RESEARCH ARTICLE

Exploiting the fungal highway: development of a novel tool for the *in situ* isolation of bacteria migrating along fungal mycelium

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ABSTRACT

Fungi and bacteria form various associations that are central to numerous environmental processes. In the so-called fungal highway, bacteria disperse along fungal mycelium. We developed a novel tool for the *in situ* isolation of bacteria moving along fungal hyphae as well as for the recovery of fungi potentially involved in dispersal, both of which are attracted towards a target culture medium. We present the validation and the results of the first *in situ* test. Couples of fungi and bacteria were isolated from soil. Amongst the enriched organisms, we identified several species of fast-growing fungi (*Fusarium* sp. and *Chaetomium* sp.), as well as various potentially associated bacterial groups, including *Variovorax soli*, *Olivibacter soli*, *Acinetobacter calcoaceticus*, and several species of the genera *Stenotrophomonas*, *Achromobacter* and *Ochrobactrum*. Migration of bacteria along fungal hyphae across a discontinuous medium was confirmed in most of the cases. Although the majority of the bacteria for which migration was confirmed were also positive for flagellar motility, not all motile bacteria dispersed using their potential fungal partner. In addition, the importance of hydrophobicity of the fungal mycelial surface was confirmed. Future applications of the columns include targeting different types of microorganisms and their interactions, either by enrichment or by state of the art molecular biological methods.

Keywords: soil; fungal highways; bacteria; fungi; enrichment; columns

INTRODUCTION

Many bacteria are able to move by swimming, swarming, twitching, sliding, or gliding without pili (Biais 2009; Sun *et al.* 2011). However, except for the last, these bacterial motility types are only efficient in the presence of a liquid film (Harshey 2003).

Thus, in water-unsaturated soils, active movement of bacteria is strongly limited, principally due to the discontinuity of water films (Or *et al.* 2007). Nevertheless, *in vitro* experiments performed in water-unsaturated media have demonstrated that fungal mycelia can be used as paths for the active dispersal of

bacteria. Bacteria are shown to be moving in the synaeretic film formed around the hyphae, which act as fungal highways, as termed by Kohlmeier *et al.* (2005).

Knowing that there can be up to 20 000 km of hyphae per cubic meter of soil (Moore, Robson and Trinci 2011), and that fungal hyphae can colonize both water-saturated and air-filled voids between soil particles (Wösten 2001), mycelium-driven transport might play an important role in regulating the dispersal of bacteria in unsaturated soils. Recent *in vitro* studies have highlighted ecological benefits of this dispersal mechanism. For example, an improved biodegradation of soil pollutants has been demonstrated in soil microcosm experiments (Kohlmeier *et al.* 2005; Wick *et al.* 2007; Banitz *et al.* 2013). Mycelium-driven bacterial dispersal has also been suggested as a mechanism contributing to the explanation of the maintenance of flagella in soil bacteria (Pion *et al.* 2013; Lee *et al.* 2014). Likewise, promotion of long-term carbon storage and soil fertility through fungal–bacterial interactions in the context of the oxalate–carbonate pathway has been shown in soil microcosms (Martin *et al.* 2012).

However, in spite of its potential ecological importance, evidence of active bacterial movement along mycelium in soils is still missing. Various attempts have been made to isolate selectively soil bacteria moving along hyphae. In a first approach, a microcosm model based on a compartmentalized Petri dish in which *Lyophyllum* sp. strain Karsten was pre-inoculated allowed for the identification of several bacterial species potentially migrating along the hyphae (Warmink, Nazir and van Elsas 2009). More recently, mycelium of *Pythium ultimum* was artificially inoculated in soil to be used as a path for the isolation of contaminant-degrading bacteria (Furuno *et al.* 2012) using an inverted Petri dish method. Following the same idea, a system without pre-inoculation of filamentous microorganisms was used to show the dispersal of soil oxalotrophic bacteria on fungal mycelium *ex situ* (Bravo *et al.* 2013).

The aim of this study was to develop a method for *in situ* collection of both bacterial and fungal strains potentially interacting via fungal highways in natural environments such as soils. We here describe the design and the validation of this method, a novel centimeter-scale device, hereafter called the 'fungal highway column'. The applicability of the system was evaluated with microcosm experiments in the laboratory. A further validation step was performed in a field experiment with a soil under the influence of the oxalate–carbonate pathway. This type of soil was selected because fungal-driven bacterial dispersal seems to be relevant for oxalatrophic bacterial activity in soil under the influence of the oxalate–carbonate pathway (Martin *et al.* 2012; Bravo *et al.* 2013). From the field validation, easily cultivable fungi and bacteria recovered simultaneously in fungal highway columns were identified. For the characterization of the strains, we considered features displayed by bacteria and fungi described as part of fungal highway interactions. Previous studies have postulated that bacteria swim actively inside the liquid film surrounding hydrophilic hyphae (Kohlmeier *et al.* 2005), and therefore bacterial motility and fungal hydrophobicity were tested. Additionally, as positive selection of bacteria harboring the Type III secretion system (TTSS) has been proposed on the mycosphere (Warmink and van Elsas 2008), and bacterial migration along fungal hyphae has shown a strong positive correlation to the presence of genes encoding TTSS subunits (Warmink and van Elsas 2009), the presence of a gene marker for TTSS was assessed in the bacterial isolates. Finally, as the isolated organisms constitute only potential couples of bacteria and fungi interacting via fungal highways, the ability of each bacterium to migrate along its associated fungus was tested. Our results underpin the

usefulness of the columns as an easy-to-use tool to advance in the study of fungal–bacterial interactions in soils.

MATERIALS AND METHODS

Design of a fungal highway column

The fungal highway columns were designed as a completely closed device (Ø: 15 mm, height: ∼48 mm) intended to isolate bacteria able to disperse along fungal mycelium towards a target culture medium, which can be used for enrichment and isolation of both bacteria and fungi.

Unless otherwise stated, each component was sterilized by immersion in 70% ethanol for a duration of 30 min and exposure to UV-C light for 30 min before assembling. Culture media and glass beads were sterilized by autoclaving (21 min at 121°C). Each column was assembled under a sterile laminar flow hood, as follows (Fig. 1). A 10-mL polystyrene tube (Fig. 1a; Semadeni, Ostermundigen, Switzerland), Ø ∼ 15 mm, was cut 4 cm from the top. The middle part of this 4-cm tube was heated with a flame and twisted manually in order to leave an inside aperture of Ø ∼ 2 mm; a pierced polyethylene cap (Fig. 1b; Semadeni) was used to hold a pre-cut agar-based medium (Fig. 1c; attracting medium) inside the bottom part of the twisted tube to serve as an attracting medium, with the help of a 3 × 3 cm square of a 25-mm mesh opening tissue (Fig. 1d; Sefar, Heiden, Switzerland) placed on top of the hollow cap. The attracting medium encompassed the diameter of the tube but was irregular, leaving some space to allow movement of fungi or bacteria at the edges. A polyethylene cap (Fig. 1e) was used to close the bottom of the column; glass beads (Fig. 1f; Ø 1 mm; Glaswarenfabrik Karl Hecht, Sondheim v. d. Rhön, Germany) were used to fill the tube, leaving ∼1 cm free space at the top of it; a second slice of agar-based medium (Fig. 1g; target medium) was placed inside the upper part of the twisted tube, on top of the glass beads. The top of the tube was closed with a polyethylene cap (Fig. 1h; Ø 10.5 mm; Semadeni) and held with Parafilm® (Bemis, Oshkosh, USA). The excess tissue was cut with sterile scissors and covered with Parafilm®. Colored tape was placed on the tube for labeling.

