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The type three secretion system facilitates migration of *Burkholderia terrae* BS001 in the mycosphere of two soil-borne fungi

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Abstract The type three secretion system (T3SS) is known to play a critical role in several bacterial-eukaryotic cell interactions. Recent indirect evidence has also pointed to a role of this system in bacterial-fungal interactions in soil. In the current study, we examine if the T3SS of the fungal-interactive *Burkholderia terrae* strain BS001 can aid in the interaction of this bacterium with two soil fungi, i.e., *Lyophyllum* sp. strain Karsten and *Trichoderma asperellum* 302. We first analyzed the T3SS of strain BS001 and then constructed a knockout mutant of the essential *sctD* gene. The selected *sctD* mutant strain did not show any differences to the wild-type strain with respect to its growth and nutrient utilization behavior, excluding polar effects of the mutation. Then, the migration ability of the *sctD* mutant strain along with the hyphae of *Lyophyllum* sp. strain Karsten growing through presterilized soil was tested, revealing hampered comigration as compared to the wild-type strain. The effect was also observed with *T. asperellum* 302. However, the migration impairment was only noticed in mixed-inoculation experiments, whereas it remained unnoticed when the two strains were inoculated in separate. These data demonstrate that the T3SS assists *B. terrae* BS001 in its interaction with two soil fungi, without being essential for these interactions. As far as we know, this is the first time that the role of a T3SS in the comigration of bacteria along with soil-exploring fungi is verified directly.

Keywords *Burkholderia terrae* BS001 · Bacterial migration · Type 3 secretion system · Mycosphere · Bacterial-fungal interaction

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Introduction

The type three secretion system (T3SS) is a protein delivery system in Gram-negative bacteria, which is divided into two families, i.e., (1) the flagellar and (2) the non-flagellar T3SS (NF-T3SS). The former system drives motility, and the latter is often involved in pathogenesis (Abby and Rocha 2012). In the subsequent text, we prefer to use the term T3SS (instead of NF-T3SS), as we place a focus on the NF-T3SS in this paper. The T3SS system is very complex, consisting of more than 20 proteins (Galan et al. 2014). These include extracellular components, the so-called outer membrane ring complex and an inner membrane ring complex (Yip and Strynadka 2006). The T3SS is located at the bacterial inner membrane and spans this, as well as the outer membrane, reaching out to the exterior on one side and the host cell cytoplasm on the other. It has been reported that the T3SS mediates the attachment of bacterial cells to plant or animal cell surfaces and the injection of effector proteins into the cytosol of host cells. Thus, the T3SS is involved in multiple bacterial-eukaryotic interactions that range from symbiosis (Silver et al. 2007; Correa et al. 2012), particularly in nitrogen-fixing bacterial-plant interactions (Dai et al. 2008; Okazaki et al. 2009; Saad et al. 2012; Piromyou et al. 2015) to parasitism, especially in bacterial-animal interactions (Plano and Schesser 2013; Neeld et al. 2014). Thus, T3SSs play diverse roles in the interactions of bacteria with their hosts.

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Additionally, several previous studies have suggested a role for the T3SS in bacterial-fungal interactions. Warmink and van Elsas found that the abundance of T3SS-positive isolates was dramatically higher in the mycosphere of *Laccaria proxima* than in corresponding bulk soil (Warmink and van Elsas 2008). Another study showed that T3SS-positive pseudomonads were significantly more abundant on mycorrhizal roots than on non-mycorrhizal ones (Viollet et al. 2011). Also, the intracellular bacterium *Burkholderia rhizoxinica* was found to employ its T3SS in the interaction with the fungal host *Rhizopus microsporus* (a pathogen of rice), modulating sporulation (Lackner et al. 2011). On the other hand, the T3SS can be involved in antagonistic bacterium-host interactions, as revealed by the fact that the biocontrol activity of *Pseudomonas fluorescens* against the cucumber pathogen *Pythium ultimum* was strongly reduced when a T3SS-deficient mutant strain was used instead of the wild-type (Rezzonico et al. 2005). Remarkably, mutation in the T3SS locus did not affect the growth-promoting effect of another *Pseudomonas fluorescens*, the mycorrhization helper strain BBc6R8, on *Laccaria bicolor* in vitro. However, it failed to promote the establishment of the Douglas fir-*L. bicolor* symbiosis (Cusano et al. 2011).

Burkholderia terrae BS001, an excellent colonizer of fungal hyphae (Nazir et al. 2014), can migrate along with growing hyphae of *Lyophyllum* sp. strain Karsten, as well as *Trichoderma asperellum* 302 and *Fusarium oxysporum* Fo47. It can form biofilms around the hyphae of all of these fungi (Warmink and van Elsas 2009; Nazir et al. 2014). Migration along with *Lyophyllum* sp. strain Karsten was found in various soils (Nazir et al. 2012). Strain BS001 also facilitated the movement of the non-migrator *Dyella japonica* BS013, allowing it to disperse along with the fungal hyphae (Warmink et al. 2011). Interestingly, *B. terrae* BS001 was able to survive in acid soil (pH 4.1–4.5) only in the presence of *Lyophyllum* sp. strain Karsten hyphae (Warmink and van Elsas 2009). Furthermore, it protected this fungal host against antagonists in soil microcosms (Nazir et al. 2014) but inhibited primordium setting (leading to mushroom formation) in liquid microcosms (Nazir et al. 2013). Thus, the interactions between *B. terrae* BS001 and its fungal host are very intricate, making it likely that a plethora of mechanisms are involved.

