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Research Article

RESEARCH ARTICLE

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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One sentence summary: This study focuses on the behavior of the fungal-interactive soil bacterium *Burkholderia terrae* BS001 in relation to two soil fungi.
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ABSTRACT

Burkholderia terrae BS001 has previously been proposed to be a 'generalist' associate of soil fungi, but its strategies of interaction have been largely ignored. 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 (CMHs) revealed that CMHs in both conidia and hyphae could bind strain BS001 cells. As BS001-Δ*sctD* adhered significantly less to the CMHs than BS001, the T3SS was presumed to have a role in the interaction. In contrast, such avid 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, in particular of the former.

Keywords: chemotaxis; bacterial–fungal interaction; *Lyophyllum* sp. strain Karsten; *Trichoderma asperellum* 302; ceramide monohexosides

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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 members of the other group (bacterial–fungal interactions – denoted BFIs) when living in close proximity in the soil. Thus, in 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 spaces that support bacterial life in soil. In return, 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) BFIs in which the partners may have shared ecologies, other successful BFIs 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 signaling, interactive processes of progressively higher intricacy are assumed to take place, leading to an ultimately successful (and potentially persistent) interaction. A plethora of different ecological outcomes of such BFIs exists, including (presumably) mutualistic, commensalistic and antagonistic ones, as described in several earlier studies (De Boer *et al.* 2005; Mille-Lindblom, Fischer and Tranvik 2006; Nazir *et al.* 2010; Frey-Klett *et al.* 2011; Haq *et al.* 2014b). 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, Tazetdinova and van Elsas 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). *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 its bacterial associates, resulting in an ecological 'trade-off', including reduced growth and allowing co-existence (Mille-Lindblom, Fischer and Tranvik 2006).

In a key recent theoretical treatise (Haq *et al.* 2014b), the development of BFIs in soil was postulated to involve different steps, roughly divided into (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, van Veen and de Boer 2015). However, we have hitherto understood 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 regarding which fungal cell envelope compound might have a 'receptor' role for bacterial cell adherence. 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, Barreto-Bergter and Taborda 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 (Nimrichter and Rodrigues 2011; Rhome *et al.* 2011; Guimarães *et al.* 2014). 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, Sassaki and de Souza 2011; Rhome *et al.* 2011; Calixto *et al.* 2016), and have predicted roles in fungal immunogenicity and lipid raft architecture, besides 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, Tazetdinova and van Elsas 2014). Hence, the organism was termed a 'generalist' fungal associate. Previous work has also indicated that the type 3 secretion system (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, Moebius and Hertweck 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, Urbanowski and Greenberg 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 for 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

Burkholderia 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. *Burkholderia terrae* BS001 and BS001-Δ*sctD* cultures were grown overnight in

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Luria-Bertani (LB) broth (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1} and NaCl 5 g L^{-1}; 28°C, with shaking at 180 r.p.m.). The cultures were routinely centrifuged (12 000 × g, 3 min) and the bacterial pellets double-washed with sterile saline (NaCl solution 0.85%, w/v). For the chemotaxis experiments, cells were harvested by low-speed centrifugation (1057 × g, 20 min), and the pellets washed twice with 1X MES buffer, containing 1 g L^{-1} of KH_2PO_4 and 1 g L^{-1} $(NH_4)_2SO_4$; pH 5.6 (Rudnick, van Veen and de Boer 2015). Washed cells were used for the experiments, as below.

Lyophyllum sp. strain Karsten and *T. asperellum* 302 were maintained in autoclaved distilled water for extended storage according to the protocol of Nakasone, Peterson and Jong (2004). For routine work, each fungus was refreshed once per month on oat flake agar (OFA) plates (30 g oat flake (Spar shop, Netherlands), 15 g of agar (Duchefa Biochemie, Haarlem, The Netherlands), 1 L MilliQ water). For soil extract preparation, freshly sampled soil was used (from an agricultural field in Buinen, Netherlands; denoted 'B' soil). 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 r.p.m.) for 24 h (room temperature). Soil particles were precipitated by centrifugation at 5430 × 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) supplemented with 0.5 g of yeast extract was 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 and *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. Plates were incubated at 28°C and fungal behavior (growth) was monitored over time.

Chemotaxis (swimming motility) assay

For the chemotaxis assay, two different (0.25% agar, w/v) 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 S1). In the case of oxalic acid, the pH of the medium was adjusted to 6.8 (before autoclaving). After autoclaving, 2 mL (L^{-1}) of filter-sterilized (0.2 μm) 1 M $MgSO_4$ and 100 μL (L^{-1}) of 1 M $CaCl_2$ were added to the medium.

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 (about every 12 h, daily).

Preparation of monoclonal antibodies specific for glucosylceramides

Monoclonal antibodies (mAbs) were produced as previously described (Da Silva *et al.* 2004). Briefly, rabbits were immunized with ultrapure CMH (glucosylceramides; 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 CMHs. 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 (Da Silva *et al.* 2004; Nimrichter *et al.* 2004; Calixto *et al.* 2016).

Quantification of adherence of bacterial cells to fungal propagules

Adherence assays were performed in 96-well 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 potato dextrose agar (PDA) plates were scrape-washed off with phosphate-buffered saline (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 (28°C, shaking at 180 r.p.m.) 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 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.

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Fungal–bacterial biofilm formation

To test the biofilm formation abilities of the partner organisms, a multi-well biofilm formation assay was performed (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, v/v). The extracts were then combined, after which they were dried. The resulting crude lipid extracts were partitioned according to Folch, Lees and Sloane Stanley (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 fraction(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 analysis of *T. asperellum* 302 glucosylceramides

Electrospray ionization mass spectrometry (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 LiCl and infused (3 μL min^{-1}) 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 min^{-1}. 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 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 μg was added to each well of a 96-well microtiter plate. Plates were then dried at room temperature. Subsequently, 50 μL of bacterial cell suspensions in PBS (5×10^5 CFU mL^{-1}) was 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 was 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 biofilm, attachment and CMH experiments (Student's *t*-test) were performed using RStudio Version 0.99.893 (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, Tazetdinova and van Elsas 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. Figure 1 shows the quantification of the hyphal growth of both fungi on the SEA plates.