The basic procedure to use a fungal highway column follows these steps (Fig. 2): the bottom cap is removed and the column is placed on a substrate (e.g. soil or a culture of microorganisms on solid medium) to be left in place for a duration of several days (see validation below). After removal from the substrate, the column is immediately closed with another bottom cap (previously sterilized in 70% ethanol). Directly, or after several days of incubation (see validation below), the cap on the top of the column is removed in a sterile environment (e.g. a sterile laminar flow hood), and the target culture medium is plated on a culture medium for the isolation of cultivable organisms or, alternatively, it can be used directly to extract nucleic acids.

Laboratory validation tests

A first validation test to evaluate bacterial dispersal in the presence and absence of a fungal carrier was conducted with the flagellated bacterium *Pseudomonas putida* KT2440 producing green fluorescent protein (GFP) constitutively (Table 1). This bacterium is known to disperse along hyphae of *Morchella crassipes* and *Trichoderma* sp. (Pion *et al.* 2013). *P. putida* was inoculated with *M. crassipes* or *Trichoderma* sp. in Petri dishes of malt agar (MA) medium, composed of 12 g·L^{-1} malt (Mycotec SA, La Chaux-de-Fonds, Switzerland) and 15 g·L^{-1} agar (Biolife Italiana S.r.l, Milano, Italy). As a control in the absence of a fungal network,
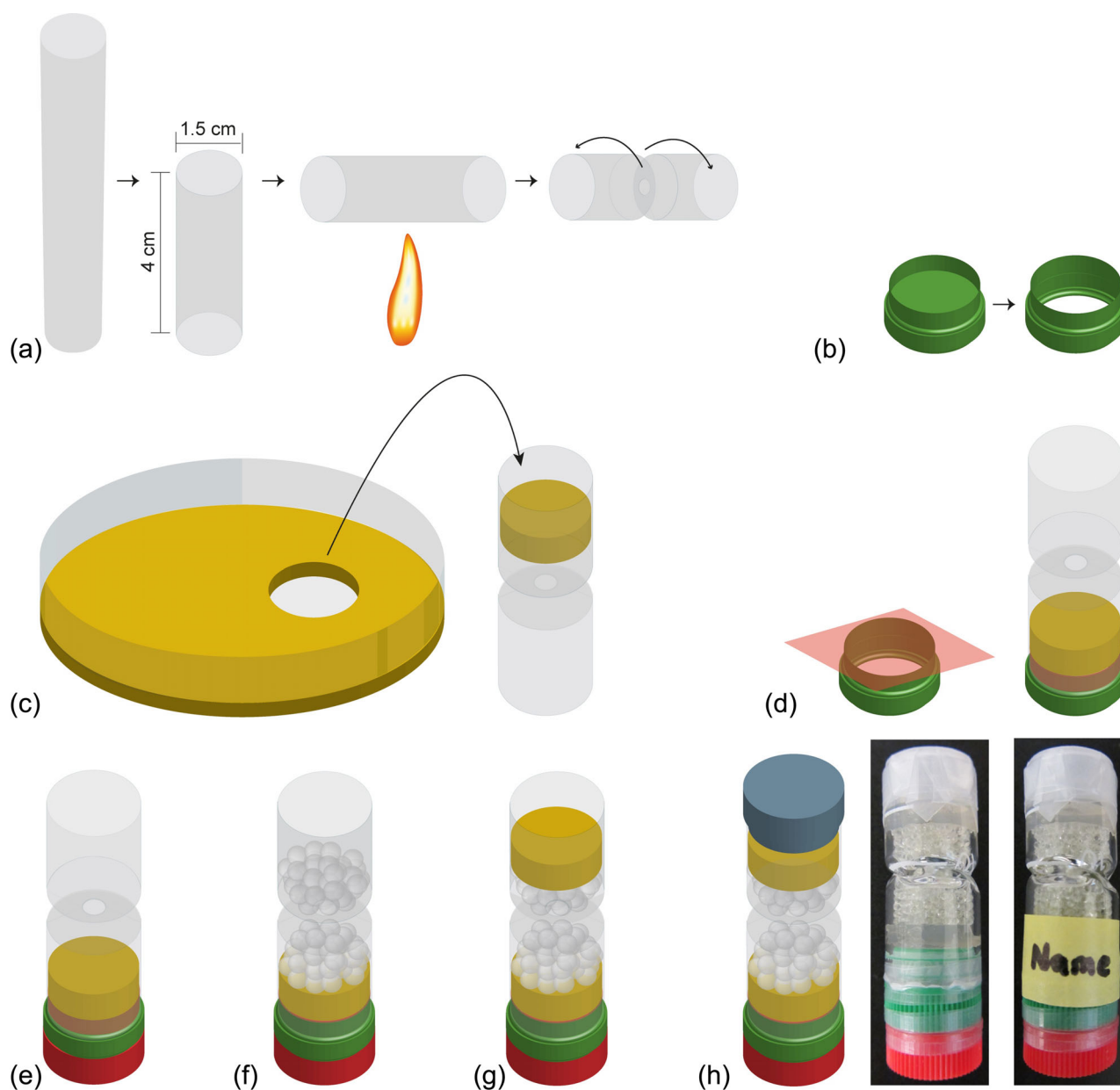
Figure 1. Components and general procedure for assembling a fungal highway column. (**a**) Polystyrene tube cut at length of 4 cm, heated with a flame and twisted. (**b**) Pierced polyethylene cap. (**c**) A slice of agar-based medium (the attracting culture medium) placed at the bottom of the tube. (**d**) The tube is placed on a 25-mm mesh opening tissue, wedging it between the hollow cap and the tube. (**e**) A polyethylene cap closing the bottom of the column. (**f**) Glass beads (Ø: 1 mm) filling the tube. (**g**) A second slice of agar-based medium (the target culture medium), placed on top of the glass beads. (**h**) A polyethylene cap closing the top of the tube.

P. putida was inoculated alone on the same medium. Both experiments were conducted in triplicate. A slice of each inoculated MA medium was placed at the bottom of a fungal highway column and left there to incubate for 6 days. After this incubation time, the target culture medium of each column was cut into four pieces. Two pieces were directly transferred onto a plate of MA medium. Two pieces were put in 1 mL of sterile physiological water (9 g·L^{-1} NaCl; Sigma-Aldrich, St Louis, MO, USA) and dispersed by shaking. From this, 100 μL was removed and streaked on a plate of nutrient agar (NA) medium, composed of 8 g·L^{-1} nutrient broth (Biolife Italiana S.r.l, Milano, Italy) and 15 g·L^{-1} agar (Fig. 3). Plates were incubated for 24 h at room temperature. After incubation, presence of colonies of GFP-

tagged *P. putida* was assessed by fluorescence microscopy (Nikon Corp., Tokyo, Japan). Presence of fungi was assessed by visual inspection.

