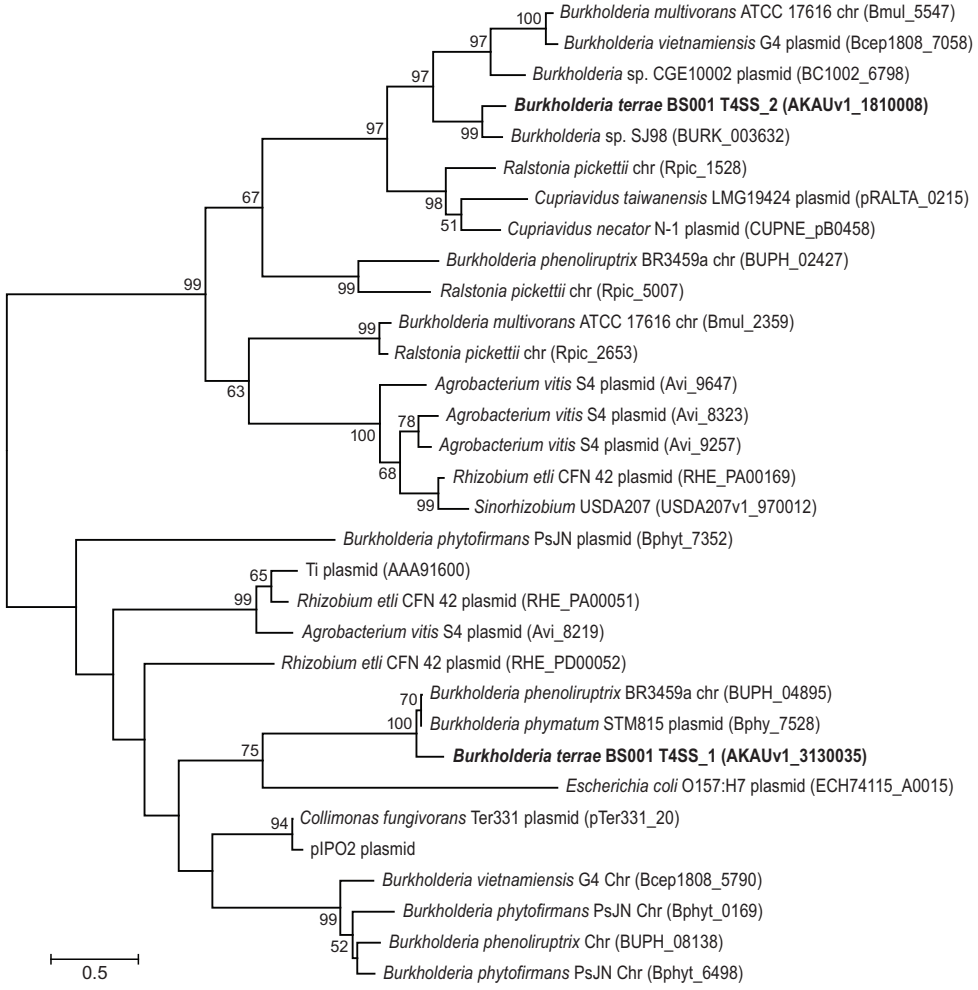

Figure S3.1 A phylogenetic tree based on amino acid sequence of *virB10* gene of T4SS cluster 1 (AKAUv1_3130035) and T4SS cluster 2 (AKAUv1_1810008) of *Burkholderia terrae* BS001, showing their respective positions and relationships.

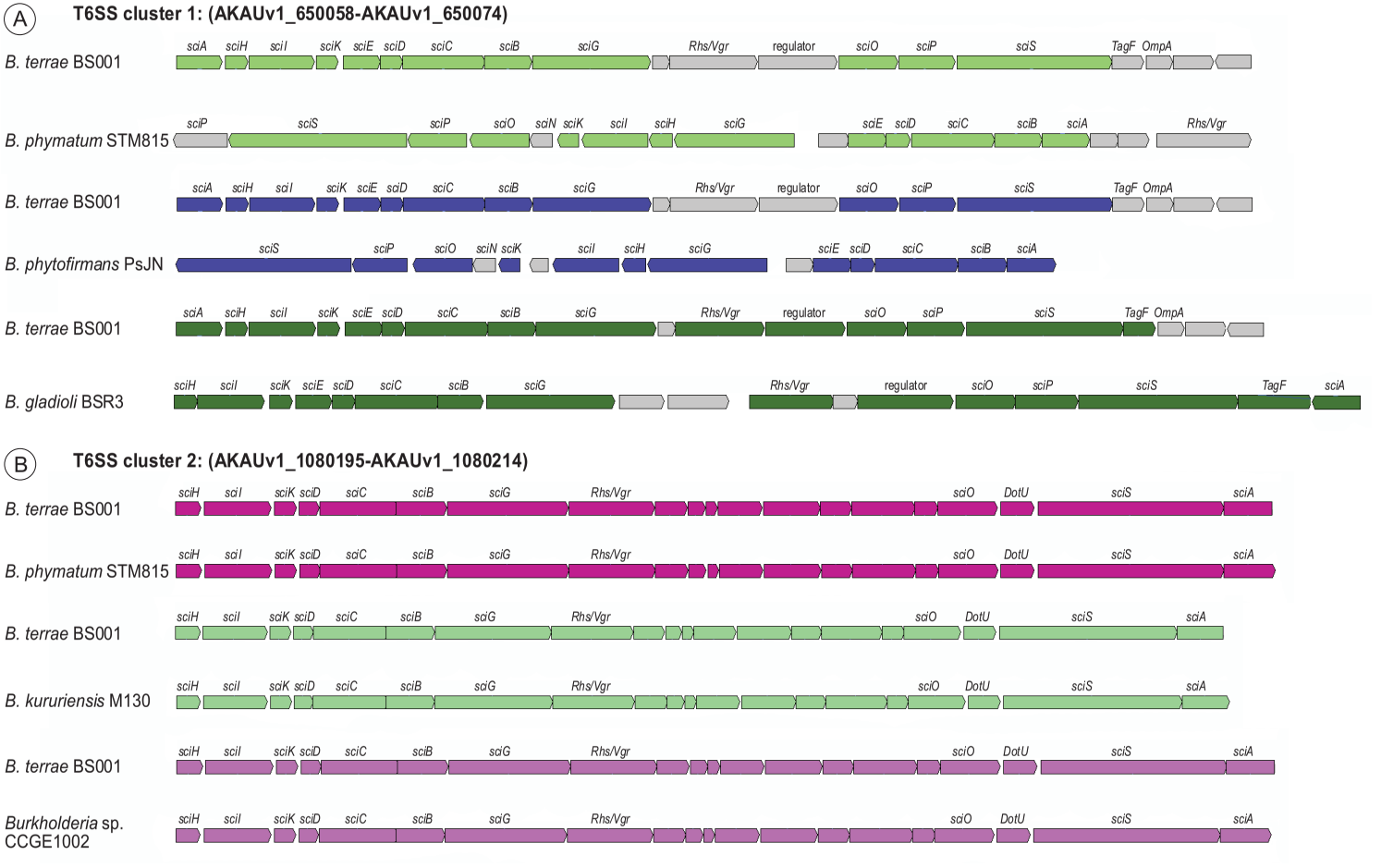
A
T6SS cluster 1: (AKAUv1_650058-AKAUv1_650074)
B. terrae BS001
sciA sciH sciI sciK sciE sciD sciC sciB sciG Rhs/Vgr regulator sciO sciP sciS TagF OmpA
B. phymatum STM815
sciP sciS sciP sciO sciN sciK sciI sciH sciG sciE sciD sciC sciB sciA Rhs/Vgr
B. terrae BS001
sciA sciH sciI sciK sciE sciD sciC sciB sciG Rhs/Vgr regulator sciO sciP sciS TagF OmpA
B. phytofirmans PsJN
sciS sciP sciO sciN sciK sciI sciH sciG sciE sciD sciC sciB sciA
B. terrae BS001
sciA sciH sciI sciK sciE sciD sciC sciB sciG Rhs/Vgr regulator sciO sciP sciS TagF OmpA
B. gladioli BSR3
sciH sciI sciK sciE sciD sciC sciB sciG Rhs/Vgr regulator sciO sciP sciS TagF sciA
B
T6SS cluster 2: (AKAUv1_1080195-AKAUv1_1080214)
B. terrae BS001
sciH sciI sciK sciD sciC sciB sciG Rhs/Vgr sciO DotU sciS sciA
B. phymatum STM815
sciH sciI sciK sciD sciC sciB sciG Rhs/Vgr sciO DotU sciS sciA
B. terrae BS001
sciH sciI sciK sciD sciC sciB sciG Rhs/Vgr sciO DotU sciS sciA
B. kururiensis M130
sciH sciI sciK sciD sciC sciB sciG Rhs/Vgr sciO DotU sciS sciA
B. terrae BS001
sciH sciI sciK sciD sciC sciB sciG Rhs/Vgr sciO DotU sciS sciA
Burkholderia sp. CCGE1002
sciH sciI sciK sciD sciC sciB sciG Rhs/Vgr sciO DotU sciS sciA

Figure S3.2 Genetic organization of the T6SS gene clusters (T6SS-I, T6SS-II, T6SS-III, and T6SS-IV) of *B. terrae* BS001 and their comparison with similar systems in other *Burkholderia* species. (A) T6SS-I (AKAUv1_650058-AKAUv1_650074), (B) T6SS-II (AKAUv1_1080195-AKAUv1_1080214), (C) T6SS-III (AKAUv1_1680089-AKAUv1_1680097), and (D) T6SS-IV (AKAUv1_920001-AKAUv1_920005).

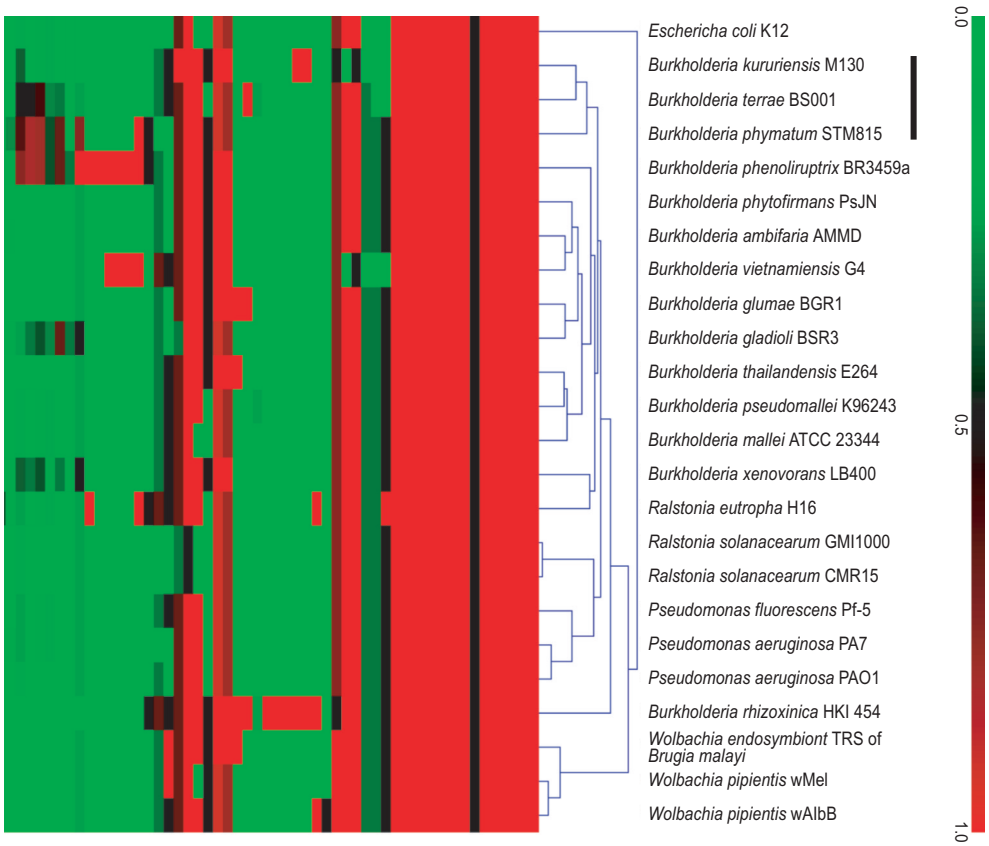

Figure S3.3 Hierarchical cluster analysis of *B. terrae* BS001 in comparison to other *Burkholderia* and outgroup species, based on their metabolic pathway completion values of the MicroCyc database.

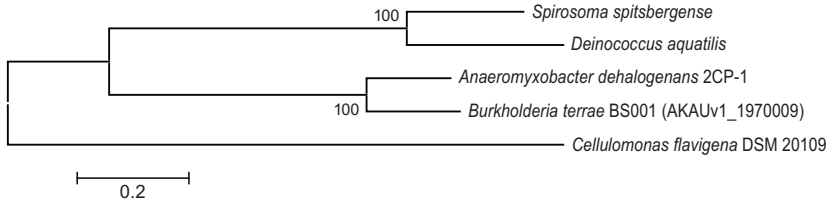

Figure S3.4 A maximum-likelihood phylogenetic tree of the tyrosinase encoding gene (AKAUv1_1970009) of *B. terrae* BS001 showing similarity with a similar gene of *Anaeromyxobacter dehalogenans* 2CP-1.

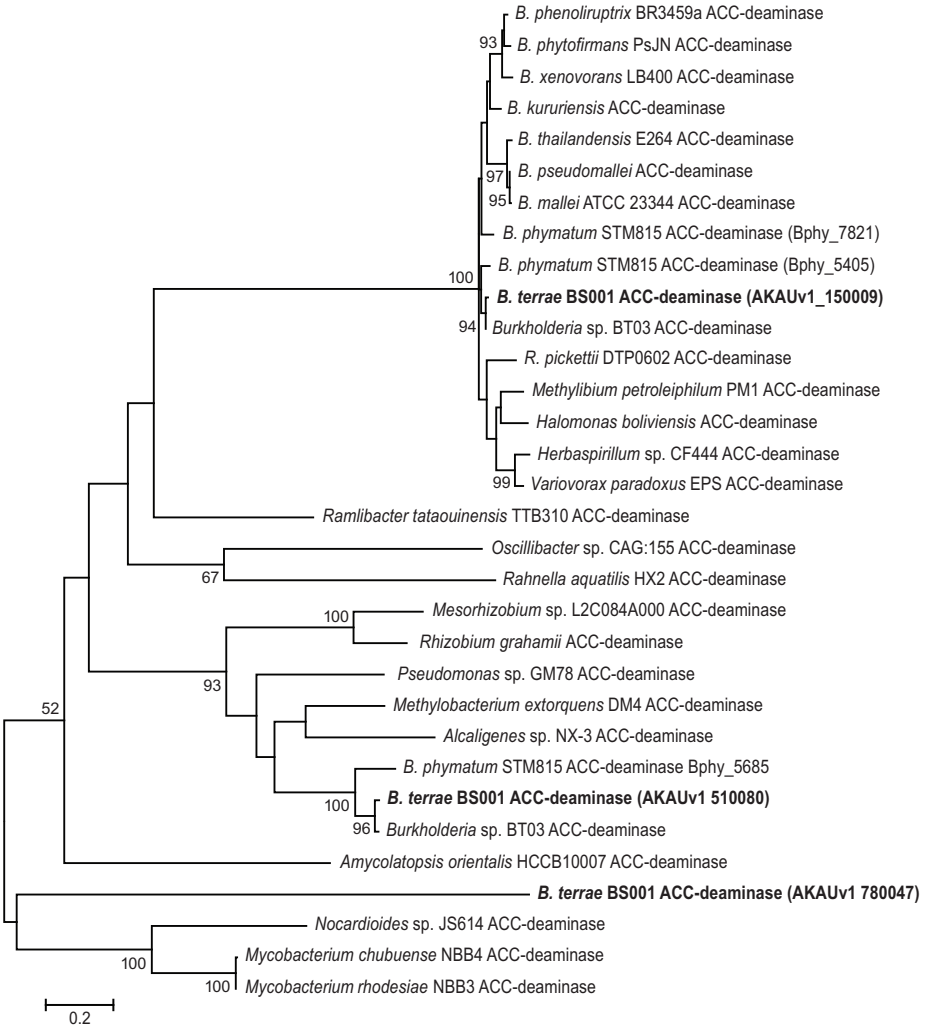

Figure S3.5 Maximum-likelihood phylogenetic tree of three genes (AKAUv1_150009, AKAUv1_510080, and AKAUv1_780047) of *B. terrae* BS001, encoding 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase). Analysis showing possible horizontal gene transfer and gene duplication events.

Chapter 4

4 Transcriptional responses of the bacterium *Burkholderia terrae* BS001 to the fungal host *Lyophyllum* sp. strain Karsten under soil-mimicking conditions

Irshad Ul Haq, Francisco Dini-Andreote, Jan Dirk van Elsas

Abstract

In this study, the mycosphere isolate *Burkholderia terrae* BS001 was confronted with the soil fungus *Lyophyllum* sp. strain Karsten on soil extract agar plates in order to examine its transcriptional responses over time. At the initial stages of the experiment (T1 – day 3; T2 – day 5), contact between both partner organisms was absent, whereas in the final stage (T3 – day 8), the two populations made intimate physical contact. Overall, a strong modulation of the strain BS001 gene expression patterns was found. First, the stationary-phase sigma factor RpoS, and numerous genes under its control, were strongly expressed as a response to the soil extract agar, and this extended over the whole temporal regime. In the system, *B. terrae* BS001 apparently perceived the presence of the fungal hyphae already at the early experimental stages (T1, T2), by strongly upregulating a suite of chemotaxis and flagellar motility genes. With respect to specific metabolism and energy generation, a picture of differential involvement in different metabolic routes was obtained. Initial (T1, T2) up- or down-regulation of ethanolamine, mandelate and tartrate uptake and utilization pathways was substituted by a strong investment, in the presence of the fungus, in the expression of three putative metabolic gene clusters (T3). Specifically at T3, five clustered genes that are potentially involved in energy generation coupled to an oxidative stress response, and two genes encoding short-chain dehydrogenases/oxidoreductases (SDR), were highly upregulated. In contrast, genes related to general stress responses, i.e. *dnaE2* (encoding error-prone DNA polymerase), were transcriptionally downregulated at this stage. This study revealed that *B. terrae* BS001, from a stress-induced state, resulting from the soil extract agar milieu, responds positively to fungal hyphae that encroach upon it, in a temporally dynamic manner. The response is characterized by phases in which the modulation of (1) chemotaxis, (2) metabolic activity and (3) oxidative stress responses are key mechanisms.

Submitted for publication

Introduction

The soil bacterium *Burkholderia terrae* BS001 was originally isolated on the basis of its capacity to interact with the basidiomycetous soil fungus *Lyophyllum* sp. strain Karsten (Nazir et al. 2014). There is mounting evidence for the contention that this interacting pair forms an ecologically relevant mutualism, which we previously have coined the *B. terrae* BS001-*Lyophyllum* sp. strain Karsten interactome (Haq et al. 2014b). A plethora of functions is presumed to be important in the processes that underlie the interactions between the two organisms (Haq et al. 2014a). Briefly, *B. terrae* BS001 was found to successfully migrate through the soil matrix along with the growing hyphae of *Lyophyllum* sp. strain Karsten (Warmink and van Elsas 2009). Moreover, this bacterium has the capacity to induce the release of glycerol by the fungus and efficiently utilize it as a carbon and energy source (Nazir et al. 2013). Finally, strain BS001 — upon confrontation with *Lyophyllum* sp. strain Karsten and several other fungi — established agglomerates of cells — i.e. 'primitive' biofilms, around the mycelium of these fungi (Nazir et al. 2014).

To shed light on the interaction between *B. terrae* BS001 and *Lyophyllum* sp. strain Karsten, the recently-sequenced 11.5-Mb strain BS001 genome (Haq et al. 2014a, Nazir et al. 2012) was investigated with respect to the presence of genetic systems that are potentially involved in the interaction. Indeed, a suite of potential 'interactome" genetic systems was present in the *B. terrae* BS001 genome, whereas other systems were suggested to be relevant for the 'free-living' modus (Haq et al. 2014a). On the basis of these findings, we hypothesized that strain BS001 might exhibit a lifestyle in soil that involves two phases; one characterized by survival as a 'loner' and a second one in which perceiving the presence of (fungal) hosts, and responding to these, constitutes the key ecological strategy. Nazir et al. (2014) recently indicated that *B. terrae* BS001 is a 'generalist' mycosphere colonizer rather than a specialist organism, as it could associate with a suite of different soil fungi. Given this facet of the lifestyle of strain BS001, particular genetic systems may have arisen that allow it to efficiently interact with diverse fungal types (Nazir et al. 2014). Interestingly, Pion et al. (2013) recently found that the fungus *Morchella crassipes* apparently 'farms' a *Pseudomonas putida* strain, allowing it to disperse and concomitantly use fungal-released compounds. The bacterium, in return, increased the resistance to stress of the fungal mycelium (Pion et al. 2013). Thus, soil-exploring saprotrophic fungi might indeed constitute hot spots for the activity and growth of bacteria that are endowed with systems that allow to explore the fungal-created novel niches (Warmink and van Elsas 2009). However, such fungi may also bring about conditions of stress (in particular oxidative stress), like suggested for the fungi *Alternaria alternata* and *Fusarium solani* in their interactions with the soil bacterium *Burkholderia glathei* (Stopnisek et al. 2015). On another notice, *Collimonas fungivorans* Ter331, upon confrontation with *Aspergillus niger* N400, did not only utilize compounds provided by the fungus, but also expressed genes responsible for the production of antifungal agents (Mela et al. 2011). Finally, the ectomycorrhizal fungus *Laccaria bicolor* S238N was found to respond

in different and quite complex ways to antagonistic, neutral and beneficial soil bacteria (Deveau et al. 2015). Notwithstanding this growing body of knowledge on bacterial-fungal interactions in soil settings, none of the aforementioned studies assessed the behaviour of bacteria with well-characterized genomes that contain — next to a core genome — a very large accessory part (such as *B. terrae* BS001; Haq et al. 2014a).

Here, we examined the transcriptional responses of *B. terrae* BS001 to the fungus *Lyophyllum* sp. strain Karsten. We hypothesized that time-dependent and dynamic responses might occur of the genetic systems that are of immediate interest to the ecological fitness of the bacterial partner of soil-exploring fungi upon confrontation with the fungus. Thus, to understand the responses that take place in the (largely carbon-limited) soil, we interrogated the transcriptional 'null' status of the bacterium under such conditions. We specifically investigated the transcriptional response of strain BS001 cell populations to the developing mycelium of *Lyophyllum* sp. strain Karsten in dual-culture systems on soil extract agar plates, using as controls fungus-less systems.

Materials and Methods

Strains, culture conditions and bacterial-fungal interactome

Burkholderia terrae BS001 maintained in the –80°C culture collection was grown overnight in Luria-Bertani (LB) medium in a shaking incubator at 28°C. The overnight culture was centrifuged and the bacterial pellet washed twice with 0.85% NaCl solution. An estimated 4×10^8 CFU mL^{-1} were used for the bacterial–fungal confrontation assay on soil extract agar (see below). For the B treatment, aliquots of 25 L were streaked in five handlings in a straight 4 mm wide line using a sterile inoculation loop onto soil extract agar plates, establishing populations of around 1×10^7 cells. For the B+F treatment, four plugs of *Lyophyllum* sp. strain Karsten grown on oat flake agar (OFA) medium were placed next to the bacterial streak line in parallel, separating both by a distance of 15 mm. For both treatments B and B+F, triplicates were used.

Soil extract agar

We used soil extract agar (SEA) to establish the *in vitro* interactome. To prepare the soil extract, we used a loamy sand soil (organic matter 5% and pH 5; Inceoğlu et al. 2010) sampled in a field in Buinen, the Netherlands. For making the extract, 500 g of soil was taken up in 1 L of sterilized MilliQ water and vigorously shaken for 24 h (room temperature). Soil particles were removed by centrifugation at maximum speed (5430R Eppendorf, Hamburg, Germany), after which the supernatant was filtered using folded qualitative filter paper (vWR European) and stored at 4°C. To prepare 1 L of medium, 500 mL of soil extract, 0.5 g of yeast extract and 15 g of agar were mixed with 500 mL of MilliQ water. The pH of the medium was adjusted to 6.8 and autoclaved. The soil extract agar (SEA) plates were prepared with about ca. 22 mL of liquid medium per plate.

Soil extract analyses

Soil extract analyses were performed at NIOZ, Yerseke, Netherlands, using a set of standard techniques. The soil extract contained 0.46 mM N-NH_4, 0.00575 mM N-NO_2, 1.67 mM N-NO_3 and 1.67 mM N-NOx. The amounts of P-PO_4 and Si-SiO_2 in the extract were 0.09 and 0.15 mM, respectively. The extract also contained ca. 0.01% of soil-extracted total organic carbon.

Bacterial RNA extraction and sequencing

At each sampling time, the entire bacterial biomass was retrieved (using a sterilized spatula) from the SEA plates and resuspended in TRIzol-chloroform (900:150 L), after which the mixture was bead-beaten for 40 sec. Total RNA was then extracted using the TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. Residual DNA was digested and removed using the TURBO DNA-free Kit (Life Technologies, Carlsbad, CA, USA) according to the instructions of the manufacturer. To remove ribosomal RNA, the MICROB*Express* Kit (Life Technologies, Carlsbad, CA, USA) was used according to the manufacturer's instructions. cDNA based library preparation and sequencing using the Illumina MiSeq platform were performed at Macrogen Inc., Seoul, South Korea.

Transcriptome sequence analysis

We employed the bioinformatics pipeline implemented in the MicroScope interface (Vallenet et al. 2013), to analyze the raw sequence data, and to perform the mapping and statistical analyses. The pipeline is a 'Master' shell script composed of different parts (collection of Shell/Perl/R scripts). The quality of the sequence data was assessed by read trimming and quality checks. The reads were then mapped onto the genome of *B. terrae* BS001 (hosted at MicroScope: http://www.genoscope.cns.fr/agc/microscope/home/) using the 'SSAHA2' package (Ning et al. 2001). This package identifies regions of high similarity using the SSAHA searching algorithm and aligns these by implementing cross-match sequence alignment (Ewing et al. 1998) based on the banded Smith-Waterman-Gotoh algorithm (Smith and Waterman 1981). For a hit to be retained, an alignment score equal to half of the read (at least) was required. The risk of false positives was reduced by extracting reliable alignments from SAM-formatted files using SAMtools (v.0.1.8) (Li et al. 2009). The 'Bioconductor-Genomic features' package (Carlson et al. 2011) was implemented to calculate the number of reads matching coding sequences [genomic objects] of the *B. terrae* BS001 genome. Differential gene expression between treatments was assessed using the Bioconductor-DESeq package (Anders and Huber 2010) with default parameters. The DESeq normalized values of genes across replicates were z-score standardized and visualized as heatmaps. All sequences are available in the short read archive (SRA) of the National Center for Biotechnology Information (NCBI), under the accession number SRP056279 and project number PRJNA278110.

Results

Biomass development of Burkholderia terrae BS001 and Lyophyllum sp. strain Karsten on soil extract agar (SEA) plates

As from introduction onto the SEA plates, *B. terrae* strain BS001 progressively developed biomass over the time of the experiment in both systems (B — bacterial strain alone and B+F — bacterial strain plus fungal inoculum). In the B+F system, fungal biomass slowly and progressively encroached upon the bacterial stripes, establishing physical contact at day 8. At three time points (T1 – day 3, T2 – day 5 and T3 – day 8), the total bacterial biomasses from B and B+F systems were sampled and subjected to bacterial RNA extraction, cDNA synthesis and high-throughput sequencing. This yielded a one-sided analysis of the transcriptional responses of *B. terrae* BS001—when under a (soil-relevant) 'null' condition—to the presence of the fungal partner organism under two conditions: physically separated (T1, T2) or in contact (T3).

Establishment of the B. terrae BS001 transcriptome

Overall, 31,831,926 sequences were produced across all replicate samples (Supplementary Table S4.1). Following sequence quality trimming and selection of the strain BS001 transcripts, a total of 5,972,111 reads were obtained (representing predicted CDSs only). This was taken as the initial dataset that was used for all downstream analyses. The raw read counts of the genes across all treatments (B and B+F) at all time points (day 3, day 5 and day 8) are provided in Supplementary Table S4.2. At the level of COG (clusters of orthologous genes) classes, representatives of all broad functional categories were found at T1, T2 and T3, in both treatments. The distribution of reads across the COG classes is provided in Supplementary Fig. S4.1A and S4.1B. Moreover, a global visualization of the differentially-expressed genes of *B. terrae* BS001 upon confrontation with *Lyophyllum* sp. strain Karsten is shown in Supplementary Fig. S4.2.

The collective data revealed a very dynamic global transcriptional response of the strain BS001 as a response to the SEA medium, over the whole temporal regime, and this dynamism was also found in the systems with *Lyophyllum* sp. strain Karsten. The transcriptional landscape was typified by: (1) a generic response to the SEA conditions, and (2) limited sets of genes being responsive to the fungus. Both types of responses were different between the physical contact (T3) versus no-contact (T1 and T2) stages, which is explored in greater detail in the following.

Analysis of the transcriptome of strain BS001 reveals a (generic) stress response on SEA and modulation of the response by the presence of Lyophyllum sp. strain Karsten

A first key observation was that the cells of strain BS001 were, apparently, in a state of (starvation) stress on the SEA medium, from T1 through T3 (Fig. 4.1). Hence, the predicted (alternative sigma factor) RpoS encoding gene (AKAUv1_1370011) was dynamically expressed in both treatments, with a trend (albeit not significant, $P > 0.05$) of

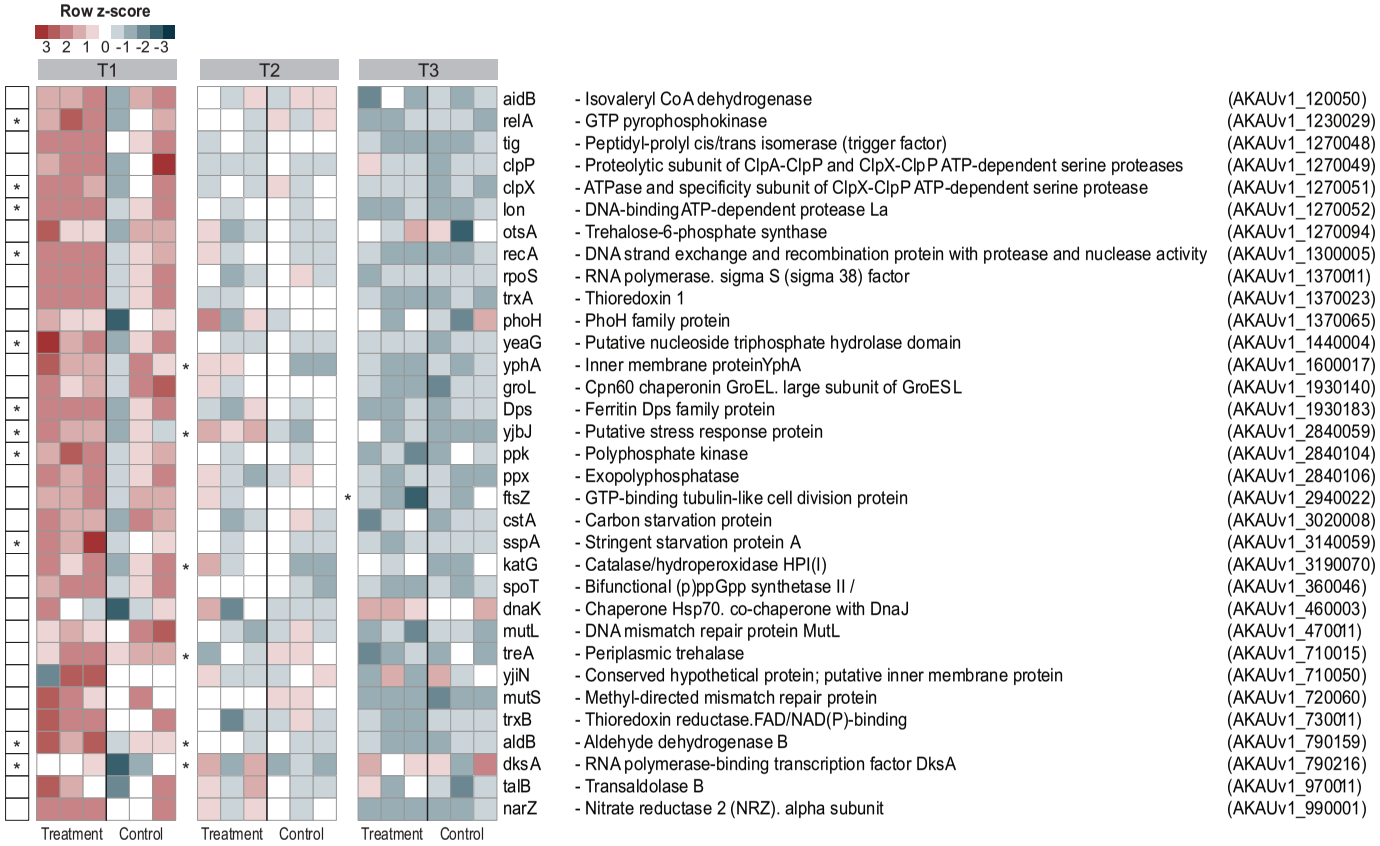

Figure 4.1 Heat map showing the expression levels of selected stress related genes. Heat maps comparing the expression levels of selected stress related genes in the B (*B. terrae* BS001) and B+F (*B. terrae* BS001 + *Lyophyllum* sp. strain Karsten) treatments at time point T1 (day 3), T2 (day 5) and T3 (day 8). Symbols (*) show statistical significant changes (statistical analysis was performed using DESeq: $P < 0.05$) between B and B+F treatments. The heat map was constructed based on normalized read counts. The standardized normalized read counts, denoted as the row Z-score, is plotted in color scale (red indicates higher expression and blue indicates lower expression). The putative gene products are given in front of each gene with their respective locus tags.

raised expression at the fungus (B: 98; B+F:129) (Fig. 4.1). Moreover, homologs of (as *per* the *Escherichia coli* annotation; Vijayakumar et al. 2004) the RpoS-regulated genes *katG* and *otsA* (cellular 'processes') were expressed similarly across all three time points (Fig. 4.1). Other transcripts for stress response-relevant proteins produced in both treatments were those of *groEL*, *dnaK*, *recA*, *ftsZ*, *mutL* and *mutS* homologs, as well as of *yeaG*, *yjbJ*, *yjiN* and *yphA* (producing as-yet-uncharacterized proteins). Similarly, transcripts of homologs of *narZ* (nitrate reductase Z; energy metabolism), *aldB* and *treA* (carbon compound metabolism), *talA* (central intermediary metabolism) and *aidB* (DNA replication/repair) were detected. Moreover, a gene *phoH* homolog, which was predicted to encode the phosphate starvation-inducible PhoH protein, had a similar expression pattern (Fig. 4.1).

Interestingly, gene *sspA*, which was predicted to encode the 'stringent starvation protein' SspA, revealed a differential upregulation at T1 (log2 fold change 1.01; $P < 0.05$) (Fig. 4.1), whereas at later stages (T2; B:29, B+F:28; T3; B:21, B+F:18) its expression remained similar across treatments. Supporting the notion that the cells were under carbon starvation stress, gene *cstA* (AKAUv1_3020008; encoding 'carbon starvation protein A') also revealed initially high expression, which gradually subsided (Fig. 4.1).

Partial alleviation of stress at later stages of the interaction

A small set of genes was found to be expressed to somewhat similar levels at T1, with subsequent different expression levels in B+F at T2 and T3. Some of these genes were upregulated as a result of potential oxidative stress response while others were downregulated (Fig. 4.2). These genes, which were all predicted to be involved in cellular responses under stress, included homologs of *rsbR* and *rsbS*, which encode "activator of sigma-B (RsbR) and anti-RsbT (RsbS)", respectively. The RsbR and RsbS proteins may play roles in the response of *B. terrae* BS001 to nutritional/environmental stresses such as salt, heat, acid or ethanol, like in *Bacillus subtilis* (Akbar et al. 2001). Surprisingly, a gene, AKAUv1_1020002, encoding error-prone DNA polymerase *dnaE2* (log2 fold change –2.03; Fig. 4.2), was downregulated at the physical contact stage. Given that error-prone polymerases enhance mutagenesis under starvation stress (Boshoff et al. 2003, Zeng et al. 2011), this might suggest partial alleviation of starvation (or other) stress. Similarly, gene AKAUv1_3010006 (encoding a metallo-beta lactamase domain protein) was also downregulated (log2 fold change –1.73). However, its exact role in the used system is not well understood.