Analysis of the genome of *B. terrae* BS001 recently yielded evidence for the contention that one functional T3SS gene cluster is present in this strain (Haq et al. 2014). In a previous study, molecular-based evidence suggested a role for the T3SS in the bacterial interaction with soil fungi (Warmink and van Elsas 2009). However, no direct evidence on the basis of tests with living bacteria has as yet been presented. In the current study, we further analyzed this system, hypothesizing that the T3SS of *B. terrae* BS001 is involved in the interaction between this organism and its host fungi in soil. To test this hypothesis, we constructed a mutant of strain BS001 with a debilitated T3SS, on the basis of the *sctD* gene.

We subsequently examined the effect of the mutation on the migration behavior of strain BS001 in the mycosphere of two selected soil fungi, i.e., the basidiomycete *Lyophyllum* sp. strain Karsten and the ascomycete *T. asperellum* 302. The results support the notion that the T3SS plays a non-essential role in the *B. terrae* BS001-fungal interaction in soil.

Materials and methods

Strains and cultural conditions

B. terrae BS001, which was isolated from the mycosphere (Warmink and van Elsas 2009), was cultured at 28 °C in LB broth (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl, Sigma-Aldrich Co., USA) with shaking, or on R2A agar (0.5 g/L yeast extract, 0.5 g/L proteose peptone no. 3, 0.5 g/L casamino acids, 0.5 g/L dextrose, 0.5 g/L soluble starch, 0.3 g/L sodium pyruvate, 0.3 g/L dipotassium phosphate, 0.05 g/L magnesium sulfate, and 15 g/L agar, Difco, USA). The fungal hosts used in this study were *Lyophyllum* sp. strain Karsten (DSM2979) and *T. asperellum* 302 (Nazir et al. 2014). Fungi were grown on oat flake agar (OFA, 30 g/L oat flake, 15 g/L agar, Warmink and van Elsas 2009) at 28 °C. Once every 4 weeks, fungal strains were transferred to fresh OFA for maintenance.

Construction of an *sctD* mutant of *B. terrae* BS001

A T3SS mutant strain was constructed by knocking out the *sctD* gene via double crossover allelic exchange using suicide vector pSUP101 (chloramphenicol [Cm] resistance, Simon et al. 1983). Figure 1 shows the strategy used. Fragments homologous to the *sctD* flanks were amplified by PCR using primer pairs sctD-LF1/sctD-LR and sctD-RF/sctD-RR1, respectively (Fig. 1). Primers were designed based on the published genome sequence of *B. terrae* BS001 (Haq et al. 2014) using Primer Premier 5 software. The two fragments were purified and fused by fusion PCR using primer pair sctD-LF1/sctD-RR1. The fused fragment was ligated into the pGEM-T vector (Promega Corporation, Madison, USA), and the new construct was verified by agarose gel electrophoresis. Then, the construct was digested with HindIII and the relevant fragment was ligated with HindIII-digested pSUP101 DNA, yielding pSUP101-Δ*sctD*. Following this, vector pSUP101-Δ*sctD* was introduced into the *B. terrae* BS001 genome by conjugation with the mobilizing strain *Escherichia coli* S17-1 (pSUP101-Δ*sctD*) (Simon et al. 1983), selecting a presumed single crossover mutant on R2A agar supplemented with nitrofurantoin (50 mg/L) and chloramphenicol (25 mg/L). Finally, five colonies grown on this medium were streaked to purity, after which one was transferred to fresh LB broth, grown (shaking, 28 °C), and transferred daily to new LB broth
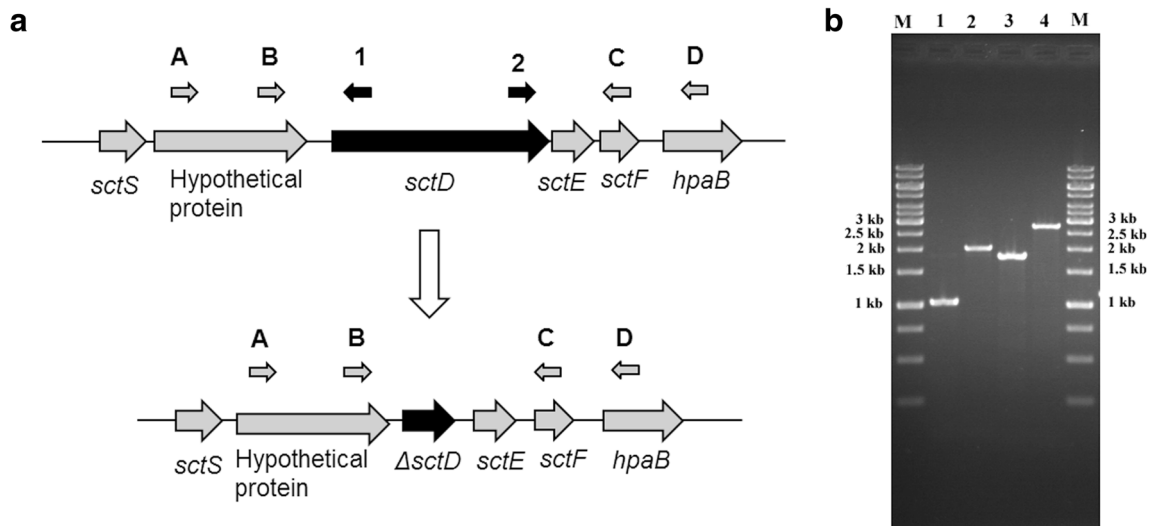
Fig. 1 Basic idea of *sctD* knockout. **a** Primer position along with *sctD* and the flanking genes. *A, B, C, D, 1, 2* are primers. *A*, sctD-LF2; *B*, sctD-LF1; *C*, sctD-RR1; *D*, sctD-RR2; *1*, sctD-LR, *2*, sctD-RF. **b** PCR verification of *sctD* knockout. *M*, GeneRuler 1-kb DNA Ladder (ThermoFisher Scientific). *1, 2*, used primer pair sctD-LF1/ sctD-RR1; *3, 4*, used primer pair sctD-LF2/ sctD-RR2; *1, 3*, used genomic DNA of *B. terrae* BS001Δ*sctD* as template; *2, 4*, used genomic DNA of *B. terrae* BS001 wild-type strain as template. The PCR product of Δ*sctD* mutant strain were 1 kb less than wild-type strain using each primer pair, respectively