A second validation test was performed in soil-containing microcosms (Table 1). A microcosm consisted of a polyethylene container with 12.5 ± 0.5 g of soil, autoclaved (21 min at 121°C), dried overnight at 60°C, and watered with 5 mL of sterile deionized water after drying (final water content of 40% of the soil water holding capacity). We used a fungal highway column containing 6 g·L^{-1} malt MA as attracting and target media for each microcosm. The microcosm tests were performed for the assessment of three properties: absence of bacterial or fungal contamination in the columns, absence of bacterial
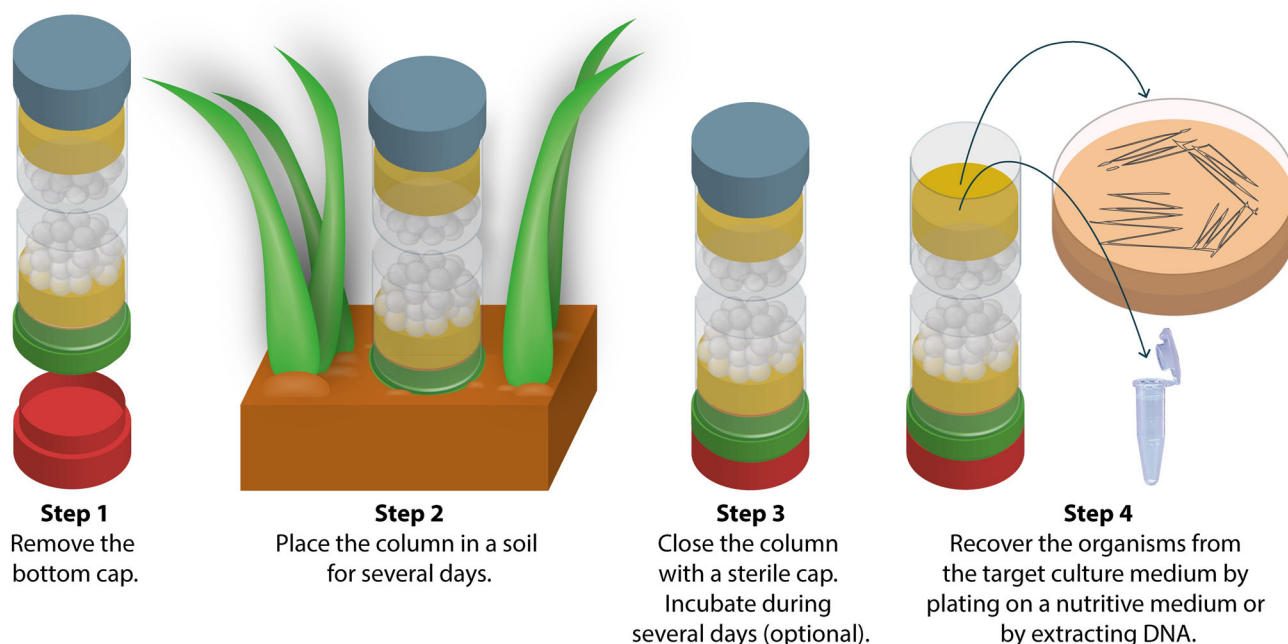
Step 1
Remove the bottom cap.

Step 2
Place the column in a soil for several days.

Step 3
Close the column with a sterile cap. Incubate during several days (optional).

Step 4
Recover the organisms from the target culture medium by plating on a nutritive medium or by extracting DNA.

Figure 2. Description of the steps involved in the use of fungal highway columns. In step 2, soil can be replaced by a culture of microorganisms on solid medium or any other substrate.

dispersal in soil without fungi, and bacterial dispersal in the presence of fungi. The organisms artificially inoculated in the soil were the same flagellated bacterium *P. putida* KT2440 and the fungi *M. crassipes* and *Fusarium oxysporum*. The fungal highway columns were placed in the soil for a duration of 4 or 10 days, and left for incubation after removal from the soil for a duration of 0, 8, or 15 days. Each condition was tested in triplicate.

For the soil inoculated with *P. putida*, cells from an overnight culture of *P. putida* on NA medium were collected in sterile physiological water, to a final OD$_{550}$ of 3, which corresponded to 25 × 10^6 cells mL^{-1}, of which 100 μL was added to the soil and mixed with it with a sterile loop. The fungal inoculum was a 7-day-old (*M. crassipes*) or 3-day-old (*F. oxysporum*) culture on MA medium. From this culture, two squares (∼3 mm^3) were cut out and mixed with the soil with a sterile loop. An individual fungal highway column was placed in each microcosm for the time indicated in Table 1. After being removed from the soil, each fungal highway column was closed and left for incubation as indicated in Table 1. After this incubation time, the target culture medium was cut into four pieces and incubated as explained above (Fig. 3). Plates were incubated for 48 h at room temperature. After incubation, presence of colonies of *P. putida* was assessed by fluorescence microscopy, and presence of fungi was assessed visually.

For the columns placed in microcosms and inoculated with both fungi and bacteria, a Hill and Smith test, allowing evaluation of the correlation between qualitative and quantitative data (Hill and Smith 1976), was performed on R (R Core Team 2013) using the package ade4 (Dray and Dufour 2007; Hervé 2013). This test was selected because it allows for the correlation of the time of incubation (in the soil and after removal; a quantitative variable) with the presence of both fungi and bacteria on the target culture medium (a qualitative variable). Pearson's chi-squared test was performed to evaluate the significance of the correlations calculated using the command chisq.test in R.

A third validation test was performed to assess the effect of water condensation that could occur inside the fungal high-

way columns during field use and the effect of transportation shaking. The hollow cap at the bottom of three fungal highway columns was filled with a sterile soil amended with *P. putida* (as indicated above) before closing the column with the bottom cap. Each column was incubated during 3 days at room temperature. After this time, columns were stored for 15 min at 4°C and left again at room temperature. After water condensation had occurred inside each column, they were shaken by hand to mimic random disturbances as likely to happen during transportation from the field site to the laboratory. The columns were then incubated horizontally at room temperature for 24 h, bringing the total time of incubation to 4 days. Target culture medium was plated on NA medium (as explained above), and presence of bacterial colonies was assessed by visual inspection after 24 h.

The same experiment was performed with *P. putida* inoculated in soil-containing microcosms (as described above). An individual fungal highway column was placed in each microcosm for 2 days, in triplicate. After this time, columns were stored for 1 h at 4°C and left again at room temperature. After water condensation had occurred inside each column, they were shaken by hand, and the columns were then incubated for a duration of 11 days at room temperature. Target culture medium was plated on NA medium (as explained above), and presence of bacterial colonies was assessed by visual inspection after 24 h.

Field tests

Fungal highway columns were used to assess the efficiency of the device to isolate bacteria dispersing using the fungal highways under natural conditions. A soil in a semi-arid area of Morocco (32°31′7′ N, 8°32′9′ W) was selected (see Supplementary Table S1 for soil properties). In total, 28 columns were placed along a depth transect in a Cambisol (WRB 2014) under *Opuntia ficus-indica* (Fig. 4 and Supplementary Table S2). This calcium oxalate-producing plant (McConn and Nakata 2004) has

Table 1. Experimental design and results of the validation tests of the columns performed at the laboratory. Results show recovery of no organisms (N), presence of bacteria (B), fungi (F), or both bacteria and fungi (BF).

