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

Pu Yang, Miaozhi Zhang, Jan Aaldrik Warmink, Miao Wang, Jan Dirk van Elsas

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 *Paraburkholderia 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 knock-out 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 pre-sterilized soil was tested, revealing hampered co-migration as compared to the wild-type strain. The effect was also observed with *Trichoderma 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 *P. 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 co-migration of bacteria along with soil-exploring fungi is verified directly.
3.1 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 (Galán 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.

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 *Paraurkholderia 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 *P. 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).

*Paraburkholderia 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, *P. 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 *Paraburkholderia terrae* BS001 and its fungal host are very intricate, making it likely that a plethora of mechanisms are involved.

Analysis of the genome of *P. 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 *P. 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 *P. terrae* BS001-fungal interaction in soil.
3.2 Materials and methods

3.2.1 Strains and cultural conditions

*P. terrae* BS001, which was isolated from the mycosphere of *Laccaria proxima* (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 four weeks, fungal strains were transferred to fresh OFA for maintenance.

3.2.2 Construction of an sctD mutant of *P. terrae* BS001

A T3SS mutant strain was constructed by knocking out the *sctD* gene via double crossover allelic exchange strategy, using suicide vector pSUP101 [chloramphenicol (Cm) resistance, Simon et al., 1983] and the donor strain *Escherichia coli* S17-1 (Simon et al., 1983). Figure 3.1 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 (Figure 3.1). Primers were designed based on the published genome sequence of *P. 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-ΔsctD. Following this, vector pSUP101-ΔsctD was introduced into the *P. terrae* BS001 genome by conjugation with the mobilizing donor strain *E. coli* S17-1 (pSUP101-ΔsctD), selecting a presumed single cross-over 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 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 Figure 3.1a and Table 3.1.

Figure 3.1 Basic idea of sctD knock-out. (a) Primer positions 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 knock out. M, GeneRuler 1 kb DNA Ladder (Thermo Fisher 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 P. terrae BS001ΔsctD as template; 2, 4, used genomic DNA of P. terrae BS001 wild type strain as template. The PCR products of ΔsctD mutant strain were 1 kb less than wild-type strain using each primer pairs respectively.
Table 3.1 Primers used in this study

<table>
<thead>
<tr>
<th>Primer names</th>
<th>Sequence (5’ – 3’)</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>sctD-LF1</td>
<td>GCTAAGCTTGCCAGGTTCGCCAGTC</td>
<td>Hind III</td>
</tr>
<tr>
<td>sctD-LF2</td>
<td>GCTAAGCTTGGACGGGCGGCTCCGG</td>
<td>Hind III</td>
</tr>
<tr>
<td>sctD-RR1</td>
<td>GCTAAGCTTGCAAGTCTCAGTGCAGGATG</td>
<td>Hind III</td>
</tr>
<tr>
<td>sctD-RR2</td>
<td>GCTAAGCTTGAAAGCAGTCAGCCGCACAT</td>
<td>Hind III</td>
</tr>
<tr>
<td>sctD-LR</td>
<td>TTCGTTTGCGGGCTTTCCAGTCGGTGATGCGAA</td>
<td>Hind III</td>
</tr>
<tr>
<td>sctD-RF</td>
<td>AAAGCCGCAACGAACCAGC</td>
<td></td>
</tr>
</tbody>
</table>

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

3.2.3 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 (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 set-ups were performed: (1) *P. terrae* BS001 wild-type strain alone, (2) *P. terrae* BS001ΔsctD mutant strain alone, (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 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.

### 3.2.4 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.

### 3.2.5 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 $P<0.05$.

### 3.3 Results

#### 3.3.1 Analysis of the *P. terrae* BS001 T3SS

Overall information on the single T3SS in the *P. 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 3.2). As suggested previously (Haq et al., 2014), the T3SS of *P. terrae* BS001 belongs to the hrp2 family, which includes most of the *Paraurkholderia* and *Ralstonia* T3SSs. It is to a large extent syntenous with the T3SS system of *P. 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 \textit{P. 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).

### 3.3.2 General properties of the \textit{P. terrae} BS001 knock-out mutant BS001\textsubscript{ΔsctD}

After the knock-out process (See Materials and Methods), 1014 bp of the \textit{sctD} gene in \textit{P. terrae} BS001 was deleted. The mutant strain, which was coined \textit{P. terrae} BS001\textsubscript{ΔsctD}, was examined by direct PCR focusing on the \textit{sctD} deletion site, and revealed amplicons of around 1 kb smaller than those
### Table 3.2 Predicted ORFs of T3SS cluster in *P. terrae* BS001

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

N.A. not applicable since the sequence of *sctC* in BS001 is not completed.
generated by the wild-type strain (two different sets of primers used, see Figure 3.1b). Furthermore, the mutant strain *P. terrae* BS001ΔsctD showed very similar growth dynamics with the wild-type in LB medium (Figure 3.2). 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 phenotypic trait 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.

