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ORIGINAL ARTICLE

Stable isotope probing reveals *Trichosporon* yeast to be active *in situ* in soil phenol metabolism

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The aim of this study was to extend the results of our previous stable isotope probing (SIP) investigation: we identified a soil fungus involved in phenol biodegradation at an agricultural field site. DNA extracts from our previous study were examined using fungi-specific PCR amplification of the 18S–28S internal transcribed spacer (ITS) region. We prepared an 80-member clone library using PCR-amplified, ^{13}C-labeled DNA derived from field soil that received 12 daily doses of ^{13}C-phenol. Restriction-fragment-length-polymorphism screening and DNA sequencing revealed a dominant clone (41% of the clone library), the ITS sequence of which corresponded to that of the fungal genus *Trichosporon.* We successfully grew and isolated a white, filamentous fungus from site soil samples after plating soil dilutions on mineral salts agar containing 250 p.p.m. phenol. Restreaking on both yeast extract–peptone–galactose and Sabouraud dextrose agar plates led to further purification of the fungus, the morphological characteristics of which matched those of the genus *Trichosporon*. The ITS sequence of our isolated fungus was identical to that of a clone from our SIP-based library, confirming it to be *Trichosporon multisporum*. High-performance liquid chromatography and turbidometeric analyses showed that the culture was able to metabolize and grow on 200 p.p.m. phenol in an aqueous mineral salts medium within 24 h at room temperature. Gas chromatography–mass spectrometry analysis of $^{13}CO_2$ respiration from laboratory soil incubations demonstrated accelerated phenol mineralization in treatments inoculated with *T. multisporum*. These findings show that *T. multisporum* actively degraded phenol in our field-based, soil experiments.
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Introduction

Phenol is a globally distributed, organic pollutant that enters the environment through both natural (decomposition of organic matter, burning wood and volatilization from manure) and anthropogenic sources (coal tar waste, production of resins, plastics, adhesives, nylon, steel, rubber and treated lumber) (Scow *et al.*, 1981; Goerlitz *et al.*, 1985; Jenkins, 1994; ATSDR, 2006). The universal toxicity and ubiquitous nature of phenol make its biodegradation a topic of great interest (ATSDR, 2006). Although the earliest studies of phenol biodegradation focused on bacteria, there have been a growing number of fungi studied for their ability to degrade phenol, including members of the genera *Trichosporon*, *Cryptococcus*, *Rhodotorula*, *Rhodococcus*, *Aspergillus*, *Penicillium*, *Cladosporium*, *Trichoderma*, *Phanerochaete*, *Candida* and *Fusarium* (Harris and Ricketts, 1962; Dagley, 1967; Neujahr and Varga, 1970; Gaal and Neujahr, 1979; Rubin and Schmidt, 1985; Alexievaa *et al.*, 2004; Atagana, 2004; Santos and Linardi, 2004; Bergauer *et al.*, 2005; Margesin *et al.*, 2005; Krallish *et al.*, 2006; Singh, 2006; Stoilova *et al.*, 2006; Jiang *et al.*, 2007). Fungi are often able to thrive under environmentally stressed conditions (low nutrient availability, low moisture, low pH and low temperature) and unlike bacteria, they can extend their biomass through environmental matrices through hyphal growth, making their potential for metabolism of organic pollutants particularly promising (Buchan *et al.*, 2003; Atagana, 2004; Margesin *et al.*, 2005; de Boer *et al.*, 2005). Singh (2006) provides a comprehensive review of phenol metabolism by fungi.

Stable isotope probing (SIP) has proven to be a valuable tool in identifying microorganisms actively degrading chemical pollutants in complex microbial communities (Jeon *et al.*, 2003; Kasai *et al.*, 2006; Leigh *et al.*, 2007; Jones *et al.*, 2008). SIP follows incorporation of a stable isotope (for example, ^{13}C, ^{15}N and ^{18}O) into cellular biomarkers (phospholipid

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fatty acids, DNA, RNA and protein) of organisms actively involved in the metabolism of a labeled substrate (Radajewski *et al.*, 2003; Neufeld *et al.*, 2007b, c; Schwartz, 2007; Whiteley *et al.*, 2007; Jehmlich *et al.*, 2008). A number of reviews are available that discuss the various applications and methodological considerations of SIP studies (Radajewski *et al.*, 2003; Dumont and Murrell, 2005; Madsen, 2006; Neufeld *et al.*, 2007c; Whiteley *et al.*, 2007).