in order to select the (desired) double-crossover mutant strain. Following 14 transfers, the culture was diluted and spread on R2A agar without antibiotics. Single colonies were randomly picked and checked for Cm resistance (25 mg/L). Five Cm-sensitive colonies were streaked to purity. Then, primer pairs sctD-LF1/sctD-RR1 and sctD-LF2/sctD-RR2 were used on colony material to identify the desired mutant strain. For the details of the strategy and the primers used, see Fig. 1a and Table 1.

Soil microcosms

For all experiments, soil from Gieterveen, the Netherlands, was used (denoted G soil). The soil was freshly sampled as topsoil (10-cm depth) and taken to the laboratory, where it was homogenized. The G soil was characterized as a sandy loam, with pH 5.1 and organic carbon (C) and total nitrogen (N) contents of 2.8 and 0.8 %. The soil was adjusted to pH 6.8 by adding 0.5 % $CaCO_3$. Then, it was autoclaved (121 °C, 27 min) three times, with intermittent incubation

at room temperature. Soil microcosms were then prepared aseptically, in three-compartment Petri dishes according to Warmink and van Elsas (2009). Briefly, one compartment was filled with OFA, and the other two with the sterile amended G soil (moisture content 17 %).

To start up the experiments, OFA plugs containing fungal growth were placed in the OFA compartments of the microcosms, and allowed to grow out at 28 °C. At the time the fungi over the barrier between the OFA and soil compartments (approx. 5 days for *Lyophyllum* sp. strain Karsten and 3 days for *T. asperellum* 302), about 5×10^5 bacterial cells were introduced at the hyphal growth fronts in each soil compartment. Three different experimental setups were performed: (1) *B. terrae* BS001 wild-type strain alone, (2) *B. terrae* BS001Δ*sctD* mutant strain alone, and (3) a 1:1 mixture of wild-type and mutant strains. The soil microcosms were closed using parafilm and incubated horizontally at 28 °C. Then, soil samples were recovered, by punching out at both the inoculation and the migration sites (i.e., at the hyphal fronts) at days 4, 7, and 14 for *Lyophyllum* sp. strain Karsten

Table 1 Primers used in this study

Primer names	Sequence (5′–3′)	Restriction enzyme
sctD-LF1	GCT<u>AAGCTT</u>CGCCCAGGTTCGCCAGTC	Hind III
sctD-LF2	GCT<u>AAGCTT</u>GACGGGCCGCTCCGG	Hind III
sctD-RR1	GCT<u>AAGCTT</u>GTGACAGTGCTCAGTGCGGATG	Hind III
sctD-RR2	GCT<u>AAGCTT</u>GAAGCAGTCGAGCCGCACAT	Hind III
sctD-LR	**TTCGTTTGCGGCTTT**CCAGTCGGTGATGCGAATGTC	
sctD-RF	**AAAGCCGCAAACGAA**CCGC	

The restriction sites at the primers are shown by underline. The nucleotides in bold represent 15-bp overlap sequences used for fusion PCR

or day 2 for *T. asperellum* 302. The samples were suspended in 0.85 % NaCl, shaken intensely (1 min, three times, with 30-s intervals), serially tenfold diluted and spread on R2A plates. For each experiment, four replicates were used.