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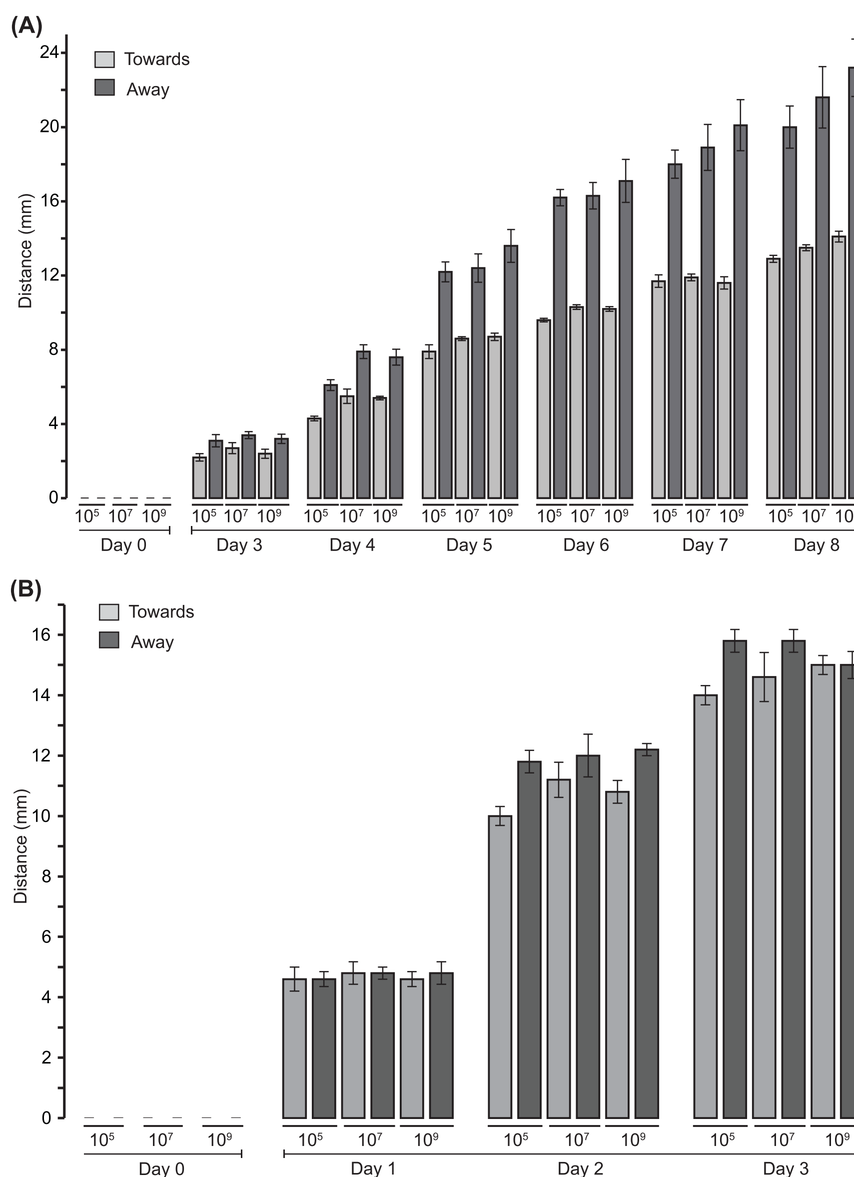

Figure 1. The behavioral response of *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 to *B terrae* BS001 populations on soil extract agar (SEA) medium. (**A**) The response of *Lyophyllum* sp. strain Karsten to *B. terrae* BS001 on SEA. The data represent the average of two different experiments. (**B**) The response of *T. asperellum* 302 to *B. terrae* BS001 on SEA medium. Initial bacterial cell densities are shown below the bars.

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; three levels), bacterial cell density (three levels, 10^5, 10^7 and 10^9), bacterial type (wild-type *vs* mutant) and movement direction (towards *vs* away from the fungus). ANOVA of all data (Supplementary Table S2) 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 and *T. asperellum* 302 mycelia at 12–15 mm distance from a cell stripe of *B. terrae* BS001 (Fig. 2A) or *B. terrae* BS001-Δ*sctD* (Fig. 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 occurred, but was significantly reduced ($P < 0.05$).

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

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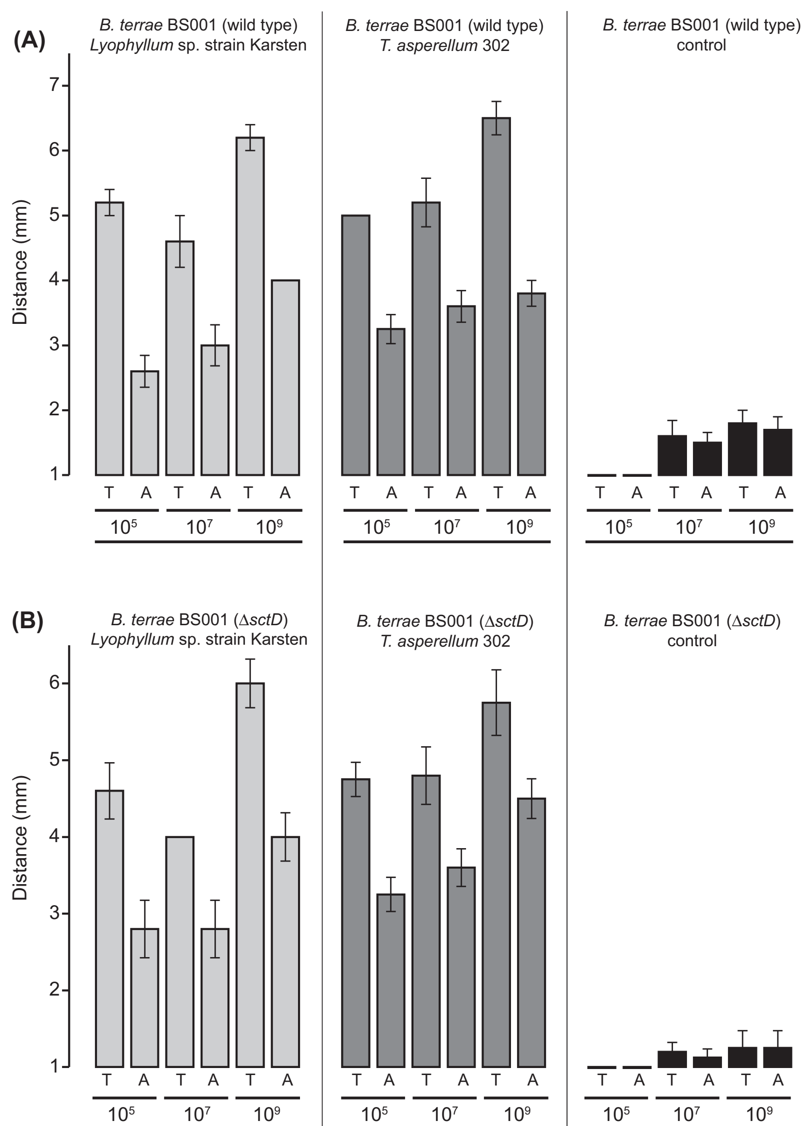