Our recent work focused on members of the domain, Bacteria, involved in phenol degradation at an agricultural field site (DeRito *et al.*, 2005). Our methods allowed us to identify a number of primary degraders of phenol as well as potential secondary consumers involved in carbon flow between members of the soil microbial community. The current study extends our inquiry to the fungal component of soil microorganisms. Using field-based DNA SIP of the 18S–28S internal transcribed spacer (ITS) region from previous centrifugation gradients (DeRito *et al.*, 2005), we identified a soil yeast actively involved in phenol biodegradation. Isolation of the phenol-degrading yeast, *Trichosporon multisporum* strain CD1, complemented our culture-independent findings and confirmed that this soil-borne fungus actively degraded phenol in our field experiments.

Materials and methods

Field study site

This study was conducted at the Cornell University Agricultural Experiment Station, Ithaca, NY, USA. The soil plot (Collamer silt loam) was level and free of vegetation. The treatments (see below) occurred in a grid of dosing points on ~10-cm centers. A table was placed over the plot (0.8 m high) to protect the experiment from rain and direct exposure to sunlight.

Prior experimental design

Five soil treatments, prepared in quadruplicate (Table 1), were designed to probe three distinct communities of phenol degraders *in situ*. Key variables for each treatment were carbon isotope of phenol (unlabeled [^{12}C] or ^{13}C) and the number of daily doses (0 or 11) prior to a final dose of phenol. For example, in Table 1, '13/13' indicates that 11 prior doses of ^{13}C-labeled phenol were delivered and the final dose was also ^{13}C-labeled. Each 20-μl dose contained 200 μg of phenol. Immediately after the final dose of ^{13}C-phenol, the plots were covered with septa-fitted chambers. Following 30 h of headspace monitoring of $^{13}CO_2$, 0.125-g soil samples from quadruplicate samples were pooled and stored at −80 °C. Subsequently, DNA extracts were prepared (DeRito *et al.*, 2005).

Fungi-specific PCR

Five ^{13}C-DNA fractions obtained from our previous phenol study (DeRito *et al.*, 2005) were PCR amplified using the primers 1406f, 5′-TGYACACACCGCCCGT-3′ (universal, 16S/18S rRNA gene), and 3126r, 5′-ATATGCTTAAGTTCAGCGGGT-3′, by methods described earlier (Sequerra *et al.*, 1997; Fisher and Triplett, 1999; Ranjard *et al.*, 2001; Hansgate *et al.*, 2005). These primers target the last 120 bp of 18S rRNA gene, ITS region 1 (ITS1), 5.8S rRNA gene and ITS2, with the 3126r primer matching the 5′ end of the 28S rRNA gene. Each 50 μl PCR reaction contained 10 μl of template DNA, 400 nM of each primer and 1 × *Taq* PCR Master Mix (ABGene, Rochester, NY, USA).

Cloning, restriction digestion and sequencing

Molecular cloning of the resulting fungal amplicon (from the 2×10^{-2} dilution) was carried out using the TOPO Cloning Kit (TA cloning; Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommended protocol. Following transformation of plasmids into host cells and blue/white screening, colonies with inserts were verified by PCR with vector-specific primers (5′-GTAACGGCCGCCAGTGTGCT-3′ and 5′-CAGTGTGATGGATATCTGCA-3′) that flanked the cloning region. Amplicons were digested with *Hae*III and *Hha*I and restriction fragment length polymorphism (RFLP) patterns were analyzed on 3% MetaPhore agarose gels (BioWhittaker; Molecular Applications, Rockland,

Table 1 Five soil treatments applied to field plots that varied the type (^{13}C- or unlabeled) of phenol and number of doses to soil (DeRito *et al.*, 2005)