Interestingly, at T2 another gene, AKAUv1_1070012 (encoding a homolog of a DNA-binding transcription antiterminator with a cold shock domain) was downregulated (log2 fold change –1.63) (Fig. 4.2). The gene product may regulate chromosome condensation and antitermination of transcription (Bae et al. 2000, Johnston et al. 2006), cold shock response (Phadtare et al. 2006) and even modulate RpoS (Phadtare et al. 2002). Notably, gene AKAUv1_1700025 (encoding a DNA cytosine methylase) was also downregulated (log2 fold change –1.68) at T2. In *E. coli*, such a gene modulates (limits) the expression of ribosomal protein genes during stationary phase (Militello et al. 2011)

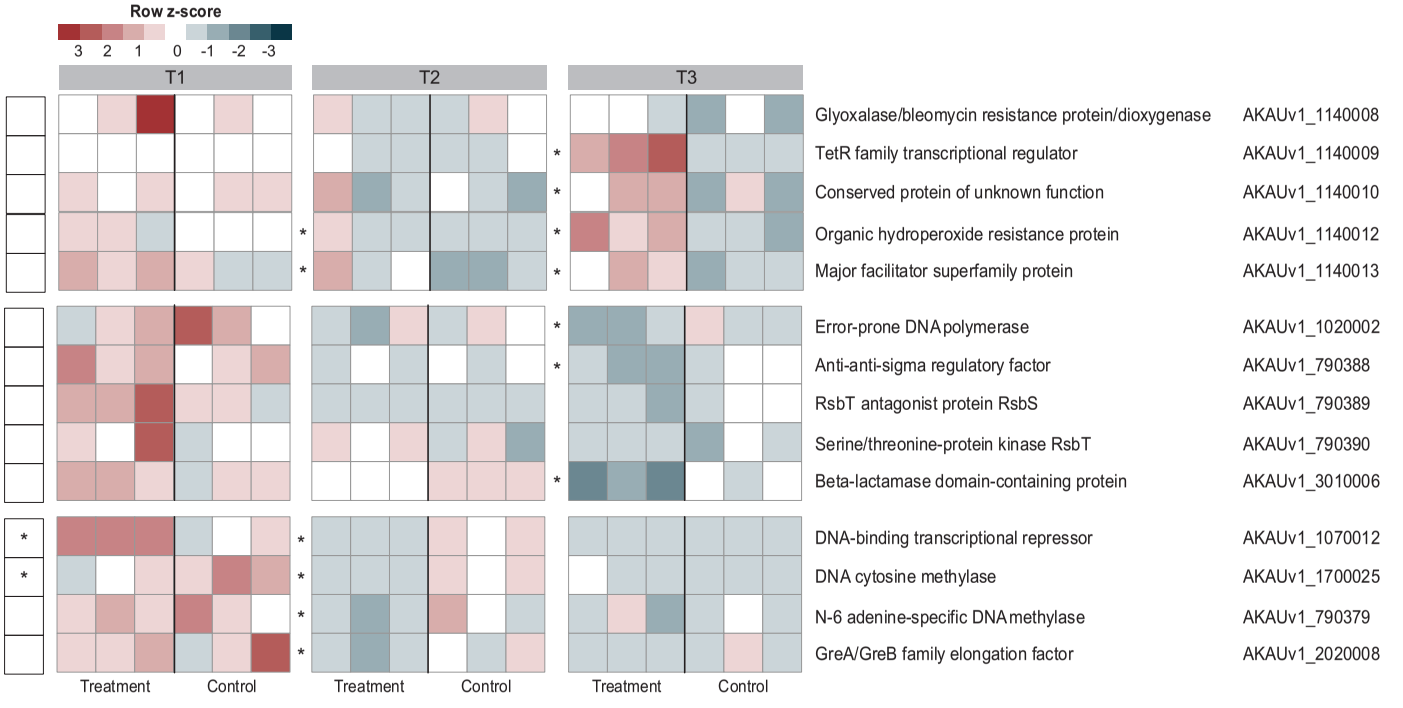

Figure 4.2 Heat map showing the expression levels of genes possibly related to stress alleviation. Heat maps comparing the expression patterns of potential stress alleviation genes in the B (*B. terrae* BS001) and B+F (*B. terrae* BS001 + *Lyophyllum* sp. strain Karsten) treatments at time point T1 (day 3), T2 (day 5) and T3 (day 8). The heat map was constructed based on normalized read counts. The standardized normalized read counts, denoted as the row Z-score, is plotted in color scale (higher expression is indicated by red color and lower expression by blue). The putative gene products are given in front of each gene with their respective locus tags. Symbols (*) show statistical significance (statistical analysis was performed using DESeq: $P < 0.05$) between control and treatment.

and the expression of the *rpoS* gene (Kahramanoglou et al. 2012). Genes AKAUv1_790379 (encoding adenine specific DNA methylase; log2 fold change –2.09) and AKAUv1_2020008 (encoding GreA/GreB elongation factor; log2 fold change –2.40) were also downregulated (Fig. 4.2). The latter protein is known to have chaperone activity and resolve the undesirable aggregation of proteins (Li et al. 2012).

However, the *ohr* gene (AKAUv1_1140012; log2 fold change 2.05), which encodes an 'organic hydroperoxide resistance' protein— a key organic peroxide scavenger (Li et al. 2014)— was upreguated (Fig. 4.2). In *Shewanella oneidensis*, a similar protein scavenges organic peroxides (tertiary butyl hydroperoxide) (Kim et al. 2012), thus being a key element in the oxidative stress response.

Chemotaxis is a prime response of B. terrae BS001 on SEA to the presence of Lyophyllum sp. strain Karsten

At both 'no-physical-contact' phases T1 and T2, the expression of a suite of genes, classed to COG class N (encompassing genes for cell motility and flagellar movement, next to secretion systems of type 2 and 3), was significantly enhanced in the B+F as compared to the B treatment. In particular, the expression of the *cheA* gene (AKAUv1_790006; histidine autokinase, assisting in the onset of chemotaxis) was significantly ($P < 0.05$) raised (Fig. 4.3). Similarly, fungal-incited upregulation of other chemotaxis-related genes, i.e. *cheW*, *cheC*, *motA*, *motB* and 'chemotaxis-related' (*tsr*) genes, AKAUv1_1760105 and AKAUv1_790004 (both encoding 'methyl-accepting chemotaxis protein I') was observed, most strongly at no–physical contact stages (Fig. 4.3; significance at $P < 0.05$ indicated by *). Concurrent with this upregulation, homologs of the flagellar biosynthesis genes *flhD*, *fliS*, *fliM*, *fliG*, *fliH*, *flil* and *ycgR* were also significantly upregulated ($P < 0.05$).

In the light of the potential importance of secretion systems in the bacterial-fungal interactome, we then examined the expression levels of type-2, type-3 and type-6 secretion systems (T2SS, T3SS, T6SS). Only low expression levels were found for the genes in these systems across both treatments. However, the genes in the whole T6SS cluster 1 (Haq et al. 2014a) (log2 fold increase 0.40–0.74) were upregulated at T1, with the differences for ten genes being statistically significant ($P < 0.05$) (Supplementary Table S4.3). Moreover, gene AKAUv1_2840083 (encoding a lytic transglycosylase) was also expressed in both treatments over the whole, with a slight upregulation in the B+F treatment at T1 (log2 fold change 0.51; (Supplementary Table S4.3).

Metabolic responses of B. terrae BS001 occur dynamically and differentially at Lyophyllum sp. strain Karsten

With respect to putative metabolic up- and/or down-shifts in *B. terrae* BS001, a dynamic picture of gene expression was obtained that pointed to a time-dependent metabolic response to (1) the SEA and (2) the presence of fungal hyphae (Fig. 4.4).

Probably as a response to a small metabolite secreted by *Lyophyllum* sp. strain Karsten into the SEA at T1, we observed a strong upregulation of three clustered genes

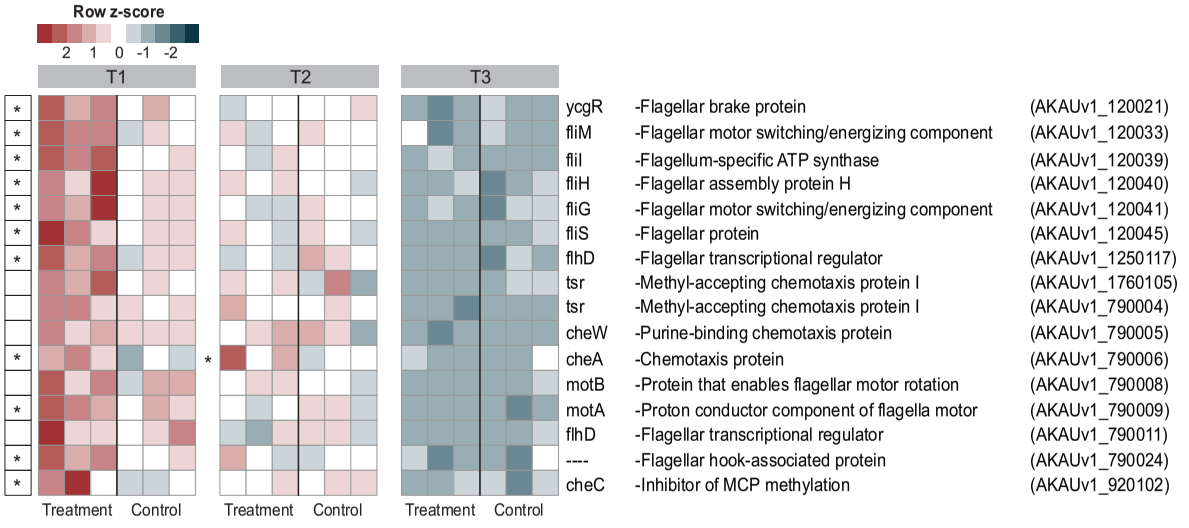

Figure 4.3 Heat map showing the expression patterns of chemotaxis and flagellar motility related genes. Heat maps showing the expression patterns of chemotaxis and motility related genes in the control *(B. terrae* BS001) and treatment (*B. terrae* BS001 + *Lyophyllum* sp. strain Karsten) at time point T1 (day 3), T2 (day 5) and T3 (day 8). Symbols (*) show statistical significance (statistical analysis was performed using DESeq: $P < 0.05$) between control and treatment. The heat map was constructed based on normalized read counts. The standardized normalized read counts, denoted as the row Z-score, is plotted in color scale (red indicates higher expression and blue indicates lower expression). The putative products are given in front of each gene with their respective locus tags.

(AKAUv1_790155, AKAUv1_790156 and AKAUv1_790157) that are predicted to encode an ethanolamine transporter and the large and small subunits of ethanolamine ammonia lyase, respectively and involved in the transport and metabolism of compounds like ethanolamine (Fig. 4.4). However, this differential response evened out quite dramatically at T2 and T3. In contrast, three (contiguous) genes predicted to encode proteins involved in the metabolism of the aromatic soil compound mandelate were highly expressed at T1 in the B, but less so in the B+F treatment (Fig. 4.4), whereas at T2 and T3 this response diminished significantly (Fig. 4.4). Interestingly, gene AKAUv1_1010100, which is predicted to encode a putative tartrate transporter, was downregulated at T1, and quite strongly upregulated at T2, thus indicating the potential utilization of a tartrate-like compound initially from the SEA and later from the fungus.

Several other genes, collectively belonging to three different clusters and possibly involved in metabolic routes for energy generation, were strongly upregulated at T2 and T3, but not at T1 (Fig. 4.4). Notably, these clusters each contained at least one gene that was predicted to encode a short-chain dehydrogenase (SDR), which suggested they were part of energy generation modules. SDRs are known to catalyze the oxidation (or reduction) of sugars, alcohols, steroids, diverse xenobiotic and aromatic compounds in an NADP(H)–dependent fashion (Kallberg et al. 2002). One such gene, AKAUv1_2490031, was upregulated at T2 and more strongly so (log2 fold change 4.64) at T3 (Fig. 4.4). The downstream gene AKAUv1_2490033 (encoding an NAD-dependent sugar epimerase/dehydratase, probably involved in the conversion of UDP-α-D-glucose to UDP-alpha-D-galactose; log2 fold change 2.84) was also upregulated, whereas the intervening gene (AKAUv1_2490032) encoding a conserved hypothetical protein, as well as the up- and downstream genes did not show differential expression. In a second cluster, two SDR-encoding genes (AKAUv1_2440023; AKAUv1_2440024), next to one encoding a transcriptional regulator (AKAUv1_2440025), were also upregulated. Interestingly, gene AKAUv1_2440024 (log2 fold change 2.69) had a keto reductase domain. The third cluster encompassed genes AKAUv1_3190155, AKAUv1_3190156 and AKAUv1_3190157 (encoding a putative FAD-dependent pyridine nucleotide-disulphide oxidoreductase (log2 fold change 3.22), a putative phosphatidylethanolamine binding protein and a short-chain dehydrogenase/reductase, respectively). These were also significantly upregulated at T3 (Fig. 4.4).

A five-gene cluster with relevance for energy generation unveiled

With respect to metabolism and energy generation, five clustered genes, AKAUv1_2870056 through AKAUv1_2870060, were dynamically modulated and significantly upregulated at T3. BLAST-N searches of the whole region revealed nucleotide identities of 95% (coverage of 91%) to a similar region of the *B. caribiensis* MBA4 2,555,069 bp replicon. The first four genes of the cluster are located on the same strand and represent an operon (Fig. 4.5A), as predicted using Rockhopper (McClure et al. 2013, Tjaden 2015). Of this operon, the first gene encodes a predicted alkyl hydroperoxidase (AHP) (log2 fold change 4.51) (Fig. 4.5B) and the second one (log2fold change 3.81) a protein that belongs to the cupin superfamily. These two gene products might reflect a combina-

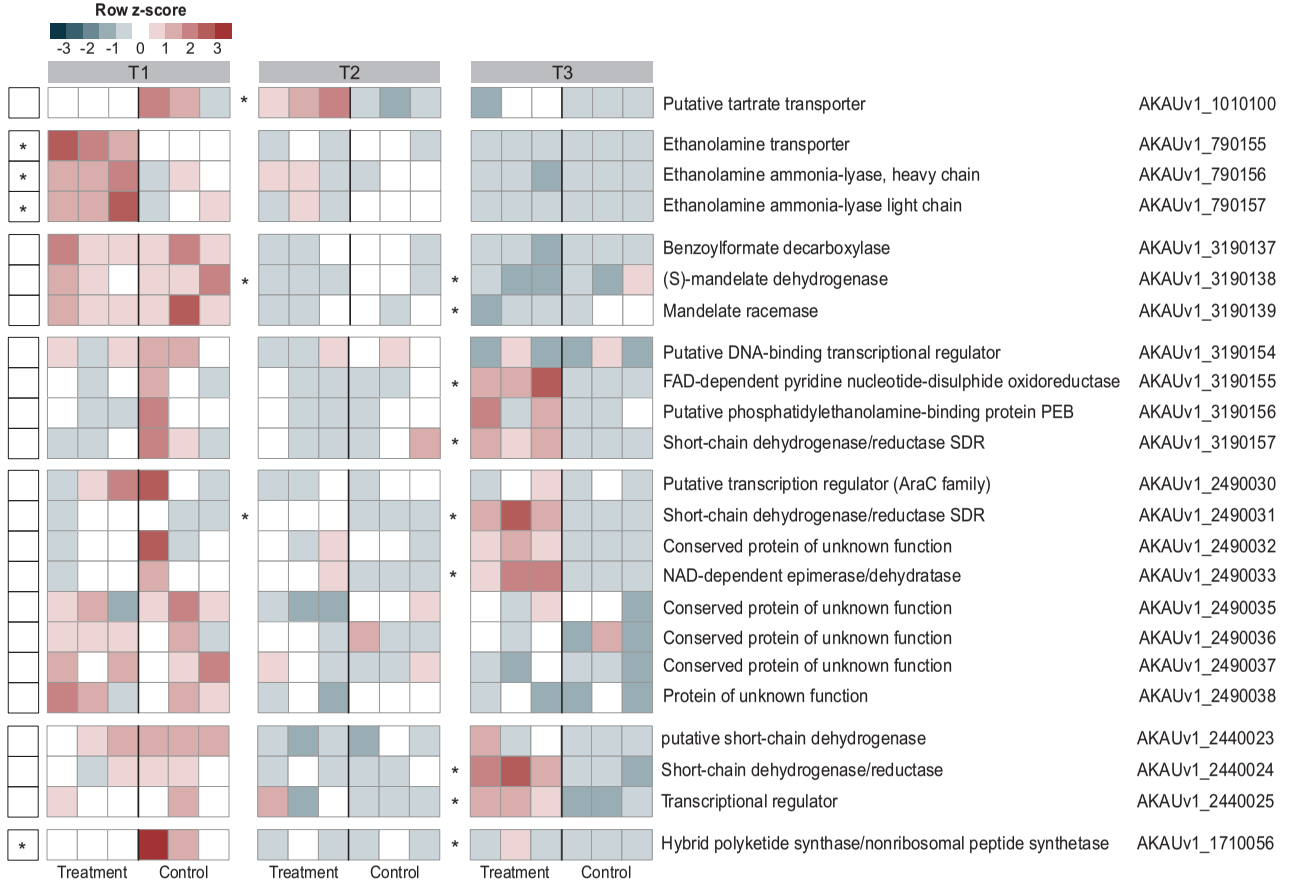

Figure 4.4 Heat map showing the expression patterns of gene clusters involved in diverse metabolic and energy generation pathways. Heat maps showing the expression patterns of genes in the control (*B. terrae* BS001) and treatment (*B. terrae* BS001 + *Lyophyllum* sp. strain Karsten) at time point T1 (day 3), T2 (day 5) and T3 (day 8). Symbols (*) show statistical significance (statistical analysis was performed using DESeq: $P < 0.05$) between control and treatment. The heat map was constructed based on normalized read counts. The standardized normalized read counts, denoted as the row Z-score, is plotted in color scale (red indicates higher expression and blue indicates lower expression). The putative products are given in front of each gene with their respective locus tags.

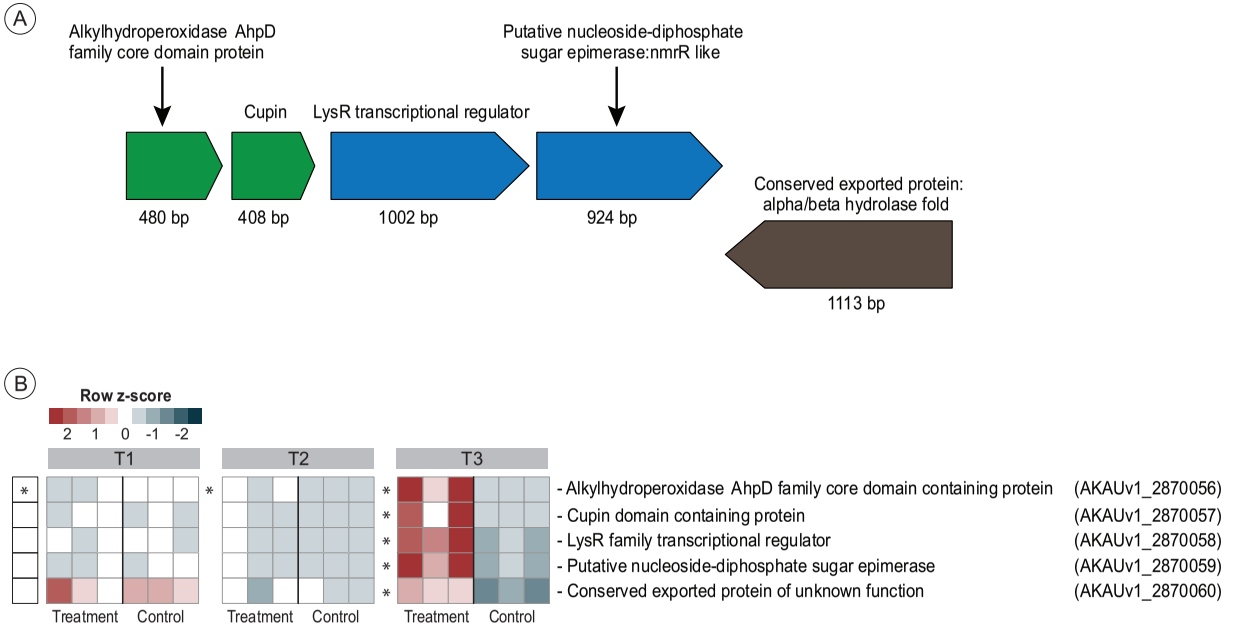

Figure 4.5 Heat map showing the genetic organization and expression pattern of a gene cluster of *B. terrae* BS001 involved in presumed metabolism and energy generation. Figure 4.5A represents the genetic organization of the cluster of genes that are significantly upregulated at T3 (day 8). Figure 4.5B represent a heat map of the expression patterns of the genes in the control (*B. terrae* BS001) and treatment (*B. terrae* BS001 + *Lyophyllum* sp. strain Karsten) at time point T1 (day 3), T2 (day 5) and T3 (day 8). Symbols (*) indicate statistical significance (statistical analysis was performed using DESeq: $P < 0.05$) between control and treatment. The heat map was constructed based on normalized read counts. The standardized normalized read counts, denoted as the row Z-score, is plotted in color scale (red indicates higher expression and blue indicates lower expression). The putative products are given in front of each gene with their respective locus tags.

tion of energy generation, i.e. from a small molecule such as oxalic acid, and a concomitant oxidative stress response, as has been found in the interaction of *Brachypodium distachyon* with *Fusarium graminearum* (Dunwell et al. 2004, Pasquet et al. 2014). The response is possibly regulated by a LysR family transcriptional regulator encoded by gene AKAUv1_2870058, which was also upregulated (log2fold change 4.10). The fourth upregulated gene, AKAUv1_2870059, was predicted to encode a putative nucleoside-diphosphate sugar epimerase (log2fold change 4.28). Finally, the fifth gene of the cluster, AKAUv1_2870060, possibly encodes a conserved (exported) 'alpha/beta hydrolase fold' protein. Using BLAST-P against the ESTHER database we found that the predicted protein was ca. 60% similar to a poly (aspartate) hydrolase from *B. glumae*.

Oxalate metabolism and energy generation

Several gene clusters with putative relevance for the uptake and utilization of oxalate were found to be dynamically modulated over time, in the B+F treatment. First, a cluster of genes containing an oxalate/formate antiporter (AKAUv1_1160010), oxalyl-CoA decarboxylase (AKAUv1_1160013) and two formyl-CoA transferases (AKAUv1_1160014; AKAUv1_1160019) was upregulated at T1 and T2 (Supplementary Fig. S4.3). Moreover, two genes in another cluster, encoding an oxalate/formate antiporter (AKAUv1_2660032) and a formyl-CoA transferase (AKAUv1_2660028), were also upregulated at T1. Strain BS001 further showed the upregulation of genes of the glycerate pathway (Sahin 2003), encoding tartronic semialdehyde reductase (AKAUv1_2390002), glyoxylate carboligase (AKAUv1_2390004), indicating further processing of oxalate.

Glycerol uptake and utilization

Given its presumed importance as an accelerator of metabolism, we examined the expression of the glycerol uptake (GUP) gene AKAUv1_1930108 (Haq et al. 2014a) across all treatments. Our analyses did not reveal any differential response, indicating the GUP trait had a minor impact, if any at all. Concerning the utilization of glycerol, we investigated the expression of gene AKAUv1_1300029 encoding glycerol kinase across treatments, however, we did not find any differential responses.

Detailed view of differentially expressed genes at T1, T2 and T3

We here provide a brief account of other differentially expressed genes at each time point, which were not discussed in the foregoing.

Differentially expressed genes at T1 – A total of 651 genes were found to be differentially expressed between treatments B+F and B at T1 (Supplementary Table S4.4), 584 of these were statistically significant ($P < 0.05$). Only 33 genes were upregulated, with the remainder (618) being downregulated.

Upregulated genes – Thirteen of the 33 upregulated genes were assigned to COG classes, R (3 genes), S (2), P (2) and M, N, T, L, E and CHR (1 each), whereas the remaining

(20) could not be assigned to any class (Supplementary Table S4.4). As indicated in the foregoing, chemotaxis regulatory gene (*cheA*; belonging to class 'N') was strongly upregulated. Furthermore, the COG class M gene AKAUv1_220060, which is predicted to encode a D-heptose-1-phosphate adenylyltransferase (DHPA), was upregulated (log2 fold change of 1.82; Supplementary Table S4.4). DHPA is involved in the biosynthesis of the lipopolysaccharide (LPS) precursor ADP-heptose, potentially of the inner core LPS (Desroy et al. 2009). Then, gene AKAUv1_710037, which is predicted to encode a protein involved in the biosynthesis of pyrroloquinoline quinone (PQQ), was upregulated (log2 fold change 1.82; Supplementary Table S4.4). PQQ is a cofactor involved in cellular processes such as phosphate solubilization and the scavenging of reactive oxygen species, as well as in stress responses, in *Pseudomonas* (Misra et al. 2012, Fernández et al. 2012b). Furthermore, genes for iron acquisition and storage, notably AKAUv1_2280031, AKAUv1_2280033, AKAUv1_2440020 and AKAUv1_2280030 (encoding respectively bacterioferritins and a TonB family protein), were upregulated (log2 fold changes 0.52, 0.64 and 0.74; $P < 0.05$; Supplementary Table S4.3). Bacterioferritins sequester iron that may be toxic to cells and release it when iron becomes limiting (Andrews et al. 2003). Finally, gene AKAUv1_690018 and three other genes, which were predicted to encode peptidases involved in the maturation and processing of a peptide antibiotic (like microcin B17 in *E.coli*; Allali et al. 2002) were upregulated (log2 fold change 0.89; Supplementary Table S4.3).

Downregulated genes – Supplementary Table S4.4 lists the main downregulated genes. About 20% of these (that is, 125 of 618) were predicted to encode conserved proteins of unknown function, whereas about 40% (260 genes) represented various 'core metabolism' enzymes. In addition, about 78 genes encoded various membrane-bound transporters and another 36 transcriptional regulators. With respect to the transporters, ATP-binding cassette (ABC) (23) and major facilitator superfamily (MFS) (11) class transporters were downregulated (Supplementary Table S4.5). Notably, the *cusA* gene (log2 fold change –3.51), which encodes a metal (copper/silver) membrane efflux system, was strongly downregulated (Supplementary Table S4.5). CusA is a member of the Resistance-Nodulation-Cell Division (RND) proton-driven cation antiporter/symporter family that is involved in the efflux of heavy metals (Conroy et al. 2010). Another 70 downregulated genes encoded proteins that make part of various KEGG metabolic pathways (Supplementary Table S4.6). These pathways encompass the transformation of carbohydrates, amino acids and fatty acids. Here, glycolysis/gluconeogenesis, citrate cycling and pyruvate and amino acid metabolic processes stood out. In addition, several genes (Supplementary Table S4.6) involved in methane, nitrogen and aromatic hydrocarbon transformation pathways were also downregulated.

Interestingly, significant downregulation (log2 fold change –1.61) of a hybrid polyketide synthase/nonribosomal peptide synthetase (PKS/NRPS; AKAUv1_1710056) gene (Haq et al. 2014a) was found, suggesting that modulation of this specific bacterial function might be key to interaction with the fungal host (Fig. 4.4).

Differentially expressed genes at T2 – A total of 155 genes were found to be differentially expressed at T2 (threshold: >1.5 log2 fold change). Of these, 66 were statistically significant ($P < 0.05$). Fifteen of the latter genes were up- and 51 downregulated in the B+F as compared to B treatment (Supplementary Fig. S4.4). These are described hereunder.

Upregulated genes – As discussed above, the chemotaxis regulator *cheA* continued to be upregulated at T2, indicating a persisting response to stimuli from the fungus. However, the expression of other genes related to flagellar motility was not differential (Fig. 4.3). Interestingly, gene AKAUv1_1000027, which was predicted to encode a 'Suppressor of variegation-Enhancer of zeste-Trithorax (SET) domain containing protein' (log2 fold change 1.68; Supplementary Fig. S4.4) was upregulated. This may suggest that the predicted 16.46 kDa SET domain protein, a potential T3SS-secreted effector, might be 'early' induced, to serve as a potential modulator of fungal gene expression (Alvarez-Venegas 2014). Notably, transcription of a gene for a putative oxidoreductase, AKAUv1_530022, was upregulated (log2 fold change 1.93), next to that of a gene for a putative cytochrome c552 (log2 fold change 2.83; AKAUv1_2920083) (Supplementary Fig. S4.4). Another upregulated gene, AKAUv1_540146, encoding a predicted alcohol dehydrogenase (log2 fold change 1.91), indicated (diverse) metabolic processes were active. Moreover, a gene encoding lactoylglutathione lyase (glyoxalase I, possibly detoxifying the metabolic byproduct methylglyoxal), AKAUv1_540002, next to gene AKAUv1_540003 (log2 fold change 1.35; $P < 0.05$), was also upregulated.

With respect to potential metabolism, two genes predicted to be involved in purine and valine/leucine metabolic pathways were upregulated (≥ 1.5 log2 fold change; Supplementary Table S4.7). Gene AKAUv1_1780022 is predicted to encode an allantoicase with a potential role in the transformation of purines or the nitrogen-rich derivative allantoin (Navone et al. 2014), releasing nitrogen for anabolism (Werner and Witte 2011, Puggioni et al. 2014). Moreover, the upregulated gene AKAUv1_3070011, that was predicted to encode 3-hydroxy isobutyrate dehydrogenase, might be involved in the valine/leucine degradation pathway (Supplementary Table S4.7).

Downregulated genes – Eight of the 51 downregulated genes at T2 were involved in central metabolic processes, with four being part of one KEGG pathway each (for glutathione, vitamin B6, glycerophospholipids, cysteine/methionine, respectively). The remaining four genes were potentially involved in more than one pathway, i.e. glycolysis/gluconeogenesis, glyoxylate/dicarboxylate, galactose and pentose/pyruvate (Supplementary Table S4.7).

Interestingly, gene AKAUv1_2870089, predicted to encode a glutathione-dependent formaldehyde-activating protein (involved in the detoxification of formaldehyde; Goenrich et al. 2002), was downregulated (log2 fold change –2.72), with its upstream gene AKAUv1_2870088, predicted to encode a methyltransferase, also being downregulated (log2 fold change –1.83; Supplementary Fig. S4.4). In *Saccharomyces cerevisiae*, such genes are reported to play roles in responses to stress (Lissina et al. 2011). Furthermore,

two other (contiguous) genes, AKAUv1_990014 and AKAUv1_990015 (both predicted to encode conserved proteins of unknown function), were strongly downregulated (log2 fold changes –2.34 and –2.99; Supplementary Fig. S4.4). Secondary structure prediction of the latter protein revealed it to resemble a *p*-amino benzoate *N*-oxygenase (AurF) from *Streptomyces thioluteus*, which has a role in the oxidation of aromatic hydrocarbons such as aminoarenes to nitroarenes (Choi et al. 2008, Winkler and Hertweck 2005).

Interestingly, gene AKAUv1_110198 (encoding a 'conserved protein of unknown function') was downregulated (log2 fold change –1.59). The predicted gene product was homologous (34% amino acid identity) to the *purD* gene product (phosphoribosylamine - glycine ligase), which is involved in purine metabolism and is upregulated in response to butanol and butyrate stress in *Clostridium acetobutylicum* (Wang et al. 2013). Moreover, the predicted ATP-dependent carboligase-encoding gene AKAUv1_110221 was also downregulated (log2 fold change –1.66).

Differentially expressed genes at T3 – Upon physical contact of strain BS001 with the fungal hyphae at T3, a total of 136 bacterial genes were differentially expressed. Ninety-six of these genes were statistically significant ($P < 0.05$). Out of these 96 genes, 62 were up- and 34 downregulated (Supplementary Fig. S4.5).

Upregulated genes – Next to the metabolic genes discussed in the foregoing, three genes representing three KEGG metabolic pathways were upregulated at T3 (Supplementary Table S4.8). The predicted proteins were likely involved in the transformation of aminoacyl-tRNA and of arginine/proline. Moreover, gene AKAUv1_2480024, encoding an enzyme of the chlorocyclohexane/chlorobenzene and toluene degradation pathway, was also upregulated (log2 fold change 1.57). Regarding ionic homeostasis, gene AKAUv1_2180021, encoding chloride channel protein EriC, an H^+/Cl^- antiporter, was upregulated (log2 fold change 1.58; Supplementary Fig. S4.5). The EriC protein may be involved in (acid) stress tolerance, as found in *E. coli* (Iyer et al. 2002).

Moreover, the aforementioned PKS/NRPS biosynthetic gene AKAUv1_1710056 was upregulated at T3 (log2 fold change 1.68), indicating potential modulation of *Lyophyllum* sp. strain Karsten physiology upon physical contact. Finally, two genes, AKAUv1_ 2420001 and AKAUv1_2490034 (predicted to encode transposases), were also upregulated (log2 fold change 1.62 and 2.39). A similarly enhanced expression of mobility of genetic elements was reported for *C. fungivorans* Ter331 in its interaction with *A. niger* (Mela et al. 2011).

Downregulated genes – With respect to general KEGG metabolic pathways, the expression of six genes was downregulated (Supplementary Table S4.8). These included two genes each, involved in purine and aminobenzoate metabolism, and one each in galactose and tryptophan metabolism. The gene AKAUv1_110190 (encoding coenzyme PQQ synthesis protein C) was downregulated (log2 fold change –1.90; $P < 0.05$; Supplementary Fig. S4.5). In *Pseudomonas*, the *pqqC* protein is involved in the response to stress

(Fernández et al. 2012b), being that in the rhizosphere of pine, *P. putida* KT2440 preferentially activates it (Fernández et al. 2013). Thus, the downregulation of the strain BS001 *pqqC* may relate to the alleviation of stress. Similarly, gene AKAUv1_2820103, predicted to encode a NodT family efflux transporter, was downregulated (log2 fold change –1.47). Similarly, the aforementioned (Supplementary Fig. S4.5) methyltransferase encoding gene, AKAUv1_2870088, was also downregulated (log2 fold change –1.70).

Discussion

So far, only few studies have unraveled the complex nature of bacterial-fungal interactions at the transcriptional level (Stopnisek et al. 2015, Mela et al. 2011, Deveau et al. 2015, Deveau et al. 2007, Maligoy et al. 2008). Recently, *C. fungivorans* strain 331 was shown to invest substantial cellular resources into the capacity to utilize compounds provided by its host fungus *A. niger*, as well as in the production of antifungal agents (Mela et al. 2011). In the current study, *B. terrae* BS001 was interrogated with respect to its response to the fungus *Lyophyllum* sp. strain Karsten on SEA plates mimicking soil conditions. Under the selected conditions, heterotrophs such as *B. terrae* BS001 are expected to express responses to scarce resources, in particular sources of carbon and energy. We hypothesized that the presence of fungal hyphae would drive additional responses, resulting in the potential exploration of fungal-derived resources, most likely along an approximation to, followed by a physical association with, the fungus. The existence of physical association of *B. terrae* BS001 with *Lyophyllum* sp. strain Karsten was recently demonstrated using fluorescence microscopy (Nazir et al. 2014).

Gene expression patterns of *B. terrae* BS001 on the SEA medium – The observations at T1 and T2 (no physical contact between partners) versus the physical contact phase T3 enabled the examination of the bacterial responses to either 'distant' or 'proximate' fungal hyphae. Thus, effects of highly diffusible (or even volatile) fungal-released compounds or of changes in the (nutritional) status of the medium versus those of more physical types of interaction, were assessed. Overall, upon introduction, strain BS001 was clearly confronted with (starvation) stress conditions on the SEA plates, which was consistent with the reduced amount of total carbon in the medium. Given that the stress-relevant transcript densities in the B+F treatments at T1 were initially as high as, or higher than, those in the B treatment indicated that little, if any, relief of the stress by the fungus occurred at this time. Key to the contention that the bacterial population was under (generic) stress were the high expression levels of *rpoS* and numerous RpoS-driven genes. We hypothesized that, following initial bacterial growth, heterogeneous cell populations emerged on the SEA plates, with different levels of growth (frontier cells) versus stress (backward cells). Such situations very likely occur in the soil, where spatial constraints foster the coexistence of both growing and growth-arrested (starvation-stressed) cell populations. Similar observations have been reported for other bacterial systems (Boylen and Ensign 1970, Matin et al. 1989).

The high expression of genes encoding lytic transglycosylases in both treatments over time was intriguing. Such enzymes are possibly involved in cell wall recycling/turnover, cell division and insertion of membrane-spanning structures (i.e. secretion systems and flagella) (Lee et al. 2013, Scheurwater et al. 2008). They are expressed under elevated stress (Bernal-Cabas et al. 2015, Betzner et al. 1990), degrading peptidoglycan and remodeling the cell wall (Van Heijenoort 2011). It appears, that due to this action, strain BS001 probably restructured its cellular make-up, when confronted with the conditions of SEA, as a result of the nutrient-limited conditions.