Analysis of strain BS001 population composition in the mixed-inoculant experiments

The percentages of the mutant strain in the mixed culture samples were measured by colony PCR using primer pair sctD-LF1/sctD-RR1. Following incubation, 24 colonies were picked randomly from suitable-diluted R2A agar plates and subjected to PCR analysis with the above primers. The PCR products were checked by agarose gel electrophoresis, and the numbers of colonies showing either wild-type or mutant amplicon sizes were quantified.

Statistical analysis of the data

All data obtained were subjected to analysis of variance (ANOVA) using the statistical program SPSS. Differences of the means were considered to be significant at $P < 0.05$.

Results

Analysis of the *B. terrae* BS001 T3SS

Overall information on the single T3SS in the *B. terrae* BS001 genome was obtained from Haq et al (2014). A more detailed analysis of this system, like performed here, revealed the system to be 18.0 kb in length, showing the presence of genes for 22 predicted proteins (Table 2). As suggested previously (Haq et al 2014), the T3SS of *B. terrae* BS001 belongs to the hrp2 family, which includes most of the *Burkholderia* and *Ralstonia* T3SSs. It is to a large extent syntenous with the T3SS system of *B. rhizoxinica* HKI 0454 (Lackner et al. 2011; Haq et al. 2014).

The predicted protein HpaB (Haq et al. 2014) was found to be identical to HpaB encoded by *B. rhizoxinica* HKI 0454 (Lackner et al. 2011). BLAST-P analysis showed that it contains a conserved domain which belongs to the Tir chaperone protein (CesT) family. CesT serves as a chaperone to maintain the stability of secreted proteins and their presentation in a secretion-competent state in the cytosol (Delahay et al. 2002). Secondly, the predicted 73 amino acid protein denoted SctF, a possible functional homologue of SctF (Haq et al. 2014), potentially represents the main component of the pilus of the T3SS injectisome (Hoiczyk and Blobel 2001), also being required in host cell detection (Torruellas et al. 2005). SctE has been proposed to be involved in needle assembly (Lackner et al. 2011); however, the exact function of this protein has not been elucidated yet. SctD is predicted to be

located on the inner membrane, forming the basal body together with SctJ and SctC. Furthermore, the SctR, SctS, SctT, SctU, and SctV proteins were predicted to be involved in formation of the main effector export apparatus (Diepold et al. 2011), linking with SctD as mediated by SctJ (Diepold et al. 2010). Furthermore, we found all other predicted T3SS proteins in the canonical order. These are SctQ (formation of cytosolic complex C-ring, Diepold et al. 2015), SctP (controlling the length of the needle; Journet et al. 2003), SctI (inner rod protein; Wood et al. 2008), SctL (combines SctQ and SctK to form a "sorting platform" that governs the order of substrate export; Jackson and Plano 2000; Diepold et al. 2015), SctN (ATPase, essential for stabilization of C-ring; Jackson and Plano 2000; Diepold et al. 2015), and SctO (unknown function). The last one gene was not complete since it was at the end of the contig. However, it is homologous to SctC (BLASTP).

General properties of the *B. terrae* BS001 knockout mutant BS001Δ*sctD*

After the knockout process (see Materials and methods), 1014 bp of the *sctD* gene in *B. terrae* BS001 was deleted. The mutant strain, which was coined *B. terrae* BS001Δ*sctD*, was examined by direct PCR focusing on the *sctD* deletion site and revealed amplicons of around 1 kb smaller than those generated by the wild-type strain (two different sets of primers used, Fig. 1b). Furthermore, the mutant strain *B. terrae* BS001Δ*sctD* showed very similar growth dynamics to the wild-type in LB medium (Fig. 2). Additionally, we tested the phenotypic trait patterns of the two strains by BIOLOG GEN III microplates. In this analysis, the mutant strain showed exactly the same substrate utilization pattern as the wild-type strain, as detailed below. The BIOLOG GEN III plates consist of two parts, i.e., (1) 71 carbon source utilization assays (columns 1–9), and (2) 23 chemical compound sensitivity assays (columns 10–12). Concerning the results from (1), these were consistent with results reported before, using the GN2 plate (Nazir et al. 2012). With respect to (2), the wild-type and mutant strains revealed the same tolerance to low pH, NaCl, and particular antibiotics.

Population dynamics of *B. terrae* BS001 in the mycosphere of *Lyophyllum* sp. strain Karsten

Single strain inoculations Both the wild-type and mutant strain survived well at the inoculation site in the mycosphere of *Lyophyllum* sp. strain Karsten, reaching around 10^8 cells/g dry soil (Fig. 3a). Moreover, they were also capable of migrating through soil along with the growing fungal hyphae (Fig. 3b), reaching up to 10^9 cells/g dry soil at the migration site. No large differences were found between the two strains that would point to a migration-abolishing role of the T3SS.