Figure 2. Chemotaxis (swimming) of wild-type and Δ*sctD* mutant *B. terrae* BS001 towards *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 hyphae on 0.25% (w/v) soil extract agar. **(A)** The chemotactic responses of wild-type *B. terrae* BS001. **(B)** The chemotactic responses of *B. terrae* BS001-Δ*sctD*. Cell densities are shown below the bars. A: away; T: towards.

increasing concentration of glycerol in the M9 medium, both wild-type (Fig. 3A) and mutant strains (Fig. 3B) showed strong chemotactic responses towards *Lyophyllum* sp. strain Karsten, whereas the responses towards *T. asperellum* 302 were less pronounced (Fig. 3C and D). ANOVA of the data (Supplementary Tables S3 and S4) 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. 4A and B). Both the wild-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. 4). ANOVA of all the data (Supplementary Table S5) 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.

Structural analysis of *T. asperellum* 302 CMH/glucosylceramides

To better understand the potential of physical interaction of *B. terrae* BS001 with soil fungi, we focused 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 Figs S1 and S2, 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 chains (Figs S1 and S2).When subjected to tandem MS/MS fragmentation, the ion species *m/z* 762 and 778 generated

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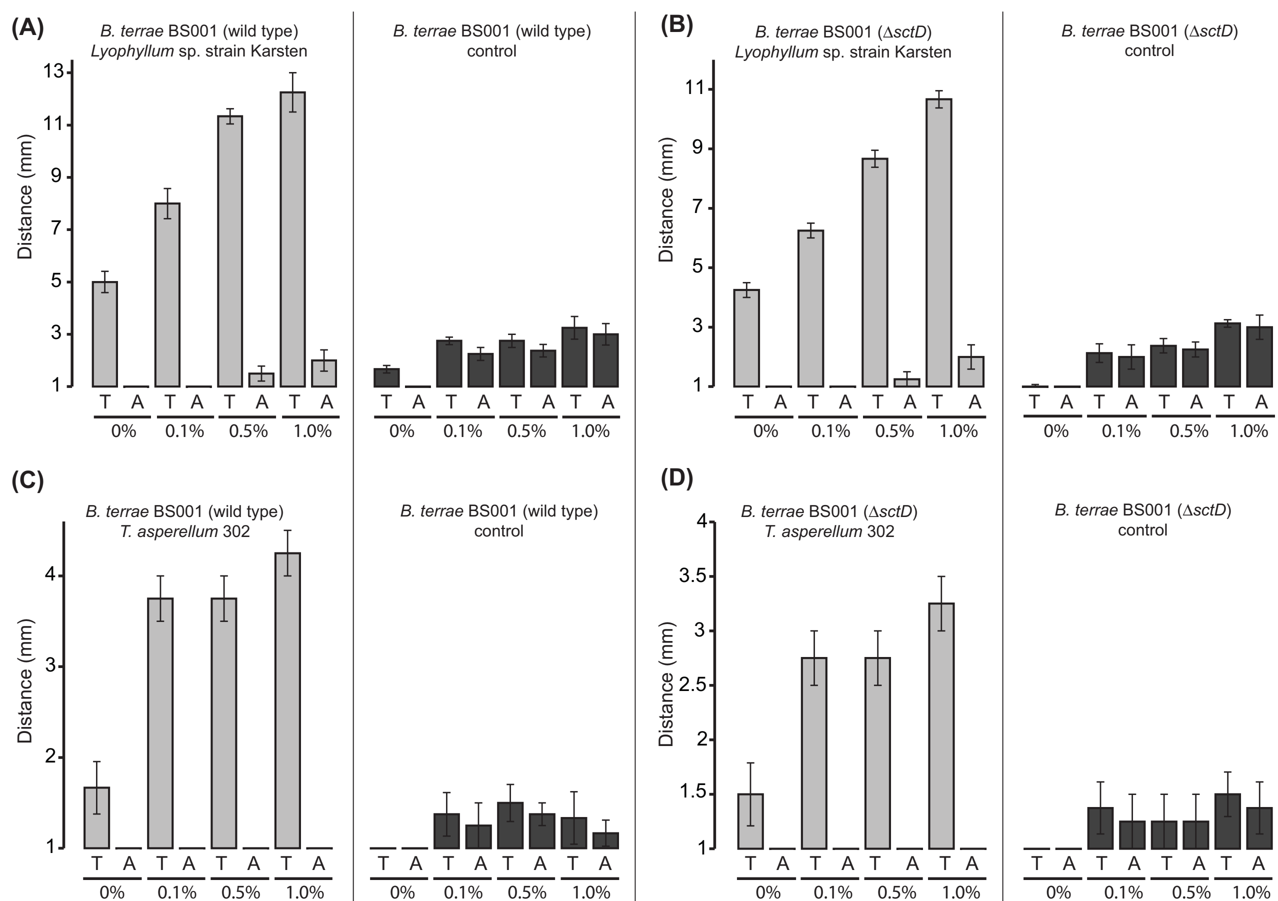