Treatment designation[a]	*No. of prior doses*	*Prior isotope*	*Final isotope*	*^{13}C-probed microbial population*
N/12	0	None	^{12}C	None
N/13	0	None	^{13}C	Unenriched primary phenol degraders
12/12	11	^{12}C	^{12}C	None
12/13	11	^{12}C	^{13}C	Enriched primary phenol degraders
13/13	11	^{13}C	^{13}C	Mixed, trophically related (^{13}C cross-feeders)

[a]Nomenclature specifies the prior dose regimen ('N' (none) versus the carbon isotope in 11 prior doses) followed by the carbon isotope of the final (12th) dose. See Materials and methods.

ME, USA) with a 100-bp ladder (Promega, Madison, WI, USA) as a marker. Clones containing unique RFLP patterns were selected for sequencing, grown overnight in 5 ml of Luria–Bertani broth with kanamycin (50 $\mu g\,\mu l^{-1}$) and pelleted, and plasmids were purified (QiaPrep spin miniprep kit; Qiagen, Santa Clarita, CA, USA). Sequencing (Cornell University DNA Sequencing Facility) was conducted using the vector-specific M13 forward (5′-TGTAAA ACGACGGCCAGT-3′) and M13 reverse (5′-AACAG CTATGACCATG-3′) primers. Raw sequence data were assembled into full-length contigs (516–629 bp) using the program SEQMAN II (DNASTAR Inc., Madison, WI, USA). After assembly, the consensus sequence was verified manually by referring to the corresponding ABI chromatograms of the sequencing reactions. A BLAST search (http://ncbi.nlm.nih.gov/BLAST) was used to identify the closest relatives for each clone, which are included in the resulting dendrogram. Sequences were aligned using the Clustal W option in the program MEGALIGN (DNASTAR Inc.) and sequence relatedness was calculated using the same program. Phylogenetic analysis was conducted using ClustalX version 1.83.

Isolation and identification of phenol-degrading fungi

For the isolation of phenol-degrading fungi, a modified version of medium from Shoda *et al.* (1980) was prepared with the following composition, per liter of distilled water: phenol 0.250 g, NH_4Cl 5 g, KH_2PO_4 5 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $CaCl_2 \cdot 2H_2O$ 0.01 g, $FeCl_3 \cdot 6H_2O$ 0.01 g, pyridoxine HCl 0.4 g, thiamine HCl 0.2 g, riboflavin 0.2 g, calcium pantothenate 0.2 g, thioctic acid 0.2 g, ρ-aminobenzoic acid 0.2 g, vitamin B_{12} 0.2 g, mercaptoethanesulfonic acid 0.2 g, biotin 0.08 g and folic acid 0.08 g. To inhibit bacterial growth, kanamycin and streptomycin were added to a final concentration of 100 p.p.m. Agar plates were prepared using the above medium containing 1.5% Agar Noble (Difco).

Soil (5 g) was added to 100 ml of liquid medium containing 250 p.p.m. phenol and agitated on a rotary shaker (300 r.p.m.) for 2 days at room temperature. During the incubation, phenol loss was confirmed by high-performance liquid chromatography (HPLC) analysis and optical density (OD) increased. The enrichment culture was diluted with phosphate-buffered saline and 100 µl aliquots of each dilution (10^{-2}–10^{-6}) were spread onto agar plates containing 250 p.p.m. phenol and incubated at room temperature. White filamentous colonies appeared after 2 days and were homogenous at each dilution. Two representative colonies were selected and purified on yeast extract–peptone–galactose (composition per liter: 10 g Bacto yeast extract, 20 g Bacto peptone and 20 g galactose) and Sabouraud dextrose agar plates. Phenol-degrading capacity of these two isolates was confirmed by restreaking on agar plates containing phenol as the carbon source. Isolates were identified morphologically, microscopically and by sequence analysis of the fungal intergenic spacer region (Kurtzman, 1988; Cullings and Vogler, 1998; Redecker *et al.*, 1999; Buchan *et al.*, 2003).