**Table 2** Predicted ORFs of T3SS cluster in *B. terrae* BS001

ORF	Gene	Gene length (bp)	Protein length (aa)	Predicted function	Homologous to *B. rhizoxinica* HKI0450 (BLASTP)
1	*hpaB*	456	151	Chaperone	60 %
2	*sctF*	222	73	Needle	–
3	*sctE*	243	80	Putative needle chaperone	45 %
4	***sctD***	1251	416	Needle complex inner rings	35 %
5	Hypothetical protein	882	293	Unknown	–
6	*sctS*	264	87	Export apparatus	59 %
7	*sctR*	657	218	Export apparatus	66 %
8	*sctQ*	1185	394	Cytoplasmic sorting plantform	31 %
9	*sctP*	561	186	needle length regulator	30 %
10	*sctV*	2085	694	Export apparatus	69 %
11	*sctU*	1074	357	Export apparatus	52 %
12	*sctG*	522	173	Unknown	42 %
13	*sctI*	393	130	Inner rod component	36 %
14	*sctJ*	801	266	Needle complex inner rings	64 %
15	*sctK*	741	246	Cytoplasmic sorting platform	36 %
16	*sctL*	765	254	Links ATPase to sorting platform	45 %
17	*sctN*	1359	452	ATPase	77 %
18	*sctO*	498	165	Unknown	38 %
19	*sctT*	858	285	Export apparatus	54 %
20	Hypothetical protein	429	142	Unknown	–
21	*HrpB*	1455	484	Unknown	44 %
22	*sctC*	>646	>215	Needle complex outer rings	N.A.

N.A. not applicable since the sequence of *sctC* in BS001 is not completed

*sctD*: *sctD* gene was knocked out to construct T3SS mutant strain

We thus surmised that more subtle effects of the *sctD* mutation might only become visible when using mutant and wild-type cells in direct competition with each other.

Joint inoculations Upon joint introduction, at the inoculation site, the proportion of the mutant strain in the mix remained at around 50 %, i.e., 48.7 ± 7.3 % at day 0 to 45.3 ± 3.6 % at day

Fig. 2 Growth curves of *B. terrae* BS001 and *B. terrae* BS001Δ*sctD* in LB broth medium
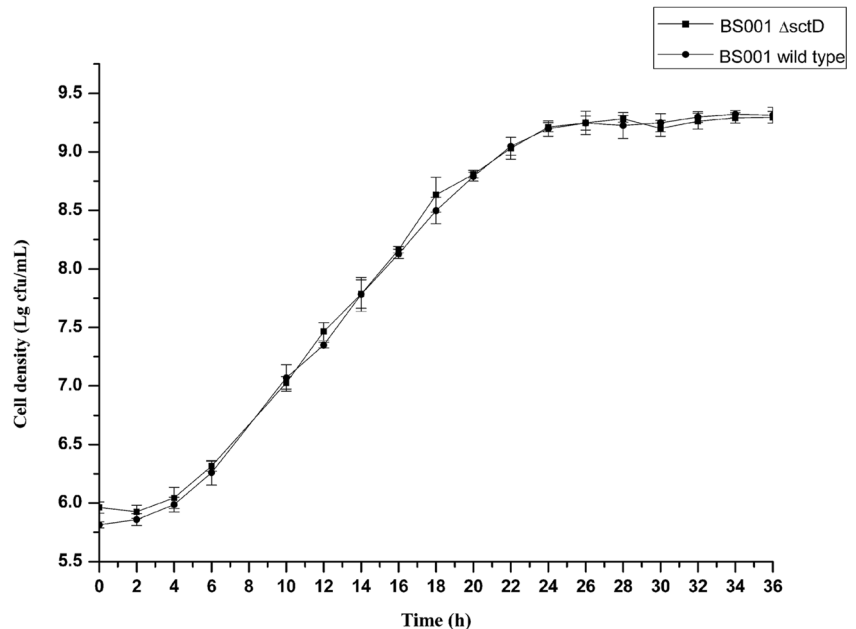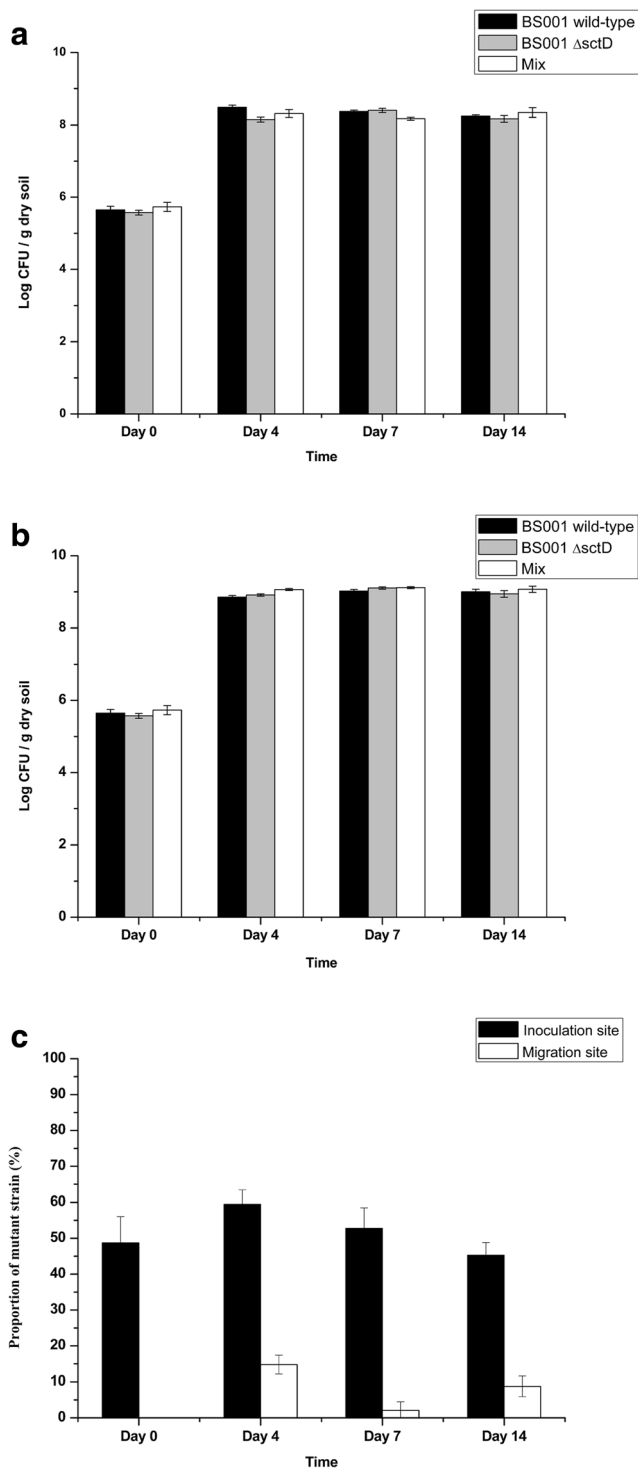
Fig. 3 Population dynamics of bacterial cells in mycosphere of *Lyophyllum* sp. strain Karsten. **a** Cell abundance (as indicated by editor) at inoculation site. **b** Cell abundance at migration site. **c** Abundance of mutant strain in the mixed-inoculant experiment in the mycosphere

