Interactions of Burkholderia terrae with soil fungi
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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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*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-Δsctd 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-ΔscD 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-ΔscD 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-ΔscD 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.
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-ΔsctD mutant strains were used throughout. Briefly, the ΔsctD 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 ΔsctD 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-ΔsctD 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 KH₂PO₄ and 1 g L⁻¹ (NH₄)₂SO₄; 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 10⁵, 10⁷ and 10⁹ 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 Na₂HPO₄, 3 g L⁻¹ of KH₂PO₄, 0.5 g L⁻¹ NaCl and 1 g L⁻¹ of NH₄Cl 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 μm) 1 M MgSO₄ and 100 μL (L⁻¹) of 1 M CaCl₂ were added to the medium after autoclaving.

Washed cells of B. terrae BS001 and B. terrae BS001-ΔsctD in MES buffer were used. Using the optical density (OD) 600, selected cell densities (10⁵, 10⁷ and 10⁹ 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 μg) 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-ΔsctD 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 NaH₂PO₄, 10 mM Na₂HPO₄, 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 (10⁶ 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-ΔstcD grown overnight in LB broth (28°C, shaking at 180 rpm) were set at about 10⁷ CFU mL⁻¹ (using the OD 600 value). Then, the conidia-loaded wells were inoculated with 5×10⁵ bacterial cells per well, after which the plates were incubated for 1–4 h at 30°C. 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 (3×10⁴ conidia per well) or bacterial cells (3×10⁵ 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 µL 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 µL 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 µg 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 µL 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 µL 3 M H₂SO₄ 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 µg was added to each well of a 96-well plate. Plates were then dried at room temperature. Subsequently, 50 µL of bacterial cell suspensions in PBS (5 × 10⁵ 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 µg 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-ΔsctD, 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-ΔsctD (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-AsctD 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 ΔsctD 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-ΔsctD. 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 ΔscD 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 ΔscD 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 ΔscD 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 ΔsctD 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 ΔsctD 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 (µg/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 (µg/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′-hydroxyoctadecanoyl-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,
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-ΔsctD 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-ΔsctD showed lowered adherence (*P* < 0.05; Fig. 5.7A).

![Graph](image)

**Figure 5.7** The attachment of *B. terrae* BS001 and *B. terrae*-ΔsctD to fungal conidia. (5.7A) Represents attachment of *B. terrae* BS001 and *B. terrae*-ΔsctD to the conidia of *Lyophyllum* sp. strain Karsten. (5.7B) Shows the attachment of *B. terrae* BS001 and *B. terrae*-ΔsctD 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-ΔactD 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. 5.8).

**Figure 5.8** Adherence assay of *Burkholderia terrae* BS001 and ΔactD 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* ΔactD 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-ΔstcD 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 ΔctD 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 ΔsctD 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 ΔsctD 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-*ΔcstD* 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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**References**


Supplementary tables and figures

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

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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 ΔstD 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
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*ctD* 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

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Table S5.4 ANOVA testing for the effects of fungus (Trichoderma asperellum 302) and absence (control), taxis direction, B. terreus BS001 strain type (wild type and ΔcctD mutant), glycerol 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

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<td>Direction:Glycerol conc.</td>
<td>1</td>
<td>1.78</td>
<td>1.78</td>
<td>4.448</td>
<td>0.0372</td>
<td>*</td>
</tr>
<tr>
<td>Strain:Glycerol conc.</td>
<td>1</td>
<td>0.02</td>
<td>0.02</td>
<td>0.053</td>
<td>0.8182</td>
<td></td>
</tr>
<tr>
<td>Fungus:Direction:Strain</td>
<td>1</td>
<td>1.03</td>
<td>1.03</td>
<td>2.576</td>
<td>0.1113</td>
<td></td>
</tr>
<tr>
<td>Fungus:Direction:Glycerol conc.</td>
<td>1</td>
<td>1.39</td>
<td>1.39</td>
<td>3.463</td>
<td>0.0654</td>
<td>.</td>
</tr>
<tr>
<td>Fungus:Strain:Glycerol conc.</td>
<td>1</td>
<td>0.75</td>
<td>0.75</td>
<td>1.863</td>
<td>0.1751</td>
<td></td>
</tr>
<tr>
<td>Direction:Strain:Glycerol conc.</td>
<td>1</td>
<td>0.14</td>
<td>0.14</td>
<td>0.342</td>
<td>0.5598</td>
<td></td>
</tr>
<tr>
<td>Fungus:Direction:Strain:Glycerol conc.</td>
<td>1</td>
<td>0.12</td>
<td>0.12</td>
<td>0.302</td>
<td>0.5838</td>
<td></td>
</tr>
<tr>
<td>Residuals</td>
<td>112</td>
<td>44.93</td>
<td>0.40</td>
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</tr>
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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 ΔcctD 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

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