Early gene expression by *B. terrae* BS001 as a response to *Lyophyllum* sp. strain Karsten – The disproportionate expression of the *cheA* gene in the presence of the fungus at T1 as well as T2, highlighted the likely role of chemotaxis and flagellar motility in the early stages of interaction of *B. terrae* BS001 with *Lyophyllum* sp. strain Karsten. This was a striking observation, as at T3 no significant difference in the expression levels of the respective genes was observed between the treatments. Although migration of bacterial cells on the agar plate surface did not visibly take place, *Lyophyllum* sp. strain Karsten most likely released compounds into the SEA that acted as chemoattractants for *B. terrae* BS001. Data obtained via RT-qPCR analysis of the *cheA* gene validated the transcriptome observations; these data make part of an upcoming manuscript. Hence, chemotaxis is likely central to the behavior of *B. terrae* BS001 in soil when confronted with a (distant) attracting fungus. In *Sinorhizobium meliloti*, chemotaxis also drives the movement of cells towards plant-secreted chemoattractants (Meier et al. 2007), whereas a study with *Fusarium oxysporum* identified fusaric acid as an attractant for *Pseudomonas fluorescens* WCS365 (De Weert et al. 2004).

Rather unexpectedly, we also observed an upshift in the expression of the T6SS at T1. Indeed, the T6SS has been reported to mediate the bacterial response to oxidative stress (Goldovà et al. 2011), regulating RpoS, modulating general stress response regulators (Weber et al. 2009) and playing a role in osmotolerance and pH homeostasis (Zhang et al. 2013, Gueguen et al. 2013). Thus, a rather early response to SEA induced stress conditions was apparent.

Metabolic response and energy generation – several putative compounds implicated – The overall metabolic responses of *B. terrae* BS001 in the B and B+F treatments may be characterized by a quick depletion of the carbonaceous compounds present in the SEA, followed by a progressively stronger response to the fungus as a potential provider of such resources. Thus, multiple sets of genes for a suite of generic energy generation pathways, as well as the specific mandelate utilization pathway, were suppressed by the fungus. In *Pseudomonas*, mandelate/mandelic acid (derived from the soil metabolite amygdalin) can serve as carbon and energy sources (Tsou et al. 1990, Dewanti et al. 2004). Next to repressed gene sets, others were highly expressed, indicating inductive events. For instance, the dynamic expression pattern of the ethanolamine (*eut*) utilization operon over time may indicate that ethanolamine or similar compounds were being

actively captured and metabolized, up to their depletion from the medium. The molecule, much like ethanolamine, may have constituted a metabolic cue in the system, in a temporally-defined manner (Garsin 2010, Stojilkovic et al. 1995). The upregulation of putative tartrate transporter encoding gene was also notable as several (ectomycorrhizal) fungi have been found to release tartrate and other low-molecular-weight organic compounds under nutrient-poor conditions (Van Schöll et al. 2006a). In *Rhizobium leguminosarum* biovar *viciae*, a similar protein acts in the utilization of tartrate in the rhizosphere of pea and alfalfa (Ramachandran et al. 2011).

Glycerol – Nazir et al. (2013) previously reported that glycerol is a main compound that is released by *Lyophyllum* sp. strain Karsten in liquid systems, constituting a resource for *B. terrae* BS001. However, the GUP system, previously hypothesized to serve in the capturing of extracellular glycerol, as well as a glycerol kinase gene, were not significantly upregulated at the fungus. Although glycerol may have become available, it may have been usurped by passive diffusion across the membrane. Alternatively, the switch-on of the *gup* gene may have only occurred in the frontier cells that were faced with the highest glycerol levels, so that it did not stand out as significant in the overall analyses.

Oxalate and oxalate transformation – The enhanced expression of several systems that are potentially involved in oxalate capture and transformation indicates that strain BS001 may assimilate fungal-released C_2 compounds such as oxalate (Quayle 1961) as sources of carbon and energy. Strain BS001 may exhibit a biphasic response mechanism towards oxalate in combination with other molecules that are released by the fungus, where genes for degradation are upregulated at earlier or later stages. A similar trend was reported for *C. fungivorans* Ter331 in its interaction with *A. niger* (Mela et al. 2011). We hypothesized that gene AKAUv1_2870057 (encoding a protein of the cupin superfamily) has a role as an oxidase, potentially of released oxalate, thus catalyzing its breakdown to carbon dioxide and hydrogen peroxide. The effects of the resulting hydrogen peroxide may have been neutralized by a peroxidase encoded by the adjacent gene (AKAUv1_2870056). Apart from this, the expression of genes involved in the degradation of oxalate, both at earlier and later stages of the interaction, likely indicates a complex scenario, in which strain BS001 may switch on/off the expression of genes, subject to the availability of potential oxalate.

Partial relief of stress by *Lyophyllum* sp. strain Karsten at T3 – At the 'physical–contact' phase T3 the stress-related genes *dnaE2*, *rsbR*, *rsbS*, *rsbT* and *pqqC* and a gene (AKAUv1_2870088) encoding a methyltransferase were downregulated suggesting a—possibly partial—relief of the stress by the fungus. This partial relief of stress by the presence of *Lyophyllum* sp. strain Karsten may be due to the delivery of palatable compounds such as glycerol and oxalate. Many fungi are known to release such compounds (Nazir et al. 2013, Rudnick et al. 2015). For instance, a recent study on the interaction of *B. glathei* with *A. alternata* and *F. solani* reported an attenuation of bacterial stress due to phos-

phate and carbon (SspA and CstA) starvation as a result of fungal presence (Stopnisek et al. 2015). We surmised that, in addition to the general SEA-incited (nutrient) stress, competition for nutrients in the interactome may have been fierce at T1, whereas the two partners differentiated the niche as the interaction progressed to physical contact.

An oxidative 'burst' or bacterial metabolism-generated oxidative stress – Given that strain BS001 revealed fungal-incited upregulation of several genes involved in the oxidative stress response, in particular at T3, suggests that either the fungus or the bacterium itself produced H_2O_2 as a metabolic by-product. The latter is known to occur in the oxalate oxidative pathway. Possibly, a partial alleviation of medium-induced stress resulted in metabolism concomitant with an enhancement of oxidative stress. This effect has also been observed in the interaction of *B. glathei* with *A. alternata* and *F. solani* (Stopnisek et al. 2015). Indeed, metabolic activities were clearly enhanced at T3, and the SDRs expressed match the expectation of their involvement in energy production by strain BS001 from fungal-released substrates. RT-qPCR analysis of genes encoding SDR (AKAUv1_2490031) and NAD-dependent epimerase (AKAUv1_2490033) validated the trends of expression obtained with RNA-seq (data not shown). In *P. fluorescens* BBc6R8, SDR-like proteins were recently shown to be upregulated in the presence of *L. bicolor* S238N (Deveau et al. 2015). Moreover, SDR mutants of *S. meliloti* have deficiencies in the catabolism of particular carbonaceous compounds, affecting its symbiosis with *Medicago sativa* (Jacob et al. 2008).

Potential role for a *B. terrae* BS001 secondary metabolite – The downregulation of the hybrid PKS/NRPS gene at T1 — in contrast to T3 — in the presence of the fungus was interesting. It is possible that perception of the fungal hyphae at a distance allowed subpopulations of the strain BS001 cells to divert energy into the repression of production of the antifungal compound, thus allowing the fungus to get physically close to the bacterial growth. Upon physical contact, upregulation occurred, by which the bacterium possibly modulated the fungal mode of growth to its own benefit. However, the nature of the natural product synthesized by the gene cluster is as yet unknown. *B. rhizoxinica*, an endosymbiont of the fungus *Rhizopus microsporus*, produces the secondary metabolite 'rhizoxin' that acts on rice seedling cells, destroying these (Partida-Martinez et al. 2007). A large PKS/NRPS operon was found to be involved in rhizoxin biosynthesis (Partida-Martinez and Hertweck 2007). We suggest that *B. terrae* BS001 expresses the NRPS/PKS gene cluster, under nutrient-limited conditions, in a different manner according with the relative 'sphere of influence' of its fungal associate.

Conclusion

Overall, our analyses reveal that the interplay between *B. terrae* BS001 and *Lyophyllum* sp. strain Karsten under soil-mimicking conditions is highly complex and dynamic. Clearly, *B. terrae* BS001 encounters stress conditions on the SEA medium used early on

in the experiment, with several genetic systems, including chemotaxis and flagellar motility, being responsive to the fungal hyphae, perceived at a distance. The early responses also included metabolic up- and down-shifts, which is probably in line with the resources encountered in the system without/with the fungus. Then, the organism likely entered a different physiological state upon contact with the fungus, in which limited sets of particular metabolic genes, next to oxidative stress responsive genes, were activated, at the expense of other metabolic genes. On the basis of this observational study, it is clear that a more focused insight into each of the mechanisms underlying the interaction of *B. terrae* BS001 with its host fungus is required. Possibly, mutational analyses of the key genetic systems unveiled here should be combined with specific transcriptome and metabolic profiling approaches.

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Supplementary tables and figures

Note: Due to large sizes, supplementary tables S4.1, S4.2, S4.4, S4.5 and S4.6 are available at the data repository of Microbial Ecology group, GELIFES, University of Groningen.

Table S4.3 Some of the type 6 secretion system, peptidase, bacterioferritin, and lytic transglycosylase encoding genes T1 (day 3).

Label	Name	Product	baseMean	baseMeanA	baseMeanB	log2 Fold Change	*P*-value
AKAUv1_650058	-	Type VI secretion-associated protein, ImpA family	175	138	212	0.62	0.003
AKAUv1_650059	-	Putative secretion system protein (EvpA-like)	86	74	98	0.4	0.100
AKAUv1_650060	-	Putative secretion system protein (EvpB-like)	245	201	288	0.52	0.015
AKAUv1_650061	hcp	Protein hcp1	346	287	405	0.5	0.013
AKAUv1_650062	-	Virulence protein SciE type	70	60	79	0.39	0.120
AKAUv1_650064	-	Type VI secretion protein, VC_A0110 family	116	95	137	0.53	0.023
AKAUv1_650065	-	Protein of unknown function	21	16	25	0.62	0.048
AKAUv1_650066	-	Type VI secretion protein	32	26	38	0.52	0.074
AKAUv1_650067	-	putative ClpA/B-type chaperone (evpH-like)	151	125	177	0.5	0.017
AKAUv1_650068	-	Conserved protein of unknown function	22	16	27	0.74	0.022
AKAUv1_650069	-	ImpA family type VI secretion-associated protein	119	93	145	0.63	0.005
AKAUv1_650070	-	Regulatory protein	87	71	104	0.56	0.024
AKAUv1_650071	-	Type VI secretion protein	51	42	60	0.52	0.045
AKAUv1_650072	-	Conserved protein of unknown function	44	39	49	0.33	0.220
AKAUv1_650073	-	Conserved membrane protein of unknown function	139	120	158	0.4	0.066
AKAUv1_650074	-	Type VI secretion-associated protein TagF	61	53	70	0.41	0.097
AKAUv1_790112	-	General secretion pathway protein D	46	35	57	0.71	0.014
AKAUv1_2470010	-	Type IV pilus assembly PilZ	23	17	29	0.75	0.031
AKAUv1_650054	-	Conserved exported protein of unknown function	140	120	161	0.42	0.042
AKAUv1_650057	-	Protein of unknown function	166	132	200	0.6	0.004
AKAUv1_160051	-	Peptidase M12A astacin	23	21	26	0.31	0.370
AKAUv1_720101	-	Peptidase M22 glycoprotease	15	14	16	0.14	0.550
AKAUv1_550004	-	Peptidase M23	8.66	5.83	11	0.98	0.063
AKAUv1_520047	-	Peptidase M23B	15	14	17	0.31	0.220
AKAUv1_750037	-	Peptidase M24	51	43	58	0.42	0.110
AKAUv1_690018	pmbA	Peptidase required for the maturation and secretion of the antibiotic peptide MccB17	63	44	82	0.89	0.001
AKAUv1_1970035	-	Peptidase S8/S53 subtilisin kexin sedolisin	89	70	109	0.64	0.007
AKAUv1_760031	-	Peptidase S8/S53 subtilisin kexin sedolisin	25	20	29	0.54	0.091
AKAUv1_1560012	-	Peptidase S10 serine carboxypeptidase	57	45	69	0.61	0.014
AKAUv1_580050	-	Peptidase S16, lon domain protein	29	26	33	0.35	0.320
AKAUv1_1560011	-	Peptidase S53 propeptide	35	31	39	0.33	0.200
AKAUv1_920062	-	Peptidase U61 LD-carboxypeptidase A	19	17	21	0.31	0.370
AKAUv1_2440020	bfr	Bacterioferritin, iron storage and detoxification protein	99	78	121	0.64	0.007
AKAUv1_2280031	-	Bacterioferritin-associated ferredoxin	112	92	132	0.52	0.024
AKAUv1_2280030	-	TonB family protein	19	15	24	0.74	0.030
AKAUv1_2840083	-	Lytic transglycosylase	3820	3150	4490	0.51	0.006

Table S4.7 List of differentially expressed genes involved in various KEGG pathways at time point 2 (day 5).

Label	Gene	Product	Log2 fold change	*P*-value	E.C. number	KEGG Pathways
AKAUv1_1780022	alc	Allantoicase	1.98	0.0750	3.5.3.4	230: Purine metabolism
AKAUv1_3070011	mmsB	3-hydroxyisobutyrate dehydrogenase	1.78	0.0019	1.1.1.31	280: Valine, leucine and isoleucine degradation
AKAUv1_930036	_	Beta-galactosidase	-1.58	0.0096	3.2.1.23	52: Galactose metabolism 531: Glycosaminoglycan degradation 600: Sphingolipid metabolism 604: Glycosphingolipid biosynthesis - ganglio series
AKAUv1_1080072	dhaS	Putative aldehyde dehydrogenase DhaS	-1.72	0.0561	1.2.1.3	10: Glycolysis/Gluconeogenesis 40: Pentose and glucuronate interconversions 53: Ascorbate and aldarate metabolism 71: Fatty acid metabolism 280: Valine, leucine and isoleucine degradation 310: Lysine degradation 330: Arginine and proline metabolism 340: Histidine metabolism 380: Tryptophan metabolism 410: beta-Alanine metabolism 561: Glycerolipid metabolism 620: Pyruvate metabolism 625: Chloroalkane and chloroalkene degradation 640: Propanoate metabolism 903: Limonene and pinene degradation
AKAUv1_160081	OPLAH	5-oxoprolinase	-2.06	0.0076	3.5.2.9	480: Glutathione metabolism
AKAUv1_1700025	dcm	DNA cytosine methylase	-1.68	0.0016	2.1.1.37	270: Cysteine and methionine metabolism
AKAUv1_1760084	tiaE	2-oxo-carboxylic acid reductase (glyoxalate reductase) (2-ketoaldonate reductase)	-1.55	0.0216	1.1.1.81, 1.1.1.79, 1.1.1.215	30: Pentose phosphate pathway 260: Glycine, serine and threonine metabolism 620: Pyruvate metabolism 630: Glyoxylate and dicarboxylate metabolism 680: Methane metabolism
AKAUv1_2570103	_	Putative glutathione S-transferase	-1.94	0.0755	2.5.1.18	480: Glutathione metabolism 980: Metabolism of xenobiotics by cytochrome P450 982: Drug metabolism - cytochrome P450
AKAUv1_2780017	pld	Pyridoxal 4-dehydrogenase	-1.77	0.0161	1.1.1.107	750: Vitamin B6 metabolism
AKAUv1_2840066	pgsA	Phosphatidyl-glycerophosphate synthetase	-1.62	0.0097	2.7.8.5	564: Glycerophospholipid metabolism

Table S4.8 List of differentially expressed genes involved in various KEGG pathways at time point 3 (day 8).

Label	Gene	Product	Log2 fold change	*P*-value	E.C. number	KEGG Pathways
AKAUv1_1020002	dnaE	Error-prone DNA polymerase	–2.03	0.0046	2.7.7.7	230: Purine metabolism 240: Pyrimidine metabolism
AKAUv1_1930128	apaH	Bis(5'-nucleosyl)-tetraphosphatase, symmetrical	–1.66	0.0325	3.6.1.41	230: Purine metabolism
AKAUv1_2940080	dgoA	2-oxo-3-deoxygalactonate 6-phosphate aldolase	–1.58	0.0034	4.1.2.21	52: Galactose metabolism
AKAUv1_3190138	mdlB	(S)-mandelate dehydrogenase	–2.25	0.0001	1.1.99.31	627: Aminobenzoate degradation
AKAUv1_3190139	mdlA	Mandelate racemase	–1.96	0.0257	5.1.2.2	627: Aminobenzoate degradation
AKAUv1_500073	kynB	Kynurenine formamidase	–1.54	0.0320	3.5.1.9	380: Tryptophan metabolism630: Glyoxylate and dicarboxylate metabolism
AKAUv1_1600011	_	4-hydroxyproline epimerase	2	0.0018	5.1.1.8	330: Arginine and proline metabolism
AKAUv1_2480024	_	Phenol 2-monooxygenase	1.57	0.0336	1.14.13.7	361: Chlorocyclohexane and chlorobenzene degradation 623: Toluene degradation627: Aminobenzoate degradation
AKAUv1_3170015	glyQ	glycine tRNA synthetase, alpha subunit	1.52	0.0329	6.1.1.14	970: Aminoacyl-tRNA biosynthesis

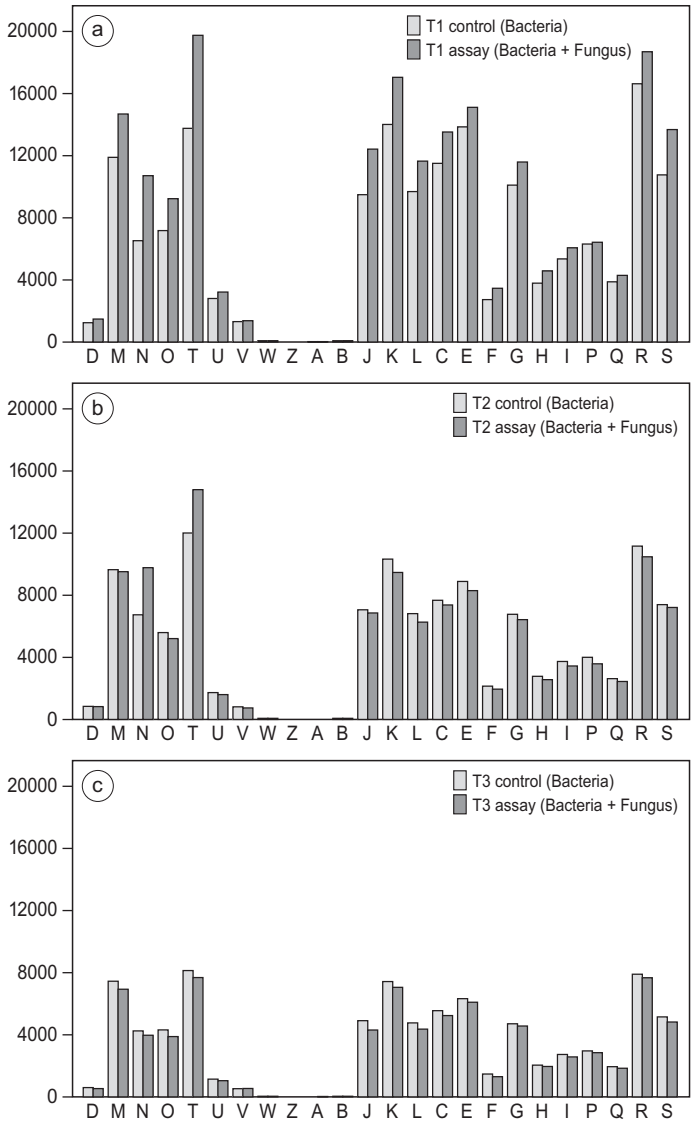

Figure S4.1A Distribution of normalized read number over COG classes. The distribution of numbers of reads (normalized) of transcripts belonging to different COG classes is shown, for treatments B (*B. terrae* BS001, in grey color) and B+F (*B. terrae* BS001 + *Lyophyllum* sp. strain Karsten, in black color). The X-axis represents COG classes and the Y-axis shows the number of normalized reads. a) T1= day 3, b) T2= day 5 and c) T3= day 8. COG classes: C– energy production and conversion; E– amino acid transport and metabolism; G– carbohydrate transport and metabolism; H– coenzyme transport and metabolism; I– lipid transport and metabolism; K– transcription; L– replication, recombination and repair; M– cell wall/membrane/envelope biogenesis; O– posttranslational modification, protein turnover, chaperones; P– inorganic ion transport and metabolism; Q– secondary metabolites biosynthesis, transport and catabolism; R– general function prediction; S– functions unknown; T– signal transduction mechanisms; U– intracellular trafficking, secretion, and vesicular transport; V– defense mechanisms.

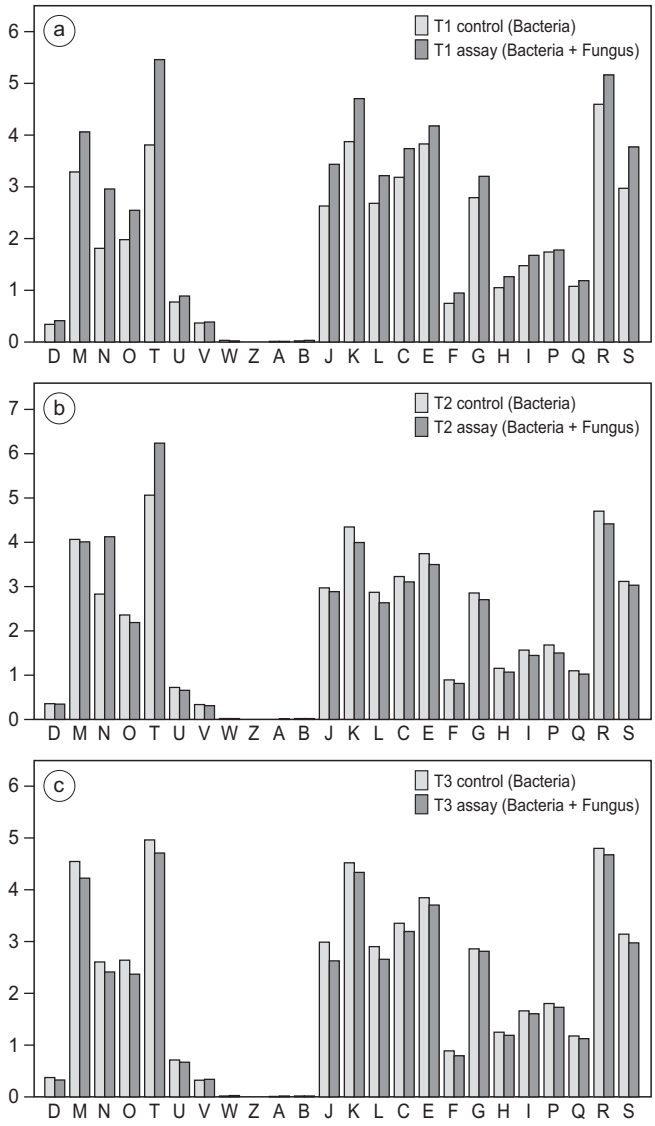

Figure S4.1B Percentage distribution of normalized read number over COG classes. The percentage distribution of normalized reads of transcripts belonging to different COG classes is shown, for treatments B (*B. terrae* BS001, in grey color) and B+F (*B. terrae* BS001 + *Lyophyllum* sp. strain Karsten, in black color). The X-axis represents COG classes and the Y-axis shows the percentage of normalized reads. a) T1= day 3, b) T2= day 5 and c) T3= day 8. COG classes: C– energy production and conversion; E– amino acid transport and metabolism; G– carbohydrate transport and metabolism; H– coenzyme transport and metabolism; I– lipid transport and metabolism; K– transcription; L– replication, recombination and repair; M– cell wall/membrane/envelope biogenesis; O– posttranslational modification, protein turnover, chaperones; P– inorganic ion transport and metabolism; Q– secondary metabolites biosynthesis, transport and catabolism; R– general function prediction; S– functions unknown; T– signal transduction mechanisms; U– intracellular trafficking, secretion, and vesicular transport; V– defense mechanisms.

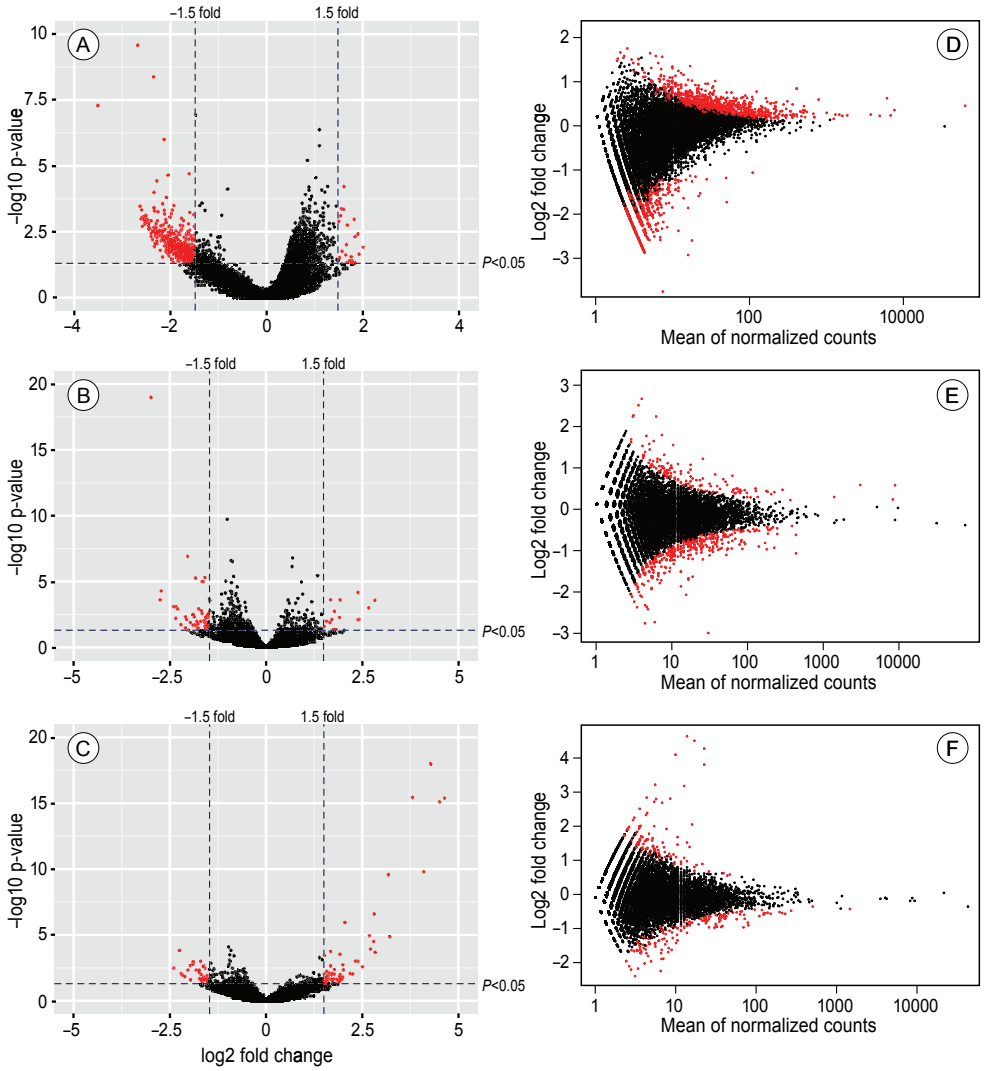

Figure S4.2 Volcano plots showing the log2 fold changes (X-axis) with −log10 (*P value*) on the Y-axis for time point 1 (day 3) (A), time point 2 (day5) (B) and time point 3 (day8) (C). Dashed vertical lines shows log2 fold change thresholds (>1.5 or <1.5; red dots). Horizontal dashed line shows the boundary line of $P < 0.05$. MA plots showing overall distribution of differentially expressed genes of *B. terrae* BS001 (red dots $P < 0.05$) at (D) time point 1 (day 3), (E) time point 2 (day5), and (F) time point 3 (day8), in the presence of *Lyophyllum* sp. strain Karsten. Y-axis represent the log2 fold change (strain BS001 alone and strain BS001 with *Lyophyllum* sp. strain Karsten), whereas the X-axis shows mean of normalized reads counts for both treatments.

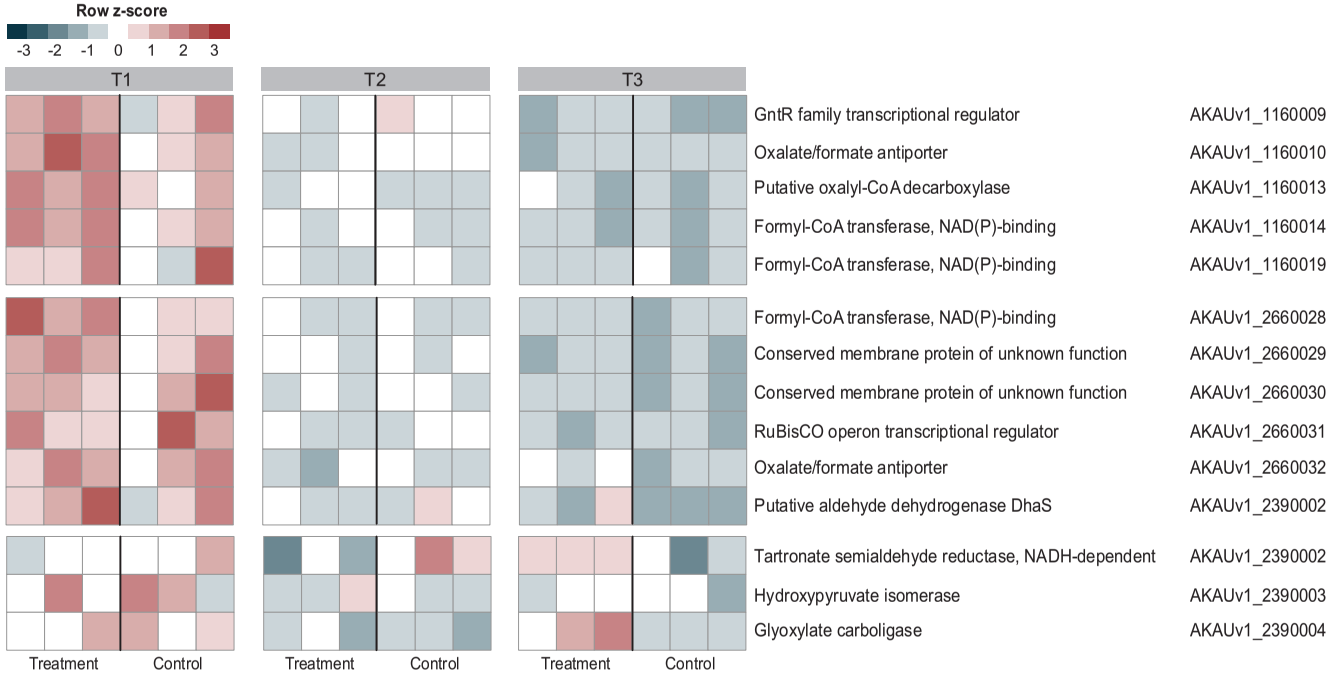

Figure S4.3 Three gene clusters potentially involved in the metabolism of oxalate. Heat map showing the expression pattern of genes belonging to different clusters presumed to be involved in the degradation and uptake of oxalate at three different time points (T1, T2 and T3). The heat map was constructed based on normalized read counts. The standardized normalized read counts, denoted as the row Z-score, is plotted in color scale (red indicates higher expression and blue indicates lower expression). The names of the putative gene products are given in front of each gene with their respective locus tags.

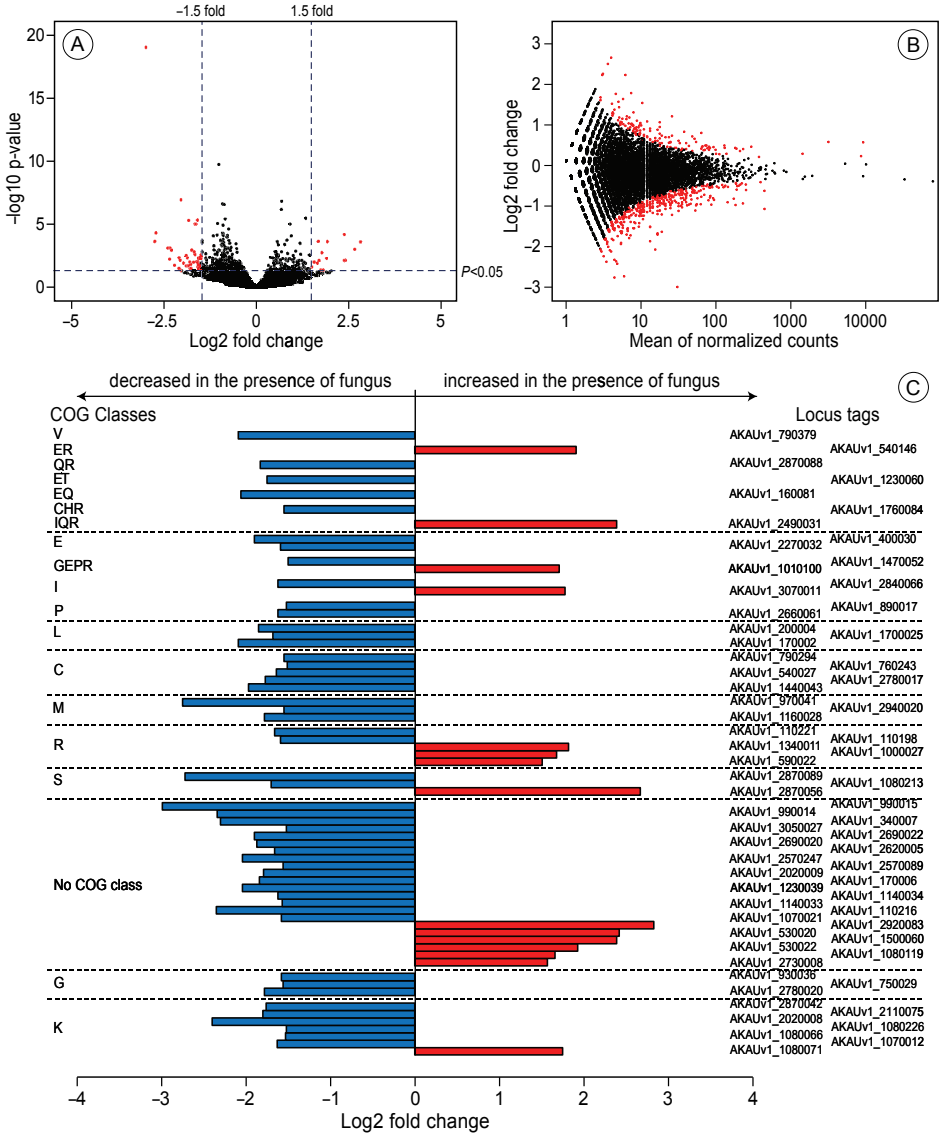

Figure S4.4 Differentially expressed genes of strain BS001 at T2 (day 5) following confrontation with *Lyophyllum* sp. strain Karsten. (A) shows volcano plot, (B) shows MA plot and (C) shows bar charts indicating fold changes on log scale for individual genes (red: upregulated, blue: downregulated) grouped into broad functional COG classes, showing differential expression after fungal confrontation ($P < 0.05$). COG classes: C– energy production and conversion; E– amino acid transport and metabolism; G– carbohydrate transport and metabolism; H– coenzyme transport and metabolism; I– lipid transport and metabolism; K– transcription; L– replication, recombination and repair; M– cell wall/membrane/envelope biogenesis; O– posttranslational modification, protein turnover, chaperones; P– inorganic ion transport and metabolism; Q– secondary metabolites biosynthesis, transport and catabolism; R– general function prediction; S– functions unknown; T– signal transduction mechanisms; U– intracellular trafficking, secretion, and vesicular transport; V– defense mechanisms.