Figure 3. Chemotaxis (swimming) of wild-type and Δ*sctD* mutant *B. terrae* BS001 towards *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 hyphae on 0.25% (w/v) M9 medium supplemented with 0%, 0.1%, 0.5% and 1% glycerol (w/v). **(A,B)** The chemotactic responses of wild-type *B. terrae* BS001 (A) and Δ*sctD* mutant *B. terrae* BS001 (B) towards *Lyophyllum* sp. strain Karsten. **(C,D)** The chemotactic responses of wild-type *B. terrae* BS001 (C) and Δ*sctD* mutant *B. terrae* BS001 (D) towards *T. asperellum* 302. Concentrations of the glycerol are shown below the bars. A: away; T: towards.

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 Figs S1 and S2). The MS-MS spectrum of ion species *m/z* 778 is shown in Supplementary Fig. S3. 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 Figs S1 and S2.

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. Figure 5A reveals the presence of CMH in both, whereas the controls revealed low background signals. Figure 5B shows a comparison of CMH detection across *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 conidia. 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. 6A and B). 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. 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. 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 and 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*

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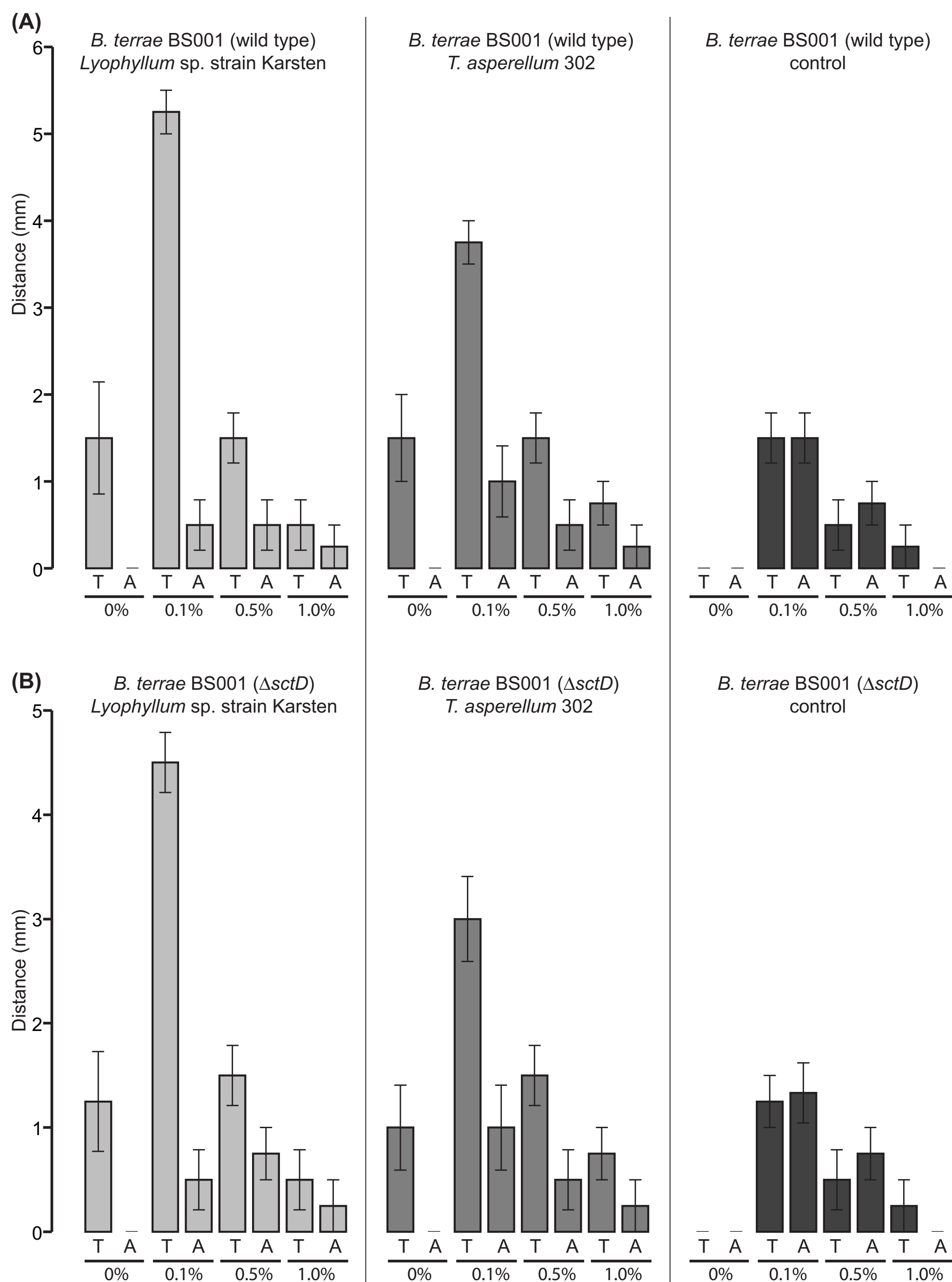

Figure 4. Chemotaxis (swimming) of wild-type and Δ*sctD* mutant *B. terrae* BS001 towards *Lyophyllum* sp. strain Karsten and *T. asperellum* 302 hyphae on 0.25% (w/v) M9 medium supplemented with 0%, 0.1%, 0.5% and 1% (w/v) oxalic acid. (**A**) The chemotactic responses of wild-type *B. terrae* BS001. (**B**) The chemotactic responses of Δ*sctD* mutant *B. terrae* BS001. Concentrations of oxalic acid are shown below the bars. A: away; T: towards.