Metabolism of phenol by T. multisporum *strain CD1*

One of the purified *Trichosporon* isolates (see above) was inoculated into a flask containing 100 ml of liquid media and 200 p.p.m. phenol (see above). The flask was shaken at room temperature for 48 h. Next, 200 µl of this cell culture (OD ~ 0.1) was inoculated into 200 ml of fresh media containing 200 p.p.m. phenol. Cell growth was measured by OD at 600 nm with a spectrophotometer (Bausch and Lomb, Rochester, NY USA). Phenol degradation was measured by HPLC analysis (see below).

T. multisporum strain CD1 was also tested for its ability to degrade phenol in soil. Two different cell inocula were prepared by growing strain CD1 to an OD_{600} ~0.4 in both 10% Sabouraud dextrose broth and in minimal media containing 0.4% glucose, washing the cells twice with phosphate-buffered saline and resuspending the cells to an OD_{600} of 0.4 in phosphate-buffered saline. OD measurements were calibrated against microscopic estimation of total length of filamentous cells (data not shown). Here, 200 µl of the resulting cell preparations (~10 cm of cells) was added, in triplicate, to 5 g of site soil in a 38 ml serum bottle sealed with a rubber septum. Accompanying treatments consisted of (i) an uninoculated control (soil + 200 µl phosphate-buffered saline) and (ii) a sterile control (soil + 200 µl of the 10% Sabouraud dextrose-grown cell inoculum, sterilized by autoclaving). Next, 200 µl of filter-sterilized, aqueous ^{13}C-phenol was added to each serum bottle resulting in a final soil concentration of 200 p.p.m. and an 8% soil moisture content. The dosed microcosms were stirred using a sterile spatula to ensure even distribution of added cells and labeled substrate. Bottles were then crimp sealed and incubated at 20 °C for 3 days. Headspace was analyzed by gas chromatography–mass spectrometry for $^{13}CO_2$ production (Jeon *et al.*, 2003; Padmanabhan *et al.*, 2003; DeRito *et al.*, 2005).

HPLC analysis of phenol

Phenol was analyzed by HPLC. Samples (1.0 ml) of culture medium were collected at various time points, immediately diluted with an equal volume of methanol, sealed and stored in screw-capped glass vials at 4 °C until analyzed. Samples were filtered through nylon acrodisc filters (0.2 µm; Acrodisc 25 mm syringe filter; Gelman, Ann Arbor, MI, USA). Phenol was separated using a Varian Microsorb-MV 100-5 C18 HPLC column (250 mm × 4.6 mm). A Waters model 590 HPLC pump was used to pump a mobile phase of

methanol:40 mM acetic acid (60:40) at a flow rate of 1.0 ml min^{-1} (DeRito *et al.*, 2005). Eluents were monitored by UV–VIS detection (ABI Analytical Absorbance Detector, Spectroflow 757) at a wavelength of 270 nm and quantified using external standard calibration curves.

Microscopy
A Nikon Eclipse E600 phase-contrast microscope equipped with a Hamamatsu color-chilled 3CCD camera (model C5810) was used to examine the isolates and to carry out direct cell counts. Images were collected using the software ATI Multimedia Center version 7.5 (ATI Technologies Inc., Sunnyvale, CA, USA). Aliquots of liquid culture (8 µl) were dispensed onto glass slides and Sabouraud dextrose agar slides (Murray *et al.*, 1994). Slide cultures were incubated in the dark at room temperature for 24–48 h and then covered with glass cover slips prior to analysis.

Nucleotide sequence accession numbers
The nucleotide sequence data reported here have been submitted to GenBank under accession nos. EU675972–EU675994.