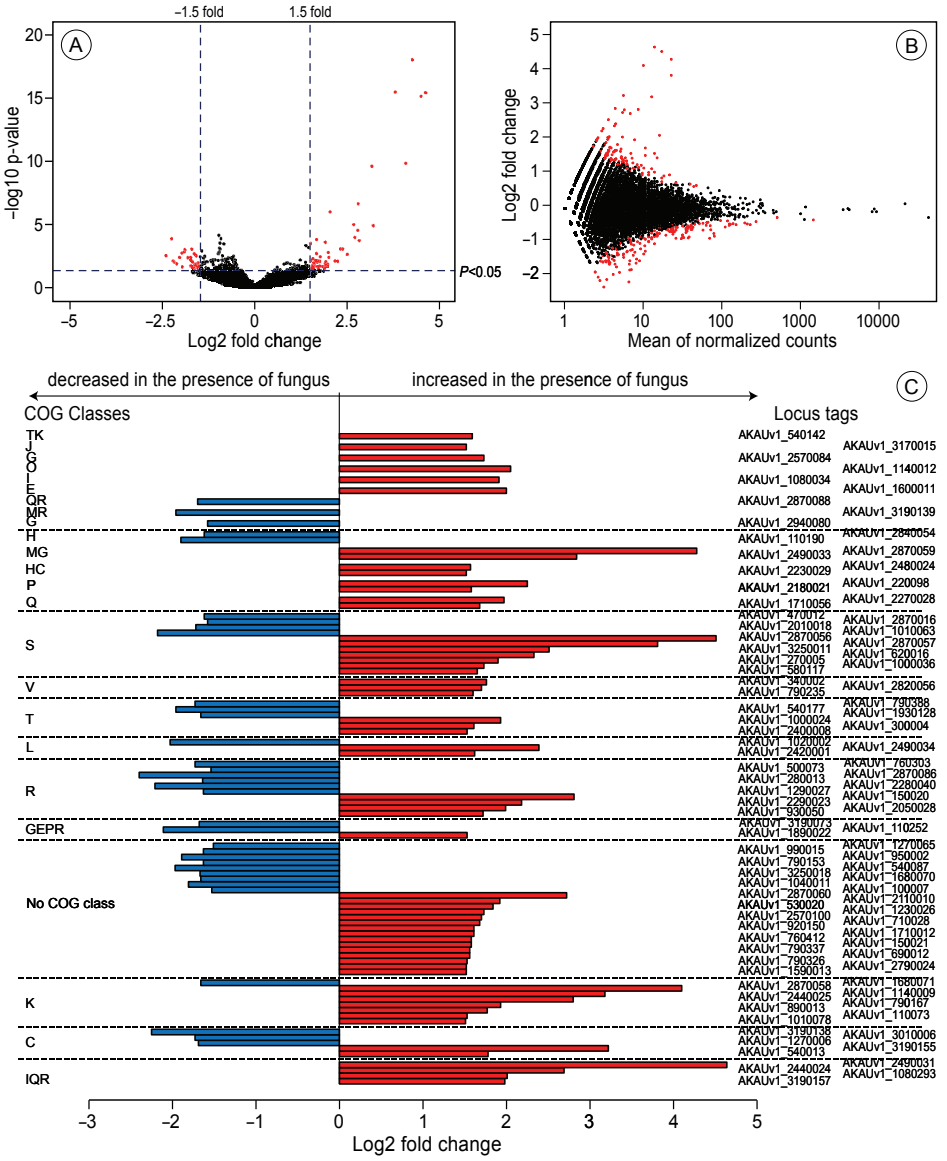

Figure S4.5 Differentially expressed genes of strain BS001 at T3 (day 8) following *Lyophyllum* sp. strain Karsten confrontation. (A) shows volcano plot, (B) shows MA plot and (C) shows bar charts that are indicating fold changes on log scale for individual genes (red: upregulated, blue: downregulated) grouped into broad functional COG classes, showing differential expression after fungal confrontation ($P < 0.05$). COG classes: C– energy production and conversion; E– amino acid transport and metabolism; G– carbohydrate transport and metabolism; H– coenzyme transport and metabolism; I– lipid transport and metabolism; J– Translation, ribosomal structure and biogenesis; K– transcription; L– replication, recombination and repair; M– cell wall/membrane/envelope biogenesis; O– posttranslational modification, protein turnover, chaperones; P– inorganic ion transport and metabolism; Q– secondary metabolites biosynthesis, transport and catabolism; R– general function prediction; S– functions unknown; T– signal transduction mechanisms; U– intracellular trafficking, secretion, and vesicular transport; V– defense mechanisms.

Chapter 5

5 Chemotaxis and adherence to fungal surfaces are key components of the behavioral response of *Burkholderia terrae* BS001 to two selected soil fungi

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Abstract

Burkholderia terrae BS001 has previously been proposed to be a 'generalist' associate of soil fungi, of which we largely ignore its strategies of interaction. Here, we studied the chemotactic behavior of *B. terrae* BS001 towards *Lyophyllum* sp. strain Karsten and *Trichoderma asperellum* 302 and the role of fungal surface molecules in their physical interaction with the bacteria. To assess the involvement of the type 3 secretion system (T3SS), wild-type strain BS001 and T3SS mutant strain BS001-Δ*sctD* were used in the experiments. First, the two fungi showed divergent behavior when confronted with *B. terrae* BS001 on soil extract agar medium. *Lyophyllum* sp. strain Karsten revealed slow growth towards the bacterium, whereas *T. asperellum* 302 grew avidly over it. Both on soil extract and M9 agar, *B. terrae* BS001 and BS001-Δ*sctD* moved chemotactically towards the hyphae of both fungi, with a stronger response to *Lyophyllum* sp. strain Karsten than to *T. asperellum* 302. The presence of a progressively increasing glycerol level in the M9 agar enhanced the level of movement. Different oxalic acid concentrations exerted varied effects, with a significantly raised chemotactic response at lower, and a subdued response at higher concentrations. Testing of the adherence of *B. terrae* BS001 and BS001-Δ*sctD* to *Lyophyllum* sp. strain Karsten and to cell envelope extracted ceramide monohexosides (CMH) revealed that CMH in both conidia and hyphae could bind strain BS001 cells. As BS001-Δ*sctD* adhered significantly less to the CMH than BS001, the T3SS was presumed to have a role in the interaction. In contrast, such adherence was not detected with *T. asperellum* 302. Thus, *B. terrae* BS001 shows a behavior characterized by swimming towards *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 and attachment to the CMH moiety in the cell envelope of the former fungus.

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Introduction

Fungi and bacteria are major groups of soil microorganisms that are responsible for important ecosystem functions (De Boer et al. 2005). Several members of these two organismal groups can develop ecological interactions with each other (bacterial-fungal interactions – denoted BFI) when living in close proximity in the soil. Thus, in the light of their mycelial mode of growth, fungi can provide hospitable sites to bacteria in soil. Such sites, characterized by the presence of newly-emerged colonizable surfaces that are rich in potential nutrients may serve as 'oasis-like' extra habitable space that supports bacterial life in soil. In response, the ensuing bacterial activities can trigger physiological responses, of diverse nature, in the fungal partner (De Boer et al. 2005, Nazir et al. 2010, Frey-Klett et al. 2011).

Apart from the very tight (obligate) BFI in which partners may have shared ecologies, other successful BFI in soil are presumed to originate from situations in which the two partner organisms initially occur in separate soil patches. When these occur at a distance that allows effective organism-to-organism signalling, interactive processes of progressively higher intricateness are assumed to take place, leading to an ultimately successful (and potentially persistent) interaction. A plethora of different ecological outcomes of such BFI exists, including (presumably) mutualistic, commensalistic and antagonistic ones, as described in several earlier studies (Haq et al. 2014b, Frey-Klett et al. 2011, Nazir et al. 2010, Mille-Lindblom et al. 2006, De Boer et al. 2005). Thus, the interaction of *Burkholderia terrae* BS001 with its fungal host *Lyophyllum* sp. strain Karsten was denoted as mutualistic, as the bacterium was found to thrive on metabolites (in particular glycerol) released by the fungus, whereas, in return, strain BS001 protected the fungus against adverse conditions in the soil (Nazir et al. 2013, Nazir et al. 2014). Antagonisms were also found, exemplified by the countering of the production and secretion of antibiotics by *Streptomyces* sp. (strain AcH505) through the synthesis of organic acids by the soil fungus *Amanita muscaria* (Riedlinger et al. 2006). Vice versa, *Collimonas fungivorans* Ter331 was found to synthesize secondary metabolites (collimomycins) in its competition with *Aspergillus niger* (Fritsche et al. 2014). Also, temporally– and spatially–explicit competition between bacteria and fungi may ensue if the same (carbonaceous) substrates are required for growth of both partners. In such cases, and dependent on spatial and/or temporal factors, the fungal partner may exhibit a degree of tolerance of their bacterial associates, resulting in an ecological 'trade-off': reduced growth rate allowing co-existence (Mille-Lindblom et al. 2006).

In a key recent theoretical treatise (Haq et al. 2014b), the development of BFI in soil was postulated to involve different steps, roughly divided in (1) approximation by chemotaxis and (2) physical contact/adherence. In this respect, an early study with the interactive pair *Pseudomonas fluorescens* WCS365 and *Fusarium oxysporum* indicated that fungal-released fusaric acid can act as a chemotactic signal that attracts strain WCS365 (De Weert et al. 2004). Oxalic acid was recently found to potentially attract different *Collimonas* types (Rudnick et al. 2015). However, we hitherto understand very little with

respect to the range of other interactive pairs and the compounds that may serve as chemoattractants.

Following a successful chemotactic response of soil bacteria to fungal signals, at some point physical contact between the two partners is necessary for the intimate interactions as reported by Warmink and van Elsas (2009). Thus, the question arises which fungal cell envelope compound might have a 'receptor' role for bacterial cell adhesion. Fungal cell walls are composed of, mainly, chitin, β-glucans, (glyco)proteins and glyco (sphingo)lipids (Rodrigues et al. 2000, Da Silva et al. 2004, Bowman and Free 2006, Latgé 2007, Pinto et al. 2008, Santos et al. 2009, Latgé 2010, Kumar and Shukla 2015). Some of these compounds may be involved in the interaction with bacterial associates (Benoit et al. 2015). In particular glycosphingolipids such as ceramide monohexosides (CMHs) may play unexpected roles in fungal cell envelopes, as recently proposed in several articles (Guimarães et al. 2014, Rhome et al. 2011, Nimrichter and Rodrigues 2011). These compounds usually contain glucose or galactose end-groups, with β-glycosidic linkages to the primary alcohol of an N-acyl sphingoid base (ceramide). They can be, to a certain extent, exposed on fungal surfaces (Barreto-Bergter et al. 2011, Calixto et al. 2016, Rhome et al. 2011), and have predicted roles in fungal immunogenicity and lipid raft architecture, next to the regulation of fungal growth, differentiation and virulence (Da Silva et al. 2004, Nimrichter and Rodrigues 2011, Zhu et al. 2014).

The aforementioned *B. terrae* strain BS001 has previously been found to form a tight association with the fungal host *Lyophyllum* sp. strain Karsten (Warmink and van Elsas 2009), as well as with five other fungal hosts, including *Trichoderma asperellum* 302 (Nazir et al. 2014). Hence, the organism was coined a 'generalist' fungal associate. Previous work has also indicated that the T3SS is selected for in bacteriomes that dwell in mycosphere habitats (Warmink and van Elsas 2008). However, we have as yet no clue as to what the exact mechanistic involvement of the T3SS is. Potentially, the system is critical for the colonization of (fungal) hosts by bacterial associates (Lackner et al. 2011) or there is a role for it, i.e. of any of the putative effector molecules (Haq et al. 2014a), in the very first step of the associative process, i.e. the migration. Quite surprisingly, recent work (Gibbs et al. 2008, Alteri et al. 2013) found the type 6 secretion system to be key to aspects of the migrational process in bacteria including *Proteus mirabilis*.

In this study, we further examine the interaction between *B. terrae* BS001 and the aforementioned two fungal hosts, i.e. the basidiomycete *Lyophyllum* sp. strain Karsten and the ascomycete *Trichoderma asperellum* 302, in *in vitro* soil-mimicking conditions. We hypothesized that (1) *B. terrae* BS001 affects the growth of these two fungal partners differently, (2) the ecological context (growth medium) matters to the scope of the interaction, (3) chemotaxis is at the basis of the intimate interaction of strain BS001 with the two fungal hosts and the T3SS plays a role in this process, and (4) strain BS001 adheres to fungal-borne ceramide monohexosides, potentially aided by the T3SS.

Materials and Methods

Strains, culture conditions and growth media

B. terrae BS001 and *B. terrae* BS001-Δ*sctD* mutant strains were used throughout. Briefly, the Δ*sctD* mutant *B. terrae* BS001 strain was constructed using the suicide plasmid system (pSUP101 vector) of Simon, Priefer and Pühler (1983). In the mutant strain, part of the Δ*sctD* gene was deleted, which led to a destabilization of the T3SS. A description of the T3SS mutation strategy and resulting mutant is reported by Yang et al. (2016). All bacterial strains used in this study were maintained in the –80°C culture collection of the Microbial Ecology group at the University of Groningen, the Netherlands. *B. terrae* BS001 and BS001-Δ*sctD* cultures were grown overnight in Luria-Bertani (LB; tryptone 10 g L^{-1}, yeast extract 5 g L^{-1} and sodium chloride 5 g L^{-1}) broth (28°C, with shaking at 180 rpm). The cultures were routinely centrifuged (12,000×g, 3 min) and the bacterial pellets double-washed with sterile saline [NaCl solution 0.85% (weight by volume)]. For the chemotaxis experiments, cells were harvested by low-speed centrifugation (1,057×g, 20 min) the pellets washed twice with morpholine ethane sulfonic acid, containing 1 g L^{-1} of KH_2PO_4 and 1 g L^{-1} $(NH_4)_2SO_4$; pH 5.6 (MES buffer, Rudnick et al. 2015). Washed cells were used for the experiments, as below.

Lyophyllum sp. strain Karsten as well as *T. asperellum* 302 were maintained in autoclaved distilled water for extended storage according to the Nakasone et al. (2004) protocol. For routine work, each fungus was refreshed once per month on oat flake agar (OFA; 30 g oat flake (Spar shop, Netherlands), 15 g of agar (Duchefa Biochemie, Haarlem, Netherlands); 1 L MilliQ water) plates. For soil extract preparation, freshly sampled soil (from an agricultural field in Buinen, Netherlands; soil denoted 'B' soil) was used. The method described by Hamaki et al. (2005) was used, with modifications. Briefly, 1 L of sterile MilliQ water was added to 500 g of fresh soil and the resulting suspension was shaken (200 rpm) for 24 h (room temperature). Soil particles were precipitated by centrifugation at 5,430×g (10 min) in an Eppendorf centrifuge (Hamburg, Germany), after which the supernatant was filtered using folded qualitative filter paper (vWR European, Cat. no. 516-0304). The resulting extract was then stored at –80°C for stocking and at 4°C for direct further use. To prepare soil extract agar (SEA), 500 mL of soil extract (stored at 4°C) amended with 0.5 g of yeast extract were added to 500 mL of fresh MilliQ water to get a final volume of 1 L, after which solidifying agar (15 g) was added, and the final mixture autoclaved (121°C; 15 min). The pH of the SEA medium was adjusted to 6.8 before it was autoclaved.

Confrontation assay

In order to observe the interaction of *B. terrae* BS001 with *Lyophyllum* sp. strain Karsten as well as *T. asperellum* 302, we designed an experiment on SEA. Washed cell suspensions of *B. terrae* BS001 were set at 10^5, 10^7 and 10^9 cells mL^{-1}, after which bacterial stripes were introduced onto agar surfaces at a distance of about 12–15 mm from fungal-mycelium-containing agar (1.5%) plugs aligned in parallel.

Chemotaxis (swimming motility) assay

For the chemotaxis assay, 2 different (0.25% [w/v] agar) media, either containing soil extract (see above) or M9 medium, were employed. Briefly, M9 medium contained 6.76 g L^{-1} of Na_2HPO_4, 3 g L^{-1} of KH_2PO_4, 0.5 g L^{-1} NaCl and 1 g L^{-1} of NH_4Cl in MilliQ water (adapted from Sambrook and Russell 2001). It was supplemented with either glycerol (0%, 0.1%, 0.5% and 1% [w/v]) or oxalic acid (0%, 0.1%, 0.5% and 1.0 % [w/v]), corresponding with, respectively, 0, 15.6, 78.2 and 156.4 mM (glycerol) and 0, 7.9, 39.6 and 79.3 mM (oxalic acid) (Supplementary Table S5.1). In the case of oxalic acid, the pH of the medium was adjusted to 6.8 (before autoclaving). Two mL (L^{-1}) of filter-sterilized (0.2 m) 1 M $MgSO_4$ and 100 L (L^{-1}) of 1 M $CaCl_2$ were added to the medium after autoclaving.

Washed cells of *B. terrae* BS001 and *B. terrae* BS001-Δ*sctD* in MES buffer were used. Using the optical density (OD) 600, selected cell densities (10^5, 10^7 and 10^9 cells mL^{-1}) were established for the chemotaxis assays. The cells were introduced – in a line – onto the plates at distances of about 12–15 mm from the fungal plugs. Controls contained plugs without fungal hyphae. Following the inoculation, the Petri plates were incubated at 25°C (24–36 h) and readings (distance in millimeters) were taken manually at regular time intervals (every about 12 h, daily).

Preparation of monoclonal antibodies specific for glucosylceramides (GlcCer)

Monoclonal antibodies (mAbs) were produced as previously described (Da Silva et al. 2004). Briefly, rabbits were immunized with ultrapure CMH (GlcCer) fractions (50 g) obtained from *Aspergillus fumigatus*, using complete and incomplete Freund's adjuvant. Then, hybridoma producing antibodies against GlcCer were expanded and cloned using limiting dilution in a 96-well microtiter plate, provided with a feeder layer of macrophages (Da Silva et al. 2004). Antibody-producing cells were injected into the peritoneal cavity of BALB/c mice and the GlcCer-specific mAbs were obtained and purified by protein-G affinity chromatography. The mAbs were isotyped as IgG2b, using the Sigma ISO/2 kit. They were shown to be specific for the CMH components of fungal cell envelopes, as in extensive work with *A. fumigatus* cell envelope fractions, they only produced enzyme-linked immunosorbent assay (ELISA) signals with the *A. fumigatus* derived CMH. Using ELISA and fluorescence microscopy, they were then tested against CMH extracts of different fungi, showing positive signals with CMH purified from mycelia and conidia of *Lyophyllum* sp. strain Karsten (this work), next to the CMH of other fungi (Calixto et al. 2016, Nimrichter et al. 2004, Da Silva et al. 2004).

Quantification of adherence of bacterial cells to fungal propagules

Adherence assays were performed in microtiter plates, in which conidia were fixed after which strain BS001 or strain BS001-Δ*sctD* cells were added. The adhering cells were quantified as in Ramírez-Granillo et al. (2015), with minor modifications. To prepare the fungal conidia, mycelia grown on PDA plates were scrape-washed off with PBS– pH 7.2 (10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 150 mM NaCl). The resulting suspension was then

filtered through cotton cloth to remove hyphal fragments and debris, and washed three times in PBS (pH 7.2), after which conidia were counted in a Neubauer chamber. Conidial suspensions in PBS (10^6 conidia mL^{-1}) were then added to the 96-well plates and left to attach (4 h, 37°C) to the wall surface. Supernatants were then removed and the wells washed with PBS, establishing a wall-adhering layer of conidia, as detected by microscopy.

Washed cells of *B. terrae* BS001 and *B. terrae* BS001-Δ*sctD* grown overnight in LB broth (28ºC, shaking at 180 rpm) were set at about 10^7 CFU mL^{-1} (using the OD 600 value). Then, the conidia-loaded wells were inoculated with 5×10^5 bacterial cells per well, after which the plates were incubated for 1–4 h at 30ºC. All incubations were done in PBS in order to limit microbial growth. After incubation, the supernatants were removed and each well was washed twice with PBS. To quantify the adhering cells, 0.5% of crystal violet was then applied for 5 min. The excess crystal violet was then eliminated by washing with sterile distilled water. The water was subsequently removed and 95% ethanol added. The plate was then shaken softly for 1 min and subsequently read at 595 nm using a microplate reader.

Fungal-bacterial biofilm formation

To test the biofilm formation abilities of the partner organisms, a multi-well biofilm formation assay was done (Ramírez-Granillo et al. 2015). Fungal (3×10^4 conidia per well) or bacterial cells (3×10^5 CFU per well) were incubated for 6 h in separate wells (28°C), after which the complementing organism was added at a similar concentration. The mixtures were then incubated at 28°C for 24 h with PYG medium (peptone 1 g L^{-1}, yeast extract 1 g L^{-1} and glucose 3 g L^{-1}). Following incubation, the non-adherent cells were removed by gentle washing, after which the wells were washed twice with PBS. Biofilm formation was then measured using crystal violet, as described above.

Extraction and analysis of glycosphingolipids from T. asperellum 302

Lipids were successfully extracted from intact hyphae of *T. asperellum* 302 (harvested from different plates) with chloroform and methanol (2:1 and 1:2, volume by volume [v/v]). The extracts were then combined, after which they were dried. The resulting crude lipid extracts were partitioned according to Folch and coworkers (1957).

Isolation and purification of neutral glycosphingolipids from T. asperellum 302

The Folch's lower phase was collected, and the recovered lipids were purified via silica gel chromatography as described (Calixto et al. 2016). Neutral lipids, glycolipids and phospholipids were recovered via elution with chloroform, acetone and methanol. The acetone and methanol fractions, which contained glycosphingolipids, were further purified on a silica gel column, which was sequentially eluted with chloroform and methanol with increasing concentrations of methanol (95:5, 9:1, 8:2, and 1:1, v/v) and finally with 100% methanol. Thin layer chromatography (TLC) was performed using chloroform, methanol and 2 M ammonia hydroxide (40:10:1, v/v/v) to determine which frac-

tion(s) contained glycosphingolipids. Spots were visualized with iodine and by spraying with orcinol-sulfuric acid. Glycosphingolipid-positive fractions were then collected and used in the experiments.

Electrospray ionization-mass spectrometry (ESI-MS) analysis of T. asperellum 302 glucosylceramides

ESI-MS analysis was performed using a quadrupole time-of-flight (QTOF) mass spectrometer (Impact HD, Bruker, Germany) in the positive mode. Samples were dissolved in chloroform/methanol (1:1 v/v) containing 1 mM lithium chloride and infused (3 L per min) into the ESI source using a microsyringe pump. The temperature of the nitrogen gas in the collision cell was 200°C at a flow rate of 4 L per min. A potential of 4.5 kV was applied to the capillary tip. For the fragmentation analysis collision–induced dissociation tandem MS studies, a precursor ion was selected in the quadrupole analyzer, with a collision energy of 30 eV.

Detection of CMH at the surface of fungal conidia by indirect ELISA

To detect the presence of CMH on fungal surfaces, we used indirect ELISA based on the anti-CMH mAbs (Lopes et al. 2010). Briefly, 5×10^4 conidia in 100 L PBS were introduced into each well, followed by incubation for 1 h at 37°C and then overnight at 4°C. Plates were washed three times with PBS and blocked with 2% bovine serum albumin (BSA) in PBS (blocking buffer). For blocking, serial two-fold dilutions of a 200 g mL^{-1} solution of different antibodies (mAbs α-CMH and a specific IgG) in blocking buffer were added [in triplicate] to the wells, and plates were incubated at 37°C for 1 h. After three washes, the plates were incubated at 37°C for 2 h with anti-mouse IgG peroxidase conjugate (Sigma-Aldrich) diluted 1:200 in blocking buffer (final volume 50 L per well). Plates were washed three times, after which O-Phenylenediamine (OPD) was added in substrate buffer (room temperature, 20 min). The reaction was stopped by adding 50 L 3 M H_2SO_4 to each well. Absorbances were then measured on a microplate reader at 490 nm.

The involvement of CMH in bacterial adherence to fungal tissue

CMH was dissolved in ethanol:methanol 1:1 (v/v), after which 5 g was added to each well of a 96-well plate. Plates were then dried at room temperature. Subsequently, 50 L of bacterial cell suspensions in PBS (5×10^5 CFU mL^{-1}) were added to each dry well. Following 2 h of incubation, supernatants were removed and the wells washed another three times with PBS to remove non-adhering or poorly-adhering cells. Then, strong mixing and shaking were applied to the material of each well, which was followed by dilution of the suspensions in PBS and plating on LB agar plates. The plates were incubated for 72 h at 25°C after which colonies were counted. As a control, anti-GlcCer monoclonal antibody (mAbs α-CMH; 100 g mL^{-1}) was used to block the bacterium – CMH interaction. Bacterial suspensions and mAbs alone were used as the controls.

Statistical analyses

All the statistical analyses, including full factorial ANOVA for the chemotaxis experiment, as well as biofilms, attachment and CMH experiments, (Student t-test) were performed using RStudio Version 0.99.893 – © 2009–2016 RStudio, Inc. The 95% confidence interval was used to indicate significance in all experiments.

Results

Different fungal behavior towards Burkholderia terrae BS001 during a confrontation assay

In a first experiment, the dynamics of hyphal development of *Lyophyllum* sp. strain Karsten and *T. asperellum* 302, in relation to *B. terrae* BS001 at a physical distance of 12–15 mm, was monitored on SEA plates. We analyzed the fungal growth in two directions, i.e. towards the bacterial cells and away from it. *B. terrae* BS001 has previously been shown to prolifically co-migrate with both fungi through soil microcosms, and to form cell agglomerates on the hyphae (Nazir et al. 2014). In the experiment on SEA plates, *B. terrae* BS001 affected the growth of *Lyophyllum* sp. strain Karsten, but not that of *T. asperellum* 302, in a time-dependent fashion. From the plugs, *Lyophyllum* sp. strain Karsten hyphae visually grew out as from day 3. Hyphal growth of this fungus away from the bacterial stripe occurred progressively, whereas growth towards it was slower. In contrast, *T. asperellum* 302 grew prolifically on the SEA plates, with no apparent growth retardation by *B. terrae* BS001. Fig. 5.1A and Fig. 5.1B shows the quantification of the hyphal growth of both fungi on the SEA plates.

Burkholderia terrae BS001 shows chemotactic movement towards the hyphae of Lyophyllum sp. strain Karsten and Trichoderma asperellum 302 on SEA plates

We then tested the response of *B. terrae* BS001 cell populations to the presence of the hyphae of *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 on 0.25% (w/v) SEA plates. In the assay, we included *B. terrae* BS001-Δ*sctD*, to examine the putative relevance of the T3SS for bacterial behavior during chemotaxis. A full factorial experiment was carried out, with the factors fungus (type and absence; 3 levels), bacterial cell density (three levels, 10^5, 10^7 and 10^9), bacterial type (wild-type versus mutant) and movement direction (towards versus away from the fungus). ANOVA of all data (Supplementary Table S5.2) established the factors fungus, bacterial cell density and movement direction (towards/away) as strong effectors of the migrational responses. In contrast, the factor bacterial type had virtually no effect. Thus, the presence of *Lyophyllum* sp. strain Karsten as well as *T. asperellum* 302 mycelia at 12–15 mm distance of a cell stripe of *B. terrae* BS001 (Fig. 5.2A) or *B. terrae* BS001-Δ*sctD* (Fig. 5.2B) prompted a significant chemotactic response of the two strains towards these, as the movement towards the two fungi was significantly higher ($P < 0.05$) than that in the control (i.e. a plug from an OFA plate). Moreover, movement in the opposite direction was also significantly lower ($P < 0.05$).

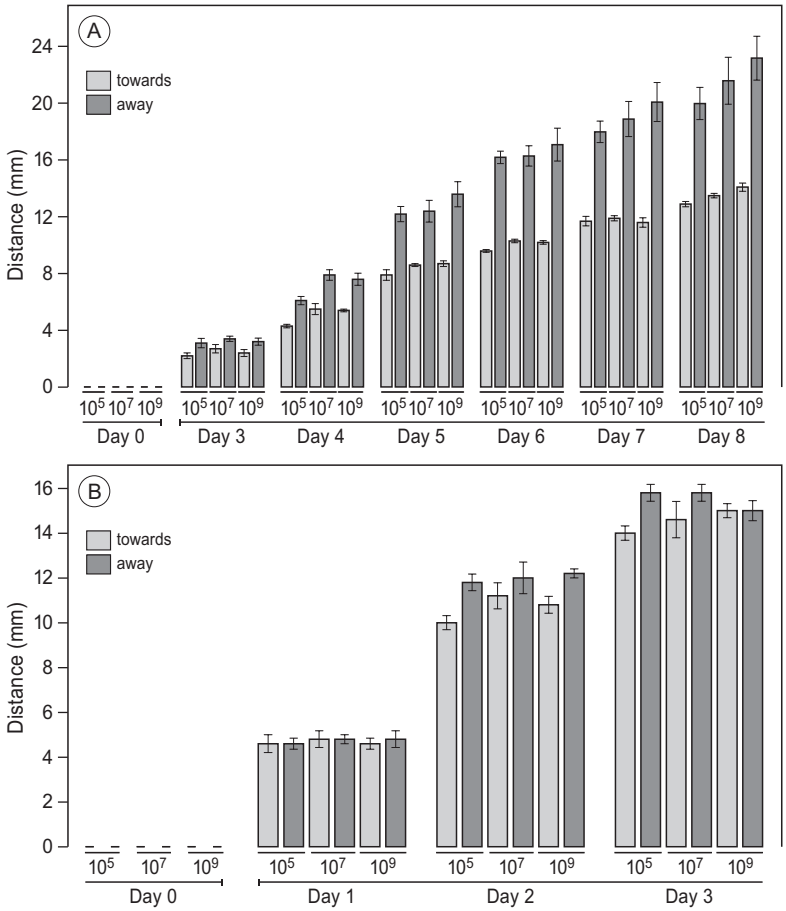

Figure 5.1 The behavioral response of *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 to *B. terrae* BS001 population on soil extract agar (SEA) medium. (5.1A) shows the response of *Lyophyllum* sp. strain Karsten to *B. terrae* BS001 on SEA. The data represent average of two different experiments. (5.1B) shows the response of *T. asperellum* 302 to *B. terrae* BS001 on SEA medium. The Y-axis represent distance in mm, whereas X-axis show the cell density and the measurement days.

Bacterial chemotaxis towards fungal hyphae in the presence of glycerol or oxalic acid

A chemotactic response was also noted for *B. terrae* BS001 and *B. terrae* BS001-Δ*sctD* interacting with *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 on M9 medium supplemented with glycerol (M9+glycerol; 0, 0.1%, 0.5% and 1%, [w/v]) agar. With an increasing concentration of glycerol in the M9 medium, both wild-type (Fig. 5.3A) and mutant strains (Fig. 5.3B) showed strong chemotactic responses towards *Lyophyllum* sp. strain Karsten, whereas the responses towards *T. asperellum* 302 were less pronounced

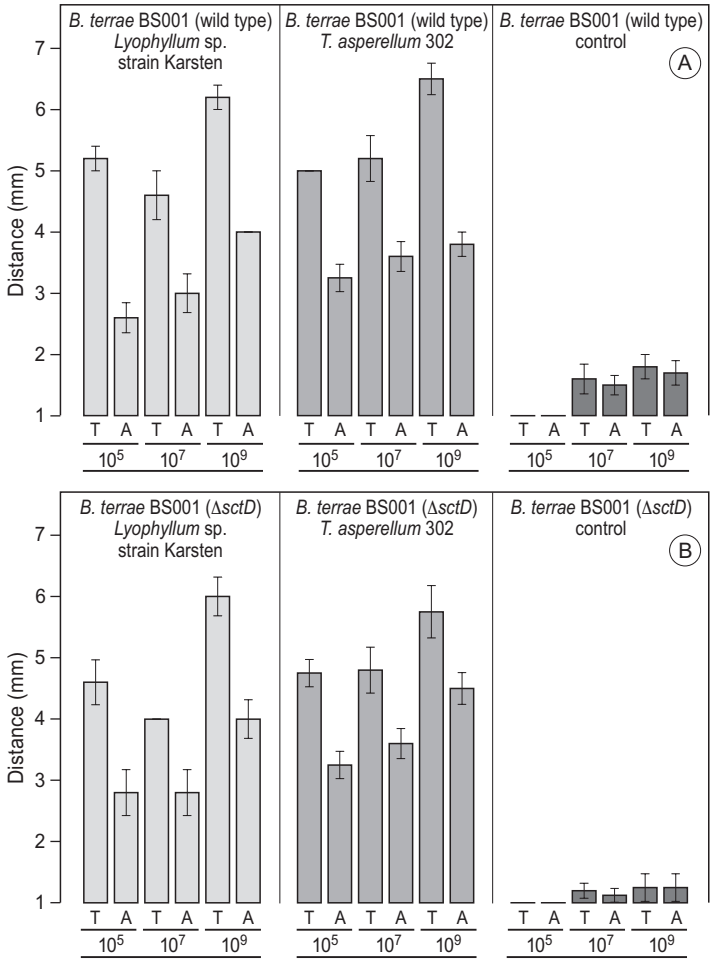

Figure 5.2 Chemotaxis (swimming) of wild-type and Δ*sctD* mutant *B. terrae* BS001 towards *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 hyphae on 0.25% [w/v] soil extract agar. (5.2A) represents the chemotactic responses of wild-type *B. terrae* BS001 cells population, whereas (5.2B) shows the chemotactic responses of *B. terrae* BS001-Δ*sctD*. Y-axis shows the distance in mm, while on the X-axis bacterial cell densities are shown. 'T' means towards and 'A' means away.

(Fig. 5.3C; Fig.5.3D). ANOVA of the data (Supplementary Table S5.3 and S5.4) showed that fungus, glycerol concentration and movement direction (towards/away) were the determinative parameters of the chemotactic responses of both the wild-type and mutant strains. The migrational response towards the hyphae increased with increasing glycerol concentrations, whereas that in the opposite direction was significantly lower ($P < 0.05$). Movement towards the control (fungus-less) plugs was minimal.

We then studied the possible effect of different levels of oxalic acid on the chemotactic responses of both strains towards the fungi (Fig. 5.4A and Fig. 5.4B). Both the wild-

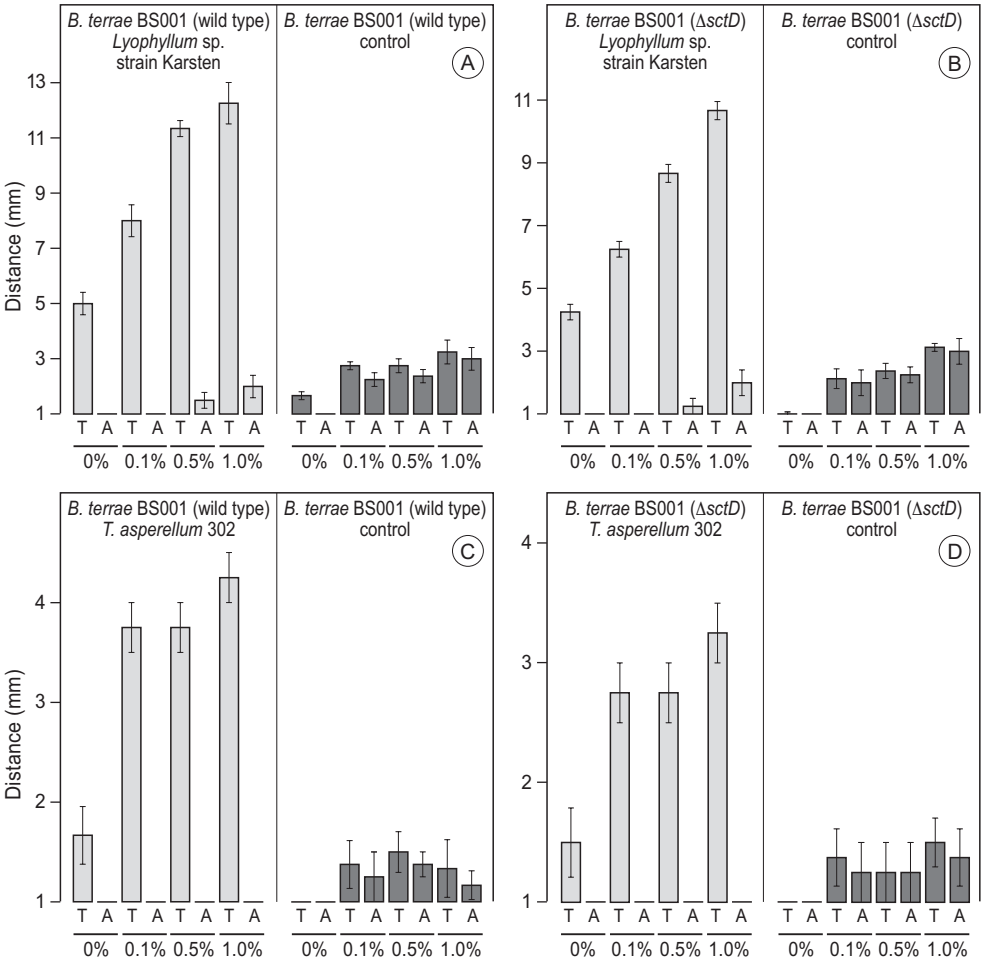

Figure 5.3 Chemotaxis (swimming) of wild-type and Δ*sctD* mutant *B. terrae* BS001 towards *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 hyphae on 0.25% [w/v] M9 medium supplemented with 0%, 0.1%, 0.5% and 1% glycerol [w/v]. (5.3A) represents the chemotactic responses of wild-type *B. terrae* BS001 cells population whereas (5.3B) shows the chemotactic responses of Δ*sctD* mutant *B. terrae* BS001 towards *Lyophyllum* sp. strain Karsten. (5.3C) represents the chemotactic responses of wild-type *B. terrae* BS001 cells population whereas (5.3D) shows the chemotactic responses of Δ*sctD* mutant *B. terrae* BS001 towards *T. asperellum* 302. Y-axis shows the distance in mm. X-axis represents respective concentrations of the glycerol. 'T' means towards and 'A' means away.

type and the mutant strain showed similar chemotactic responses towards both fungi, and oxalic acid level was critical with respect to the magnitude of the responses (Fig. 5.4). ANOVA of all the data (Supplementary Table S5.5) showed that fungus, oxalic acid level (0%, 0.1%, 0.5% and 1%, [w/v]) and movement direction (towards/away) were significant ($P < 0.05$) and strong effectors of the swimming behavior of both the wild-type and the mutant strain, with virtually no difference between the bacterial types.