Validation test	Condition tested	Microorganisms inoculated in substrate		Incubation in substrate (days)	Post-incubation (days)	Organisms recovered		
		P. putida	Fungus					
Agar substrate	Absence of bacterial dispersal in absence of fungi	+	N	6	0	N	N	N
	Presence of bacterial dispersal in presence of fungi	+	*M. crassipes*	6	0	FB	FB	FB
		+	*Trichoderma* sp.	6	0	FB	FB	FB
Soil microcosm	Absence of contamination	N	N	4	15	N N	N N	N N
		N	N	10	15	N N	N N	C N
	Absence of bacterial dispersal in absence of fungi	+	N	4	0	N	N	N
		+	N	4	15	N	N	N
		+	N	10	0	N	N	N
		+	N	10	15	N	N	N
	Presence of bacterial dispersal in presence of fungi	+	*M. crassipes*	4	0	N	N	N
		+	*F. oxysporum*	4	0	N	N	N
		+	*M. crassipes*	4	8	F	FB	N
		+	*F. oxysporum*	4	8	N	B	B
		+	*M. crassipes*	4	15	FB	B	N
		+	*F. oxysporum*	4	15	N	B	B
		+	*M. crassipes*	10	0	N	N	N
		+	*F. oxysporum*	10	0	N	N	N
		+	*M. crassipes*	10	8	N	FB	FB
		+	*F. oxysporum*	10	8	F	N	B
		+	*M. crassipes*	10	15	FB	N	N
		+	*F. oxysporum*	10	15	F	F	FB
Transport and condensation	Bacterial dispersal in water film, with shaking, in absence of fungi	+	N	4	0	N	N	N
		+	N	2	11	N	N	N

been considered as a model for pedogenic carbonate accumulation related to the oxalate–carbonate pathway (Cailleau *et al.* 2005), in which fungal-driven dispersal seems to be relevant for the activity of oxalotrophic bacteria (Martin *et al.* 2012; Bravo *et al.* 2013). In order to attract fungi but avoid a nutrient shock (Oliver 2005), the columns contained a lower amount of carbon (6 g·L^{-1} MA). Furthermore, the target culture medium of each column was either supplemented with 4 g·L^{-1} CaC$_2$O$_4$.H$_2$O (Sigma-Aldrich) or included a 1-mm layer of modified Schlegel medium (Braissant, Verrecchia and Aragno 2002) with 4 g·L^{-1} CaC$_2$O$_4$.H$_2$O, in order to attract oxalotrophic bacteria. This combination of attracting/target media was tailored to the oxalate–carbonate pathway in order to favor the isolation of oxalotrophic organisms. However, the set-up can be modified depending on the specific organisms studied (for example, the same medium can be used as both attracting and target medium). Variable times of installation (4, 5 and 8 days) were tested. Most of the columns (22 out of 28 columns) were placed at a depth of 15 cm in response to observations by Bravo *et al.* (2013) indicating an abrupt decrease of oxalotrophic bacteria at depths below 20 cm. However, one column was also placed at 35 cm and five more at 60 cm depth. After being removed from the soil and closed, the columns were incubated for a duration of 15–37 days (cor-

responding to the time of transport back to the laboratory). The columns were transported fixed into a sealed container. After this time, the target culture medium of each column was inoculated on MA and NA medium, as indicated for the laboratory microcosm assays. After 72 h, development of colonies was visually checked and individual organisms were isolated for further identification and characterization.

Identification of enriched microorganisms

Cultivable microorganisms isolated from columns containing both bacterial and fungal growth were identified by DNA sequencing. For bacteria, DNA extraction was performed according to the instructions of the InnuPREP bacteria DNA kit (Analytic Jena, Jena, Germany). DNA extracts were quantified using a Qubit® fluorometer (Life Technologies Corp., Carlsbad, CA, USA). DNA concentration ranged from 1.21 to 177 ng·μL^{-1}. PCR amplification was performed of a partial fragment of the 16S rRNA gene with primers EUB 9–27f (5′-AGAAAGGAGGTGATCCAGCC-3′) and EUB 1542r (5′-AGAAAGGAGGTGATCCAGCC-3′; Liesack, Weyland and Stackebrandt 1991). PCR master mix contained (in a final volume of 50 μL): 10 μL buffer (with 1.5 mM MgCl$_2$), 0.2 mM dNTPs
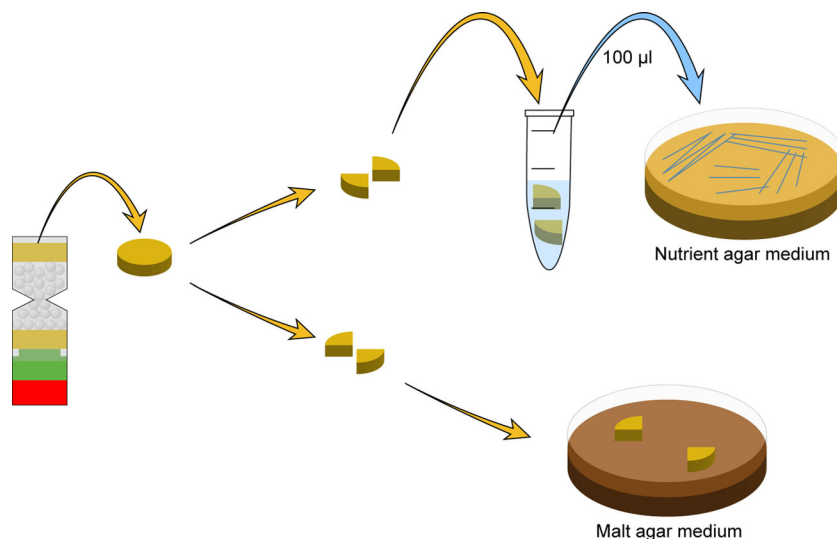
Figure 3. Recovery of cultivable microorganisms from a fungal highway column. The target culture medium at the top of the column is cut into four pieces. Two pieces are transferred onto a plate of MA medium, and two pieces are placed in physiological water, of which 100 μL are streaked on a plate of culture medium (e.g. nutrient agar).
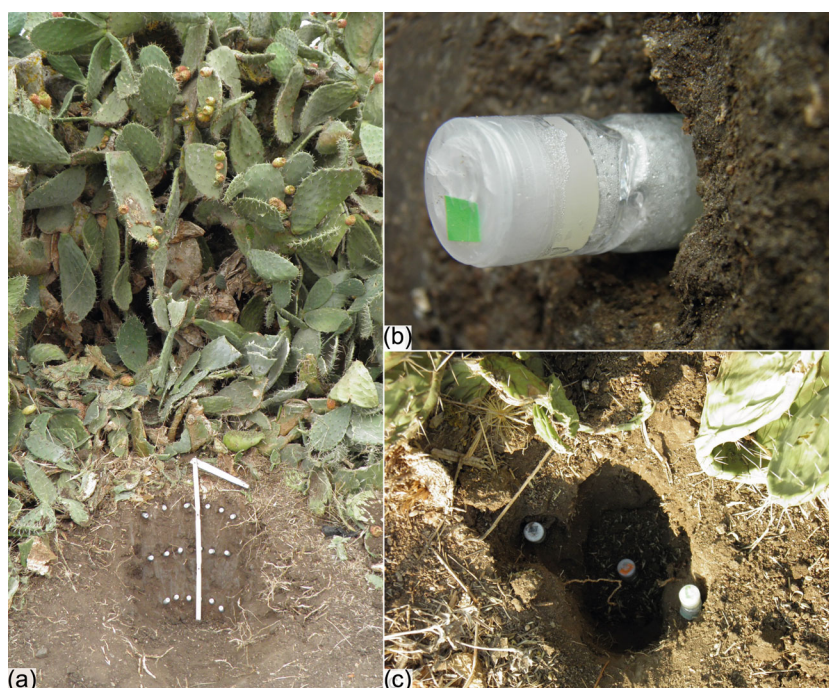

Figure 4. Fungal highway columns in soils under *Opuntia ficus-indica*. (**a**) Columns placed at various depths in the soil. (**b, c**) close-up images of the columns placed in soil.