Results

The methodologies established in our previous study (DeRito *et al.*, 2005) sought to identify soil microbial populations in the domain *Bacteria* involved in phenol degradation at an agricultural field site. These populations included (i) unenriched, primary degraders, (ii) enriched, primary degraders and (iii) trophically related organisms (carbon cross-feeders). In this study, DNA centrifugation gradients from our previous investigation were examined for fungal populations. PCR primers, originally designed for qualitative analysis of fungal communities by F-ARISA (fungal-automated rRNA intergenic spacer analysis) (Sequerra *et al.*, 1997; Fisher and Triplett, 1999; Ranjard *et al.*, 2001; Hansgate *et al.*, 2005), but lacking the fluorescence label, were used to amplify sediment-derived ^{13}C-DNA from five key soil treatments (Table 1; DeRito *et al.*, 2005). Compared with treatments that received unlabeled phenol and only one dose of ^{13}C-phenol, we obtained robust amplification of the targeted 18S–28S ITS gene region from diluted DNA extracted from the treatment which received multiple doses of ^{13}C-phenol (Supplementary Figure 1, lanes 17–20). To avoid (minimize) the possibility of mistaking spurious PCR amplicons from SIP (that may result from background nucleic acid contamination in reagents) for ^{13}C-labeled DNA, we cloned from dilutions from the ^{13}C treatments that failed to yield amplicons in the corresponding ^{12}C control treatment (DeRito *et al.*, 2005). Additional steps for validating SIP results include isolation of microbial cultures, the genotype and phenotype of which match those found with SIP (see below).

We prepared a clone library (a total of 80 white colonies) from the 13/13 field treatment-derived 18S–28S ITS amplicon. RFLP analysis of the resulting clone library revealed 15 patterns. Representatives of these were sequenced and compared with members of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). The resulting dendrogram, which includes amplicon sizes for each clone, is shown in Figure 1. DNA sequences retrieved from the clone library were dominated by those identical to (100% sequence similarity) a member of the fungal species *Trichosporon dulcitum* from the NCBI database—41% of the clones analyzed shared identical RFLPs. In total, 3% of the clones fell within a closely related clade, *T. multisporum* (Figure 1). Other organisms represented in this dendrogram included members of the genera *Cryptococcus*, *Rhodotorula*, *Nectria*, *Fusarium*, *Ascomycete* and *Acremonium*. Because several prior studies that have found *Trichosporon* and other fungi to have phenol-degrading capacities (Neujahr and Varga, 1970; Rubin and Schmidt, 1985; Alexievaa *et al.*, 2004; Margesin *et al.*, 2005), we suspected that some, if not all, of the fungi identified in the 13/13 treatment may be primary degraders.

To address this hypothesis, we attempted to grow and isolate phenol-degrading fungi from the site soil samples. After plating a dilution series (10^{-2}–10^{-6}) of a phenol-enrichment culture on minimal salts medium containing 250 p.p.m. phenol, 95 white filamentous colonies appeared on the 10^{-2} plate after 2 days. All colonies observed at each dilution were phenotypically indistinguishable. Next, 15 isolates were randomly selected and their ITS DNA was amplified by PCR and screened by RFLP. All 15 amplicons produced identical RFLP patterns, thus confirming population homogeneity (data not shown). Restreaking of two isolated colonies (on both yeast extract–peptone–galactose and Sabouraud dextrose agar plates) yielded several white/cream-colored, cone-shaped, wrinkled colonies. Microscopic observation of cultures grown on Sabouraud dextrose agar slides showed the presence of pseudohyphae and true hyphae (Figure 2)—characteristics of the genus *Trichosporon* (Kurtzman, 1988; Barnett *et al.*, 2000). Moreover, the ITS sequence of our isolate was identical to that of a clone from our SIP-based library, confirming it to be a member of the species *T. multisporum* (Figure 1). One of these isolates (CD1) was examined for its ability to degrade phenol. HPLC analysis combined with spectrophotometric analysis showed that the isolate was able to metabolize and grow on 200 p.p.m. phenol within 24 h at 20 °C (Supplementary Figure 2).