showed lowered adherence ($P < 0.05$; Fig. 7A). Whereas the effect was evident over the first 3 h, 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. 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

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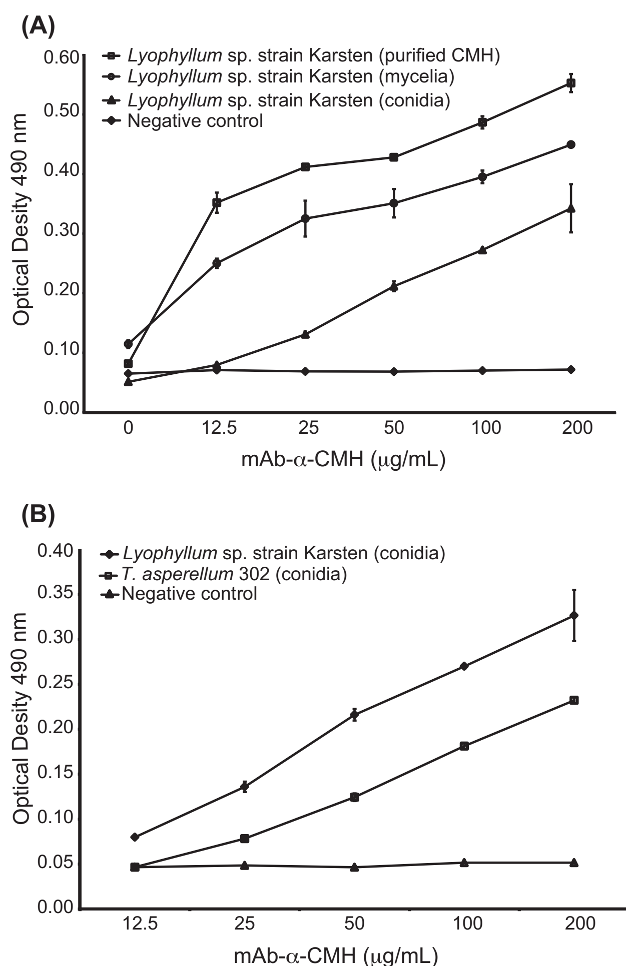

Figure 5. Reactivity of anti-CMH monoclonal antibodies (mAbs) to fungal CMH. (**A**) ELISA results for evaluating binding of mAbs at 200, 100, 50, 25, 12.5 or 0 μg mL^{-1} to wells coated with purified CMH, mycelia and conidia of *Lyophyllum* sp. strain Karsten. (**B**) Comparison of the binding of mAbs at different concentrations (200, 100, 50, 25, 12.5 or 0 μg mL^{-1}) to wells coated with conidia of *Lyophyllum* sp. strain Karsten and *T. asperellum* 302.

significantly ($P < 0.05$) upon the use of mAbs α-CMH (Fig. 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. 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 recognized the *T. asperellum* 302 CMH, to a similar extent, with the α-CMH mAbs showing poor blocking of the association (data not shown).

DISCUSSION

Although the *B. terrae* BS001–*Lyophyllum* sp. strain Karsten association and 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, Tazetdinova and van Elsas 2014), there are still questions about the precise mechanisms that play roles in the initial phases of the interaction. Thus, the occurrence of antagonistic *vs* 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 signaling and chemotaxis. Thirdly, although *B. terrae* BS001 has been found to form biofilms with the two fungal counterparts (Warmink and van Elsas 2009; Nazir, Tazetdinova and van Elsas 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 (T. V. M. 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, Fischer and Tranvik (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, Klein Gunnewiek and Woldendorp 1998). The growth retardation effect exerted on *Lyophyllum* sp. strain Karsten does not preclude 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.

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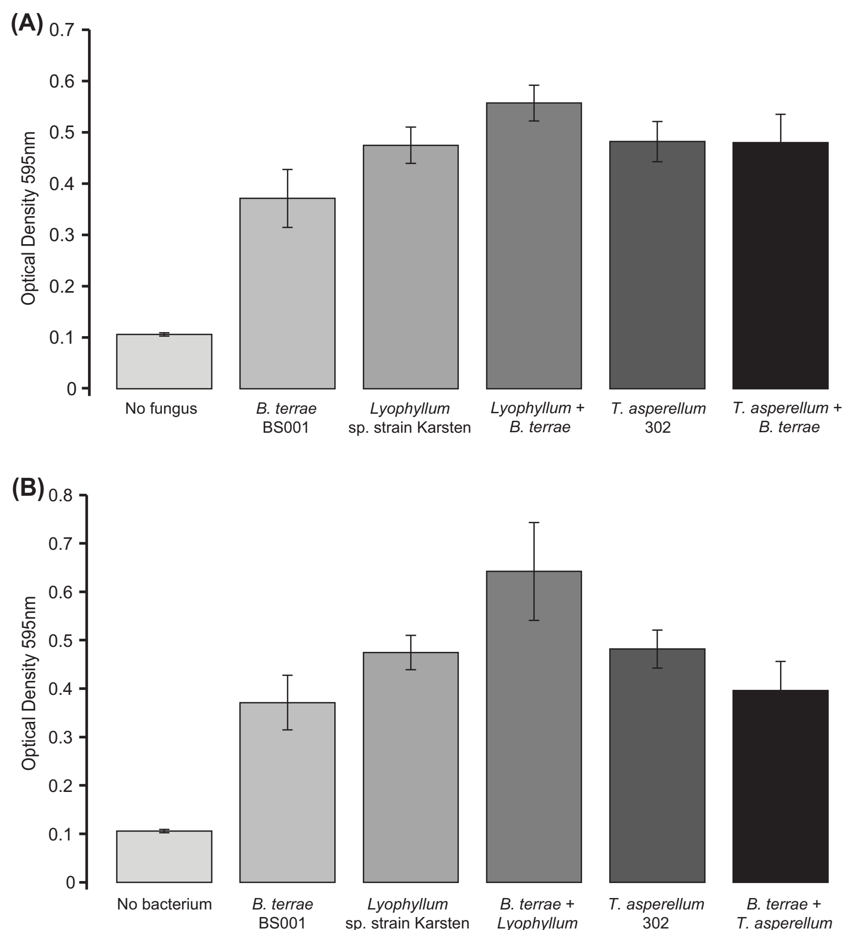

Figure 6. Bacterial–fungal behavior during mixed biofilm formation. (**A**) Biofilm formation when fungi were added as first colonizers. (**B**) Biofilm formation when *B. terrae* BS001 was added as first colonizer. Y-axis shows biomass quantified indirectly by the Crystal Violet method.