mix, 0.2 mM of each primer and 1 U GoTaq DNA Polymerase (Promega Corp, Madison, WI, USA); 1 μL of diluted DNA template was added (c. 1.21–2 ng$\cdot\mu$L^{-1} of DNA). PCR was carried out in a Sensoquest Labcycler thermocycler (Witec AG, Göttingen, Germany), with an initial denaturation at 95°C for 5 min, followed by 10 cycles consisting of denaturation at 95°C for 30 s, annealing at 60°C (−0.5°C per cycle) for 45 s, and elongation at 72°C for 1 min, then 25 cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 45 s and elongation at 72°C for 1 min. Final extension was performed at 72°C for

5 min. Purification of PCR products was carried out using a MultiScreen PCRμ96 Filter Plate (Millipore AG, Zug, Switzerland). PCR products were premixed in 50 μL of sterile nanopure water and filtered for 15 min, adding 10–25 μL of sterile nanopure water afterwards. PCR products were quantified using a Qubit® fluorometer (DNA concentrations ranged from 51 to 86 ng$\cdot\mu$L^{-1}) and sent for Sanger sequencing to GATC Biotech AG (Konstanz, Germany). The sequences were deposited in GenBank under accession numbers KT634058-KT634071. Search for similarity against sequences of the 16S rRNA gene was performed using

the combination of BLAST (Basic Local Alignment Search Tool; Altschul *et al.* 1990) and pairwise sequence alignment, comparing the query sequence with the non-redundant GenBank database using the online services of EzTaxon (Kim *et al.* 2012).

For some of the isolates, direct sequencing of the PCR products failed and thus the amplicon was cloned prior to sequencing. The TOPO TA cloning kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to produce this plasmid in One Shot TOP10F′ chemically competent *Escherichia coli* cells (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's guidelines. Plasmid DNA was extracted with the Wizard Plus SV Miniprep DNA purification system (Promega Corp, Madison, WI, USA) following the manufacturer's instructions. Amplification, purification, sequencing and analysis were conducted as indicated above.

For fungi, DNA extraction was performed according to the instructions of the PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA) with a bead-beating step of 5 min at 50 beats·s^{-1} (Qiagen, Hilden, Germany). DNA extracts were quantified using a Qubit fluorometer. DNA concentration ranged from 1.14 to 8.03 ng·μL^{-1}. A PCR amplification of the internal transcribed spacer (ITS) region of the 5.8S rRNA gene was performed with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; Anderson, Prosser and Campbell 2003). PCR master mix contained (in a final volume of 50 μL): 5 μL buffer, 0.25 mM dNTPs mix, 0.2 mM of each primer and 2.5 U Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA). One microliter of DNA template (c. 1.14–2 ng·μL^{-1} of DNA) was added. The PCR program consisted of an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 68°C for 30 s. Final extension was performed at 68°C for 5 min. Purification of PCR products was carried out using a MultiScreen PCRμ96 Filter Plate. PCR products were premixed in 50 μL of sterile nanopure water and filtered for a duration of 15 min, adding 10–25 μL of sterile nanopure water afterwards. Amplicons were quantified using a Qubit fluorometer (DNA concentrations ranged from 29 to 52 ng·μL^{-1}) and sent for Sanger sequencing to GATC Biotech AG. The sequences were deposited in GenBank under accession numbers KT634072-KT634079. The search for similarity against sequences from the ITS region on 5.8S rRNA gene was performed using BLAST comparing the query sequence with the International Nucleotide Sequence Database (INSD) at UNITE (Unified system for the DNA based fungal species linked to the classification; Kõljalg *et al.* 2013).

Hydrophobicity, motility, bacterial dispersal and Type III secretion system

Since previous research on fungal–bacterial interactions proposed that bacterial dispersal along mycelia was most efficient for motile bacteria moving along hydrophilic fungal surfaces (Kohlmeier *et al.* 2005), and that bacteria from the mycosphere often present a Type III secretion system (Warmink and van Elsas 2008), we tested these properties on the microorganisms isolated from the field trials with the fungal highway columns.

In order to measure the hydrophilic properties of the surface of the fungal mycelia, Whatman® circular membranes of nitrocellulose (ref. NC45) or cellulose acetate (ref. OE67) were placed at the center of MA plates. Each fungus was inoculated in triplicate on each type of filter, and incubated at room temperature for 8 days before measuring the contact angle according to Smits *et al.* (2003). Motility of bacteria was measured on swim and swarm agar (composition according to Deziel, Comeau and Villemur [2001]). Swim agar: 1% tryptone (Merck, Darmstadt,
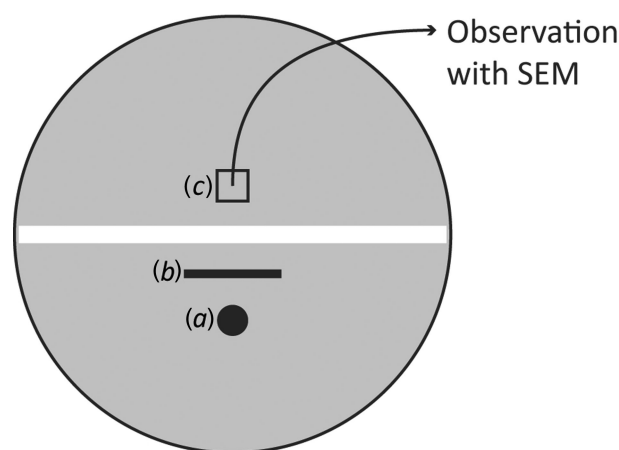

Figure 5. Separated Petri dish used for the confirmation of bacterial dispersal along fungal mycelium. Fungus (**a**) and bacteria (**b**) were inoculated at one side of the separation, and a square of medium (**c**) was cut off beyond the separation after 8 days of incubation and observed by scanning electron microscopy (SEM).

Germany), 0.5% NaCl (Panreac Quimica S.L.U., Barcelona, Spain) and 0.3% agar; swarm agar: 0.8% nutrient broth, 0.5% dextrose (Merck, Darmstadt, Germany) and 0.5% agar. Each bacterium was inoculated in triplicate on a single point at the center of a swim and a swarm agar plate. Diameter of the colonies was measured after 48 h. *Micrococcus luteus* was used as a negative and *P. putida* as a positive control.

In order to confirm that the migratory bacteria recovered were able to disperse on their associated fungal mycelium, we co-cultivated each fungal-bacterial couple on a separated Petri dish (Fig. 5). Fungal and bacterial colonization beyond the separation was assessed using scanning electron microscopy (SEM). Separated Petri dishes contained either MA or NA medium separated in the middle by a 3-mm wide strip without culture medium that encompassed the entire dimension of the Petri dish. This area was created by cutting out a strip of medium from the middle of the Petri dish. A cube ($\sim$3 mm^3) of a fungal pre-culture on MA medium was inoculated 1 cm away from the separation. A loop of an overnight bacterial pre-culture on NA medium was streaked on a 2.5 cm line between the fungus and the separation. After 8 days of incubation at room temperature, a cube ($\sim$3 mm^3) was cut out beyond the separation and fixed during at least 1 h with an aldehyde fixative (1 mL paraformaldehyde 20%, 1 mL glutaraldehyde 25% (Agar scientific, Stansted, UK), 5 mL sodium cacodylate trihydrate buffer 0.2 M (pH 7.4; Merck, Darmstadt, Germany), 3 mL H$_2$O), and during at least 1 h with an osmium fixative (1 mL OsO$_4$ 4% (Merck, Darmstadt, Germany), 2 mL sodium cacodylate trihydrate buffer 0.2 M (pH 7.4), 1 mL H$_2$O). Fixed samples were dehydrated in successive acetone (VWR international, Radnor, PA, USA) baths and dried in CO$_2$ at the critical point (BalTec, Pfäffikon, Switzerland). Finally, each sample was placed on a support, and covered with a 23 nm gold layer (Baltec, Pfäffikon, Switzerland). A scanning electron microscope (ESEM-FEG XL30, Philips, Amsterdam, the Netherlands) was used to assess the presence of fungi and bacteria in the fixed agar plugs (high-vacuum, 10–15 kV, distance 10 mm).