14. However, at the migration site, this proportion decreased to 14.8 ± 2.6 % at day 4 and 2.5 ± 2.4 % at day 7, after which it increased slightly to 8.8 ± 2.9 % at day 14 (Fig. 3c). These values were all significantly lower than the initial 48.7 ± 7.3 %

($P < 0.001$), as well as the further values measured at the inoculation site, suggesting that, whereas the mutant had no ecological fitness disadvantage at the older hyphae, it did so at the hyphal migration front. At the latter site, younger (growing) hyphae were prevalent.
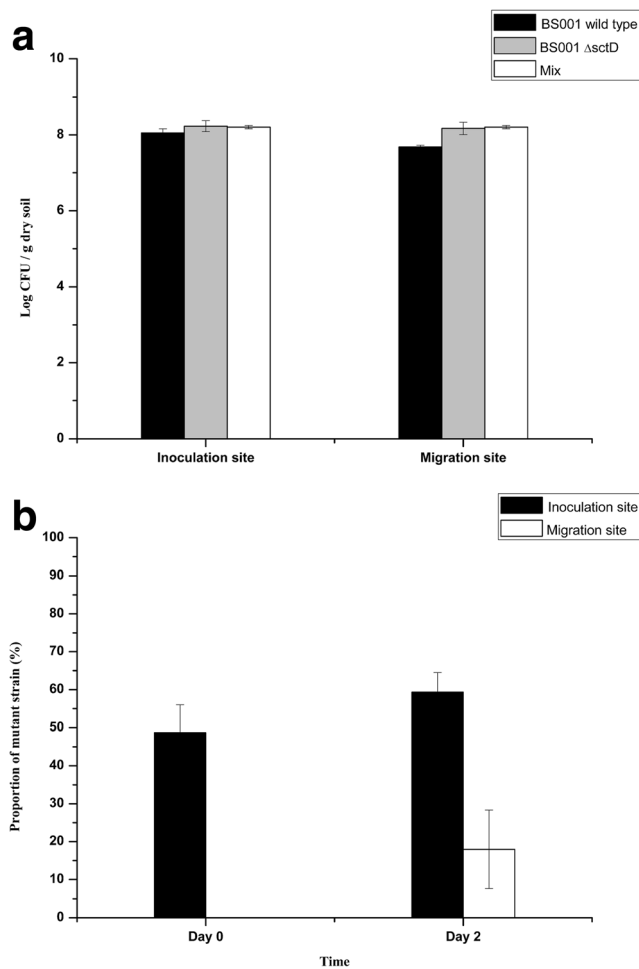
Population dynamics of *B. terrae* BS001 in the mycosphere of *T. asperellum* 302