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, van Veen and de Boer (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, van Veen and de Boer (2015) recently found oxalic acid to act as a signaling 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, van Veen and de Boer 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 signaling molecule and may also have a growth promotion effect on *B. terrae* BS001. We are currently further pursuing the role of oxalic acid, in terms of whether it serves as merely a signaling 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

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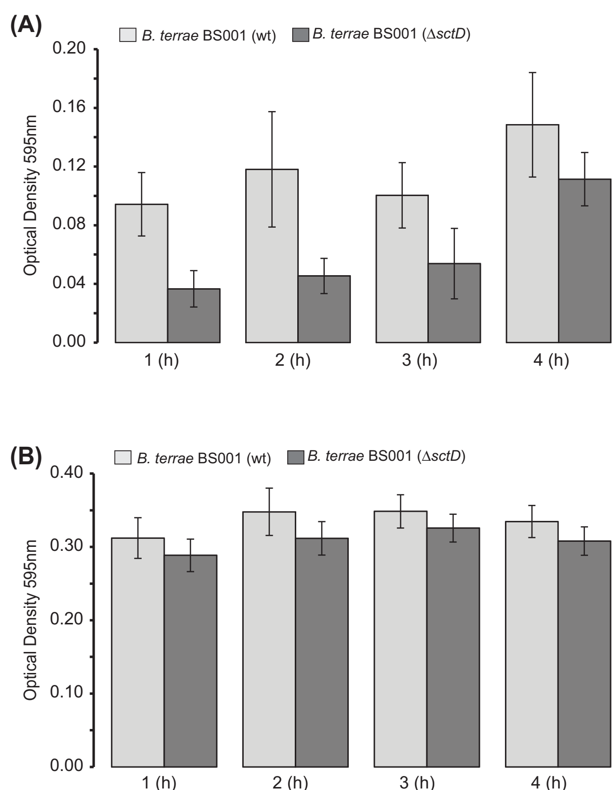

Figure 7. The attachment of *B. terrae* BS001 and *B. terrae*-Δ*sctD* to fungal conidia. (**A**) The attachment of *B. terrae* BS001 and *B. terrae*-Δ*sctD* to the conidia of *Lyophyllum* sp. strain Karsten. (**B**) The attachment of *B. terrae* BS001 and *B. terrae*-Δ*sctD* to conidia of *Trichoderma asperellum* 302. The Y-axis shows biomass quantified indirectly by the Crystal Violet method, while the duration of adherence is shown below the bars. Biofilm quantification was noted at different incubation times (1, 2, 3 and 4 h).

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 set-up 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 reproductive 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 (Paris *et al.* 2003; Beauvais *et al.* 2014). They have also been shown to contain multiple CMH molecules on their surfaces (Da Silva *et al.* 2004; Nimrichter *et al.* 2004; Rollin-Pinheiro *et al.* 2014; Calixto *et al.* 2016), 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 (Da Silva *et al.* 2004; Rodrigues *et al.* 2000) as well as by the monoclonal antibodies used by us in this study (M. I. D. S. 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 molecular 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 a CMH in the surface of the latter fungus that occurred in a more exposed form than the one expressed in *T. asperellum* 302.

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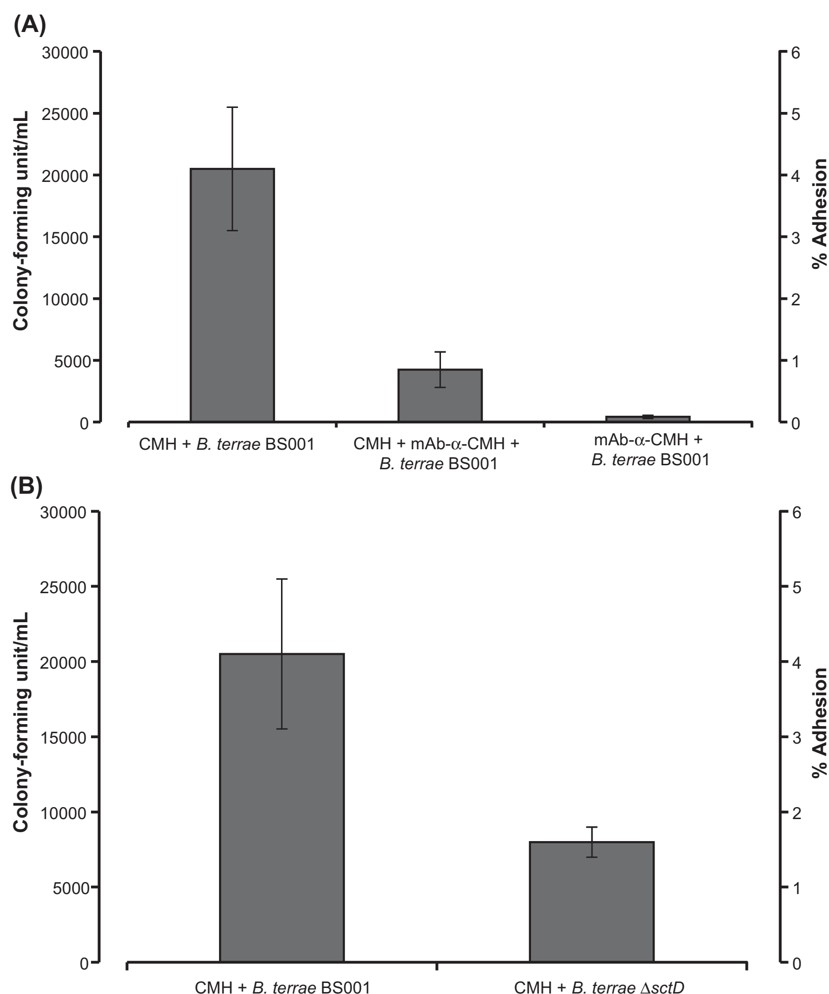