In order to confirm that the recovered bacteria were not able to reach the target culture medium of a column in the absence of fungi, a validation test was performed in soil-containing microcosms, inoculated with each isolated bacterial strain. A bacterial lawn was prepared on NA medium and incubated for 48 h. Cells from half of the plate were streaked out and resuspended

in 1 mL of sterile physiological water. The final OD_{550} of this solution varied from 1 to 10. One hundred microliters was added to 12.5 g of soil and mixed with it with a sterile loop. An individual fungal highway column was placed in each microcosm for 5 days. After being removed from the soil, the columns were closed and left for incubation for 15 days. After this incubation time, the target culture medium was cut into four pieces and incubated as explained above (Fig. 3). Plates were incubated for 48 h at room temperature. After incubation, presence of bacterial colonies was visually assessed.

Presence of the *hrc*R gene, coding for one of the elements of the Type III secretion system, was detected by PCR amplification with primers hrcRF (5′-ATCGGGGTGCAGCAGGTRC-3′) and hrcRR (5′-CGAACAGCAGCAGCTTKARYG-3′; Warmink and van Elsas 2008). We used the same DNA extracts employed in bacterial identification. PCR master mix contained (in 25 μL of final volume): 5 μL buffer B, 0.2 mM dNTPs mix, 0.2 mM of each primer, 5 μL enhancer, 0.25 μL BSA and 0.1 U KAPA2G Robust DNA polymerase (Kapa Biosystems, Wilmington, MA, USA); 1 μL of DNA template was added. The PCR program consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles consisting of denaturation at 94°C for 45 s, annealing at 56°C for 45 s, and extension at 72°C for 25 s. Final extension was performed at 72°C for 5 min. Presence of amplified DNA fragments was assessed by a 30 min gel electrophoresis at 100 V on a 1.5% agarose gel (Bioconcept, Allschwil, Switzerland). DNA was stained for a duration of 30 min in a GelRed bath (Biotium, Hayward, CA, USA) before observation with a Transilluminator (VWR International). DNA isolated from *Burkholderia terrae* (BS001) was used as a positive control.

RESULTS AND DISCUSSION

Design of the fungal highway column

A novel tool (fungal highway column) was designed for the collection of fungal–bacterial associations from soils or similar water-unsaturated ecosystems by both attracting fungi and selecting for bacteria moving along their hyphae towards a target culture medium. It thereby exclude bacterial isolation by other means than migratory dispersal along the hyphae (i.e. transport by soil fauna, turbulent dispersal in the soil air and mobilization in continuous films of condensing water). Three elements in the columns ensured this. First, a 25-μm mesh tissue was placed between the soil and the attracting culture medium to avoid the entrance of soil fauna, including mites as their body length generally corresponds to around 500 μm (Goddard 2002; Tixier *et al.* 2012). Second, glass beads inside the columns prevented bacterial aerial dispersal, in particular of bacterial spores (Yamaguchi, Ichijo and Sakotani 2012). Third, the columns were shrunk in the middle in order to avoid formation of a continuous water film in which bacteria could swim when the columns are held horizontally (i.e. during transportation or when placed horizontally in a soil profile).

The first slice of culture medium, placed between the tissue and the glass beads, was carbon rich intended for attracting fungi into the columns. The second slice of culture medium, placed at the top of the glass bead layers, acted as a target medium that could be used later for isolation (Fig. 2). When columns are placed in a substrate, for example a soil, the latter is in direct contact with the 25-μm mesh tissue and the attracting culture medium leads fungi into the column (Supplementary Fig. S1). The column is left on the substrate, in order to allow fungi and their associated bacteria to reach the attract-

ing culture medium. The additional incubation is intended to allow more time for the organisms to migrate inside the column towards the target culture medium. In this study, we focused on cultivable organisms. However, the target culture medium could also be used directly for DNA extraction for use with high-sensitivity molecular ecology methods (Fig. 2).

Fungal highway columns are low-cost, low-weight, small devices that were created to be easy to build and transport. We did not use any glass component (except for the beads) in order to make the device robust. Furthermore, as the composition of the culture media intended to attract the organisms inside the columns can vary, these devices can be used for targeting different kinds of fungi and bacteria with specific metabolic capabilities.

Validation tests with GFP-tagged *Pseudomonas putida*

The first validation test was performed in order to determine if fungal highway columns were efficient at attracting fungi as well as collecting bacteria moving along their mycelium and to assess that this was not possible in the absence of the fungi. In co-cultures of the flagellated GFP-tagged bacterium *P. putida* with either *M. crassipes* or *Trichoderma* sp., the fungi–bacteria couple was recovered from the target culture medium after 6 days of incubation, and bacterial fluorescence was observed on the target medium in all columns. In contrast, when columns were inoculated with *P. putida* in the absence of fungi, no bacterium was recovered from the target culture medium.

The second validation test was performed with microcosms containing soil in which columns were placed for variable durations. No fungal growth was observed in the tests for the absence of fungal or bacterial contamination inside the columns. One column, placed for a duration of 10 days into the soil and left closed for another 15 days of incubation, showed bacterial growth (Table 1). Thus, fungal contamination during the assembly of the columns is very unlikely, whereas bacterial contamination occurred in 1/12 of the cases tested under the lengthiest incubation conditions. In the tests assessing bacterial dispersal in the absence of fungi, no bacterium was recovered in any of the columns (Table 1). These results show that motile bacteria cannot reach the target culture medium in the absence of fungi. In the tests assessing bacterial dispersal in the presence of fungi, no growth was observed when the target medium was used for plating without post-incubation after taking the columns from the soil regardless of the time they were left in place (4 or 10 days). However, we did not find any significant correlation between the time columns spent in soil with respect to the time of incubation, and the recovery of organisms (*p*-value = 0.11 for the time in soil, and *p*-value = 0.12 for the time of incubation). In the remaining 24 columns that were post-incubated for 8 or 15 days, the bacterium alone was recovered from six columns, the fungus alone was recovered from 4 columns, and both fungus and bacterium were recovered from six columns (Table 1).

For the third validation test, we tested whether a water film produced by condensation could allow motile bacteria to reach the target culture medium in the absence of fungal mycelium. Similarly, as the columns may be shaken during transportation from the field to the laboratory, we tested if severe shaking could accidentally bring bacteria to the target culture medium. No bacterium was recovered from the target culture medium in either case. For the test with shaking and longer incubation time, no bacterium was recovered from the target culture medium.

Overall, the validation tests underpin the usefulness of these columns to prevent bacteria from reaching the target culture

medium in the absence of fungal hyphae. Based on the validation tests demonstrating the absence of bacterial colonization in the absence of fungi, the most likely mechanism explaining the presence of bacteria in the target medium, even in the absence of the fungal partner, is the facilitated dispersal by the fungal mycelium.