We used a laboratory study to confirm that *T. multisporum*'s ability to metabolize phenol could be expressed in the same soil used in the field SIP

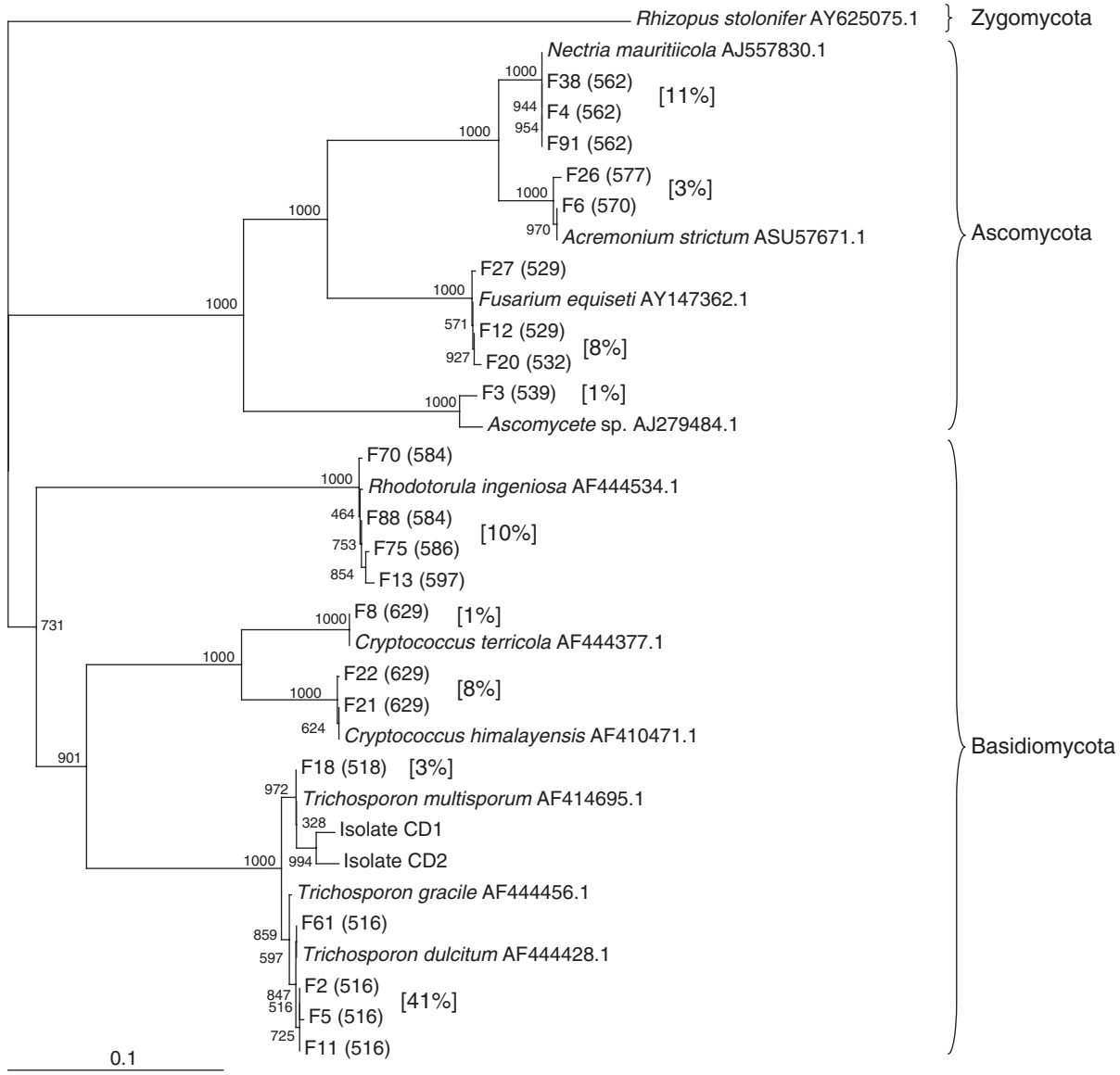

Figure 1 Phylogenetic analysis of cloned fungal genes (partial 18S rRNA, complete internal transcribed spacer region 1 (ITS1), 5.8S rRNA and ITS2, and partial 28S rRNA) from the sediment-derived ^{13}C-DNA fraction of the 13/13 treatment. Clones were screened by restriction fragment length polymorphism (RFLP) and 21 were sequenced. Phylogenetic relationships were completed using the computational tools of DNASTAR Inc. and ClustalX version 1.83. Numbers at nodes are bootstrap values based on a neighbor-joining bootstrap analysis with 1000 replicates. Numbers in parentheses ( ) indicate the amplicon size for each clone in base pairs. Numbers in brackets [ ] indicate the percentage of clones analyzed with identical RFLPs (for example, 41% of the clones had identical RFLPs and are most closely identified with the species *Trichosporon dulcitum*. *Rhizopus stolonifer* is the outgroup.