Figure 8. Adherence assay of *Burkholderia terrae* BS001 and Δ*sctD* mutant to fungal CMH. (**A**) The adherence of *B. terrae* BS001 to CMH of *Lyophyllum* sp. strain Karsten in the presence or absence of mAbs α-CMH. (**B**) The adherence of *B. terrae* BS001 and *B. terrae* Δ*sctD* to CMH of *Lyophyllum* sp. strain Karsten. Time required for adherence was 2 h. Percentage adherence was calculated in relation to the initial cell density added in the start of the experiment.

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 with a faster one in the latter. On the other hand, at lower concentrations of oxalic acid the response was stronger than that with glycerol. *Burkholderia terrae* BS001 showed 'partial competitive' behavior 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.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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REFERENCES

Alteri CJ, Himpsl SD, Pickens SR *et al*. Multicellular bacteria deploy the type VI secretion system to preemptively strike neighboring cells. *PLoS Pathog* 2013;**9**:e1003608.

Barreto-Bergter E, Sassaki GL, de Souza LM. Structural analysis of fungal cerebrosides. *Front Microbiol* 2011;**2**:239.

Beauvais A, Fontaine T, Aimanianda V *et al*. *Aspergillus* cell wall and biofilm. *Mycopathologia* 2014;**178**:371–7.

Benoit I, van den Esker MH, Patyshakuliyeva A *et al*. *Bacillus subtilis* attachment to *Aspergillus niger* hyphae results in mutually altered metabolism. *Environ Microbiol* 2015;**17**:2099–113.

Bowman SM, Free SJ. The structure and synthesis of the fungal cell wall. *Bioessays* 2006;**28**:799–808.

Calixto RO, Rollin-Pinheiro R, Da Silva MI *et al*. Structural analysis of glucosylceramides (GlcCer) from species of the *Pseudallescheria/Scedosporium* complex. *Fungal Biol* 2016;**120**: 166–72.

Da Silva AF, Rodrigues ML, Farias SE *et al*. Glucosylceramides in *Colletotrichum gloeosporioides* are involved in the differentiation of conidia into mycelial cells. *FEBS Lett* 2004;**561**:137–43.

De Boer W, Folman LB, Summerbell RC *et al*. Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS Microbiol Rev* 2005;**29**:795–811.

De Boer W, Klein Gunnewiek PJA, Woldendorp JW. Suppression of hyphal growth of soil-borne fungi by dune soils from vigorous and declining stands of *Ammophila arenaria*. *New Phytol* 1998;**138**:107–16.

De Weert S, Kuiper I, Lagendijk EL *et al*. Role of chemotaxis toward fusaric acid in colonization of hyphae of *Fusarium oxysporum* f. sp. radicis-lycopersici by *Pseudomonas fluorescens* WCS365. *Mol Plant Microbe Interact* 2004;**17**:1185–91.

Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957;**226**:497–509.

Frey-Klett P, Burlinson P, Deveau A *et al*. Bacterial-fungal interactions: hyphens between agricultural, clinical, environmental, and food microbiologists. *Microbiol Mol Biol Rev* 2011;**75**:583–609.

Fritsche K, van den Berg M, de Boer W *et al*. Biosynthetic genes and activity spectrum of antifungal polyynes from *Collimonas fungivorans* Ter331. *Environ Microbiol* 2014;**16**:1334–45.

Gibbs KA, Urbanowski ML, Greenberg EP. Genetic determinants of self identity and social recognition in bacteria. *Science* 2008;**321**:256–9.

Guimarães LL, Toledo MS, Ferreira FA *et al*. Structural diversity and biological significance of glycosphingolipids in pathogenic and opportunistic fungi. *Front Cell Infect Microbiol* 2014;**4**:138.

Hamaki T, Suzuki M, Fudou R *et al*. Isolation of novel bacteria and actinomycetes using soil-extract agar medium. *J Biosci Bioeng* 2005;**99**:485–92.

Haq IU, Graupner K, Nazir R *et al*. The genome of the fungal-interactive soil bacterium *Burkholderia terrae* BS001—a plethora of outstanding interactive capabilities unveiled. *Genome Biol Evol* 2014a;**6**:1652–68.

Haq IU, Zhang M, Yang P *et al*. The interactions of bacteria with fungi in soil: emerging concepts. *Adv Appl Microbiol* 2014b;**89**:185–215.

Kost T, Stopnisek N, Agnoli K *et al*. Oxalotrophy, a widespread trait of plant-associated *Burkholderia* species, is involved in successful root colonization of lupin and maize by *Burkholderia phytofirmans*. *Front Microbiol* 2014;**4**:421.

Kumar A, Shukla PK. A monoclonal antibody against glycoproteins of *Aspergillus fumigatus* shows anti-adhesive potential. *Microb Pathog* 2015;**79**:24–30.

Lackner G, Moebius N, Hertweck C. Endofungal bacterium controls its host by an *hrp* type III secretion system. *ISMEJ* 2011;**5**:252–61.

Latgé JP. Tasting the fungal cell wall. *Cell Microbiol* 2010;**12**: 863–72.

Latgé JP. The cell wall: a carbohydrate armour for the fungal cell. *Mol Microbiol* 2007;**66**:279–90.

Levy A, Merritt AJ, Mayo MJ *et al*. Association between *Burkholderia* species and arbuscular mycorrhizal fungus spores in soil. *Soil Biol Biochem* 2009;**41**:1757–59.