To verify whether the *ΔsctD* effect reflected a "generalist" type of interaction of strain BS001 with fungal surfaces, we extended our study to the mycosphere of *T. asperellum* 302. *T. asperellum* 302 is a biocontrol fungus with which *B. terrae* BS001 has previously been found to form a migratory association (Nazir et al. 2014). Since this organism grows very rapidly through the amended G soil, we sampled the microcosm systems at just one early time point, i.e., day 2 (Fig. 4). In separate inoculation experiments, the mutant behavior was found to be similar to that of the wild-type strain, with similar CFU abundance at the inoculation and migration sites between the two strains, reaching up to 10^8 CFU/g dry soil (Fig. 4a). In the microcosms inoculated with (1:1) mixed cultures, the proportion of the mutant strain at the inoculation site remained at the initial level, i.e., around 59.3 ± 5.2 %. In contrast, it decreased significantly ($P < 0.01$), to 18.0 ± 10.4 %, at the migration site (day 2; Fig. 4b). These data are in line with those obtained with *Lyophyllum* sp. strain Karsten, indicating that also the *T. asperellum* 302 growing hyphal front selectively carried more wild-type (T3SS-positive) than mutant (T3SS-negative) cells through the soil.

Discussion

Fungal mycelium developing in soil allows bacterial cells to move to new microhabitats in which local nutrients can be utilized (Bravo et al. 2013), "remote" pollutants can be accessed (Wick et al. 2007; Zhao et al. 2016) and survival can be supported (Warmink and van Elsas 2009). Additionally, in plant-associated soil environments, fungal hyphae can transport bacteria, such as plant-growth-promoting or biocontrol organisms, to reach distant rhizosphere or rhizoplane sites, thus promoting effects of these (Shao et al. 2015). Then, the composition of the soil microbiome can be directed (Yu et al. 2015), rhizosphere niches, in which root-microbiome associations were disrupted, filled (Qiu et al. 2014), and phytopathogens antagonized (Tan et al. 2016). Thus, bacterial dispersal along with fungal hyphae in soil is very important for a number of key soil functions.

The T3SS has previously been suggested to play key roles in several bacterial-plant, bacterial-animal, and, incidentally, bacterial-fungal interactions (Lackner et al. 2011; Viollet et al. 2011). In this paper, we explore the role of the T3SS in the

Fig. 4 Population dynamics of bacterial cells in the soil microcosm of *Trichoderma asperellum* 302. **a** Cell abundance at day 2 in the mycosphere of *T. asperellum* 302. **b** Abundance of mutant strain in the mixed-inoculant experiment in the mycosphere

interaction of the mycosphere dweller *B. terrae* BS001 with two saprotrophic soil fungi, *Lyophyllum* sp. strain Karsten and *T. asperellum* 302. To achieve this aim, we successfully produced an *sctD*-minus mutant of strain BS001.

In previous work, an *sctD* (YscD in *Yersinia pestis*) mutant strain failed to secrete the effector protein YopM, indicating its key role in the T3SS (Plano and Straley 1995). Also, another *sctD* mutant strain (*prgH*, lacking a functional SpI-1 T3SS in *Salmonella enterica*) showed significantly lower recovery rate than the wild-type strain inside *Macrosteles quadrilineatus* (Dundore-Arias et al. 2015). According to the recent literature, the assembly of the T3SS has four steps, assembly of basal body rings and the export apparatus is a key first one (Burkinshaw and Strynadka 2014). SctD is a main component of the basal body ring (Burkinshaw and Strynadka 2014), linking the SctC (outer ring) and SctJ protein (component of inner ring) (Diepold et al. 2010). The subsequent recruitment of the cytoplasmic components SctK, SctL, SctN, SctO, and SctQ, and their linkage to the basal body, are thought to be

dependent on the first step (Galan et al. 2014). Thus, SctD plays a key role in the assembly of the T3SS, and we predict that a functional T3SS complex is not being built in the absence of SctD in *B. terrae* BS001Δ*sctD*.

One key requirement for sound mutant effect testing is that it did not undergo any other changes than the intended one. As far as we could see, this was the case for strain BS001Δ*sctD*, as it showed similar growth dynamics to the wild-type strain and did not reveal any capability to utilize the substrates encompassed in the BIOLOG GEN III plates (columns 1–9). Moreover, there was no impact of the mutation on the capacity of the strain to deal with stress (BIOLOG GEN III plates, columns 10–12). These data demonstrate that, within the confines of the experiment, the absence of the functional *sctD* gene (affecting the stability of the T3SS) had no significant effect on bacterial development and physiological status. In the sterilized soil, nutrients were expected to be available, first as a result of the autoclaving (releasing microbial based nutrients, Juarez et al. 2013) and secondly as a result of the release by active fungal hyphae. Thus, growth of bacterial cells in soil, along with the developing fungal hyphae, was expected, resulting in the increases of cell abundance from about 10^5 cfu/g dry soil at day 0 to 10^8 cfu/g dry soil at day 4.