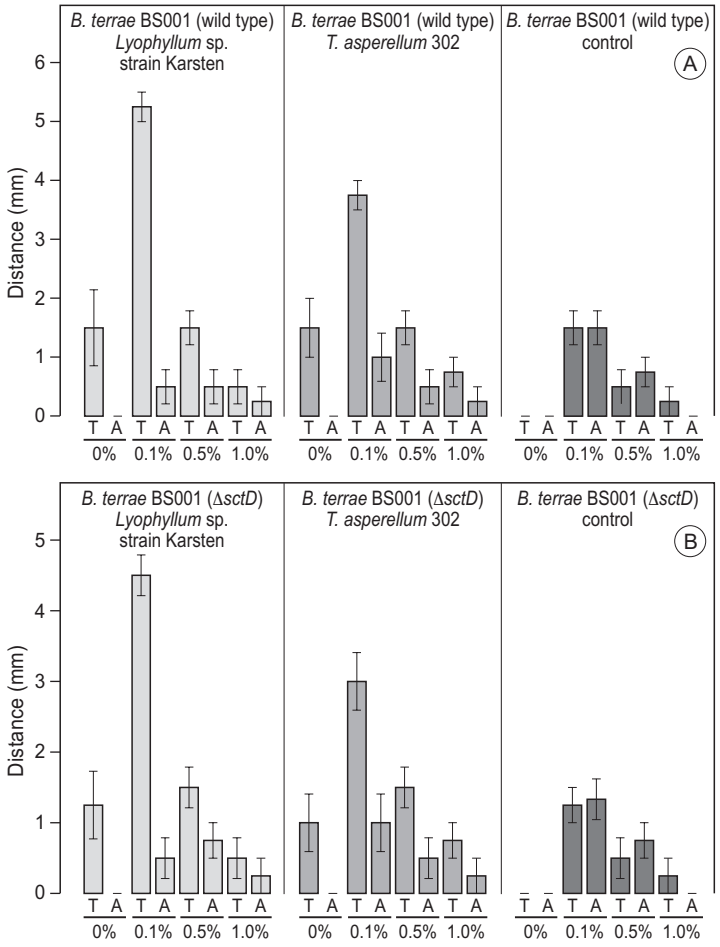

Figure 5.4 Chemotaxis (swimming) of wild-type and Δ*sctD* mutant *B. terrae* BS001 towards *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 hyphae on 0.25% [w/v] M9 medium supplemented with 0%, 0.1%, 0.5% and 1% [w/v] oxalic acid. (5.4A) represents the chemotactic responses of wild-type *B. terrae* BS001 cells population, whereas (5.4B) shows the chemotactic responses of Δ*sctD* mutant *B. terrae* BS001. Y-axis shows the distance in mm, while the X-axis shows respective concentrations of the oxalic acid. 'T' means towards and 'A' means away.

Structural analysis of T. asperellum 302 CMH / glucosylceramides

To better understand the potential of physical interaction of *B. terrae* BS001 with soil fungi, we placed a focus on the fungal glucosylceramide moieties (CMH). Here, we determined the nature of the *T. asperellum* 302 CMHs. Two major, lithiated, singly-charged ion species at m/z 762 and 778 were observed in the MS1 spectrum (Supplementary Fig. S5.1 and S5.2, respectively). Two other minor species were detected at m/z 760 and 776 that represent differences in the degree of unsaturation of the fatty acid

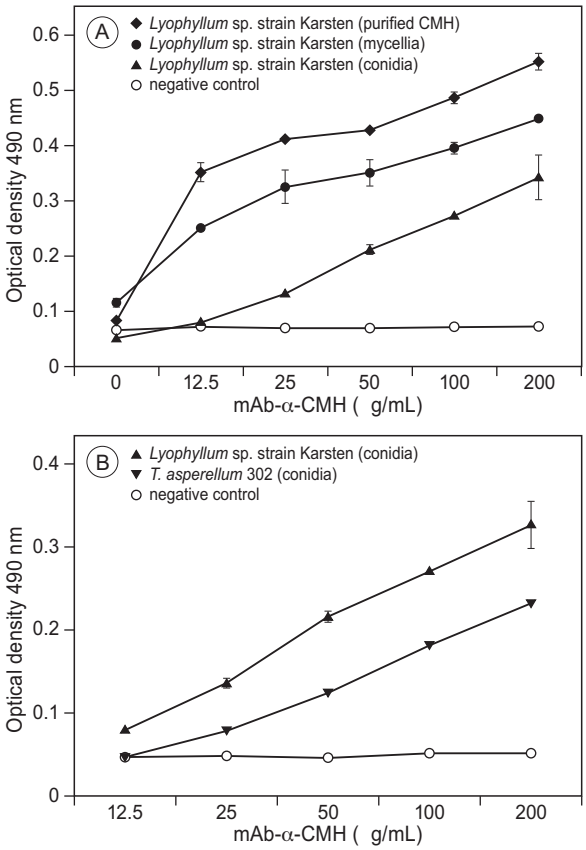

Figure 5.5 Reactivity of anti-CMH monoclonal antibodies (mAbs) to fungal CMH. (5.5A) shows ELISA results to evaluate binding of mAbs at 200, 100, 50, 25, 12.5 or 0 (g/mL) to wells coated with purified CMH, mycelia and conidia of *Lyophyllum* sp. strain Karsten. (5.5B) represents the comparison of the binding of mAbs at different concentrations (200, 100, 50, 25, 12.5 or 0 (g/mL)) to wells coated with conidia of *Lyophyllum* sp. strain Karsten and *T. asperellum* 302.

chains (Supplementary Fig. S5.1 and S5.2).When subjected to tandem MS/MS fragmentation, the ion species m/z 762 and 778 generated fragment ions at m/z 600 and 616, respectively, 162 mass units shorter than the parent ions, which corresponds to the loss of a hexose residue. A prominent ion at m/z 496 from species m/z 778 and 762 is consistent with the loss of an OH-C_{18} fatty acid present in the ceramide (Supplementary Fig. S5.1 and S5.2). The MS-MS spectrum of ion species m/z 778 is shown in supplementary Fig. S5.3. The ceramide ion species at m/z 616 and 600 can be assigned as N-2´-hydroxy-octadecanoyl-4-OH-9-methyl-4,8-sphingadienine and N-2´-hydroxy-octadecanoyl-9-methyl-4,8-sphingadienine, respectively. Based on these data, the proposed structures of the *T. asperellum* 302 CMHs are shown in Supplementary Fig. S5.1 and S5.2.

Detection of CMH on the surface of mycelia and conidia of Lyophyllum sp. strain Karsten and T. asperellum 302, and interaction with Burkholderia terrae BS001

For CMH to act as an anchoring molecule for *B. terrae* BS001, it should be exposed at the fungal surface. Using ELISA with anti-CMH mAbs, we first screened for the presence of CMH at the surface of both the mycelia and conidia of *Lyophyllum* sp. strain Karsten. Fig. 5.5 reveals the presence of CMH in both the conidia and the hyphae of *Lyophyllum* sp. strain Karsten, whereas the controls revealed low background signals. Given the likelihood of surface-exposed CMH, we hypothesized that such CMH-rich cell envelope regions may represent anchoring sites for *B. terrae* BS001.

In a subsequent experiment, the association between *B. terrae* BS001 cells and the conidia of the two fungi was tested *in vitro* by assaying the amounts of biofilm formed (Fig. 5.6A and Fig. 5.6B). Remarkably, for *Lyophyllum* sp. strain Karsten, differences in biofilm formation were found in relation to the order of colonization, but this was not the case for *T. asperellum* 302. In the 'fungi-first' systems, fungal biofilms were formed,

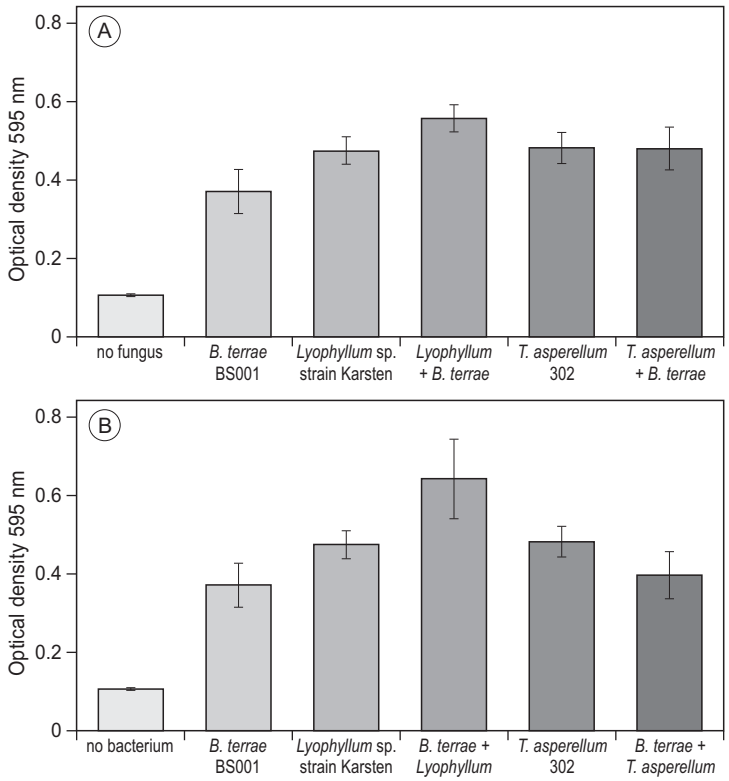

Figure 5.6 Bacterial-fungal behavior during mixed biofilm formation. (5.6A) Represents biofilm formation when fungi were added as first colonizer. (5.6B) shows biofilm formation when *B. terrae* BS001 was added as first colonizers. Y-axis shows biomass quantified indirectly by Crystal Violet method.

and the addition of *B. terrae* BS001 enhanced the total biomass, albeit slightly, with *Lyophyllum* sp. strain Karsten (Fig. 5.6A) but not with *T. asperellum* 302. Biofilm formation by *Lyophyllum* sp. strain Karsten was significantly raised ($P < 0.05$) when bacteria were already present on the well walls (Fig. 5.6B). Hence, *B. terrae* BS001 interacted with *Lyophyllum* sp. strain Karsten in a positive way, enhancing the biofilm, whereas it possibly affected *T. asperellum* 302 negatively.

Burkholderia terrae BS001 adherence to Lyophyllum sp. strain Karsten and T. asperellum 302 conidia

We first tested if *B. terrae* BS001 as well as BS001-Δ*sctD* can attach to the conidia of the two selected fungi, as conidia were found to contain surface-exposed CMH. The adherence of *B. terrae* BS001 to *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 over time was clearly different. Strain BS001 did adhere to *Lyophyllum* sp. strain Karsten conidia, whereas the mutant strain BS001-Δ*sctD* showed lowered adherence ($P < 0.05$; Fig. 5.7A).

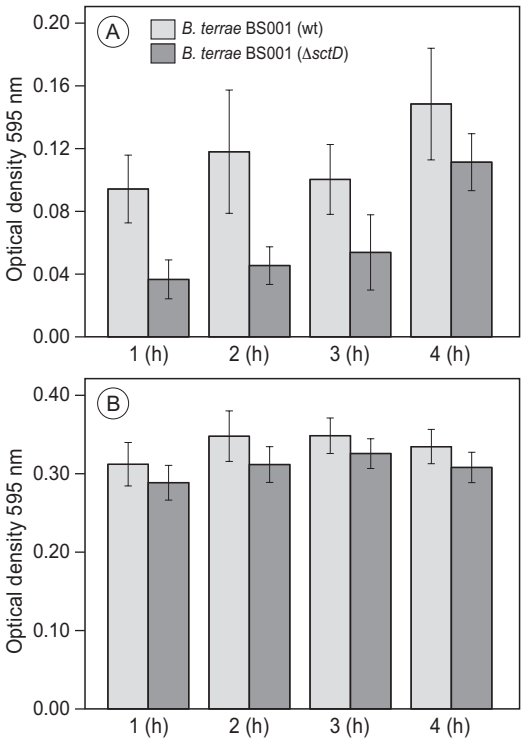

Figure 5.7 The attachment of *B. terrae* BS001 and *B. terrae*-Δ*sctD* to fungal conidia. (5.7A) Represents attachment of *B. terrae* BS001 and *B. terrae*-Δ*sctD* to the conidia of *Lyophyllum* sp. strain Karsten. (5.7B) Shows the attachment of *B. terrae* BS001 and *B. terrae*-Δ*sctD* to conidia of *Trichoderma asperellum* 302. Y-axis shows biomass quantified indirectly by Crystal Violet method, while X-axis represents the duration for adhesion. Biofilm quantification was noted at different incubation times (1, 2, 3, and 4 hours).

Whereas the effect was evident over the first three hours, it became less apparent at 4 h. With respect to *T. asperellum* 302, the *B. terrae* wild-type and mutant strains were both able to attach to the conidia (Fig. 5.7B), without any difference between them or the time of attachment. Thus, the dynamics of adherence of *B. terrae* BS001 to *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 was clearly different, which may relate to differences in the surface properties between the two fungi.

CMH is an anchoring site for Burkholderia terrae BS001 in Lyophyllum sp. strain Karsten

To examine whether the CMH moiety is involved in the attachment of *B. terrae* BS001 and *B. terrae* BS001-Δ*sctD* to *Lyophyllum* sp. strain Karsten, we tested the role of *Lyophyllum* sp. strain Karsten purified CMH as a potential anchoring site. The analyses revealed that *B. terrae* BS001 adheres to the CMH as a 'receptor' moiety, as it revealed high signals, which decreased significantly ($P < 0.05$) upon the use of mAbs α-CMH (Fig.

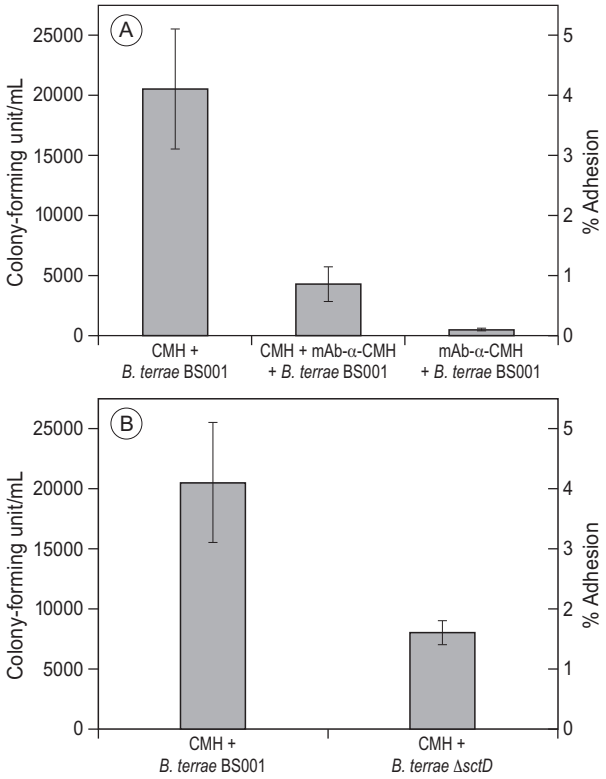

Figure 5.8 Adherence assay of *Burkholderia terrae* BS001 and Δ*sctD* mutant to fungal CMH. (5.8A) represents the adhesion of *B. terrae* BS001 to CMH of *Lyophyllum* sp. strain Karsten in the presence or absence of mAbs α-CMH. (5.8B) shows the adhesion of *B. terrae* BS001 and *B. terrae* Δ*sctD* to CMH of *Lyophyllum* sp. strain Karsten. Time required for adherence was 2h. Percentage of adherence was calculated in relation to the initial cell density added in the start of the experiment.

5.8A). A comparison of the adherence of *B. terrae* BS001 and *B. terrae* BS001-Δ*sctD* cells to the purified CMH showed the former strain to bind significantly better to the CMH than the latter ($P < 0.05$). Specifically, the mutant adhered about 40% less than the wild-type (Fig. 5.8B), suggesting that the type-3 pilus may play a role in the bacterial adherence to CMH under the conditions applied.

CMH as an anchoring site for strain BS001 to T. asperellum 302?

To examine the adherence of *B. terrae* BS001 to *T. asperellum* 302 conidia, in particular with respect to the CMH moiety, we used CMH extracted from *T. asperellum* 302 with *B. terrae* BS001 cells in the adherence assays. Both the *B. terrae* BS001 wild-type and the mutant strain did recognize the *T. asperellum* 302 CMH, to a similar extent, with the mAbs α-CMH showing poor blocking of the association (data not shown).

Discussion

Although the *B. terrae* BS001 - *Lyophyllum* sp. strain Karsten association, as well as the association of *B. terrae* BS001 with *T. asperellum* 302, have been described in previous papers (Warmink and van Elsas 2009, Nazir et al. 2013, Nazir et al. 2014), there are still questions about the precise mechanisms that play roles in the initial phases of the interaction. Thus, the occurrence of antagonistic versus synergistic effects between the bacterial and fungal partners has not been adequately addressed in previous work. Moreover, although motility was suggested to play a role (Warmink and van Elsas 2009), there are no exact data on this process, which may include signalling and chemotaxis. Thirdly, although *B. terrae* BS001 has been found to form biofilms with the two fungal counterparts (Warmink and van Elsas 2009, Nazir et al. 2014), virtually nothing is known about the onset of the physical interactions in which a 'landing apparatus – anchoring site' interaction is presumably at work (Haq et al. 2014b). It may be argued that the adherence properties of *B. terrae* BS001 cells are governed by particular fungal cell wall structures including exposed CMH moieties. At the start of the current study, no information was available about the type and level of CMH in *T. asperellum* 302, whereas that in *Lyophyllum* sp. strain Karsten has been communicated (Vila, pers comm.). In this study, in accord with Haq et al. (2014b), we thus hypothesized that all of the aforementioned phenomena were operational in the interaction of *B. terrae* BS001 with the two selected soil fungi. We deliberately included the strain BS001 T3SS mutant in the analyses in order to shed further light on the presumed involvement of this system in the interactive processes in the mycosphere, as indicated by Warmink and van Elsas (2008), here focusing on the two major steps leading towards the biofilm, i.e. (1) chemotaxis, and (2) adherence.

We thus first assessed the onset of the association between *B. terrae* BS001 and two soil-derived fungi, using SEA plates, mimicking the nutrient (carbon)-limited conditions that are commonly encountered in soil. The divergent fungal responses to the presence of strain BS001 were striking, as they pointed to interaction mechanisms that differed

between the two fungi. Mille-Lindblom et al. (2006) reported that the competition for substrate between bacteria and fungi is context-dependent, having temporal and spatial constraints. For instance, fungi fare better if they become established before bacterial colonization than when confronted with bacteria during hyphal growth. Here, an 'exploitation competition' seems unlikely. In a key study, the suppression of fungi in soil was attributed to the production of inhibiting compounds by other microorganisms (De Boer et al. 1998). The growth retardation effect exerted on *Lyophyllum* sp. strain Karsten does not preclude the physical contact, as in a previous experiment (in which transcriptional responses of strain BS001 on SEA with *Lyophyllum* sp. strain Karsten were studied) final confluence of both partners was found (data not shown). With respect to the (migratory) behavior of *B. terrae* BS001, our data indicate that it was attracted to the living hyphae of both *Lyophyllum* sp. strain Karsten and *T. asperellum* 302, moving towards these hyphae in a progressive manner. With respect to our hypothesis that the T3SS might have a role in any step of the migration process, given its presumed ability to secrete putative effector molecules (Haq et al. 2014a), we conclude from the data that this effect appears to be extremely unlikely, at least under the experimental conditions applied. The Δ*sctD* mutant, with disabled T3SS, did not, in any generic sense, affect the migrational behavior of strain BS001.

The effect of the initial cell densities on the chemotactic response of *B. terrae* BS001 was striking. We here posit that the initial cell density/level of crowding was determinative for the overall response, as higher initial cell densities may have led to an earlier potentially orchestrated response to the fungal trigger. Indeed, consistent with current understanding, the swimming behavior of *B. terrae* BS001 may constitute a form of collective behavior in which bacterial cells communicate before/upon migrating. Here, quorum sensing (systems are present on the BS001 genome; Haq et al. 2014a) might be involved. Recently, Rudnick et al. (2015), also suggested that quorum sensing might be at the basis of coordinated collimonad taxis towards the hyphal tips of fungi.

Given that the swimming response of *B. terrae* BS001 towards the fungal hyphae increased progressively with increasing glycerol levels, we surmised that glycerol acted as a generic carbon and energy source (Nazir et al. 2013) rather than as a specific attractant. This brings forth the possibility of secretion of a different signal molecule (attractant) by the respective fungal type. As Rudnick et al. (2015) recently found oxalic acid to act as a signalling molecule for *Collimonas* spp., we tested whether this compound might be an attractant. Clearly, the effect of oxalic acid on the chemotactic responses of *B. terrae* BS001 was different from that of glycerol. At low concentrations, strong responses were recorded, while these became weaker at higher concentrations. This is consistent with the observation (Rudnick et al. 2015) that *C. fungivorans* Ter331 showed varied (swarming) behavior with increasing oxalic acid levels. Recently, Kost et al. (2014) also showed that plant-associated *Burkholderia* spp., such as *B. phytofirmans* PsJN have the ability to utilize oxalic acid (while pathogenic *Burkholderia* species lack such traits). However, they did not confirm that oxalic acid could be toxic when used in higher concentrations. Here, we postulate that oxalic acid secreted from *Lyophyllum* sp. strain

Karsten is acting as a signalling molecule and may also have a growth promotion effect for *B. terrae* BS001. We are currently further pursuing the role of oxalic acid, in terms of whether it serves as merely a signalling molecule or as a potential carbon source, in the interaction of *B. terrae* BS001 with *Lyophyllum* sp. strain Karsten and *T. asperellum* 302.

B. terrae BS001 can form biofilms on the hyphal surfaces of *Lyophyllum* sp. Karsten, most likely attaching to their hyphal tips, as suggested by Warmink and van Elsas (2009). Here, we found evidence for the contention that *B. terrae* BS001 cells can also attach to the conidia of this fungus. We used conidia, as they are an important facet of the fungal lifestyle in soil. Our findings are consistent with those of Levy et al. (2009), who found adherence of *Burkholderia pseudomallei* cells to spores of arbuscular mycorrhizal fungi (Levy et al. 2009). Possibly, such adhering cells are well-placed for rapid colonization upon germination of the conidia as a response to favorable conditions in soil. In the biofilm formation experiment with *Lyophyllum* sp. strain Karsten, the total biofilm mass was most robust when the bacterium was the first colonizer, but in the reverse setup the biofilm also formed. Given the fact that no such effect was noted with *T. asperellum* 302, we suggest that the robustness of the association of *B. terrae* BS001 with different soil fungi, such as used here, is fungus-specific rather than generic.

With respect to the physical interaction of *B. terrae* BS001 with the two soil fungi, we suggest a role for a chemical moiety involving CMH expressed at the conidial surface in the attachment to fungal conidia. Conidia are vegetative reproduction structures that are different from mycelia and serve dispersion purposes. With their limited metabolism, they contain molecules that potentially give survival advantages, such as hydrophobins and melanin (Beauvais et al. 2014, Paris et al. 2003). They have also been shown to contain multiple CMH molecules on their surfaces (Calixto et al. 2016, Rollin-Pinheiro et al. 2014, Da Silva et al. 2004, Nimrichter et al. 2004), much like hyphae.

As the attachment of bacterial cells to fungal surfaces depends on surface-expressed molecules, we here studied if CMHs might constitute 'anchors' for *B. terrae* BS001 at fungal surfaces. Indeed, CMH molecules can be localized at fungal cell envelopes as minor components, as shown by immunogold and other antibody assays (Rodrigues et al. 2000, Da Silva et al. 2004, Rhome et al. 2011). Rodrigues et al. (2000) provided initial evidence for the tenet that CMH is transported by vesicles from the plasma membrane towards the cell wall, being deposited locally in the latter. This supports the contention that CMH in fungal cell envelopes is – to some extent – surface-exposed. Moreover, Guimarães et al. (2014) recently proposed that the glycans in the cell wall close to microdomain regions of the cell membrane can become 'loose', which, in turn, can expose the membrane glycosphingolipids to the surface of the cell envelope (next to glycoproteins and adhesins). More evidence for the surface exposure of CMHs has come from independent work by Rhome et al. (2011), who studied the surface of *Cryptococcus neoformans* during host infection (Rhome et al. 2011). With respect to the validity of data obtained with (monoclonal) anti-CMH antibodies, Toledo et al. (2001) described a monoclonal antibody denoted MEST-2 that was strictly specific to the glucosylceramide moieties of fungi as well as plants. The minimum epitope for antibody binding turned

out to be a β-D-Glc residue plus another site represented by a 2-hydroxy group present in the fatty acid (Toledo et al. 2001). In several concurrent studies, the specificity to such epitopes was confirmed by immunostaining of fungal-envelope-extracted total lipids and purified CMH with human– as well as rabbit–derived polyclonal antibodies (Rodrigues et al. 2000, Da Silva et al. 2004) as well as by the monoclonal antibodies used by us in this study (Xisto, pers. comm.). Overall, these data indicate that anti-CMH antibodies enable the specific detection of epitopes presented by CMH upon exposure at (fungal) cell surfaces. In contrast, in the classical Bowman and Free review (2006), only major fungal cell wall components are mentioned and so the presence of CMHs is easily overlooked. We here posit that – on the basis of our data – CMH represents a binding site on the *Lyophyllum* sp. strain Karsten envelope for *B. terrae* BS001 cells. Given that the extent of binding to CMH was lower – but not zero – for the Δ*sctD* mutant strain, the T3SS of strain BS001 may be involved as a helper structure in the cellular binding to the CMH moiety at the *Lyophyllum* sp. strain Karsten surface. In contrast, according to the data shown herein, an involvement of the T3SS system in the attachment of strain BS001 to *T. asperellum* 302 is unlikely. First, both wild-type and Δ*sctD* mutant strains could recognize, to a similar but reduced extent, the *T. asperellum* 302 CMH. The ceramide moiety of the *T. asperellum* 302 CMH had an additional hydroxyl group at C-4, which is lacking in the CMH of *Lyophyllum* sp. strain Karsten. However, CMH recognition by mAbs – and potentially also by bacterial cell surface compounds – occurs through specific epitopes in the molecule, the ideal conformation including the sugar in the ceramide moiety and the α-hydroxyl group in fatty acid (Nakakuma et al. 1989, Villas-Boas et al. 2005). Also, the intramolecular interactions between the lipid and sugar moieties (Villas-Boas et al. 2005) and some restrictions (due to an engulfing lipid bilayer) to molecule exposure in the cellular membrane (Nyholm and Pascher 1993) are important. In the case of *T. asperellum* 302, a shielding of the CMH by other cell envelope compounds such as melanin may have occurred, although this is open for further research. Clearly, the exact cellular structure holding the CMH moiety in *T. asperellum* 302 is unknown so far, whereas that in *Lyophyllum* sp. strain Karsten may enable surface exposure.

Nimrichter et al. (2004) indicated that CMH extrusions from the membrane might direct a modulation of fungal physiology 'from the outside'. We here posit that the attachment of *B. terrae* BS001 cells to *Lyophyllum* sp. strain Karsten may have involved specific CMH-bacterial surface interactions. For that to happen, the bacterium possibly attached to the CMH in the surface of the latter fungus that occurred in a more exposed form, than the one expressed in *T. asperellum* 302.

Concluding, we here provide glimpses of the mechanisms that underlie the interaction between *B. terrae* BS001 and the soil fungi *Lyophyllum* sp. strain Karsten and *T. asperellum* 302. First, a chemotactic response towards the two fungi was unveiled, which had divergent patterns with the supplemented compounds oxalic acid and glycerol. The increasing concentration of the former compound incited a subdued movement compared to a faster one in the latter. On the other hand, at lower concentration of oxalic acid the response was stronger than that with glycerol. *B. terrae* BS001 showed 'partial

competitive' behaviour towards *Lyophyllum* sp. strain Karsten, contrary to *T. asperellum* 302, on SEA plates. Our observations also revealed the attachment of strain BS001 to the cell envelope extracted molecule CMH of *Lyophyllum* sp. strain Karsten but not to that of *T. asperellum* 302. The attachment efficiency to the former fungus was reduced when strain BS001-Δ*sctD* was used, suggesting a potential role of the T3SS in the physical interaction, although this needs further investigation. Overall, our data add to the broader understanding of bacterial-fungal interactions in soil, in which bacterial chemotaxis towards and attachment to fungal tissue play key roles.

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Supplementary tables and figures

Table S5.1 Contains weight by volume (concentrations) and mM (concentrations) of oxalic acid and glycerol.

S.No.	Oxalic acid [%]	Oxalic acid [mM]	Glycerol [%]	Glycerol [mM]
1	0.1 % [w/v]	7.9 mM	0.1 % [w/v]	15.6 mM
2	0.5 % [w/v]	39.6 mM	0.5 % [w/v]	78.2 mM
3	1.0 % [w/v]	79.3 mM	1.0 % [w/v]	156.4 mM

Table S5.2 Full factorial ANOVA testing for the effects of fungal type and absence (control), cell density, movement direction, bacterial strain type (wild type and Δ*sctD* mutant), and their interaction on the distance travelled in the chemotaxis experiment on soil extract agar.
Statistical significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Code
Fungus	2	401.3	200.66	201.071	< 2e-16	***
Cell density	1	35.2	35.22	35.296	1.78e-08	***
Direction	1	58.4	58.37	58.488	1.97e-12	***
Strain	1	4.5	4.51	4.522	0.035	*
Fungus:Cell density	2	3.5	1.76	1.763	0.175	
Fungus:Direction	2	28.1	14.04	14.072	2.41e-06	***
Cell density:Direction	1	0.3	0.27	0.273	0.602	
Fungus:Strain	2	0.2	0.10	0.104	0.901	
Cell density:Strain	1	0.6	0.63	0.634	0.427	
Direction:Strain	1	2.1	2.11	2.117	0.148	
Fungus:Cell density:Direction	2	0.1	0.07	0.075	0.928	
Fungus:Cell density:Strain	2	1.1	0.56	0.558	0.573	
Fungus:Direction:Strain	2	1.1	0.55	0.555	0.575	
Cell density:Direction:Strain	1	0.0	0.02	0.024	0.877	
Fungus:Cell density:Direction:Strain	2	0.4	0.21	0.209	0.812	
Residuals	156	155.7	1.00			

Table S5.3 ANOVA testing for the effects of fungus (*Lyophyllum* sp. strain Karsten) and absence (control), movement direction, *B. terrae* BS001 strain type (wild type and Δ*sctD* mutant), glycerol concentrations and their interaction on the distance travelled in the chemotaxis experiment on M9 agar.
Statistical significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Code
Fungus	1	178.1	178.1	204.004	< 2e-16	***
Direction	1	465.1	465.1	532.678	< 2e-16	***
Strain	1	10.1	10.1	11.596	0.000919	***
Glycerol conc.	1	176.9	176.9	202.642	< 2e-16	***
Fungus:Direction	1	371.3	371.3	425.205	< 2e-16	***
Fungus:Strain	1	3.8	3.8	4.330	0.039717	*
Direction:Strain	1	8.5	8.5	9.743	0.002290	**
Fungus:Glycerol conc.	1	22.5	22.5	25.821	1.51e-06	***
Direction:Glycerol conc.	1	13.5	13.5	15.431	0.000148	***
Strain:Glycerol conc.	1	0.1	0.1	0.157	0.692546	
Fungus:Direction:Strain	1	3.4	3.4	3.946	0.049434	*
Fungus:Direction:Glycerol conc.	1	22.8	22.8	26.066	1.36e-06	***
Fungus:Strain:Glycerol conc.	1	0.6	0.6	0.727	0.395527	
Direction:Strain:Glycerol conc.	1	0.1	0.1	0.076	0.782830	
Fungus:Direction:Strain:Glycerol conc.	1	0.7	0.7	0.769	0.382397	
Residuals	112	97.8	0.9			

Table S5.4 ANOVA testing for the effects of fungus (*Trichoderma asperellum* 302) and absence (control), taxis direction, *B. terrae* BS001 strain type (wild type and Δ*sctD* mutant), glycerol concentration and their interaction on the distance travelled in in the chemotaxis experiment on M9 agar.
Statistical significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Code
Fungus	1	10.41	10.41	25.948	1.43e-06	***
Direction	1	66.85	66.85	166.646	< 2e-16	***
Strain	1	0.71	0.71	1.758	0.1876	
Glycerol conc.	1	15.70	15.70	39.144	7.41e-09	***
Fungus:Direction	1	59.81	59.81	149.117	< 2e-16	***
Fungus:Strain	1	1.64	1.64	4.095	0.0454	*
Direction:Strain	1	1.22	1.22	3.043	0.0838	.
Fungus:Glycerol conc.	1	0.50	0.50	1.247	0.2666	
Direction:Glycerol conc.	1	1.78	1.78	4.448	0.0372	*
Strain:Glycerol conc.	1	0.02	0.02	0.053	0.8182	
Fungus:Direction:Strain	1	1.03	1.03	2.576	0.1113	
Fungus:Direction:Glycerol conc.	1	1.39	1.39	3.463	0.0654	.
Fungus:Strain:Glycerol conc.	1	0.75	0.75	1.863	0.1751	
Direction:Strain:Glycerol conc.	1	0.14	0.14	0.342	0.5598	
Fungus:Direction:Strain:Glycerol conc.	1	0.12	0.12	0.302	0.5838	
Residuals	112	44.93	0.40			

Table S5.5 Full factorial ANOVA testing for the effects of fungal type and absence (control), direction of taxis, bacterial strain type (wild type and Δ*sctD* mutant), oxalic acid concentration and their interaction on the distance travelled in the chemotaxis experiment on M9 agar.
Statistical significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Code
Fungus	2	17.17	8.58	9.694	0.000104	***
Direction	1	49.01	49.01	55.346	4.96e-12	***
Strain	1	0.63	0.63	0.712	0.400061	
Oxalic acid conc.	1	19.68	19.68	22.225	5.07e-06	***
Fungus:Direction	2	24.54	12.27	13.859	2.69e-06	***
Fungus:Strain	2	0.04	0.02	0.024	0.976749	
Direction:Strain	1	0.42	0.42	0.476	0.490981	
Fungus:Oxalic acid conc.	2	3.15	1.57	1.776	0.172403	
Direction:Oxalic acid conc.	1	11.88	11.88	13.413	0.000335	***
Strain:Oxalic acid conc.	1	0.54	0.54	0.607	0.436939	
Fungus:Direction:Strain	2	0.38	0.19	0.212	0.809373	
Fungus:Direction:Oxalic acid conc.	2	9.47	4.73	5.347	0.005608	**
Fungus:Strain:Oxalic acid conc.	2	0.03	0.02	0.019	0.981578	
Direction:Strain:Oxalic acid conc.	1	0.23	0.23	0.257	0.613169	
Fungus:Direction:Strain:Oxalic acid conc.	2	0.22	0.11	0.123	0.884045	
Residuals	168	148.75	0.89			

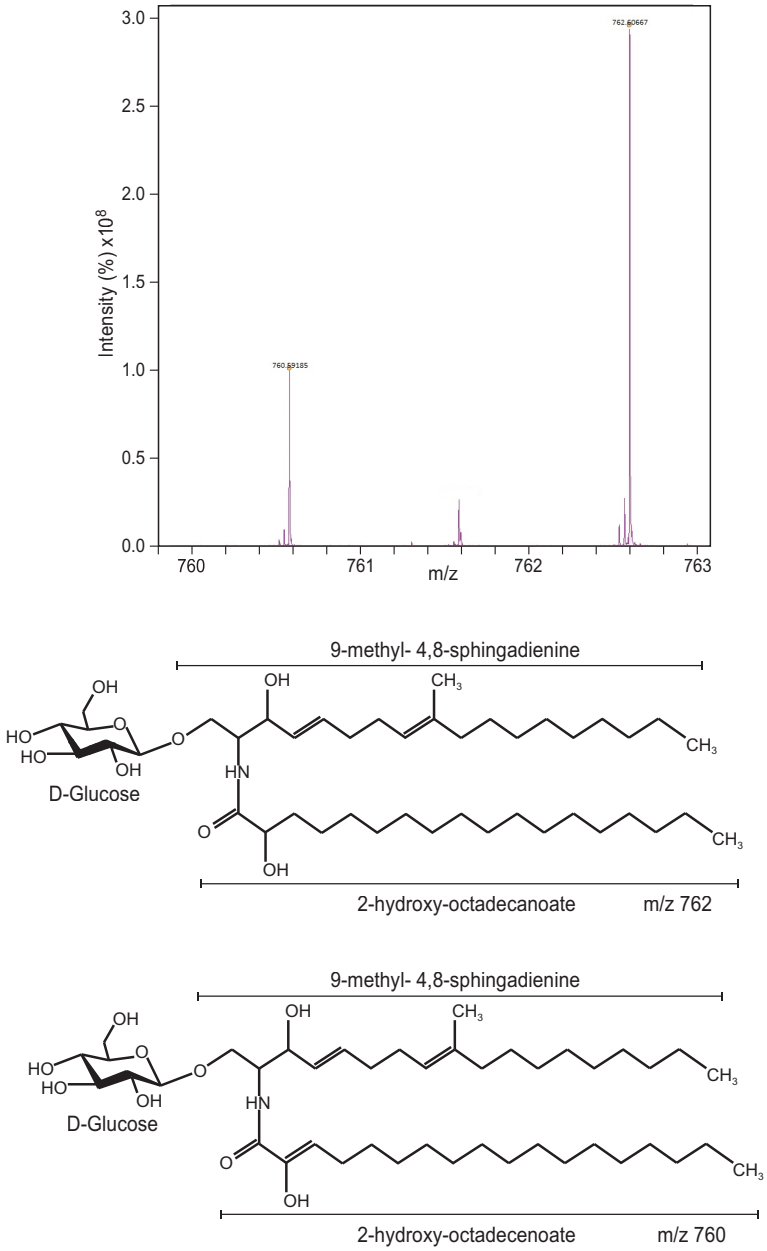

Figure S5.1 ESI-QTOF-MS analysis in the positive mode of glucosylceramide (GlcCer) species from *Trichoderma asperellum* 302 m/z 762 and 760 and proposed structures for each GlcCer species.