Field tests

A soil located in a semi-arid area of Morocco was chosen for the first field test of the columns. The latter were placed at different depths along a soil transect under the oxalogenic plant *Opuntia ficus-indica*. After various time of both placement into the soil and incubation, presence of easily cultivable fungi and bacteria on the target culture medium of each column was assessed. As observed in the validation tests in microcosms, bacteria and fungi (together or alone) were not recovered from all the columns. Both fungi and bacteria were isolated from 8 out of the 28 columns, while bacteria alone were isolated from three columns, and fungi alone from eight columns. We were not able to cultivate any organism from the remaining nine columns (Supplementary Table S2). Concerning the depth, most of the columns were placed at 15 cm and thus the results of the other depths need to be considered with caution. From those columns placed at 35 or 60 cm, fungi and bacteria were recovered from only one column and fungi alone from another.

For the columns from which we isolated bacteria alone, it is possible that these did use a mycelial path, but that the associated fungus did not develop in the target culture medium after reaching the medium serving as dispersal vector for those bacteria. In the case of columns colonized by fungi alone, the processes that impaired bacterial dispersal are unclear, but one possible explanation is that the challenging trophic and physical conditions inside the unsaturated part of the column (glass beads) might modify fungal physiology and interaction with bacteria. For example, we have observed in culture experiments that under conditions that are favorable to the fungus (for example growth in MA), the number of fungi transporting bacteria decreases (Table 2). More importantly, our results confirm the ability of fungal highway columns to attract fungal–bacterial couples onto a target culture medium. Although it is likely that the composition of the culture media in the columns could affect the amount and diversity of recovered organisms, evaluating this was not the aim of these validation tests in the field.

Identification of microorganisms enriched

Among the microorganisms isolated, 12 bacterial strains (*Achromobacter mucicolens*, *Achromobacter spanius*, *Acinetobacter calcoaceticus, Acinetobacter* sp., *Ochrobactrum pecoris, Ochrobactrum* sp., *Olivibacter soli*, *Pseudomonas frederiksbergensis*, *Stenotrophomonas humi*, *Stenotrophomonas maltophilia*, *Stenotrophomonas rhizophila*, and *Variovorax soli*) and five fungal species (*Fusarium chlamydosporum*, *Fusarium equiseti*, *Fusarium nygamai, Fusarium oxysporum,* and *Chaetomium globosum*) were identified (Table 2). Three more bacterial strains were isolated, but all our attempts either to sequence directly a PCR product or to clone and sequence their DNA failed. Thereby, we named them 'Unidentified bacterium a', 'Unidentified bacterium b' and 'Unidentified bacterium c'. The identified fungi corresponded almost exclusively to *Fusarium* spp., a genus of fast-growing and competitive fungi producing diverse mycotoxins (Gutleb, Morrison and Murk 2002). Moreover, this genus is known to contain species that resist water stress, which can be a limiting

ecological factor in semi-arid soils (Deacon 2006). Indeed, fungal species of the genus *Fusarium* usually show an r-strategy (ruderal strategy, consisting of fast-growing organisms producing many offspring), but in addition, they can also show an S-strategy (stress-tolerance, whether to low water potential or to plant phenols in this case) and a C-strategy (combative behavior, or antagonistic propensity; Dix 2012; Deacon 2006). Although other fungal genera such as *Penicillium* present a better water-stress tolerance than *Fusarium*, species of the former generally show a much lower growth rate. In fact, a *Penicillium* strain was isolated in one of the columns without bacteria, and thus was not characterized further. In addition, the presence of an easily accessible carbon source in the form of the attracting and target medium could select for opportunistic fungi with an r-strategy. This does not mean that other fungi are not able to enter the columns (and thus bring bacteria along their hyphae), but the latter appear to have been outcompeted in the culturing approach conducted here.

Most of the isolated bacteria belonged to groups that are likely to be associated with soil fungi. These 'fungiphile' bacteria belong to the orders Rhizobiales (Alphaproteobacteria), Burkholderiales (Betaproteobacteria), Xanthomonadales and Pseudomonadales (Gammaproteobacteria; Folman *et al.* 2008; Bonfante and Anca 2009; Warmink, Nazir and van Elsas 2009). In addition to these groups, we have identified *Olivibacter* sp. (Bacteroidetes), as well as bacteria from the families Comamonadaceae (*Variovorax* sp.) and Alcaligenaceae (*Achromobacter* sp.), which have not been reported in previous studies. The latter two have been recently identified *ex situ* from soils under the influence of *Milicia excelsa* (another oxalogenic plant) in Cameroon (Bravo *et al.* 2013), as well as in a culture-based survey of bacterial diversity in three different oxalogenic tree species (Bravo *et al.* 2015).

Hydrophobicity, motility, bacterial dispersal and Type III secretion system

The hydrophobicity of the fungal mycelium measured by the contact angle method gave origin to two possible interpretations of the results. As there are discrepancies regarding the interpretation of the contact angle value for the determination of the hydrophobicity of a surface, we compared two references for the analysis of our results. According to Chau *et al.* (2009), all fungal mycelia are hydrophilic (hydrophilic surface: contact angle <90°; hydrophobic surface: contact angle >90°), but according to Smits *et al.* (2003), one fungal mycelium is hydrophobic and all the others intermediate (hydrophilic surface: contact angle <30°; intermediate surface: contact angle between 30° and 60°; hydrophobic surface: contact angle >60°). Nonetheless, migration was demonstrated, supporting a hydrophilic nature.

As the growth of fungal hyphae is apical (Deacon 2006), passive bacterial transport by the fungus (a dispersal mechanism dubbed 'subway'; Kohlmeier *et al.* 2005) is improbable. Bacterial dispersal along fungal hyphae can thus be expected to be an active process involving a type of bacterial motility (Kohlmeier *et al.* 2005). In fact, swimming was a common feature observed in a diverse group of flagellated bacteria able to disperse along fungal hyphae (Pion *et al.* 2013). Therefore, we were expecting that motile bacteria would be recovered from the target culture medium of the columns. However, not all the isolates were positive in the swimming and swarming tests in agar media. The capabilities of the isolated bacteria to swim or swarm varied amongst the isolates and also within the same bacterial species (Table 2). For example, *A. calcoaceticus* isolated from column 5

Table 2. Identification and properties of the organisms isolated from eight columns presenting simultaneously fungal and bacterial growth in a soil under the oxalogenic plant *Opuntia ficus-indica*. The percentage of identity to the indicated group is shown in brackets.

Column[a]	Fungus	Contact angle (°)[b]	Associated bacteria	Motility test Swimming	Motility test Swarming	TTSS	Confirmation of migration MA	Confirmation of migration NA	Migration in a column in absence of fungus
1	Chaetomium globosum (99.8%)	58	Variovorax soli (98.4%)	–	–	–	–	–	–
			Olivibacter soli (99.1%)	+	+	–	+	–	–
			Unidentified bacterium a	–	+	+	+	+	–
			Acinetobacter sp.	+	+	–	–	–	–
3	Fusarium oxysporum (99.8%)	47	Stenotrophomonas maltophilia (99.0%)	+	+	–	+	+	–
			Ochrobactrum sp. (99.6%)	+	+	–	–	+	–
4	Fusarium chlamydosporum (99.4%)	40	Ochrobactrum pecoris (98.8%)	+	+	–	+	+	–
			Pseudomonas frederiksbergensis (99.3%)	–	+	–	–	+	–
5	Fusarium chlamydosporum (99.6%)	50	Acinetobacter calcoaceticus (99.8%)	+	+	–	–	–	–
			Stenotrophomonas rhizophila (99.6%)	+	+	–	–	+	–
			Unidentified bacterium b	+	+	–	+	+	–
7	Fusarium oxysporum (99.8%)	72	Unidentified bacterium c	N/A	N/A	–	N/A	N/A	N/A
8	Fusarium chlamydosporum 99.8%	56	Acinetobacter calcoaceticus (99.4%)	–	–	–	–	–	–
18	Fusarium equiseti (99.4%)	42	Achromobacter spanius (99.9%)	+	+	–	–	+	–
			Stenotrophomonas humi (99.2%)	+	+	–	–	+	–
26	Fusarium nygamai (99.8%)	55	Achromobacter mucicolens (99.9%)	–	–	–	–	+	–