Lopes LC, Rollin-Pinheiro R, Guimarães AJ *et al*. Monoclonal antibodies against Peptidorhamnomannans of *Scedosporium apiospermum* enhance the pathogenicity of the fungus. *PLoS Negl Trop Dis* 2010;**4**:e853.

Mille-Lindblom C, Fischer H, Tranvik LJ. Antagonism between bacteria and fungi: substrate competition and a possible tradeoff between fungal growth and tolerance towards bacteria. *Oikos* 2006;**113**:233–42.

Nakakuma H, Arai M, Kawaguchi T *et al*. Monoclonal antibody to galactosylceramide: discrimination of structural difference in the ceramide moiety. *FEBS Lett* 1989;**258**:230–2.

Nakasone KK, Peterson SW, Jong SC. Preservation and distribution of fungal cultures. In: Muller GM, Bills G, Foster MS (eds). *Biodiversity of Fungi: Inventory and Monitoring Methods*. Amsterdam: Elsevier, 2004, 37–47.

Nazir R, Tazetdinova DI, van Elsas JD. *Burkholderia terrae* BS001 migrates proficiently with diverse fungal hosts through soil and provides protection from antifungal agents. *Front Microbiol* 2014;**5**:598.

Nazir R, Warmink JA, Boersma H *et al*. Mechanisms that promote bacterial fitness in fungal-affected soil microhabitats. *FEMS Microbiol Ecol* 2010;**71**:169–85.

Nazir R, Warmink JA, Voordes DC *et al*. Inhibition of mushroom formation and induction of glycerol release-ecological strategies of *Burkholderia terrae* BS001 to create a hospitable niche at the fungus *Lyophyllum* sp. strain Karsten. *Microb Ecol* 2013;**65**:245–54.

Nimrichter L, Barreto-Bergter E, Mendonça-Filho RR *et al*. A monoclonal antibody to glucosylceramide inhibits the growth of *Fonsecaea pedrosoi* and enhances the antifungal action of mouse macrophages. *Microbes Infect* 2004;**6**:657–65.

Nimrichter L, Rodrigues ML. Fungal glucosylceramides: from structural components to biologically active targets of new antimicrobials. *Front Microbiol* 2011;**2**:212.

Nyholm PG, Pascher I. Steric presentation and recognition of the saccharide chains of glycolipids at the cell surface: favoured conformations of the saccharide-lipid linkage calculated using molecular mechanics (MM3). *Int J Biol Macromol* 1993;**15**:43–51.

Paris S, Debeaupuis JP, Crameri R *et al*. Conidial hydrophobins of *Aspergillus fumigatus*. *Appl Environ Microbiol* 2003; **69**:1581–8.

Pinto MR, Barreto-Bergter E, Taborda CP. Glycoconjugates and polysaccharides of fungal cell wall and activation of immune system. *Braz J Microbiol* 2008;**39**:195–208.

Ramírez-Granillo A, Canales MG, Espíndola ME *et al*. Antibiosis interaction of *Staphylococcus aureus* on *Aspergillus fumigatus* assessed *in vitro* by mixed biofilm formation. *BMC Microbiol* 2015;**15**:15–33.

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Rhome R, Singh A, Kechichian T *et al*. Surface localization of glucosylceramide during *Cryptococcus neoformans* infection allows targeting as a potential antifungal. *PLoS One* 2011;**6**:e15572.

Riedlinger J, Schrey SD, Tarkka MT *et al*. Auxofuran, a novel metabolite that stimulates the growth of fly agaric, is produced by the mycorrhiza helper bacterium *Streptomyces* strain AcH505. *Appl Environ Microb* 2006;**72**:3550–7.

Rodrigues ML, Travassos LR, Miranda KR *et al*. Human antibodies against a purified glucosylceramide from *Cryptococcus neoformans* inhibit cell budding and fungal growth. *Infect Immun* 2000;**68**:7049–60.

Rollin-Pinheiro R, Liporagi-Lopes LC, de Meirelles JV *et al*. Characterization of *Scedosporium apiospermum* glucosylceramides and their involvement in fungal development and macrophage functions. *PLoS One* 2014;**9**:e98149.

Rudnick MB, van Veen JA, de Boer W. Oxalic acid: a signal molecule for fungus-feeding bacteria of the genus *Collimonas? Environ Microbiol Rep* 2015;**7**:709–14.

Sambrook J, Russell DW. *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press, 2001.

Santos AL, Bittencourt VC, Pinto MR *et al*. Biochemical characterization of potential virulence markers in the human fungal pathogen *Pseudallescheria boydii*. *Med Mycol* 2009;**47**:375–86.

Simon R, Priefer U, Pühler A. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Nat Biotech* 1983; **1**:784–91.

Toledo MS, Suzuki E, Levery SB *et al*. Characterization of monoclonal antibody MEST-2 specific to glucosylceramide of fungi and plants. *Glycobiology* 2001;**11**:105–12.

Villas-Boas MH, Wait R, Silva RB *et al*. Ceramide glycosylation and fatty acid hydroxylation influence serological reactivity in *Trypanosoma cruzi* glycosphingolipids. *FEMS Microbiol Lett* 2005;**244**:47–52.

Warmink JA, van Elsas JD. Migratory response of soil bacteria to *Lyophyllum* sp. strain Karsten in soil microcosms. *Appl Environ Microb* 2009;**75**:2820–30.

Warmink JA, van Elsas JD. Selection of bacterial populations in the mycosphere of *Laccaria proxima*: Is type III secretion involved? *ISMEJ* 2008;**2**:887–900.

Yang P, Zhang M, Warmink JA *et al*. The type three secretion system facilitates migration of *Burkholderia terrae* BS001 in the mycosphere of two soil-borne fungi. *Biol Fertil Soils* 2016, DOI: 10.1007/s00374-016-1140-6.

Zhu C, Wang M, Wang W *et al*. Glucosylceramides are required for mycelial growth and full virulence in *Penicillium digitatum*. *Biochem Biophys Res Commun* 2014;**455**:165–71.

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