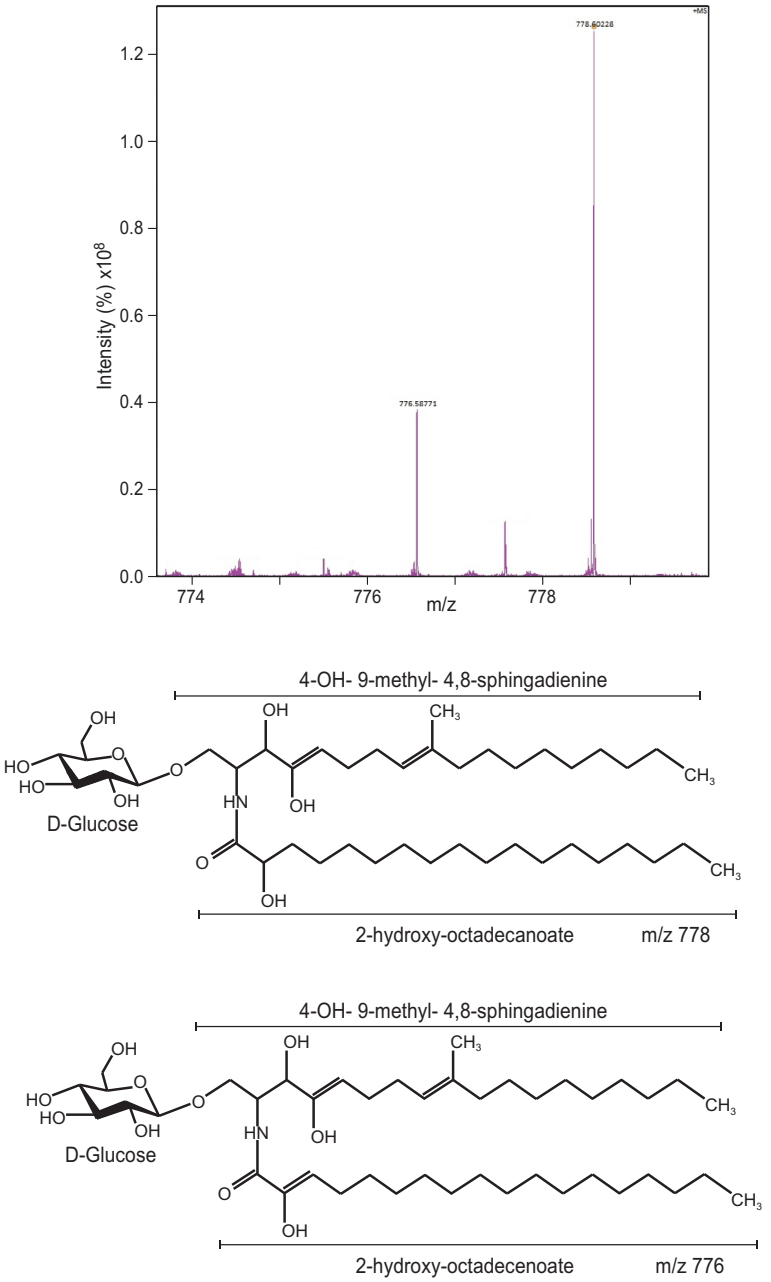

Figure S5.2 ESI-QTOF-MS analysis in the positive mode of glucosylceramide (GlcCer) species from *Trichoderma asperellum* 302 m/z 778 and 776 and proposed structures for each GlcCer species.

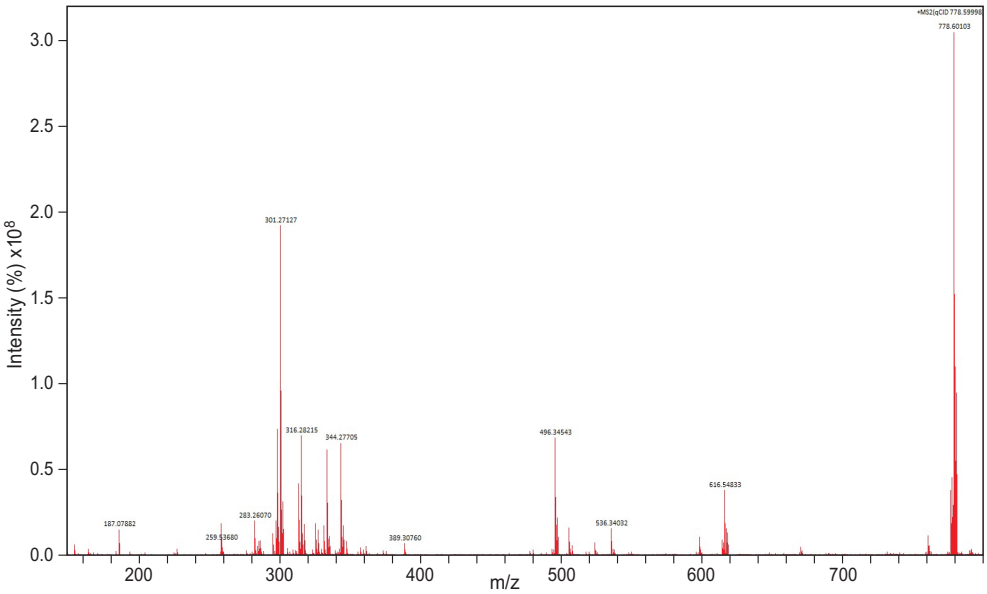

Figure S5.3 ESI-MS2 of the ion species m/z 778 of *Trichoderma asperellum* 302.

Chapter 6

6 A signalling molecule or a potential carbon source: the conundrum of oxalic acid in the interactions of *Burkholderia terrae* strains with two soil fungi

Irshad Ul Haq, Reto D. Zwahlen, Pu Yang, Jan Dirk van Elsas

Abstract

Fungal–associated *Burkholderia terrae* strains in soil have been extensively studied, but their sensing strategies to locate fungi in soil have remained largely elusive. In this study, we investigated the ecological behaviour of five *Burkholderia terrae* strains [including BS001–Δ*sctD*] and the type strain 17804^T with respect to their fungal–sensing strategies. The putative role of oxalic acid as a signalling molecule in bacterial chemotaxis towards these fungi, as well as a potential carbon source, was assessed. Our analyses of the chemotactic responses, using oxalic acid as a proxy, revealed that all *B. terrae* strains, including the type strain, sensed different levels of oxalic acid (0.1%, 0.5% and 0.8%) at a distance. Strikingly, the chemotactic responses were faster and stronger at a lower concentration of oxalic acid (0.1%) than at higher ones. We then tested the chemotactic responses of all strains towards exudates of *Lyophyllum* sp. strain Karsten and *Trichoderma asperellum* 302 used in different dilutions (0 %, 1, 1:10 1:100). All *B. terrae* strains showed significant directed chemotaxis towards the exudates, with full–strength exudates showing the strongest response. In a separate experiment, strain BS001 was shown to be able to grow on oxalate–amended (0.1% and 0.5%) M9 medium. Chemical analyses of the fungal secretomes using proton nuclear magnetic resonance (^{1}H NMR) next to high performance liquid chromatography (HPLC) revealed the presence of oxalic acid, glycerol, acetic acid, formic acid and fumaric acid for both fungi. In addition, citric acid was found only for *Lyophyllum* sp. strain Karsten. Given the fact that such compounds serve as C and energy sources for *B. terrae*, both fungi clearly offered ecological benefits to this organism *B. terrae*. We posit that oxalic acid released by the fungi primarily acts as a signalling molecule and a "second option" carbon source for *B. terrae* strain BS001.

Manuscript in preparation

Introduction

Bacteria in soil most often encounter conditions of nutrient (carbon) scarcity (Van Elsas et al. 2007). Hence, hospitable microhabitats at plants and fungi, where carbonaceous compounds become available, are attractive refuges. To localize such potential niches, soil bacteria depend on genetic traits responsible for signal perception, potentially leading to chemotaxis, guiding them through soil's microenvironments. Bacteria that have a symbiotic phase included in their lifestyle are particularly dependent on processes of recognition and positive movement. Different fungi in soil have been found to release carbon– and energy–yielding compounds like oxalic and citric acid (Sullivan et al. 2012). These are used by soil fungi to scavenge metals and release inorganic nutrients (Van Hees et al. 2006, Adeleke et al. 2012). In some plant–associated fungi and in fungi involved in the decay of litter and wood, the oxalic acid can even form complexes – in the form of oxalate – on the hyphal tips (Dutton and Evans 1996, Van Hees et al. 2006, Heller and Witt-Geiges 2013, Bhatti et al. 1998, Harrold and Tabatabai 2006). A limited range of soil bacteria is known to be able to use oxalic acid and its complexes as a potential carbon source, in sites under the direct influence of fungi (Bravo et al. 2013), or plants (Franceschi and Nakata 2005). Interestingly, oxalic acid has recently been shown to act as a signalling molecule for bacteria of the genus *Collimonas* (Rudnick et al. 2015). In addition, it was shown to serve as a carbon source for plant–associated *Burkholderia phytofirmans* PsJN (Kost et al. 2014).

The genus *Burkholderia* encompasses different species, of which some can interact with soil fungi. Examples are *B. terrae* BS001 and the related strains BS110, BS007 and BS437(Nazir et al. 2014, Nazir et al. 2012). These strains have been shown to harbor genetic systems that may furnish the capacity to obtain ecological benefits from fungi in soil (Nazir et al. 2012, Haq et al. 2014). Among such systems, those responsible for chemotaxis and flagellar movement are of prime importance, as they underlie the active exploration of the soil for nutrients, by sensing the putative chemoattractants released by fungi. Nazir et al. (2013) showed that the interaction between *Lyophyllum* sp. strain Karsten and *B. terrae* BS001 [in liquid microcosms] resulted in the release of glycerol by the fungus, spurring the growth of the bacterium. Strain BS001 was also able to grow actively in minimal medium supplemented with glycerol as the sole carbon source (Nazir et al. 2013). However, evidence for the production of oxalic acid by *Lyophyllum* sp. strain Karsten (as well as *Trichoderma asperellum* 302), and its putative role in their interactions with *B. terrae* strains BS001, BS110, BS007, and BS437, has been lacking.

In this study, we test the hypothesis that *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 exude a number of carbonaceous compounds, including oxalic acid, which offer ecological benefits to fungal–associated *B. terrae* strains. We used simple microcosm set–ups to test bacterial behaviour, and performed proton nuclear magnetic resonance (^{1}H NMR) and high performance liquid chromatography (HPLC) to investigate the release of chemical compounds. We further hypothesized that oxalic acid acts primarily as a signalling molecule for the fungal–interactive *B. terrae* strains BS001, BS110, BS007, and BS437, next to the type strain 17804^T.

Materials and methods

Bacterial strains, growth conditions, media and chemotaxis assay

In this study, we used fungal–interactive *Burkholderia* strains BS001, BS110, BS007, BS437, Δ*sctD* mutant BS001, and type strain 17804^T. All strains were maintained at –80°C. They were routinely grown on R2A agar at 25°C. All *B. terrae* cultures were grown overnight in Luria–Bertani (LB) broth at 28°C, with shaking at 180 rpm. Bacterial cultures were routinely subjected to centrifugation at 12,000×g for 3 min. The bacterial pellet was double–washed with sterile saline [0.85% NaCl solution]. For all experiments on chemotaxis, all cultures were centrifuged at 1,057×g for 20 min and the resulting pellets were washed twice with morpholine ethane sulfonic acid (MES buffer), containing 1 g of KH_2PO_4 and 1 g $(NH_4)_2SO_4$ per L; pH 5.6 (Rudnick et al. 2015). The medium used for the chemotaxis assay was M9 agar, where the final concentration of the agar was set at 0.25% (M9 agar for testing of swimming motility[M9A–S]). Glycerol (0.5%) was provided as the sole carbon source. M9 was comprised of 6.76 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g NaCl and 1 g of NH_4Cl per L MilliQ water. The pH was adjusted at 6.8 before autoclaving. The medium was supplemented with two mL (per L) of filter–sterilized (0.2 m) 1 M $MgSO_4$ and 100 L (per L) of 1 M $CaCl_2$.

For testing oxalic acid and the exudates of both fungi as potential chemoattractants, we used M9 agar (1.2% [w/v]) and included either oxalic acid (0%, 0.1% 0.5% and 0.8% [w/v]) or different levels of fungal exudates (no exudates, undiluted exudates, 1:10 and 1:100 dilutions). We cut rectangular (15×5 mm) stripes from the resulting plates and used these in all the experiments.

Washed cells were resuspended in MES buffer and the OD 600 was adjusted to have an approximate number of 10^7 CFU/mL. The resulting cell suspensions were introduced onto the Petri plates at distances of about 15 mm from the stripes containing either oxalic acid (0% [controls], 0.1%, 0.5% and 0.8% [w/v]) or fungal–exudates (undiluted, 1:10 and 1:100). The Petri plates were incubated at 25°C (24–36 h) and readings (distance in millimeters) were taken manually at set times.

Fungal strains, growth media and conditions to obtain exudates

For long–term maintenance, *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 were maintained in autoclaved distilled water. For routine use, the fungi were refreshed once a month on oat flake agar (OFA) plates. For the analysis of their respective secretomes, both fungal strains were first grown for 48–72 h in oat flake medium at 28°C with shaking. The well–grown fungal cultures were then centrifuged at 2,655×g (FA–45–30–11 rotor, Eppendorf) for 10 min and the pellets washed twice with M9. M9 medium supplemented with 1% [w/v] sodium–propionate (pH 4.8), was then used for the preparation of fungal secretomes. Erlenmeyer flasks containing 50 mL of medium were inoculated with the respective strains and incubated for two weeks at 28°C, with shaking.

Harvesting fungal exudates and sample preparation for 1H NMR

Well–grown 50 mL cultures of both *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 were harvested by centrifugation at 4,226×g (F–35–6–30 rotor, Eppendorf) for 10 min The resulting supernatants were then filtered through 0.2 m filters (Whatman; FP 30/ 0.2 CA–S), to remove remaining cells and debris. The filtrates containing fungal exudates were stored at –20°C. For ^{1}H NMR analyses, samples were subsequently defrosted at room temperature. To 570 L of filtrate, 30 L of D2O containing 3–(trimethylsilyl)–1–propanesulfonic acid sodium salt (DSS; 0.1 mM final concentration) were added (as internal standard). To minimize pH–based peak shifts, the pH for all samples was adjusted to 7.0 with either HCl or NaOH. For ^{1}H NMR acquisition, aliquots of 600 L were transferred to Bruker NMR tubes.

^{1}H NMR data acquisition and analysis

The ^{1}H NMR spectra were acquired on a Bruker DRX400 spectrometer 500–MHz (Bruker Spectrospin, Toronto, Canada). The ^{1}H NMR spectra were further processed and analyzed using MestReNova (version 9.0.0–12821) software. For identification, spectra obtained were compared with spectra of known compounds using the Human Metabolome Database (HMDB).

High performance liquid chromatography (HPLC) based analysis of the fungal exudates

Oxalic acid levels were determined in the exudates of both fungi, using a C18 reverse phase column (id 5 m; d 4.6×250–mm) on a Shimadzu UPLC device (Shimadzu Corp., Kyoto, Japan). An isocratic method was chosen, using a 25 mM K–phosphate buffer with a pH of 2.4 as the mobile phase. The pump flow was set at 1.5 mL/min and the column temperature at 30°C. Then, and samples at 4°C (20 L) were run [sample injection followed 30 sec after run initiation with a total length of 14 min]. The chromatogram was subsequently evaluated at 210 nm. Peak areas were extracted using the Shimadzu software. The oxalic acid concentration was derived using an external standard at concentrations of 0.01%; 0.05%; 0.10%; 0.25%; 0.5% and 1.0% (w/v). Corresponding peaks were further identified using 0.1% oxalic acid internal sample spiking.

Analysis of putative oxalate degradation pathways in Burkholderia genomes

We used the MetaCyc database and specifically examined the available *Burkholderia terrae* genomes for oxalate degradation pathways. We did a directed search in the aforementioned genomes hosted at MicroScope (Vallenet et al. 2013) for the presence and absence of any of the five pathways of oxalate degradation.

Statistical analyses

All chemotaxis experiments had four biological replications. Full factorial ANOVA was performed using RStudio Version 0.99.893 – © 2009–2016 RStudio, Inc.. The input data consisted of the distances (measured in mm) travelled by the bacterial strains in two

directions (towards and away). The confidence interval of 95% was used in all the analyses.

Results

Chemotactic behaviour of fungal–interactive Burkholderia terrae strains exposed to various concentrations of oxalic acids

To understand the ecological behaviour of fungal–interactive *B. terrae* strains, we tested the chemotactic responses of *B. terrae* strains BS001, BS110 (Fig. 6.1), BS007, BS437 (Fig. 6.2), 17804^T and BS001–Δ*sctD* (Fig. 6.3), on M9 agar for testing of swimming motility (M9A–S), supplemented with 0.5% glycerol as the carbon source. Oxalic acid in different concentrations [0.1%, 0.5%, 0.8% and 0% (control)] was used as the chemoattractant. Full factorial ANOVA (Supplementary Table S6.1) of the collective data suggested that the factors oxalic acid level and movement (towards/away) were the key drivers of the chemotactic responses of all *B. terrae* strains, including the type strain. First, without any added oxalate, the swimming behaviour of all strains was 'random' in the sense that movement 'towards' and 'away' were never different ($P > 0.05$). Second, the chemotactic responses of all strains towards the oxalic acid source was significantly faster and higher ($P < 0.05$) than away from it, clearly suggesting that positive chemotaxis took place. Remarkably, the responses were stronger at the low concentration of oxalic acid (0.1%) than at the two higher concentrations ($P < 0.05$).

The chemotactic responses of fungal–interactive Burkholderia terrae strains towards exudates of Lyophyllum sp. strain Karsten and T. asperellum 302

We tested the chemotactic responses of all fungal–interactive *B. terrae* strains as well as *B. terrae* type strain 17804^T when confronted with exudates from *Lyophyllum* sp. strain Karsten, as well as *T. asperellum* 302, at different levels (0, 1:10 diluted, 1:100 diluted and full–strength). First, the swimming behaviour of all strains in response to the control stripes (0%) was undirected, as they apparently explored the milieu around the inoculum similarly in both directions (towards and away; $P > 0.05$). Interestingly, all *B. terrae* strains showed a strong chemotactic response towards the M9 agar stripes containing fungal exudates (full–strength, 1:10 diluted and 1:100 diluted), as compared to the movement away ($P < 0.05$). A full factorial ANOVA (Supplementary Table S6.2) of the data showed that the movement direction (towards/away), fungal strain, bacterial strain and the exudate level in the stripes were the driving factors of the chemotactic behavior of all *B. terrae* strains (including 17804^T). The chemotactic responses were more stronger towards the exudates of *Lyophyllum* sp. strain Karsten compared to those of *T. asperellum* 302 (data of all strains are shown in Fig. 6.1, 6.2 and 6.3). There were differences in the movement of the strains towards the exudates (full–strength) of both fungi, with strains BS110, BS437, BS001, and BS007 being more 'proactive' than strain 17804^T.

With respect to *T. asperellum* 302, the different *B. terrae* strains responded differently to the same concentrations of the exudates, with strains BS110, BS437, BS001, and

BS007 being 'better' responders than strains 17804^T and BS001–Δ*sctD*. ANOVA (Supplementary Table S6.2) of the data revealed that three factors, i.e. exudate level, taxis direction (towards/away) and *B. terrae* strain, were the key variables that drove the chemotactic responses. Thus, there were significant differences ($P < 0.05$) in the responses of all *B. terrae* strains towards full–strength exudates (1%) compared to the diluted ones (0.01% and 0.1%) and the control (0%). At higher concentration (1%) the bacterial taxis was significantly one–directional (towards the stripes) and stronger ($P < 0.05$).

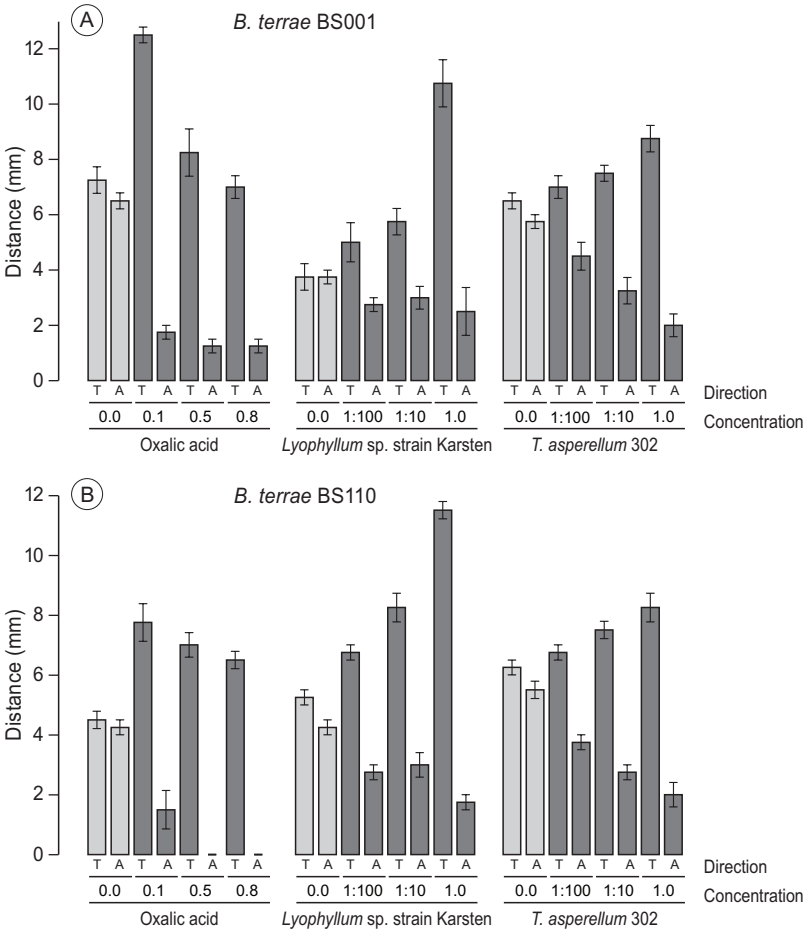

Figure 6.1 Chemotaxis (swimming) of *B. terrae* strains BS001 and BS110 towards oxalate and exudates of *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 on 0.25% [w/v] M9 medium (A) shows the response of *B. terrae* BS001 to either oxalate or exudates of both fungal strains. (B) shows the response of *B. terrae* BS110 either oxalate or exudates of both fungal strains The Y–axis represent distance in mm, whereas X–axis show the direction of taxis ('towards' is denoted by 'T' and 'away' by 'A') and concentration of oxalate or levels of fungal exudates.

Identification and quantification of oxalic acid in the exudates of Lyophyllum sp. strain Karsten and T. asperellum 302

Previous work (Boersma et al. 2010) has analyzed *Lyophyllum* sp. Karsten exudates by ^{1}HNMR, however this method cannot detect oxalic acid. Hence, here we examined the exudates of *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 for the presence of oxalic acid using HPLC analyses over a C18 reverse phase column (id 5 m; d 4.6×250–mm). Control runs of progressively increasing levels of pure oxalic acid revealed the appearance of progressively bigger peaks, at retention time 2.4 min, in the resulting chromatograms (Supplementary Fig. S6.1). The exudates from both fungi then showed the

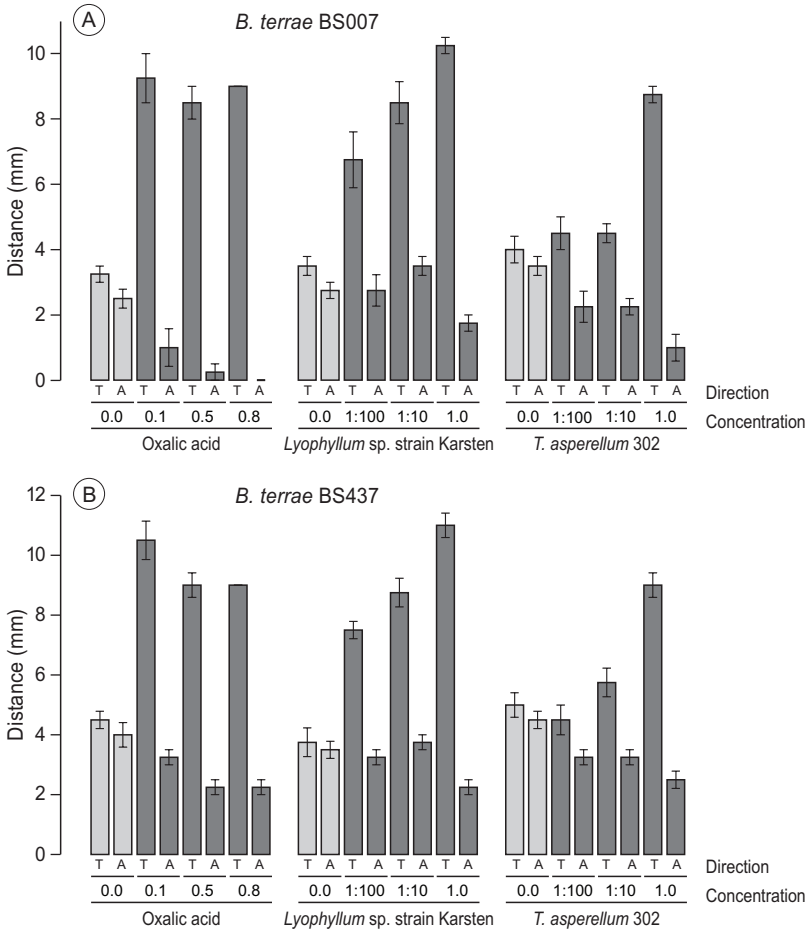

Figure 6.2 Chemotactic (swimming) behaviour of *B. terrae* strains BS007 and BS437 towards oxalate and exudates of *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 on 0.25% [w/v] M9 medium (A) shows the response of *B. terrae* BS007 to either oxalate or exudates of both fungal strains. (B) shows the response of *B. terrae* BS437 either oxalate or exudates of both fungal strains The Y–axis represent distance in mm, whereas X–axis show the direction of taxis (towards denoted by 'T' and away denoted by 'A') and concentration of oxalate or levels of exudates.

appearance of peaks at the exact same retention time. Quantification of the peaks revealed that considerable amounts of oxalic acid had been released by both fungi into the propionate–amended M9 medium. The final oxalic acid levels were in the range 0.075–0.093 % [w/v] (Table 6.1). To validate the identification of the respective peaks in the chromatogram, we spiked the respective fungal exudates with 0.1% [w/v] of oxalic acid in the same HPLC run (Supplementary Fig. S6.1). Indeed, the added 0.1% oxalic acid about doubled the peak weight and so the measured amount of oxalic acid. Together, our results suggested that oxalic acid is indeed released by both fungal types, in similar quantities, under the applied conditions.

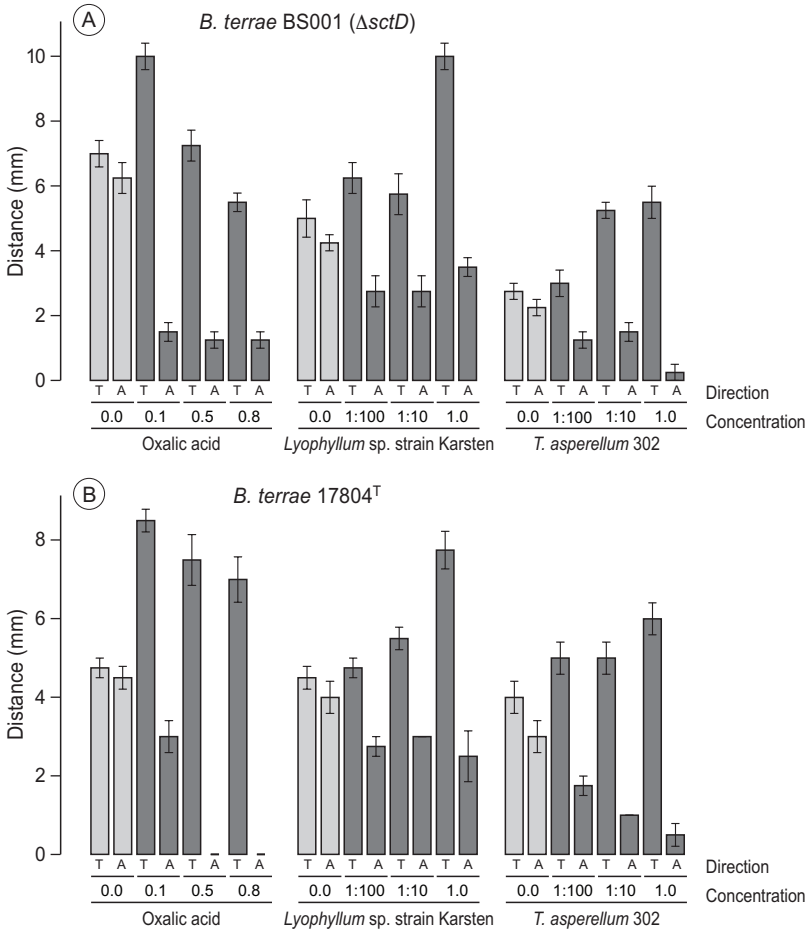

Figure 6.3 Chemotactic (swimming) behaviour of *B. terrae* strains BS001–Δ*sctD* and 17804^T towards oxalate and exudates of *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 on 0.25% [w/v] M9 medium (A) shows the response of *B. terrae* BS001–Δ*sctD* to either oxalate or exudates of both fungal strains. (B) shows the response of *B. terrae* 17804^T either oxalate or exudates of both fungal strains The Y–axis represent distance in mm, whereas X–axis show the direction of taxis (towards denoted by 'T' and away denoted by 'A') and concentration of oxalate or levels of exudates.

Table 6.1 Quantitative analysis of the exudates of *Lyophyllum* sp. strain Karsten and *Trichoderma asperellum* 302 for the identification of oxalic acid, using high performance liquid chromatography (HPLC). Oxalic acid was used as an external standard and M9 medium as a negative control.

Compounds	Retention Time (min)	Area	Height	Concentration % [w/v]
MilliQ	0.00	0	0	0.000
Oxalic acid_0.01%	2.48	386101	27450	0.010
Oxalic acid_0.01%	2.48	369726	27375	0.010
Oxalic acid_0.05%	2.39	5071290	562392	0.051
Oxalic acid_0.05%	2.39	5033482	567899	0.051
Oxalic acid_0.10%	2.39	9400274	1084105	0.099
Oxalic acid_0.10%	2.38	9960133	1074788	0.102
M9 medium	2.37	86273	12120	0.001
M9 medium	2.37	103940	14645	0.001
M9+0.1% Oxalic acid	2.37	8007944	1290413	0.092
M9+0.1% Oxalic acid	2.38	8026472	1301304	0.092
Lyophyllum sp. strain Karsten	2.41	7795023	719831	0.090
Lyophyllum sp. strain Karsten	2.41	6417830	710125	0.074
Lyophyllum sp. strain Karsten 1:5	2.36	3554342	518881	0.041
Lyophyllum sp. strain Karsten 1:5	2.36	3243405	494120	0.037
Lyophyllum sp. strain Karsten 1:10	2.36	2205528	388395	0.025
Lyophyllum sp. strain Karsten 1:10	2.35	2217147	359645	0.025
T. asperellum 302	2.40	8137144	683484	0.093
T. asperellum 302	2.40	7592703	680421	0.087
T. asperellum 302 1:5	2.36	1576896	286151	0.018
T. asperellum 302 1:5	2.35	1500720	262248	0.017
T. asperellum 302 1:10	2.36	847009	162324	0.010
T. asperellum 302 1:10	2.35	848337	162203	0.010

^{1}H NMR spectra based analyses of exudates released by Lyophyllum sp. strain Karsten in propionate–amended M9 medium

To underpin the chemotaxis findings, we then re–analyzed the exudates of *Lyophyllum* sp. strain Karsten, using ^{1}H NMR. The resulting spectra had various peaks, indicating that *Lyophyllum* sp. strain Karsten had released various compounds. The control M9 +propionate medium, as well as that containing potential fungal exudates, both showed two large peaks at 1.04 and 2.16 ppm, respectively, which were attributed to propionate (Fig. 6.4A). The potential exudate spectra further revealed a multiplet corresponding to glycerol (from 3.54 to 3.80 ppm). Expectedly, acetic acid (1.91 ppm) and formic acid (8.44 ppm) were also detected (Fig. 6.4B). Moreover, another multiplet (2.51; 2.54 and 2.65; 2.67 ppm) was identified as citric acid. Finally, a peak probably representing fumaric acid (6.51 ppm) stood out. Both citric and fumaric acid had not been detected previously (Boersma et al. 2010). Several other small peaks could not be attributed to

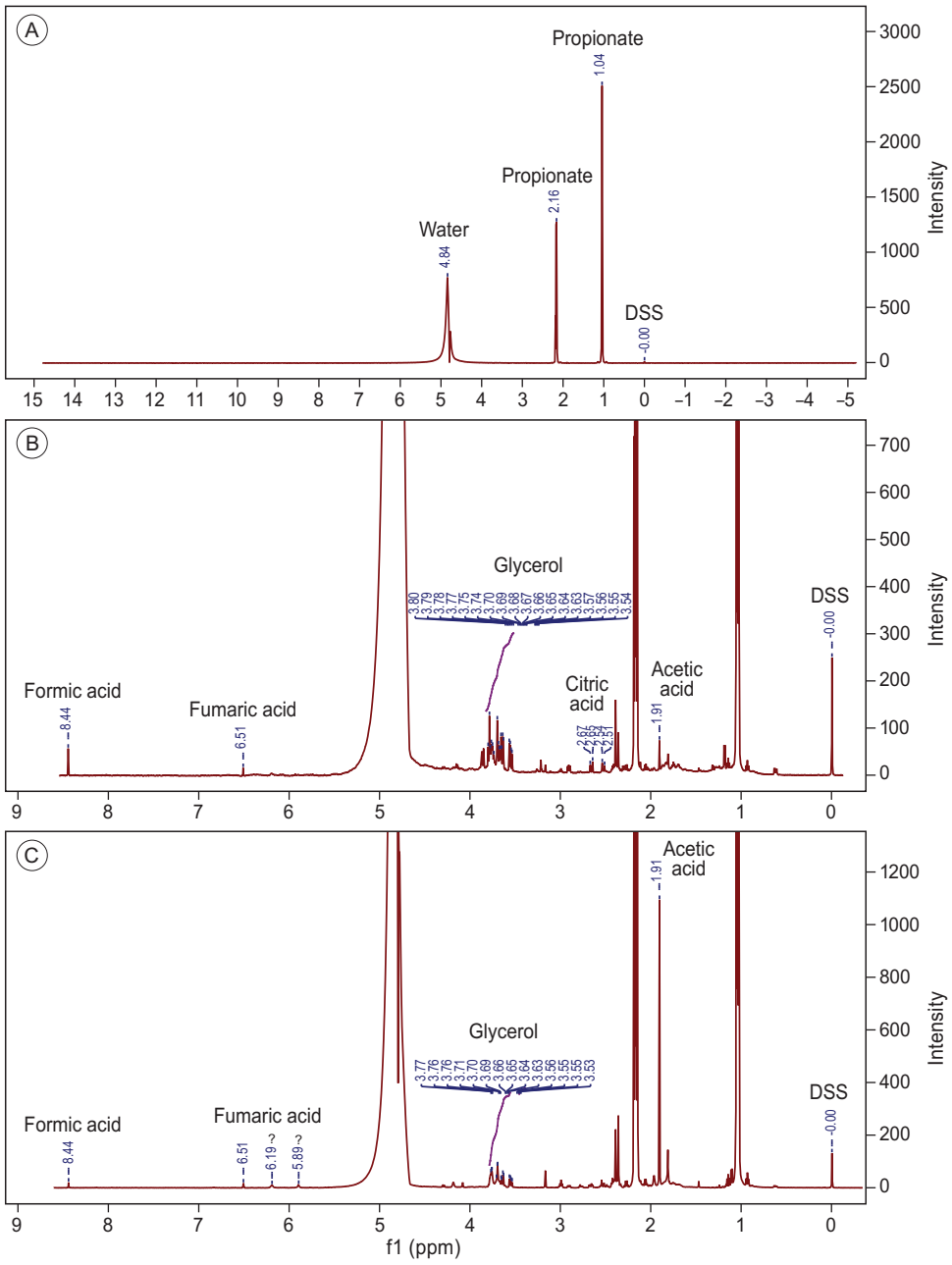

Figure 6.4 Proton NMR spectra of M9 medium, and fungal exudates (A) represents M9 spectrum showing peaks corresponding to water and propionate. (B) shows full spectrum of exudates of *Lyophyllum* sp. strain Karsten (C) depicts full spectrum of exudates of *T. asperellum* 302. 3–(trimethylsilyl)–1–propanesulfonic acid sodium salt (DSS) was used as the internal standard (0.0 ppm). Corresponding peaks of compounds in exudates of *Lyophyllum* sp. strain Karsten (B) and *T. asperellum* 302 (C) are annotated with their names. Unknown peaks are denoted by (?).

any known compound or metabolite. Thus *Lyophyllum* sp. strain Karsten releases compounds/metabolites into its milieu that might be used by the fungal–interactive bacteria *B. terrae* BS001, BS110, BS007, and BS437 as potential carbon sources.