We previously obtained circumstantial evidence for the contention that the T3SS may be involved in the *B. terrae* BS001-fungal interaction (Warmink and van Elsas 2008; 2009). In the current study, we provide direct evidence for this contention, on the basis of the data obtained with two fungi in coinoculation experiments. Thus, when both wild-type and mutant strains were introduced together, in a ratio close to 1:1, in the soil microcosms, the wild-type strain clearly outcompeted the mutant in terms of comigration with both fungal counterparts. However, this effect was not observed at the inoculation site, where mutant/wild-type ratio's remained roughly stable. Thus, the T3SS clearly plays a role, albeit a relatively minor one, in the migratory interaction of *B. terrae* BS001 with the two soil fungi. In a recent study, the NF-T3SS was considered to be derived from the flagellar T3SS, with a complex evolutionary history (Abby and Rocha 2012). However, in our study with *B. terrae* BS001, the loss of the functional T3SS (*sctD* gene, in this case) did not incur any differential motility toward fungal hyphae between the wild-type and mutant strain (Haq et al. 2016). On the contrary, the *B. terrae* BS001 Δ*sctD* mutant strain adhered to a lower extent to *Lyophyllum* sp. strain Karsten surfaces than the wild-type strain, with a potential involvement of ceramide monohexoside (CMH) in the binding (Haq et al. 2016). These novel data demonstrated that the T3SS can serve as an adhesion-enhancing mechanism, mediating or strengthening the attachment of bacterial cells to a host cell surface. Thus, the wild-type strain most likely outcompeted the mutant strain in the comigrational behavior by occupying more binding sites or by strengthening

existing bonds at the surfaces of the fungal tips. The enhanced number of wild-type versus mutant cells adhering to the extending fungal hyphae thus is at the basis of the detected mutant/wild-type ratio shifts.

The fact that the mutant strain apparently comigrated, to a lesser extent, with the growing fungal hyphae indicated that the comigration was not solely mediated by the T3SS. In other words, the comigration process is probably multifaceted, having the involvement of other (adhesion and/or movement) mechanisms, which jointly result in a superior migration ability. Examination of the 11.5 Mb BS001 genome, with 12,047 predicted coding sequences (CDSs) (Haq et al. 2014) indicated a suite of other systems that possibly mediate the adhesion of bacterial cell to host cell surfaces, i.e., systems for biofilm formation, type 4 pili, flagella, and type 4 and type 6 secretion systems (T4SS, T6SS). Type 4 pili and flagella are not only motility appendages, but they can also act as adhesins that connect bacteria to surfaces and facilitate near-surface motility prior to biofilm formation (Conrad 2012; Laverty et al. 2014). The T4SS is required in conjugation, which is cell-to-cell contact dependent. In this respect, transfers of conjugative plasmids from bacteria to fungi have been reported (Zhang et al. 2014). Additionally, in some cases, the T4SS is responsible for secretion of DNA into the external milieu, improving biofilm formation (Zweig et al. 2014). There are three T4SS gene clusters on the genome of *B. terrae* BS001. Only one of them, which is present on a 70.42-kb genomic island, contains all canonical T4SS functions (Haq et al. 2014). We assume this system may mediate *B. terrae* BS001 adhesion to the fungal surface and/or promote biofilm formation around the fungal hyphae indirectly by releasing DNA to its surroundings. Moreover, *B. terrae* BS001 carries several gene clusters that encode extracellular polysaccharide biosynthesis, including poly-beta-1, 6-N-acetyl-D-glucosamine (PGA) and Pel (a glucose-rich polysaccharide polymer) production (Haq et al. 2014). In other studies, these polymers have been shown to be able to aid biofilm formation (Vasseur et al. 2005; Itoh et al. 2008). Furthermore, cellulose has been reported to be involved in *Salmonella* biofilm formation on *Aspergillus niger* (Brandl et al. 2011), and cellulose production loci are also present in the *B. terrae* BS001 genome. From these considerations, we surmise that the migratory association of *B. terrae* BS001 with soil fungi is a multifaceted process, and the presence of the T3SS is an asset that enhances the migratory ability but is not strictly necessary.

In conclusion, this study confirms the role of a bacterial T3SS as a comigration helper mechanism through soil in the mycosphere of two soil fungi. *B. terrae* BS001 cells thus use their T3SS as helper systems that promote their migration in the mycosphere. Finally, we did not address the potential of the T3SS to deliver effector molecules into fungal cells, as our primary aim was to address the overall effect on migration. Future studies might address the potential fungal physiology

modulatory role of the strain BS001 T3SS. Our data are consistent with the contention that, besides the T3SS, other surface-exposed systems are employed by *B. terrae* BS001 in its interaction with soil fungi. Thus, strains debilitated in, for instance, type 4 pili (T4P), flagella, the T4SS, or the T6SS should be constructed for a further understanding the mechanism behind bacterial migration along with fungal hyphae.

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