[a] See Supplementary Table S2 for conditions associated with each number.
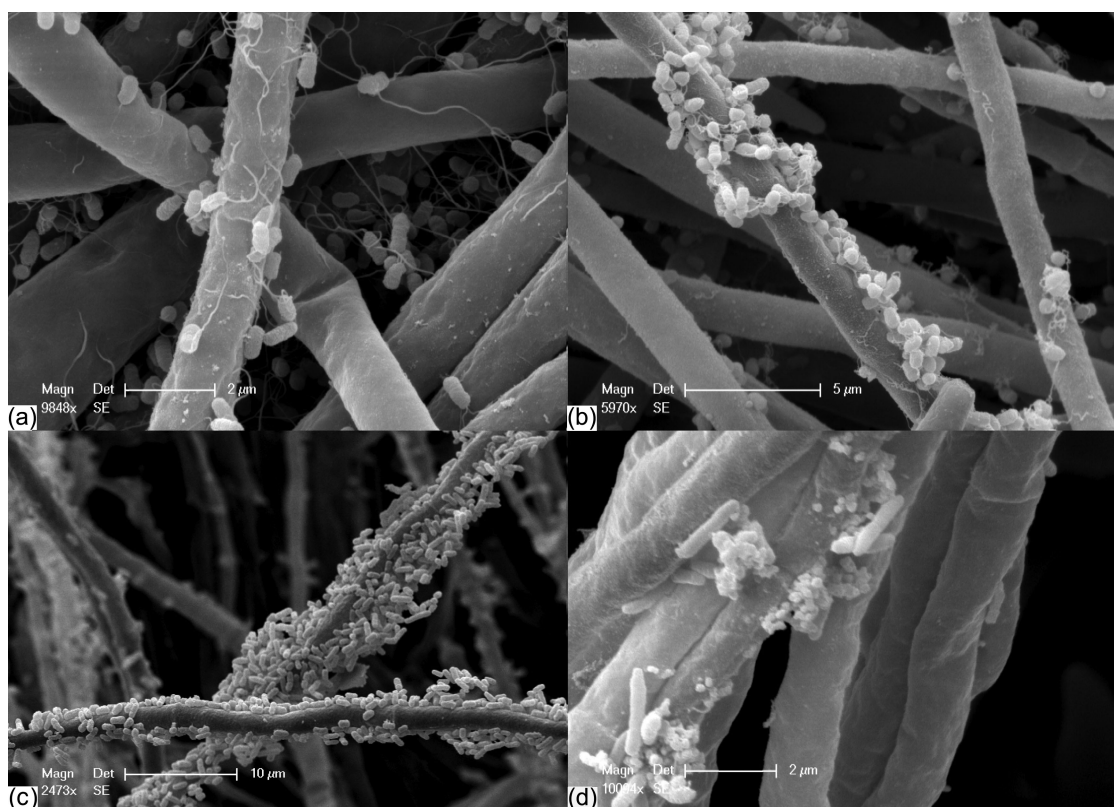[b] Mean value.

Figure 6. SEM images of bacteria moving along fungal hyphae. (**a**) *Stenotrophomonas maltophilia* on hyphae of *Fusarium oxysporum*; (**b**) *Ochrobactrum* sp. on hyphae of *Fusarium oxysporum*; (**c**) Unidentified bacterium b on hyphae of *Fusarium chlamydosporum*; (**d**) *Stenotrophomonas humi* on hyphae of *Fusarium equiseti*.

was positive in both swimming and swarming tests, whereas another isolate from the same species isolated from column 8 was negative in both swimming and swarming tests. In order to understand the relevance of motility on the dispersal of the isolated bacteria along fungal hyphae, we tested the ability of each isolated bacterium to migrate along its potentially associated fungal mycelium on the media used for isolation. For 10 out of the 14 fungal–bacterial couples tested, migration was observed (Table 2). On the SEM pictures of positive migratory couples (examples are shown in Fig. 6), bacteria surround their associated hyphae. In three cases, *O. soli*, 'Unidentified bacterium a', and *A. calcoaceticus*, the swimming and swarming test were positive but the bacteria did not migrate on the associated fungus. The opposite was observed in the case of *A. mucicolens*, which was negative in the swimming and swarming test but dispersed on the mycelium of *F. nygamai*. We have no certitude about the phenomenon that led to these contradictory results. In the case of motility, it is possible that the swimming and swarming test can lead to false negatives. For those bacteria that reached the target culture medium of the columns but did not disperse on the associated mycelium, it is important to mention that the isolated couples represent only potentially associated partners and that it is possible that these bacteria had reached the medium using hyphae from a fungal species that was not recovered in the isolation process.

An alternative explanation to the absence of dispersal for some of the isolated strains is the phenomenon known as 'hitchhikers' on the fungal highway (Warmink *et al.* 2011). Hitchhikers corresponded to bacteria able to move along fungal hyphae only in presence of other motile bacteria acting as 'community mi-grators'. From our isolates, *V. soli* (column 1) and *A. calcoaceticus* (column 26) are potential candidates for this type of community migrators. Even though the phenomenon of hitchhikers has not yet been explored extensively, species competent to migrate appear to be TTSS positive, while the community migrators appear not to be. Therefore, we investigated whether the capability of migrating along fungal mycelium was correlated to the presence of a molecular marker for TTSS. The Type III secretion system was detected in one bacterial strain (Unidentified bacterium a; Table 2), but was not observed in any of the other strains, even those that could migrate along the associated mycelium. Although negative PCR results are not conclusive since PCR bias (e.g. mismatches with the annealing sites) might have influenced the results, our findings suggest that TTSS is not essential in fungal highway interactions, but its role on community migration needs to be investigated further. As TTSS is a needle-shaped structure that generally allows for protein injection into target eukaryotic cells by pathogenic bacteria (Galan *et al.* 2014), it might not necessarily have a direct link with fungal highways, in which bacteria actively move along the cells and do not attach to them.

To confirm that all the bacteria isolated could not reach the target culture medium of a column without the presence of fungal mycelium, an additional experiment was conducted. When each bacterium was inoculated alone under a column, we were not able to recover any of these bacteria from the target culture medium, after 5 days of incubation in the soil and 15 days of post-incubation, which indicated that the bacteria we recovered from the field columns may not have been able to migrate towards the target culture medium in absence of fungi.

CONCLUSION

The validation tests we performed on the fungal highway columns show that they enable the isolation of bacteria moving along fungal hyphae from soils. These columns allowed the evaluation of the diversity of microorganisms potentially interacting through fungal highways in one specific soil. The confirmation of migration on fungal hyphae (on a Petri dish) and of the absence of bacterial dispersal in columns without the fungal partner are important steps for the validation of the enriched fungal–bacterial couples. The columns could be the basis for studying fungal highway-like associations in various natural ecosystems. Furthermore, the design of these columns opens new perspectives for targeting different kinds of microorganisms, for example by changing the culture media used as attractor or target, by changing the size of the column and by combining culture-dependent and molecular ecology methods with higher sensitivity.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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