Analysis of the exudates released by Trichoderma asperellum 302 into M9 medium using ^{1}H NMR

We then analyzed the exudates of *T. asperellum* 302, using ^{1}H NMR, in order to find any commonalities and/or differences with the exudates of *Lyophyllum* sp. strain Karsten. First, exudation of glycerol by *T. asperellum* 302 was confirmed by the finding of a multiplet in the range of 3.53 to 3.77 (Fig. 6.4C). Then, we also found peaks representative of acetic acid (1.91 ppm) and formic acid (8.44 ppm). Interestingly, fumaric acid (6.51 ppm) was also detected. Despite these broad commonalities in the metabolites released by both fungi, there were two unknown peaks (5.89 and 6.19 ppm), which were absent in the spectra obtained with *Lyophyllum* sp. strain Karsten. On the other hand, we did not find the multiplet representing citric acid. Thus the ecological opportunities offered by *T. asperellum* 302 to associated *B. terrae* strains were to a considerable extent similar to those of *Lyophyllum* sp. strain Karsten, with some conspicuous differences.

Growth of Burkholderia terrae BS001 on oxalate supplemented M9 medium

We then tested whether or not oxalic acid could serve as a sole carbon and energy source for the growth of *B. terrae* BS001 in M9 medium. M9 medium was used as the negative and M9 medium supplemented with glucose as the positive control. The negative control did not support growth of strain BS001, as the CFU counts – from the initial 5.30 log 10 CFU/mL – increased only slightly over the 66 h incubation time, indicating residual growth (Fig. 6.5A). *B. terrae* BS001 grown in M9+0.5% glucose showed a significantly higher final population density, of 7.89 log 10 CFU/mL after 66 h of incubation. On 0.5% oxalate, we observed a progressive increase in the initial cell density, from about 5.30 log 10 CFU/mL to 6.20 log 10 CFU/mL (after 18 h), to 7.68 log 10 CFU/mL (42 h) and back to 6.86 log 10 CFU/mL at 66 h of incubation (Fig. 6.5A). On M9 medium supplemented with 0.1% oxalate [w/v], the CFU/mL of strain BS001, from an initial 5.30 log 10 CFU/mL reached 7.33 log 10 CFU/mL after 66 h of incubation (Fig. 6.5B). The overall results suggest that *B. terrae* BS001 can use oxalic acid as a carbon and energy source when supplied as a single carbonaceous compound in M9 medium.

Oxalate utilization genetic potential of B. terrae strains

We analyzed the genomes of *B. terrae* BS001, BS110, BS437, and BS007 to find genes potentially involved in the utilization and uptake of oxalic acid or its complexes.

Although we did not find any genes (as per the annotation) encoding enzymes involved in pathway I, we found genes encoding the enzymes involved in pathways II and III of the oxalate degradation in all *B. terrae* strains (Table 6.2). Interestingly, we did find a cluster of five genes, which was recently reported to be upregulated in the transcriptome of strain BS001 during its interaction (physical contact stage) with *Lyophyllum*

sp. strain Karsten (Haq et al. in review; chapter 4), across all *B. terrae* strains. The cluster contains a gene encoding cupin protein that was postulated to be acting as oxalate oxidase and/or oxalate carboxylase and may potentially be involved in the degradation of oxalate to formate and H_2O_2 as per the pathway IV and V.

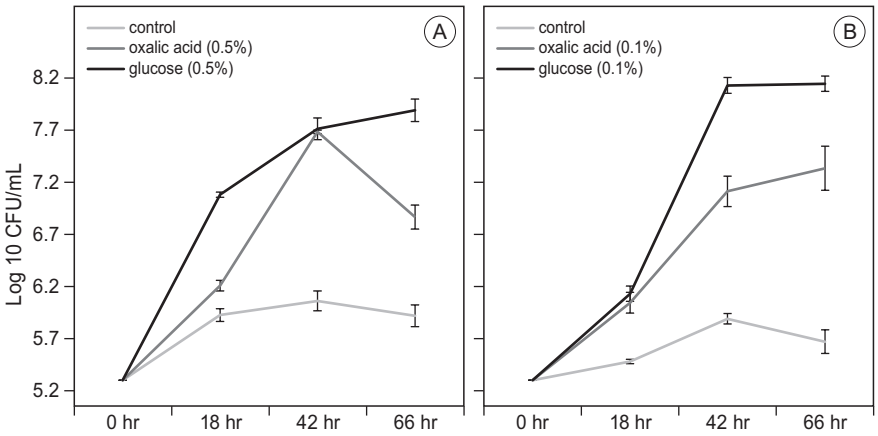

Figure 6.5 Growth curve of *B. terrae* BS001 grown on M9 medium. (A) supplemented with 0.5% of glucose, 0.5% oxalate [w/v] and no carbon source. (B) represents the growth patterns of *B. terrae* BS001 on M9 medium supplemented with 0.1% of glucose, 0.1% oxalate [w/v] and no carbon source. Y-axis represented CFU/mL on log 10 scale while X-axis shows the times in hours.

Table 6.2 The list of oxalate degrading pathways according to MetaCyc. The potential presence (+) or absence (−) of oxalate degradation pathways is shown in the respective *Burkholderia terrae* strains. Cupin domain containing proteins are postulated to be acting either as oxalate oxidase or oxalate decarboxylase.

Strains	Pathway I [(a)]	Pathway II [(b)]	Pathway III [(c)]	Pathway IV [(d)]	Pathway V [(e)]
Burkholderia terrae BS001	–	+	+	±	±
Burkholderia terrae BS110	–	+	+	±	±
Burkholderia terrae BS007	–	+	+	±	±
Burkholderia terrae BS437	–	+	+	±	±
Burkholderia terrae 17804^T	N/A	N/A	N/A	N/A	N/A

N/A= not applicable '+' indicates presence '–' indicates absence '±' presence or absence (cupin domain containing proteins; described down the line).
(a) Single reaction pathway that involves the direction conversion of oxalate to two CO_2 molecules that is catalyzed by oxalate oxidoreductase in the presence of two oxidized ferredoxin [iron-sulfur] cluster.
(b) Consists of three reactions resulting in the conversion of oxalate to formate, involving oxalate CoA-transferase, oxalyl-CoA decarboxylase and formyl-CoA transferase.
(c) The third pathway concerning oxalate degradation has two possible outcomes, where oxalyl-CoA is either converted to formate and subsequently to NADH or to glyoxalate that goes to glycolate and glyoxalate degradation pathway.
(d) The fourth pathway involves only one reaction where oxalate is converted to hydrogen peroxide via oxalate oxidase.
(e) The fifth pathway involves only one reaction where oxalate is converted to formate via oxalate decarboxylase.
Cupin: Either acting as oxalate oxidase (monocupin) or oxalate decarboxylase (bicupin) (Tanner et al. 2001). Based on the presence of cupin in the genomes of *B. terrae* strains they are proposed to have roles in oxalate degradation.

Discussion

The ecological benefits offered by soil fungi to bacteria living in their vicinity are well documented (Warmink and van Elsas 2009, Nazir et al. 2010, De Boer et al. 2005). One model 'system', featuring *B. terrae* BS001 and a range of fungi (Nazir et al. 2014), including *Lyophyllum* sp. strain Karsten, has previously been interrogated with respect to the potential mechanisms involved (Warmink and van Elsas 2008, Warmink and van Elsas 2009, Nazir et al. 2013, Haq et al. 2014). However, questions regarding other fungal–interactive *B. terrae* strains, such as BS110, BS007, and BS437, have remained unanswered. Moreover, any information regarding the type strain 17804^T has been lacking so far. In this study, we specifically looked at the chemotactic behaviour of a range (BS110, BS007, BS437, BS001, Δ*sctD*, and 17804^T) of *B. terrae* strains towards oxalic acid as well as exudates from the basidiomycete *Lyophyllum* sp. strain Karsten and the ascomycete *T. asperellum* 302. We also analyzed the fungal exudates using proton ^{1}H NMR and HPLC, in order to obtain insight into the compounds that are released by these fungi.

Concerning the role of oxalic acid for the behaviour of the tested *B. terrae* strains, we found that all *B. terrae* strains responded significantly faster at lower concentration than at higher concentrations. Thus oxalic acid may act as a signal molecule at lower concentrations and the signalling function is reduced at higher levels. Similar observations have been reported for other bacterial species. For instance, in *C. fungivorans* Ter331 an increasing concentration of oxalic acid in the medium prompts a lethargic response, compared to a more active one, at lower concentration (Rudnick et al. 2015).

The conundrum whether oxalic acid (or [sodium] oxalate, i.e. acid–base neutralized during pH adjustment) is a signalling molecule or a potential carbon source (or a two–sided sword dependent on the local concentration), may become solved, as we found *B. terrae* BS001 to grow on M9 medium supplemented with defined concentrations of oxalic acid. This in contrast to previous findings where *B. terrae* strains were reportedly unable to utilize oxalate (Nazir et al. 2012). However, using the Biolog assay, Nazir et al. (2012) showed that all *B. terrae* strains were able to utilize citric acid, acetic acid and formic acid, whereas they did not report on the utilization of fumaric acid. Kost et al. (2014) reported that a range of plant–beneficial *Burkholderia* species, including *B. phytofirmans*, *B. phymatum*, *B. terricola*, *B. kururiensis*, *B. phenoliruptrix*, *B. tuburum*, *B. xenovorans*, *B. tropica*, *B. hospita*, *B. graminis*, *B. fungorum*, *B. bryophilla*, *B. caribiensis*, *B. caldonica*, *B. glathei*, *B. sordidicola*, *B. caryophylli* and *B. sacchari*, were able to grow on medium containing oxalic acid as a sole carbon source. However, the plant–pathogenic (*B. glumae*, *B. gladioli*, *B. plantarii*) and human pathogenic strains (*Burkholderia cepacia* complex) did not use oxalate as a carbon source. We posit that *B. terrae* BS001, on the basis of its oxalate degradation potential (Table 6.2), is able to use oxalic acid as the sole carbon and energy source. Given the rather poor energetic value of oxalate (Schneider et al. 2012), its utilization may be most prominent under conditions of severe nutrient scarcity (yielding 'less privileged' niches) in soil. This seems plausible, as fungi in soil (Dutton and Evans 1996, Heller and Witt–Geiges 2013) release oxalate as a virulence

factor and as a helper in lignin degradation. Plants, on the other hand, (Franceschi and Nakata 2005) use it for calcium storage and as a herbivore repelling agent. Oxalate also serves plants to tolerate metals such as aluminum (Klug and Horst 2010). Given the potentially abundant availability of oxalate in such niches, it probably plays a major role in the selection of bacterial species with oxalotrophic traits.

Boersma et al. (2010) previously quantified the glycerol levels in the exudates of *Lyophyllum* sp. strain Karsten, and found levels of approximately 2 mM. Here, the estimated oxalic acid levels were approximately 7.5–7.9 mM, or three– to fourfold more. The fact that glycerol holds more energy than oxalate, and can easily diffuse (passively) into cells might suggest it as a preferred carbon/energy source, although this contention needs proof in future work. Thus, oxalate might have a 'secondary' feeding function for *B. terrae* BS001. On the other hand, the signalling function of oxalic acid cannot be excluded. As described, genetic systems that potentially enable the catabolism of oxalate exist in strain BS001's genome and such systems were expressed when it confronted with *Lyophyllum* sp. strain Karsten on soil extract agar (Haq et al. in review; chapter 4).

Considering the fact that also in the soil, the two fungi are expected to release small carbonaceous compounds like oxalic acid and glycerol, we analyzed the chemoattractant action of exudates of the two test species. Remarkably, all *B. terrae* strains, including type strain 17804^{T}, moved in a concerted fashion towards full–strength exudates of both fungi, and to a lesser extent to the diluted ones. The fact that the migrational behavior of the type strain was so similar to that of the other *B. terrae* strains indicated that *B. terrae*, in contrast to what was previously thought, might constitute a behaviorally – 'tight' group of organisms. This stands in contrast to the hypothesis that strain BS001 and its relatives are 'outliers' within this species, as – in contrast to the type strain – they were isolated on the basis of their fungal interactivity. Furthermore, given the fact that fungi like *Lyophyllum* sp. strain Karsten in soil likely release various C compounds, fungal–responsive bacteria (broadly speaking, the species *B. terrae*) adapted to get the ecological benefits for growth and survival. Conversely, the fungi themselves may profit by an increased level of protection (Nazir et al. 2014) or by getting easy access to an enhanced food source (Pion et al. 2013). Oxalate/oxalic acid exuded by fungi in soil may thus be used by the locally occurring *B. terrae* cells as a signalling molecule to locate the fungal source for access to nutrients of higher value such as glycerol, citric acid, acetic acid and formic acid, among others.

The fact that both *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 possessed a similar metabolic palette corroborates previous findings by Nazir et al. (2014), who found that *B. terrae* BS001 could interact with these two fungi, next to a suite of other ones. We here extended this broad–range fungal interactivity to a set of other *B. terrae* strains, including the type strain. Plausible concepts to explain these findings are (1) different fungal strains share commonalities in terms of the chemical milieu surrounding them that are 'appreciated' by *B. terrae* strains and so the latter can be characterized as 'generalist' fungal interactors and (2) the bacteria are endowed with genetic potential that allow them to utilize the metabolites offered by the fungi. Indeed, in all *B. terrae*

strains, systems for the catabolism and uptake of oxalate were found. The presence of genes encoding two key enzymes of the degradation pathways II and III, and the presumed role of the 'cupin' (oxalate oxidase/carboxylase) suggest that all strains have the potential to utilize oxalate. However, with respect to the latter, further experiments would be needed to have deeper insight into the specific role of the cupin protein.

Concluding, our results confirm that the chemical conditions offered by two diverse soil fungi had key commonalities. In the light of the finding of oxalate, glycerol, three organic acids (next to some unknown compounds), these conditions are of complex nature. Yet, they offer the baseline against which interactions of the two fungi with *B. terrae* take place. Some of the fungal–released compounds, i.e. oxalic acid, at a particular concentration might have a primary role of attracting the bacterial cells, via a chemotactic response, to micro–habitats with raised metabolite loads. In such a scenario, the attracted bacterial cells would utilize other fungal–secreted metabolites of higher nutritional value, such as glycerol. However, upon depletion of such energy–rich exuded compounds, the *B. terrae* strains may start to generate energy by catabolizing oxalic acid and its (oxalate) complexes.

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Supplementary tables

Table S6.1 Full factorial ANOVA testing for the effects of bacterial strains, oxalic acid concentration, movement direction, and their interaction on the distance travelled, in the chemotaxis experiment on M9 medium.
Statistical significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Code
Direction	1	1441.0	1441.0	524.742	< 2e-16	***
Strains (bacterial)	5	88.0	17.6	6.409	1.78e-05	***
Oxalic acid conc.	1	59.4	59.4	21.642	6.63e-06	***
Direction:Strains (bacterial)	5	18.5	3.7	1.346	0.247477	
Direction:Oxalic acid conc.	1	103.4	103.4	37.636	5.92e-09	***
Strain (bacterial) :Oxalic acid conc.	5	71.3	14.3	5.190	0.000187	***
Direction:Strains (bacterial):Oxalic acid conc.	5	28.4	5.7	2.065	0.072246	.
Residuals	168	461.4	2.7			

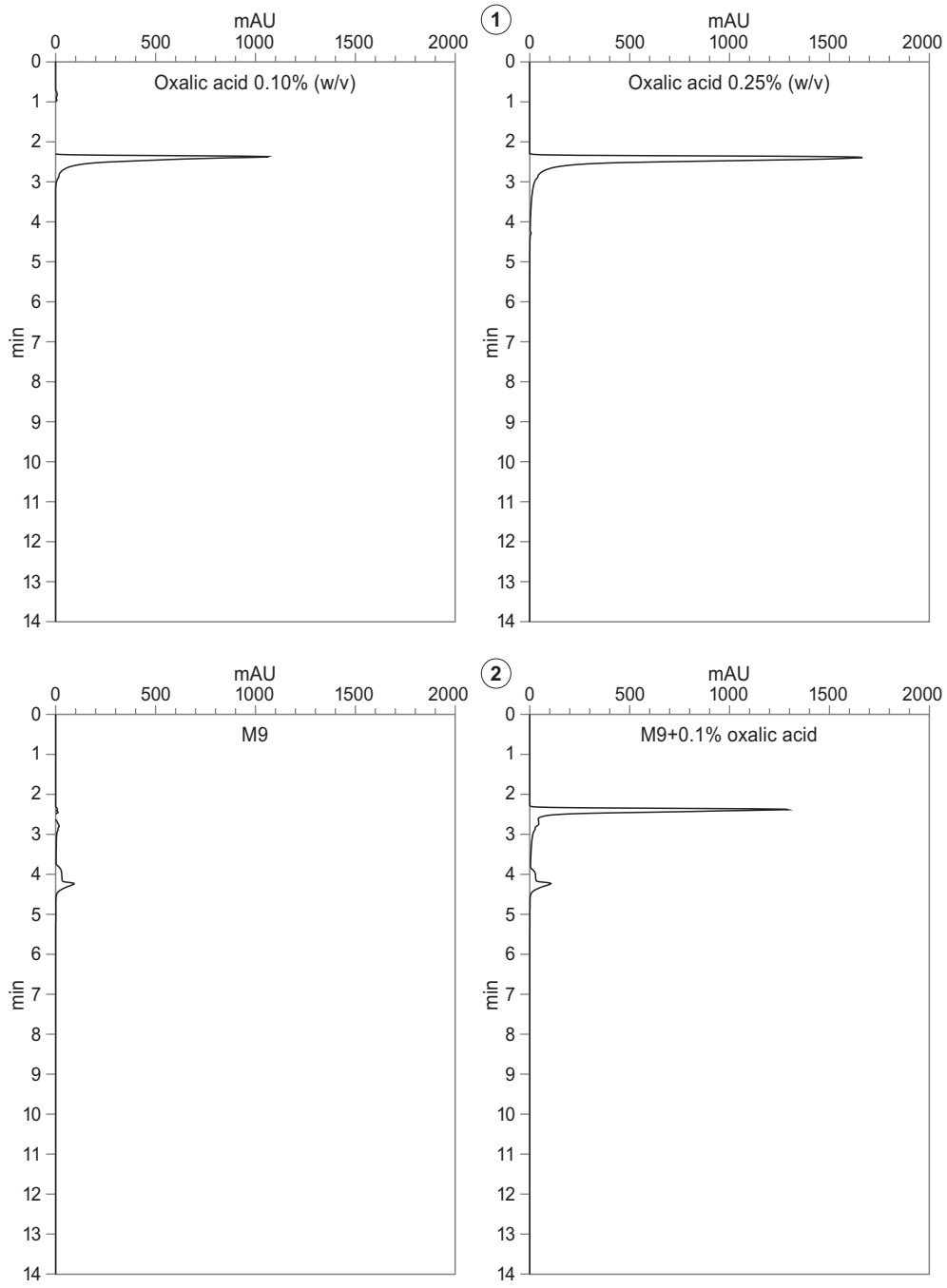
Table S6.2 Full factorial ANOVA testing for the effects of bacterial strains, fungal strains, exudates levels (conc.), movement direction, and their interaction on the distance travelled in the chemotaxis experiment on M9 medium.
Statistical significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Code
Direction	1	1211.3	1211.3	1,085.545	< 2e-16	***
Strains (bacterial)	5	146.2	29.2	26.207	< 2e-16	***
Exudates conc.	1	85.8	85.8	76.935	< 2e-16	***
Fungal strains	1	49.6	49.6	44.446	1.07e-10	***
Direction: Strain (bacterial)	5	21.6	4.3	3.869	0.00201	**
Direction:Exudates conc.	1	424.3	424.3	380.249	< 2e-16	***
Strain (bacterial) :Exudates conc.	5	10.8	2.2	1.939	0.08738	.
Direction: Fungal strains	1	11.3	11.3	10.166	0.00156	**
Strain (bacterial): Fungal strains	5	98.4	19.7	17.633	1.62e-15	***
Exudates conc. : Fungal strains	1	39.8	39.8	35.631	6.06e-09	***
Direction:Strain (bacterial):Exudate conc.	5	16.6	3.3	2.982	0.01195	*
Direction:Strain (bacterial): Fungal strains	5	21.6	4.3	3.876	0.00198	**
Direction:Exudates conc.: Fungal strains	1	4.7	4.7	4.178	0.04174	*
Strain (bacterial):Exudates conc.: Fungal strains	5	18.1	3.6	3.249	0.00703	**
Direction:Strain (bacterial):Exudates conc.: Fungal strains	5	6.5	1.3	1.157	0.33006	
Residuals	336	374.9	1.1			

Supplementary figure

Figure S6.1 The high performance liquid chromatography (HPLC) chromatograms representing oxalic acid peaks of:

1. Oxalic acid (0.1% and 0.25% [w/v]) as a positive control.
2. M9 medium as a negative control and M9 medium spiked with oxalic acid (0.1% [w/v]).
3. *Lyophyllum* sp. strain Karsten exudates and *Lyophyllum* sp. strain Karsten exudates spiked with oxalic acid (0.1% [w/v]). Note: 'sample A' in the file means *Lyophyllum* sp. strain Karsten exudates sample 'A'.
4. *Trichoderma asperellum* 302 exudates and *T. asperellum* 302 exudates spiked with oxalic acid (0.1% [w/v]). Note: 'Tri B' in the file means *T. asperellum* 302 exudates 'B'.
5. At the end the calibration curve is provided.


1
mAU
0
500
1000
1500
2000
Oxalic acid 0.10% (w/v)
Oxalic acid 0.25% (w/v)
min
0
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4
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6
7
8
9
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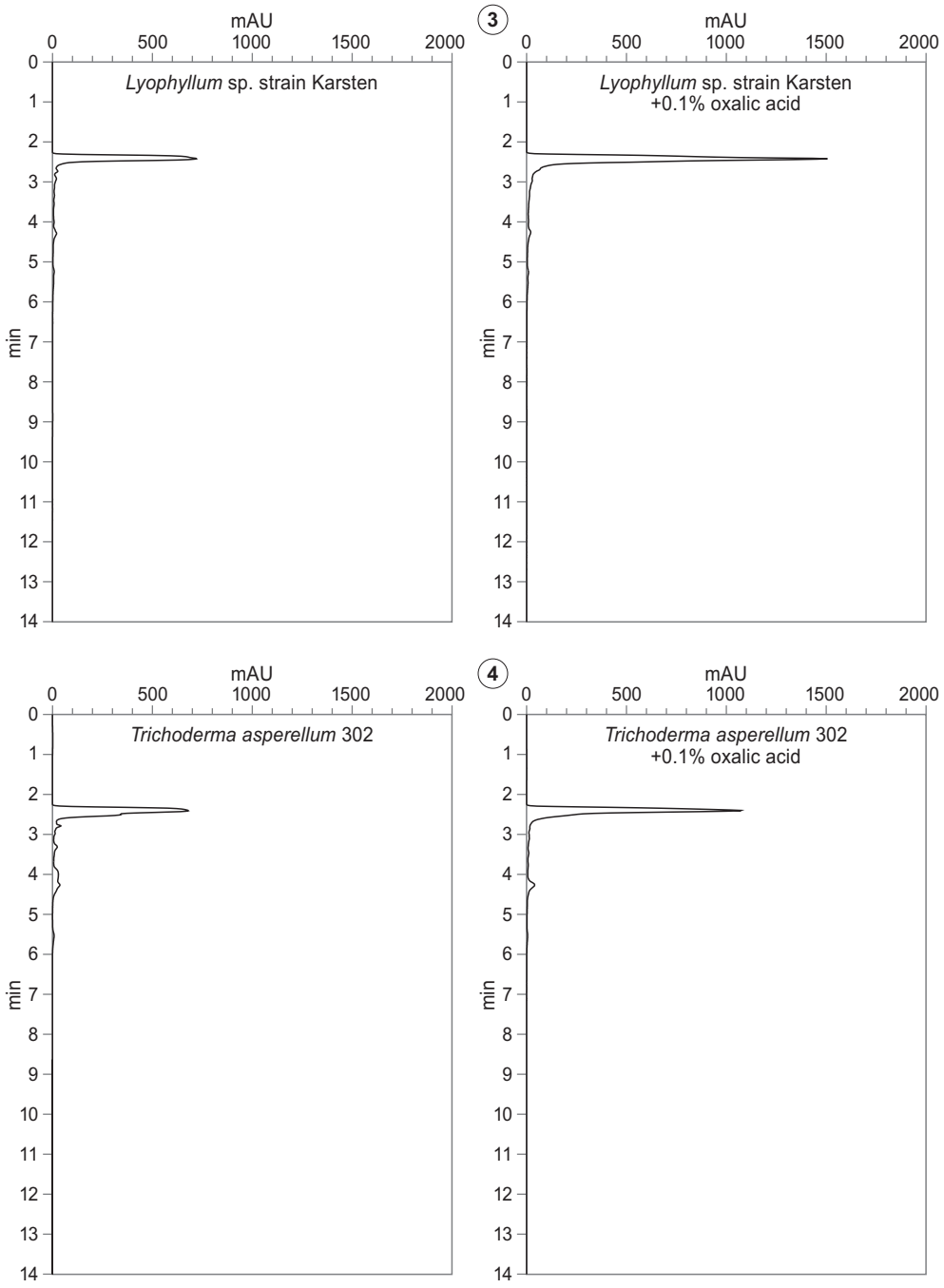
11
12
13
14
2
M9
M9+0.1% oxalic acid


3
mAU
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500
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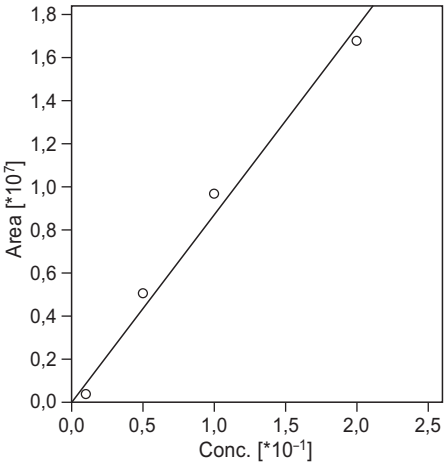
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Lyophyllum sp. strain Karsten
Lyophyllum sp. strain Karsten
+0.1% oxalic acid
4
Trichoderma asperellum 302
Trichoderma asperellum 302
+0.1% oxalic acid

ID# : 1
Name : Oxalic acid
Quantitative Method : External Standard
Function : f(x)=8,70380e+007*x+0
Rr1=0,9934920 Rr2=0,9870263 RSS=2,094493e+012
MeanRF: 7,987142e+007 RFSD: 2,688731e+007 RFRSD: 33,663247
FitType : Linear
ZeroThrough : Through
Weighted Regression : None
Detector Name : PDA



#	Conc.(ratio)	MeanArea	Area
1	0,01	377914	386101 369726
2	0,05	5052386	5071290 5033482
3	0,10	9680204	9400274 9960133
4	0,20	16768910	16764882 16772938

Chapter 7

7 General discussion and outlook

Irshad Ul Haq

The soil environment as a matrix for microbial life

Structurally, soil is an environment that developed to its current form of solids and pores as a result of mechanical, physical, chemical, and biological processes that acted together. Soil therefore varies spatially and temporally, making it a complex environment for the inhabiting microbiota (Standing and Killham 2007). This concerns in particular the local soil bacteria, in their efforts to reach resources. Basically, soil bacteria are confined to the microhabitats they inhabit, and their exploration of other microsites, even when nearby, is often spatially–limited by physical barriers that limits their migration. A second key issue is the general recalcitrancy of carbonaceous soil substrates to degradation. The two phenomena lead to a generic scarcity of easily–available and degradable C (and energy) sources for heterotrophic bacteria. This results in (average) slow or arrested bacterial growth and survival of cells in any state of 'dormancy'. Other factors that – directly or indirectly – exert pressure on the soil bacteria is the water potential of soil. The water potential, which is 'inversely congruent' with the water level in soil, is key to the motility of bacterial cells (both passive and active), the diffusion of nutrients, pH, (buffering of) temperature and aeration. Regarding the latter, the activity of microbes in soil is clearly determined by the soil's redox potential, which has a strong relationship with the local availability of oxygen. However, according to Standing and Killham (2007), soil–microbe relationships are not simply one–way, but they should be looked at as a two–way process, in which "the soil shapes the microbes and the microbial activity shapes the soil habitats". I here posit that the major challenge to bacterial life in soil is the necessity to be vigilant to adverse conditions, and thus the 'holy grail' of soil bacteria is their key ability to (continuously) monitor, explore and adapt to the local soil conditions.

Soil habitats and the role of fungi in supporting bacterial life

The living soil encompasses a system hosting a plethora of life forms, which – next to soil bacteria – also includes Eukarya such as plants and fungi. Whereas plants are clearly important primary producers, soil fungi are considered to be extremely important biological forms that are involved in major processes of organic matter decomposition and mineralization (Finlay 2007). A particular feature of the soil fungi is their capacity to cross the physical barriers of soil, mainly the air gaps that form barriers to the soil connectivity. Such gaps prevent even motile bacteria from moving far in the soil matrix. In effect, the soil fungi are able to form a connected matrix of hyphae (hyphal network), establishing bridges over the disconnected microsites (Kohlmeier et al. 2005). This property makes them very successful in exploring niches in soil, such as those under the influence of plant roots – the rhizosphere. As brought up in the introduction to this thesis, the interconnectivity of plant roots and the hyphae of plant–root–associated fungi (mycorrhizal fungi) results in a habitat that is under the combined influence of fungi and plants [denoted the mycorrhizosphere]. An enhanced microbial activity is expected to occur in this sphere (De Boer et al. 2005). Next to the mycorrhizosphere, other, rather

transient, zones of microbial activity occur at fungal hyphae such as under mushroom feet or directly at the hyphal network [denoted the mycosphere].

Given their ability to connect soil microsites, next to their capacity to spur microbial activity as a result of the provision of resources, fungi can be seen as key 'rescuers' of soil bacteria in terms of the ecological opportunities they appear to offer. For instance, some bacteria may actually rely to a large extent on their ability to establish relationships with soil fungi, in order to explore the hospitable microhabitats in the soil that are beyond their reach without a hyphal network. Such a capacity may support their survival and dispersal over large patches of area in the soil and not just confined to a ~5– m soil aggregate located microsite. Thus, for particular heterotrophic soil bacteria, the answer to C starvation, with respect to survival, dispersal and sustainability in soil, may well lie in their interactive capabilities with divergent soil partners, including fungi (as well as with plants).

The complexity of bacterial–fungal interactions in soil and the efforts to understand them

The field of bacterial–fungal interactions (BFI) is relatively nascent in terms of the understanding of the underlying principles. However, as I discussed in the previous chapters of this thesis, in particular **chapter 2**, key assets of the BFI in soil have already been pinpointed. For instance, several fungal–associated bacteria have been shown to benefit from the ecological conditions offered by their fungal hosts, thus establishing successful symbiotic relationships.

In fact, much of the earlier work in this area has highlighted several *Burkholderia* species as prime occupants of the mycospheres of diverse soil fungi and even as endofungal occupants (Warmink and van Elsas 2008, Stopnisek et al. 2015, Partida–Martinez et al. 2007). In particular the species *B. terrae* (strains BS001, BS110, BS007, and BS437), *B. phytofirmans* (strains BS455, BS421 and BS413), *B. caribiensis* (BS442), *B. xenovorans* (BS416) and *B. terricola* BS454 (Nazir et al. 2012; note that the taxonomic information is – for some strains – not final) were found to be fungal–interactive based on their 'single–strain' migratory capacity along with the hyphae of *Lyophyllum* sp. strain Karsten. Of these, *B. terrae*, being an aerobic, heterotrophic and mesophilic soil dweller, utilizes a broad range of C compounds (Yang et al. 2006); on the basis of its capacity to fix nitrogen, it might constitute an excellent partner for some soil–dwelling fungi. Nazir et al. (2012) also reported on the ability of strain BS001 (and some other strains) to use a wide range of sugars, organic acid and sugar alcohols.

On the basis of the capacities of *B. terrae* BS001 and related strains for life in the mycosphere, I selected these strains and here addressed issues of the interactivity of *B. terrae* (in particular strain BS001) with selected soil fungi. Concerning the latter, *Lyophyllum* sp. strain Karsten and, in a later stage, *Trichoderma asperellum* 302 were used, as representatives of, respectively, the basidiomycota and the ascomycota. In effect, *Lyophyllum* sp. strain Karsten was selected because it was used as a 'model' organism for saprotrophic soil–exploratory fungi and *T. asperellum* 302 was selected because strain

BS001 successfully migrated with it (Nazir et al. 2014). As outlined in the introduction, some of the outstanding research questions pertaining to the ecology and mechanisms of the interactions were answered, as follows:

1. What are the genome size and composition of *B. terrae* BS001 and to what extent do the genes and operons underpin the interactivity of BS001 with diverse fungi in soil?
2. What genes and operons of *B. terrae* BS001 are transcribed and thus important for its survival and interaction with *Lyophyllum* sp. strain Karsten at different time points during the interaction in soil?
3. How do partners in the bacterial–fungal interaction sense the presence of each other? And, are there any communication channels between the two partners?
4. What potential anchoring sites do fungi possess on their cell walls that fungal–interactive *Burkholderia* species attach to?
5. To what extent are fungal–interactive soil *Burkholderia* species attracted towards fungal–released compounds?
6. What is the role of oxalic acid/oxalate in the interaction between *Burkholderia* species and soil fungi?

These specific questions are addressed in the sections below and the data obtained are interpreted in a broader context.

Theoretical mechanisms of interaction between soil bacteria and fungi: with an emphasis on *B. terrae* BS001

In **chapter 2**, I address how the predominant bacterial types that are associated with different fungi may behave in the mycosphere, mycorrhizosphere and in the soil. I provide a detailed overview of the developing concepts of BFI in soil, with emphasis on *B. terrae* BS001 and *Lyophyllum* sp. strain Karsten. These bacterial–fungal as well as bacterial–fungal–plant interactions in soil are argued to play crucial ecological roles (transportation of bacteria along fungal hyphae, influxes of C compounds in their respective zones of influence, protection against antagonists and survival) that influence the ecophysiologies of all the partners. The considerations then zoomed in on the *L. proxima* and *Lyophyllum* sp. strain Karsten mycospheres, where the interaction of *B. terrae* BS001 with the fungal hosts occurs (Warmink and van Elsas 2008, Warmink and van Elsas 2009). A key argument provided was the importance of understanding the genome of strain BS001, as a token of past adaptive processes to the mycosphere and possibly other systems. Overall, the hypothetical concepts forwarded in this chapter provided the basis for the experimental work carried out in the following chapters.

The genome of *B. terrae* BS001 – a wealth of genetic information

For any organism to adapt to, and occupy, spatially and temporally different soil microhabitats, it is imperative to possess the genetic capacity that allows flexibility in adaptive processes to such microhabitats. Konstantinidis and Tiedje (2004) first indicated that

bacteria with larger genomes are ecologically more successful in soil, where 'nutritional scarcity exists and the penalty for slow growth is lower'. At the onset of my work, there were indications that the genome of *B. terrae* BS001 is extremely large, and so one may posit that the bacterium has adapted to a whole suite of conditions that naturally occur in the soil. This is consistent with tenets by Warmink and van Elsas (2009), who stated that *B. terrae* BS001 – although isolated as a typical mycosphere dweller – may actually well have the ability to adapt to different niches in soil (Warmink and van Elsas 2009). Nazir et al. (2012) later showed that *B. terrae* BS001 is able to utilize a wealth of carbonaceous compounds, including citric acid, acetic acid and formic acid, which further supported the metabolic versatility of the organism. In order to unravel the genetic potential of strain BS001, I performed a detailed analysis of its genome, in a comparative genomics study (using 23 *Burkholderia* genomes; **chapter 3**). This included an analysis of past horizontal gene transfer events. The exceptionally large genome of *B. terrae* BS001 (11.5 Mb) contains a range of genetic systems, including those involved in motility and chemotaxis, nutrient sensing/acquisition/transformations, stress responses, protein secretion, biofilm formation and secondary metabolite production, that may confer mycosphere competence to the organism. From the genomics data, I surmised that these systems indeed can provide selective advantages, in terms of spurring survival, dispersal and metabolic rearing, to strain BS001 in the mycosphere as well as beyond.