![Figure 3.2 Growth curves of *P. terrae* BS001 and *P. terrae* BS001ΔsctD in LB broth medium.](image)

**Figure 3.2** Growth curves of *P. terrae* BS001 and *P. terrae* BS001ΔsctD in LB broth medium.
3.3.3 Population dynamics of *P. terrae* BS001 in the mycosphere of *Lyophyllum* sp. strain Karsten

### 3.3.3.1 Single strain inoculations

Both the *P. terrae* BS001 wild-type and ΔsctD mutant strain survived well at the inoculation site in the mycosphere of *Lyophyllum* sp. strain Karsten, reaching around $10^8$ CFU/g dry soil (Figure 3.3a). Moreover, they were also capable of migrating through soil along with the growing fungal hyphae (Figure 3.3b), reaching up to $10^9$ CFU/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. We thus surmised that more subtle effects of the ΔsctD mutation might only become visible when using mutant and wild-type cells in direct competition with each other.

### 3.3.3.2 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 14 (Figure 3.3c). 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 (Figure 3.3c). 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) fungal hyphae were prevalent.

3.3.4 Population dynamics of *P. terrae* BS001 in the mycosphere of *Trichoderma 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 *P. 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
Figure 3.3 Population dynamics of bacterial cells in mycosphere of *Lyophyllum* sp. strain Karsten. (a) Cell abundance at inoculation site. (b) Cell abundance at migration site. (c) Proportion of mutant strain in the mixed-inoculant experiment in the mycosphere.
just one early time point, i.e. day 2 (Figure 3.4). In separate-inoculation experiments, the mutant behavior was found to be similar to that of the wild-type strain, with similar abundance at the inoculation and migration sites between the two strains, reaching up to $10^8$ CFU/g dry soil (Figure 3.4a). 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, Figure 3.4b). 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) cell through the soil.

### 3.4 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 and bacterial-animal interactions. The involvement of T3SS in bacterial-fungal interactions was also reported incidentally (Lackner et al., 2011; Viollet et al., 2011). In this paper, we explore the role of the T3SS in the interaction of the mycosphere dweller *Paraburkholderia 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 *S. 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 (Galán 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 _P. terrae_ BS001\(\Delta\)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 BS001\(\Delta\)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 density from about \(10^5\) CFU/g dry soil at day 0 to around \(10^8\) CFU/g dry soil at day 4.

We previously obtained circumstantial evidence for the contention that the T3SS may be involved in the _P. terrae_ BS001-fungal interaction (Warmink and van Elsas, 2009). In the current study, we provide direct evidence for this contention, on the basis of the data obtained with two fungi in co-inoculation 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 co-migration with both fungal counterparts. However, this effect was not observed at the inoculation site, where mutant/wild-type ratio was remained roughly stable. Thus the T3SS clearly plays a role, albeit a relatively minor one, in the migratory interaction of _P. 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 *P. terrae* BS001, the loss of the functional T3SS (*sctD* gene, in this case) did not incur any differential motility towards fungal hyphae between the wild-type and mutant strain (Haq et al., 2016). On the contrary, the *P. terrae* BS001Δ*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 co-migrational 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 co-migrated, to a lesser extent, with the growing fungal hyphae indicated that the co-migration was not solely mediated by the T3SS. In other words, the co-migration process is probably multi-faceted, 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 four pili, flagella and type four and type six secretion systems (T4SS, T6SS). Type four 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 *P. 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 *P. terrae* BS001 adhesion to the fungal surface and/or promote biofilm formation around the fungal hyphae indirectly by releasing DNA to
its surroundings. Moreover, *P. 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 *P. terrae* BS001 genome. From these considerations, we surmise that the migratory association of *P. terrae* BS001 with soil fungi is a multifaceted process, and the presence of the T3SS is one of the assets 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. *P. terrae* BS001 cells 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 investigate the overall effect on migration. Future studies might address the potential fungal physiology modulatory role of the T3SS in strain BS001. Our data are consistent with the contention that, besides the T3SS, other surface-exposed systems are employed by *P. terrae* BS001 in its interaction with soil fungi. Thus, strains debilitated in, for instance, type four pili (T4P), flagella, the T4SS or the T6SS should be constructed for a further understanding the mechanisms behind bacterial migration along with fungal hyphae in soil.

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