experiment (Figure 3). Phenol mineralization was dramatically accelerated in laboratory soil incubations in treatments inoculated with ~10 cm of *T. multisporum* strain CD1 cells. In treatments receiving inocula, 30% of the added ^{13}C label was recovered as $^{13}CO_2$ within 24 h compared with the 2% recovered in uninoculated treatments (Figure 3). Subsequently, 11% of the added ^{13}C label was recovered in the uninoculated control after 70 h. Thus, the ability of our isolate to grow on phenol is consistent with its role as being active *in situ* in phenol assimilation in soil.

Discussion

In this study, DNA-based SIP of the 18S–28S ITS rRNA gene region allowed us to identify a number of putative phenol-degrading fungi (basidiomycetes and ascomycetes) at our agricultural field site. We were able to discern from our electrophoresis gels, cloning, RFLP assays and sequencing procedures that the probed fungal community contained at least 12 different sized ITS1–5.8S–ITS2 amplicons, ranging from 516 to 629 bp (Figure 1). These lengths are within the range of those reported by Ranjard *et al.*

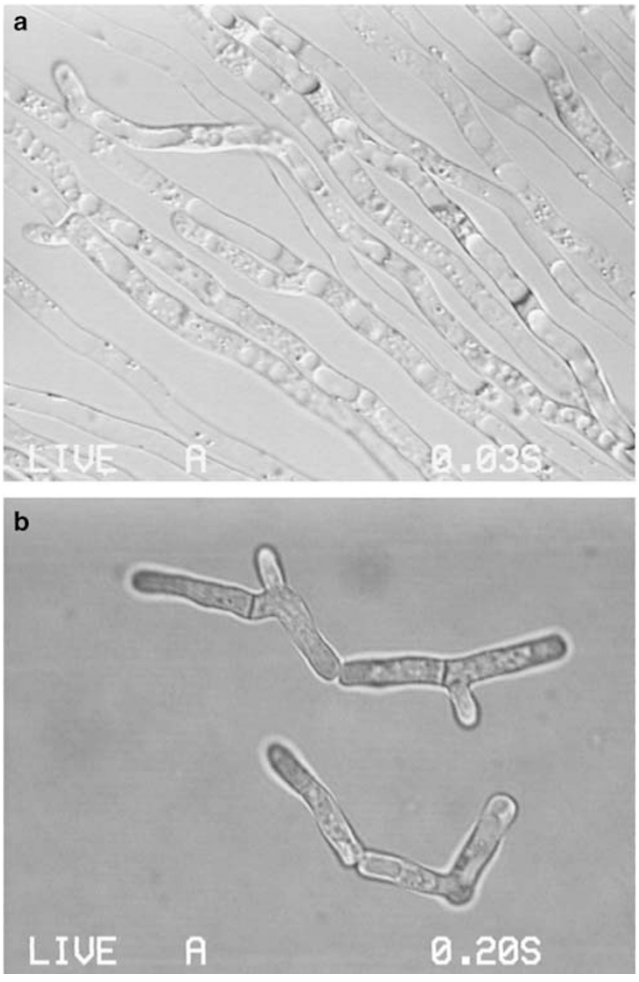

Figure 2 Phase-contrast images of *Trichosporon multisporum* strain CD1. (**a**) Sabouraud dextrose agar slide culture and (**b**) Sabouraud dextrose broth culture. Note the presence of pseudohyphae and true hyphae (characteristics of the genus *Trichosporon*) in both (**a**, **b**).