In a previous study, a preliminary tree, showing the phylogenetic neighbors of strain BS001, was shown (Nazir et al. 2012; based on partial 16S rRNA sequences). Here, I extended this preliminary analyses by making use of seven concatenated core genes (*aroE*, *dnaE*, *groeL*, *gyrB*, *mutL*, *recA* and *rpoB*) to produce a more robust phylogeny. On the basis of this novel information, I inferred that *B. terrae* BS001 belongs to the so–called non–pathogenic clade of the genus *Burkholderia*. The findings are in accordance with previous data, where the concept of pathogenic and non–pathogenic clades was previously reported (Estrada–de los Santos et al. 2013).

In particular, **chapter 3** placed emphasis on the type 3 secretion system (T3SS), as well as other protein secretion systems (T2SS, T4SS and T6SS), presumed to be important for the successful interaction of the *Burkholderia* with soil fungi. As such, I performed comparisons with such systems in other related *Burkholderia* species, including the endofungal *B. rhizoxinica* HKI454. The analyses indeed confirmed the presence of protein secretion systems of types 3, 4, 6 (**chapter 3**) in the genome of *B. terrae* BS001, as well as in other genomes within this genus.

Considering its evolutionary history, *B. terrae* BS001 may have dwelled, over time, in a suite of (geographically distinct) niches, and so established an "evolutionary metacommunity" connected through time (Leibold et al. 2004). Warmink and van Elsas (2009) reported, as a key asset, the migration of *B. terrae* BS001 along the hyphae of *Lyophyllum* sp. strain Karsten in soil. This allows to infer that strain BS001 is adapted to this mycosphere, or, in a broader perspective, these adaptations may likely be extendable to other systems that offer similar conditions. Indeed, the large genome size of strain BS001 is consistent with the tenet that the organism may even have acquired capacities

allowing multifaceted life style phases in the soil. I thus surmised that horizontal gene transfer (HGT) events have played a large role over the evolutionary trajectory of strain BS001. Indeed, evidence was found for such HGT events, as a large portion of the strain BS001 genome contained regions of genomic plasticity (RGP). Such transferred (blocks of) genes may have been selected by past niche conditions, and the fact that we still find them pleads against strong forces of de–selection. Consistent with the multi–niche hypothesis is the fact that *B. terrae* BS001 was able to become associated with a high number of fungal species (Nazir et al. 2014). Interestingly, one of the RGPs (RGP76, 70.42 Kb) harboured a T4SS and other plasmid–related genes i.e. *mobB*, *mobC*, *repB*, *korC*, *klcA*, *parA*, *parB* and *parD* (**chapter 3**), possibly indicating that HGT events may have taken place with this potential vector.

The aerobic heterotroph *B. terrae* – for its survival and growth – requires carbonaceous compounds such as sugars, sugar alcohols and organic acids. Strain BS001 has apparently invested strongly in the capacities to sense, capture and utilize a wide range of carbonaceous compounds, as large numbers of CDSs (11.1% of the genome; **chapter 3**) encode various membrane transporters with presumed involvement in such processes. The consequent large genome size, reflecting a wide range of metabolic as well as stress tolerance mechanisms, is consistent with the theory of Konstantinidis and Tiedje (2004), who showed that larger bacterial genomes tend to accumulate genes involved in regulation, secondary metabolism and energy conversion. Metabolism and compound transport–related genes tended to increase in numbers with increases in genome size (more than 15%; Konstantinidis and Tiedje 2004). Connected to the consequent metabolic activity spurs, chemically–mediated antibiosis is often prevalent. In such interaction, the release of antibiotics or other products (secondary metabolites) by microorganisms is aimed at countering other microbes. In **chapter 3**, I describe the presence of nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) gene clusters, highlighting the potential of strain BS001 to engage in such biochemical warfare. Although I did not identify the potential products of such clusters, I here postulate that these will have significant roles in the ecology of strain BS001 in soil. Interestingly, I found a varied expression pattern of the PKS/NRPS in the transcriptome analyses (see next section), where at the early 'non–physical–contact' stage the expression was downregulated contrary to an upregulation at the physical–contact stage (**chapter 4**).

B. terrae BS001 reveals differential gene expression during the interaction with *Lyophyllum* sp. strain Karsten

In **chapter 4** I investigated the transcriptional responses of *B. terrae* BS001 in the presence versus absence of *Lyophyllum* sp. strain Karsten. I hypothesized that some genetic systems of strain BS001 might respond dynamically, in a time–dependent fashion (physical and no–physical contact), to *Lyophyllum* sp. strain Karsten upon confrontation. I addressed these questions through RNA sequencing using a soil extract agar (SEA) system that was purposefully devoid of easily accessible carbon sources. The resulting data

hinted at a great complexity, and spatiotemporal variability, of the strain BS001 gene expression events that took place. Temporally speaking, there was a clear indication of the occurrence of chemotaxis and signalling (communication) at the onset of the interaction, when both counterparts were physically apart from each other (**chapter 4**). However, the conditions on the SEA plates did not allow the visualization of the taxis (due to a high agar level).

Furthermore, the use of SEA devoid of added key carbonaceous nutrients strongly impacted the dynamics of the bacterial–fungal interaction. A prime observation during this study was the overwhelming expression of stress response related genes, i.e. *rpoS*, *dnaK*, *recA*, *groEL*, *ftsZ*, *mutL* and *mutS*. This observation reflected the [mimicked] nutritionally–scarce soil conditions. Stopnisek et al. (2015) suggested that, during the interaction of *Burkholderia glathei* with *Alternaria alternata* and *Fusarium solani*, the fungi could alleviate the conditions of nutrients scarcity, however during this process, they also caused oxidative stress responses in the bacterium. I posit here that our data, revealing partial stress alleviation in strain BS001 by *Lyophyllum* sp. strain Karsten, are coupled to metabolic influxes into the bacterium concomitant with oxidative stress–at the physical contact stage. This was evident, as the transcriptional dynamics of the interactome changed when *B. terrae* BS001 and *Lyophyllum* sp. strain Karsten physically came into contact with each other. Specifically, a cluster of five genes (see Fig. 4.5 and Table 7.1), consisting of two operons, showed significantly and strongly upregulated expression

Table 7.1 Genetic systems with their potential roles in the interaction of *Burkholderia terrae* BS001 with *Lyophyllum* sp. strain Karsten and *Trichoderma asperellum* 302.

S.No.	Genetic systems/Ecological behaviour	Potential role/Relevance	References/chapter
1	Chemotaxis and flagellar motility genes and their upregulation	Chemotaxis/communication	This thesis/ 3 and 4
2	Chemotaxis towards fungal hyphae, exudates and oxalate	Niche exploration via signals	This thesis/ 5 and 6
3	Oxalate degradation genetic potential in *B. terrae*	Oxalate utilization	This thesis/ 6
4	Oxalate and other metabolites production by *Lyophyllum* sp. strain Karsten and *T. asperellum* 302	Signalling and rearing	This thesis/ 6
5	Five genes' cluster and short–chain dehydrogenases upregulation	Metabolic influxes/oxidative stress response	This thesis/ 4
6	PKS/NRPS presence, dynamic expression during *B. terrae* interaction with *Lyophyllum* sp. strain Karsten	Secondary metabolism	This thesis/ 3 and 4
7	SET domain containing protein and its upregulation	Possible roles in host physiology modulation	This thesis/ 4
8	Type 6 secretion system enhanced expression under soil mimicking conditions	Potential roles in stress response	This thesis/ 3 and 4
9	Type 4 secretion system and plasmid	Horizontal gene transfer	This thesis/ 3
10	Presence of the *gup* gene and glycerol production by fungi	Horizontal gene transfer and rearing	This thesis/3 and 6

compared to the control treatment. The aforementioned gene cluster was probably dedicated to situations where both the ecological benefits from the degradation of compounds such as oxalate or its complexes (potentially coming from the soil fungi) can be reaped, as well as potential stresses neutralized.

The information gathered in **chapter 4** revealed the high complexity of the expression patterns of *B. terrae* BS001 during its interaction with the fungus in a carbon–limited situation. I argue here that a more hypothesis–driven approach should be applied to understand each one of them in depth.

Does *B. terrae* BS001 swim towards soil fungi and what are the potential anchoring sites for the bacterium on the fungal cell wall?

Active bacterial motility is dependent on mechanisms like swimming, swarming and gliding (next to twitching), and is collective in nature (Ben–Jacob et al. 2016). Despite the fact that each bacterial cell might be equipped with flagella, a concerted movement towards a source or a signal is often seen (Ben–Jacob et al. 2016). From the transcriptome data described in **chapter 4**, and consistent with the theoretical insights stated in **chapter 2**, I derived the hypothesis that the interaction between *B. terrae* BS001 and two soil fungi (*Lyophyllum* sp. strain Karsten and *T. asperellum* 302) is driven by directed taxis towards the fungal hyphae, followed by attachment to fungal surface anchoring sites. Furthermore, the movement (swimming) of *B. terrae* cells towards fungi might be collective in nature, and there might be a communication channel among the bacterial cell population, prior to the decision of movement in a certain direction. Therefore, to understand the spread of BS001 cells in an ecological sense, in **chapter 5** I analyzed the ecophysiological behavior of *B. terrae* BS001 during its interaction with *Lyophyllum* sp. strain Karsten and *T. asperellum* 302. Indeed, positive chemotactic responses were found when both fungi were sensed. This confirmed, first of all, the attractant function of the two soil fungi. Next, it indicated a certain commonality in the responses, reflecting potentially similar signals sent out by the fungal mycelia. Interestingly, the extent of chemotaxis towards the two fungal species was different, which might reflect their different physiological behavior. One line of explanation is that different metabolites are released by the two fungi. Given that *T. asperellum* 302 was a faster grower than *Lyophyllum* sp. strain Karsten, a key driving factor may also be "timing", e.g. of the release of chemoattractants. Different compounds may have occurred at different levels or concentrations in the exudates of the fungi, which formed the gradients. Thus, such chemical cues (Chet and Mitchell 1976) may have varied between the fungal host species, suggesting a somewhat varied response towards these (Zentmyer 1961).

Chapter 5 then explored the effects of compound level on the chemotactic behaviour of strain BS001 towards the selected soil fungal extracts. Previously, glycerol was shown to be a major compound exuded by *Lyophyllum* sp. strain Karsten, at approximately 2 mM. Later, Nazir et al. (2013) also found glycerol is released by the fungus in standing liquid microcosms, into viscous droplets on top of mycelial mats. On the other hand, in

previous analyses oxalate was not measured and hence the potential role of oxalate as a signalling molecule (Rudnick et al. 2015) in the interaction of *B. terrae* and soil fungi was missing. Here, the effects of these two compounds, glycerol and oxalate, on the chemotactic responses of *B. terrae* strains towards two fungal species was assessed. The chemotactic response of strain BS001 increased as the concentration of glycerol in the medium increased. Remarkably, BS001 showed stronger/faster chemotaxis at a lower concentration and a lesser response at higher concentrations of oxalate. In *B. phytofirmans* PsJN mutant Δ*oxc* , the possible toxicity of oxalate was addressed, but the strain did not reveal any significant response (Kost et al. 2014). Hence, the potential role of oxalate as a toxic compound to *Burkholderia* sp. is not fully understood at present. I interpret our collective data of the experiments with glycerol and oxalate, as glycerol providing the energy for positive movement of strain BS001 towards the oxalate source. The latter compound thus acted as signalling molecule at the low concentration (0.1%, equivalent to 7.9 mM). At higher concentrations, the strain BS001 cells may have been 'deceived' by a 'sufficient' supply of carbon and energy, and therefore migrated towards the fungi to a lesser extent. In systems addressing the swarming motility of *Collimonas fungivorans* Ter331, oxalate (50 M) was suggested to also act as a signaling molecule (Rudnick et al. 2015). However, at higher oxalate levels (500 M), the swarming intensity receded and so it was suggested that such swarming may become arrested once the source of the signal is located (Rudnick et al. 2015).

The physical interactions between *B. terrae* BS001 and the two soil fungi were then further explored (**chapter 5**). In a collaborative work with partners in UFRJ, Brazil (group of Prof. Barreto–Bergter), I thus investigated the potential anchoring sites on the fungal cell surfaces and the mechanisms involved in the physical interactions between *B. terrae* BS001 and *Lyophyllum* sp. strain Karsten and *T. asperellum* 302. The analyses revealed that the order of colonization (bacteria first and fungus second or *vice versa*) was important, as significantly higher biomass (reflecting the co–culture) was found when bacteria were inoculated first and the *Lyophyllum* sp. strain Karsten as the second colonizer. However, the behaviour of *B. terrae* BS001 towards *T. asperellum* 302 was different, as joint biofilm formation was not easily observed. The attachment of bacterial cells to the fungi was suggested to involve surface–exposed molecules, in particular the glycolipid ceramide monohexosides (CMH). The data suggested that CMH is one of the potential binding sites on the *Lyophyllum* sp. strain Karsten's surface for strain BS001. The binding of strain BS001 to CMH molecules of *T. asperellum* 302 was lower. We hypothesized that a difference in the ceramide portion of the CMH molecules of both fungi, where an additional hydroxyl group at C–4 was present in the CMH of *T. asperellum* 302, might be at the basis of the difference, however this position needs confirmation.

Does oxalate act as a signalling molecule or a potential carbon source?

Oxalate is a highly oxidized compound which contains two carbon atoms with an oxidation number of +3 and only two electrons (Schneider et al. 2012). It is 'generally'

considered to be a 'poor' substrate compared to other carbon sources for soil bacteria such as glucose and glycerol. The precise role of oxalate in the interaction of *B. terrae* BS001 and fungi in soil was still enigmatic, in particular whether the capacity to perceive it as a signal as well as to utilize it, would be shared among different strains. In **chapter 6**, I tested the hypothesis that oxalate may be acting as a signaling molecule for such strains, to sense the presence and location of fungi in soil. I conclude that, at low concentration (0.1% [w/v]), oxalate incited a strong chemotactic response in all *B. terrae* strains, including the type strain 17804^{T}. However, such responses were 'lethargic' at higher concentrations of oxalate. I then looked at the chemotactic responses of all *B. terrae* strains to exudates of both fungal species at different levels. Interestingly, both exudates attracted all *B. terrae* strains when used undiluted. However, this occurred to varied levels by the different strains. Prior information on the use of oxalate by *Burkholderia* was scarce. Clearly, I showed that oxalate–amended M9 medium supported the growth of strain BS001, indicating it was able to use oxalate as the carbon and energy source for its active growth {at 0.1% and 0.5% [w/v]}. This becomes plausible due to the fact that the genomes of strains BS001, next to BS007, BS110 and BS437, harbor genetic systems that are potentially involved in the utilization of oxalate. I here posit that *B. terrae* BS001 is oxalotrophic, yet may use oxalate as a last–resort compound. Dijkhuizen et al. (1978) revealed that *Pseudomonas oxalaticus* (now known as *Cupriavidus oxalaticus*) follows a diauxic growth pattern when the bacterium is provided with a mixture of oxalate and formate in a batch culture, preferring formate as a substrate. This behavior is predictable, as the energetic potential of oxalate is lower than that of formate. Although this thesis did not pursue the potentially diauxic growth patterns of strain BS001, I expect a diauxic growth in cases where both glycerol and oxalate are offered. The conjecture that oxalate may not be a preferred carbon source for the growth of *Burkholderia* is corroborated by the findings of Dijkhuizen et al. (1977), where the active transport of oxalate across the membrane had a large effect on the energy budget of the cell. That is, 50% of the potential energy contained in oxalate was required for its translocation across the cell membrane, compared to 25% for formate. Hence, the energy possessed by oxalate may not be sufficient for the vigorous growth of *B. terrae* strains like BS001. Thus oxalate may be a signalling molecule in 'first place' and a 'potential' carbon source in the second (Rudnick et al. 2015; **chapter 6**). In the mycosphere, *B. terrae* BS001 may perceive released oxalate as a signal to locate the fungal source, and – being equipped with oxalate catabolic genetic systems – may also use it as a 'second–option' carbon source (i.e. following depletion of released glycerol) in the nutrient–depleted microenvironment.

Do different fungal types provide similar or different ecological opportunities to *Burkholderia terrae* strains?

Although Boersma et al. (2012) described the composition of exudates released by *Lyophyllum* sp. strain Karsten, evidence for the presence of oxalate has been lacking due to the method used for identification (^{1}H NMR). Here, I analyzed exudates of the two

selected fungi with a focus on oxalate. In **chapter 6**, I confirmed that both fungi release the compound into the M9+propionate medium in a concentration range of 0.075–0.093 % [w/v], as evidenced by the HPLC analyses of the exudates. The fungal exudates also contained other compounds such as glycerol, formic acid, acetic acid, fumaric acid and citric acid, in addition to other unidentified compounds. This further strengthened the tenet that the two fungi in soil (and possibly others as well) attract bacteria via the release of oxalate. It is important to note that many other chemical molecules are also likely to have an effect in the BFI, but these were not addressed, with respect to their potential function, in this thesis.

Overall analysis of the data and conclusions

On the basis of the data described in this thesis, I posit that key genetic systems / traits that might act as the potential cornerstones of *B. terrae* –fungal interactions in soil are multifold, and potentially act in concert. The speculative list, with a brief description of the evidence supporting this tenet, is shown in Table 7.1. Also, the questions posed in the introduction to this thesis, with the answers obtained through experimentation, are shown in Fig. 7.1.

Briefly, from the work presented in this thesis, the following conclusions are drawn:

1. The 'mycosphere' presents a particular niche to soil bacteria, providing them with resources that guarantee their survival. Inhabitants of the mycosphere, such as *B. terrae*, are endowed with genetic traits, including (integrated) plasmids, that enable them to explore the available niches.
2. The genome of the fungal–interactive *B. terrae* BS001 carries a suite of genetic traits that confer the capacity to interact with the soil fungus *Lyophyllum* sp. strain Karsten.
3. HGT events have shaped the genome of *B. terrae* BS001 over evolutionary time, as evidenced by the occurrence of a suite of RGPs. Such RGPs are hypothesized to enhance the strain BS001 ecological fitness required for (a) its successful survival in soil microhabitats and (b) its interactivity with soil organisms, including (but not confined to) soil fungi.
4. *B. terrae* BS001 expresses several genes differentially during its interaction with *Lyophyllum* sp. strain Karsten. Genetic systems involved in chemotaxis towards the growing fungal hyphae, metabolism and stress responses are positively involved. Moreover, some stress resulting from the local conditions was alleviated by the fungus.
5. *B. terrae* BS001 moves chemotactically towards two selected fungi on SEA plates and on M9 agar. The chemotactic responses towards these fungi in the presence of extra added glycerol are amplified. However, with respect to oxalate, the behaviour of strain BS001 towards fungi follows a pattern of being vigorous at a lower concentration and lethargic at higher concentrations.

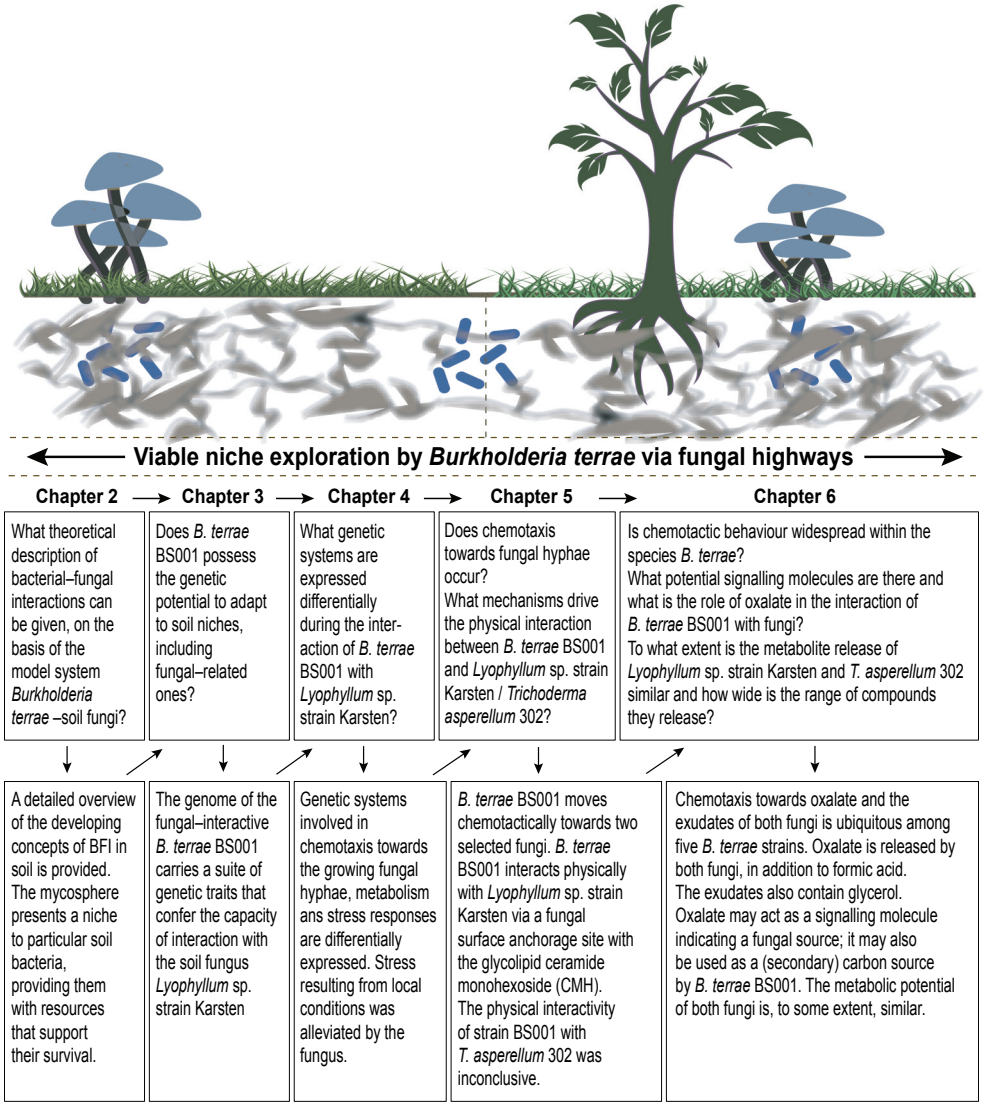


Figure 7.1 A schematic flow chart of thesis questions and the results. Graphic resources for this figure were obtained from www.freepik.com, available as free vectors and used after modifications.

6. *B. terrae* BS001 physically interacts with *Lyophyllum* sp. strain Karsten via a fungal surface anchorage site with the glycolipid ceramide monohexosides (CMH). It is at present unknown to what extent this interaction is exclusive.
7. The metabolites released by *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 are to some extent similar and so relatively similar conditions are shaped in both mycospheres.
8. The capacity of chemotactic movement towards oxalate and the exudates of both *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 is omnipresent across the *B. terrae* strains BS001, BS110, BS007, BS437 and type strain 17804^T.
9. Oxalate is released by both *Lyophyllum* sp. strain Karsten and *T. asperellum* 302, in addition to formic acid, citric acid, acetic acid and fumaric acid. These exudates also contain glycerol.
10. Oxalate or oxalic acid act as positive signalling molecules. These may also be used as a (secondary) carbon source by *B. terrae* BS001.
11. All *B. terrae* strains analyzed in this thesis possess the necessary genetic potential to utilize and degrade oxalate and its complexes.

Outlook – future perspectives

Although this thesis answered some of the outstanding research questions pertaining to the ecology and mechanisms governing the BFI involving *B. terrae* in soil, using *in vitro* studies, answers to some of the complex questions still remain at large. Thus future work should be focused on the exploration of the secondary metabolite production potential, and its regulation by ecological conditions, of both *B. terrae* strain BS001 and the associated fungi. Such an endeavor will open up new avenues that will assist in a better understanding of the *B. terrae* – fungal interactions. One may hypothesize that the production and release of such molecules has large ecological significance, potentially modulating the growth and/or development of the interaction partner. Or, of other (neighboring) organisms in the same sphere. Such studies also bear the enormous potential of finding new antibiotics, as evident from the work of others, i.e. the interaction of *B. rhizoxinica* HKI454 and *Rhizopus microsporus* (Partida–Martinez and Hertweck 2005).

In **chapter 4** I found glimpses of something unique that might play a role in the interaction of *B. terrae* BS001 with *Lyophyllum* sp. strain Karsten. It involved the upregulation of a so–called 'Suppressor of variegation–Enhancer of zeste–Trithorax' (SET) domain containing protein during the interaction. The role of SET domain containing proteins in bacterial–fungal interactions is not yet understood. However, in the light of the available literature, I surmised that such proteins may be used by the bacterium to, indeed, modulate the physiological status of the fungus. Might this modulation be at the basis of the inhibition of primordium setting, as described previously by Nazir et al. (2013)? In order to understand the exact role of the upregulated SET domain protein, a mutation–based approach is recommended. In general, I advocate the use of hypothesis–driven approaches like this one, see also Table 7.1 for more prospective confirmatory trajectories.

This thesis also yielded data pointing at the strong upregulation of a dedicated cluster of five genes in the presence of the fungus. Although a role of this cluster in the utilization of oxalate and the concomitant detoxification of reactive oxygen species is a possibility, I here did not elaborate further on the clarification of such a potential mechanism. Clearly, there is room to further explore the role of the gene cluster in the interaction, including its timing, again via hypothesis–driven approaches (and possibly using mutagenesis). Given that the five–gene cluster was present across all the tested *B. terrae* strains and is flanked by repeats and transposases (unpublished data), there is a possibility of it having been acquired horizontally in the past. Evolutionary theory then predicts it to have conferred ecological fitness to these strains, potentially in the mycosphere and beyond. In the light of this arguments, a dedicated focus should be placed on the likely origin of such gene clusters.

Further explorations of the *B. terrae* – fungal interactome should also include an examination of the dynamics and relevance of oxalate in the mycosphere and its potential role in BFI. Even though I did find some answers to the questions regarding its role as a signalling molecule and potential carbon source, a better clarification of the involvement of any or all of the oxalate degradation systems is required. Finally, in the light of the presence of glycerol, oxalate, fumaric acid, acetic acid, formic acid and fumarate in the mycosphere, a better focus should be placed on the putative diauxic growth patterns of *B. terrae* BS001 in the mycosphere, when confronted with such compounds as sources of carbon and energy.

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Summary / Samenvatting

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English Summary

Soil constitutes a complex ecosystem harboring diverse communities of bacteria. The carbon-limiting nature of soil presents bacteria with conditions of nutrient scarcity, often retarding their growth. However, soil bacteria may rely on other ecological opportunities offered by soil fungi and plants in their respective zones of influence; i.e. mycosphere, mycorrhizosphere and/or rhizosphere. Such ecological interactions benefit soil bacteria, fungi and plants in different capacities, which leads to improvement in soil health. Therefore, understanding the underlying mechanisms of bacterial-fungal interactions and their respective ecological outcomes, are of paramount importance.

This thesis starts off with a comprehensive treatise of soil microhabitats, bacterial-fungal interactions, mechanisms governing such interactions, and their ecological outcomes in terms of bacterial behavior in response to soil fungi. A particular focus is placed on the interaction of *Burkholderia terrae* BS001 and *Lyophyllum* sp. strain Karsten. Based on the arguments presented by Konstantinidis and Tiedje (2004) that bacteria possessing larger genomes are ecologically more successful in soil, **chapter 2** highlights the need to explore the genome of strain BS001 as an ultimate answer to the complexity of bacterial-fungal interactions in soil. Thus, it provides the basis for further hypothesis–driven experimental work.

Chapter 3 describes a detailed account of the genome of the model fungal-interactive bacterium—*B. terrae* BS001—with a focus on genetic systems (i.e. type 3, 4 and 6 protein secretion systems, biofilm formation, motility and chemotaxis), which are hypothesized to have key roles in bacterial-fungal interactions. In order to ascertain the commonalties and differences in terms of the coded genetic information, the genome of *B. terrae* BS001 is compared with genomes of 23 other *Burkholderia* species. A large portion of the strain BS001's genome constituted regions of genomic plasticity, suggesting that horizontal gene transfer events have taken place during the evolutionary history of this bacterium. The presence of nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) gene clusters in the genome indicate the ability of strain BS001 to synthesize secondary metabolites. The higher number of membrane transporters, and the glycerol uptake system indicate the ability of strain BS001 to be ecologically versatile.

In **chapter 4** I examine the transcriptional patterns of *B. terrae* BS001 in response to *Lyophyllum* sp. strain Karsten and the soil-mimicking environment, in a spatio-temporal context. The transcriptional responses of the bacterium are characterized by the upregulation of motility and chemotaxis related genes at the onset of interaction, when the two partners are spatially apart. However, as the physical interaction between partners established the dynamics of the gene expression patterns changed, in particular, with the upregulation of genes responsible for energy generation and oxidative stress responses.

Given that the expression of chemotaxis related genes of strain BS001 was higher in the presence of the fungus, in **chapter 5** I investigate and describe the chemotactic behavior of strain BS001 towards two selected fungi—*Lyophyllum* sp. strain Karsten and *Trichoderma asperellum* 302. A strong chemotactic response towards both fungi revealed

the existence of signalling and communication channels between the two partners. The signalling roles of glycerol and oxalic acid are sought and it is inferred that glycerol acts as a carbon source more than a signalling molecule in contrast to oxalic acid. In terms of the physical interactions between strain BS001 and fungi, a surface-exposed molecule (i.e. ceramide monohexosides — CMH) of *Lyophyllum* sp. strain Karsten is suggested to have a role in the attachment of bacteria.

In **chapter 6**, I further investigate whether or not, *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 exude oxalic acid, and if so, does it act as a signalling molecule and/or carbon source for the growth of strain BS001. The experiment revealed that both fungi exude oxalic acid and other compounds including glycerol and low-molecular-weight organic acids i.e. formic acid, acetic acid, fumaric acid, and citric acid. While the signalling role of oxalic acid in the interaction of bacteria and fungi is further corroborated by experiments in **chapter 6**, where it incited a strong chemotactic response in the *B. terrae* strains i.e. BS001, BS110, BS437, and BS007, it also supported the growth of strain BS001. Hence, it is inferred that oxalic acid 'primarily' acts as a signalling molecule—serving bacteria to spatially locate fungi in soil—and a potential 'secondary' carbon source under nutrient-limiting conditions.

Answers to the questions posed—ecological behavior of *B. terrae* BS001, gene expression patterns, and the mechanisms underlying bacterial–fungal interactions—are obtained in this book. Furthermore, considerations regarding future perspectives and research in this exciting and ground–breaking, yet, nascent area of microbiology are discussed.

Nederlandse Samenvatting

De bodem is een complex ecosysteem met diverse bacteriële gemeenschappen. De koolstoflimitatie van de bodem confronteert bacteriën met nutriëntenschaarste en vertraagt hun groei. Echter, bodembacteriën kunnen een beroep doen op ecologische mogelijkheden die geboden worden door bodemschimmels alsook planten in hun respectievelijke invloedzones, i.e. de mycosfeer, de mycorrhizosfeer en / of de rhizosfeer. Dergelijke ecologische interacties bieden mogelijk voordeel aan bodembacteriën, schimmels en planten in verschillende hoedanigheden en kunnen de bodemgezondheid verbeteren. Op grond van het voorgaande is het begrijpen van de mechanismen die onderliggen aan bacterie-schimmel interacties en de respectievelijke ecologische uitkomsten van primordiaal belang.

Dit proefschrift begint met een uitgebreide verhandeling over bodemmicrohabitats, bacterie-schimmel interacties—gericht op de interactie van *Burkholderia terrae* BS001 en *Lyophyllum* sp. stam Karsten— en de mechanismen die dergelijke interacties bepalen, alsook de ecologische gevolgen, in termen van het bacteriële gedrag in reactie op schimmels. Conform de argumenten van Konstantinidis en Tiedje (2004), die vonden dat bacteriën met grotere genomen ecologisch succesvoller zijn in de bodem, benadrukt **hoofdstuk 2** de noodzaak om het genoom van stam BS001 te bepalen als een ultiem middel om de complexiteit van bacterie–schimmel interacties in de bodem te begrijpen. Dit hoofdstuk vormde de basis voor het verdere hypothese–gedreven experimentele werk.

Hoofdstuk 3 geeft derhalve een gedetailleerde beschrijving van het genoom van de schimmel- interactieve bacterie—*B. terrae* BS001—met een focus op de genetische systemen (dat wil zeggen type 3, 4 en 6 eiwitsecretiesystemen, de vorming van biofilms, beweeglijkheid en chemotaxis), waarvan wordt aangenomen dat ze rollen spelen in bacterie-schimmel interacties. Om de gemeenschappelijke aspecten en verschillen in de gecodeerde genetische informatie te bepalen, is het genoom van *B. terrae* BS001 vergeleken met de genomen van 23 andere *Burkholderia* soorten. Een groot deel van het genoom bestond uit gebieden van genomische plasticiteit, wat suggereert dat horizontale genoverdracht frequent heeft plaatsgegrepen tijdens de evolutionaire geschiedenis van de bacteriegenomen. De aanwezigheid van non-ribosomaal-gevormde peptidesynthetase (NRPS) en polyketidesynthase (PKS) genclusters in het genoom wees op het vermogen van stam BS001 om secundaire metabolieten te synthetiseren. Het hoge aantal membraantransporteiwitten, en het glycerolopnamesysteem wees op de ecologische veelzijdigheid van stam BS001.

In hoofdstuk 4 onderzoek ik de transcriptiepatronen van *B. terrae* BS001 in reactie op de bodemschimmel *Lyophyllum* sp. stam Karsten en een bodemsimulatie, in een ruimte-tijd context. De transcriptiepatronen werden gekenmerkt door de 'upshift' (opwaartse regulatie) van expressie van motiliteit- en chemotaxisgenen bij het begin van de interactie, waarbij de twee interactiepartners ruimtelijk gescheiden waren. Bij fysiek contact van de interactiepartners veranderde de dynamiek van de genexpressiepatronen,

met name gekenmerkt door upshift van genen voor energieopwekking en de oxidatieve stressrespons.

Aangezien de expressie van chemotaxisgenen van stam BS001 hoger was in de aanwezigheid van de schimmel, onderzoek en beschrijf ik in **hoofdstuk 5** het chemotaxisgedrag van stam BS001 bij twee geselecteerde schimmels—*Lyophyllum* sp. stam Karsten en *Trichoderma asperellum* 302—op bodemextractagar en M9 medium. Een sterke chemotactische reactie op beide schimmels was indicatief voor het bestaan van signalering en communicatiekanalen tussen de twee partners. De mogelijke signaalrol van glycerol en oxaalzuur werden onderzocht en er werd geconcludeerd dat glycerol meer fungeerde als koolstofbron dan als signaalmolecuul, in tegenstelling tot oxaalzuur. Wat betreft de fysieke interactie tussen stam BS001 en de schimmels, werden er aanwijzingen gevonden voor een rol van het oppervlak-blootgestelde molecuul ceramide monohexosides (CMH)—van *Lyophyllum* sp. stam Karsten in de hechting van de bacteriën.

In **hoofdstuk 6** heb ik verder onderzocht of *Lyophyllum* sp. stam Karsten en *T. asperellum* 302 oxaalzuur uitscheiden, en, zo ja, of dit oxaalzuur kan fungeren als een signaalmolecuul en / of een koolstofbron voor de groei van stam BS001. Het experiment toonde dat beide schimmels oxaalzuur uitscheiden, naast andere verbindingen zoals glycerol, mierezuur, azijnzuur, fumaarzuur en citroenzuur. De signaalrol van oxaalzuur in de interactie tussen bacteriën en schimmels werd verder bevestigd in **hoofdstuk 6**. Met name werd gevonden dat het aanzet tot sterke chemotactische reacties in de *B. terrae* stammen BS001, BS110, BS437 en BS007; voorts ondersteunde oxaalzuur ook de groei van stam BS001. Op grond van deze gegevens werd geconcludeerd dat oxaalzuur 'primair' fungeert als een schimmelsignaalstof voor bacteriën in de bodem en daarnaast als mogelijke 'secundaire' koolstofbron.

In dit boekje worden enkele antwoorden op de gestelde vragen ten aanzien van het ecologische gedrag van *B. terrae* BS001, haar genexpressiepatronen, en de mechanismen van interactie met schimmels gegeven. Bovendien breng ik overwegingen te berde die betrekking hebben op de toekomstperspectieven van het onderzoek op dit erg spannende en baanbrekende, evenwel nog ontluikende gebied van de bodemmicrobiologie.

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