(2001) for Basidiomycota and Ascomycota. The clone library produced from the PCR amplification of the ITS region contained members of the genera *Trichosporon*, *Cryptococcus*, *Rhodotorula*, *Nectria*, *Fusarium*, *Ascomycete* and *Acremonium*. Of these seven genera, isolated strains of *Trichosporon*, *Cryptococcus*, *Rhodotorula* and *Fusarium* have been shown to degrade phenol (Neujahr and Varga, 1970; Rubin and Schmidt, 1985; Margesin *et al.*, 2003, 2005; Alexievaa *et al.*, 2004; Atagana, 2004; Santos and Linardi, 2004; Bergauer *et al.*, 2005; Krallish *et al.*, 2006). Our attempt to isolate phenol-degrading fungi revealed only *Trichosporon* as a culturable, phenol-degrading yeast from our site. We speculate that the other sequences represented in our clone library were representative of either: (i) phenol degraders that failed to grow on our culture medium, (ii) secondary consumers in a phenol food web or (iii) spurious nucleic acid contaminants of the DNA-processing reagents. We took steps aimed at guarding against the latter possibility by cloning only from the pool of ^{13}C-labeled DNA at a dilution that failed to yield a PCR amplicon in the corresponding ^{12}C treatment (Padmanabhan *et al.*, 2003; DeRito *et al.*, 2005).

Similar to all procedures in environmental microbiology, SIP has strengths and weaknesses. The major strength is that SIP can find the 'needle' of rare, metabolically active populations in the inactive 'haystack' of a complex microbial community (Madsen, 2005). The major weakness of SIP derives from the various obstacles that must be overcome to accurately interpret resulting data. Discussed at length elsewhere (for example, Manefield *et al.*, 2002a, b; Lueders *et al.*, 2004; Buckley *et al.*, 2006; Madsen, 2006; Neufeld *et al.*, 2007a, b), these obstacles include the potential for both carbon cross-feeding (DeRito *et al.*, 2005; Neufeld *et al.*, 2007a) and erroneous, reagent-borne, false-positive sequences that may occur in clone libraries of the ^{13}C-enriched nucleic acids. As discussed by Madsen (2006), routinely implemented SIP quality control procedures include short exposure to the labeled substrates (to minimize cross-feeding) and accounting for potential background contamination within the density gradients used to separate labeled from unlabeled nucleic acids. SIP procedures are successful mainly when the microbial populations that attack the labeled substrate also fully assimilate it. In many food chains, especially anaerobic ones (for example, Schink, 1997; Schmitz *et al.*, 2006), the primary degraders of the added ^{13}C-substrate may be so far removed from the final C-assimilation step that dilution of the labeled atoms may render isopycnic separation ineffective in identifying active populations (Kunapuli *et al.*, 2007). Additional well-recognized sources of potentially misleading information include: PCR bias in preparing the clone libraries (for example, Suzuki and Giovannoni, 1996; von Wintzingerode *et al.*, 1997) and small, incompletely sampled clone libraries that may not fully represent community members (for example, Hughes *et al.*, 2001; Schloss *et al.*, 2004; Tringe *et al.*, 2005; Schloss and Handelsman, 2006). A robust strategy to assure the validity of SIP-derived sequence information is to succeed in isolating a microbial culture representative of the implicated sequence, and then to verify both that the cultured microorganism possesses the expected physiological capabilities and that it is active in its native habitat (Jeon *et al.*, 2003; Madsen, 2005; Kasai *et al.*, 2006; Pumphrey and Madsen, 2008). We pursued this strategy in the present study.

One of our isolates, *T. multisporum* strain CD1, was able to metabolize 200 p.p.m. within 24 h in broth culture. This finding is consistent with a previous study in which an isolated species of *Trichosporon* (*T. cutaneum*) showed visible growth on 500 p.p.m. phenol within 18 h (Neujahr and Varga, 1970). The ITS sequence of isolate CD1 was identical to that of an organism identified in our SIP experiment (clone F18; Figure 1). The resulting

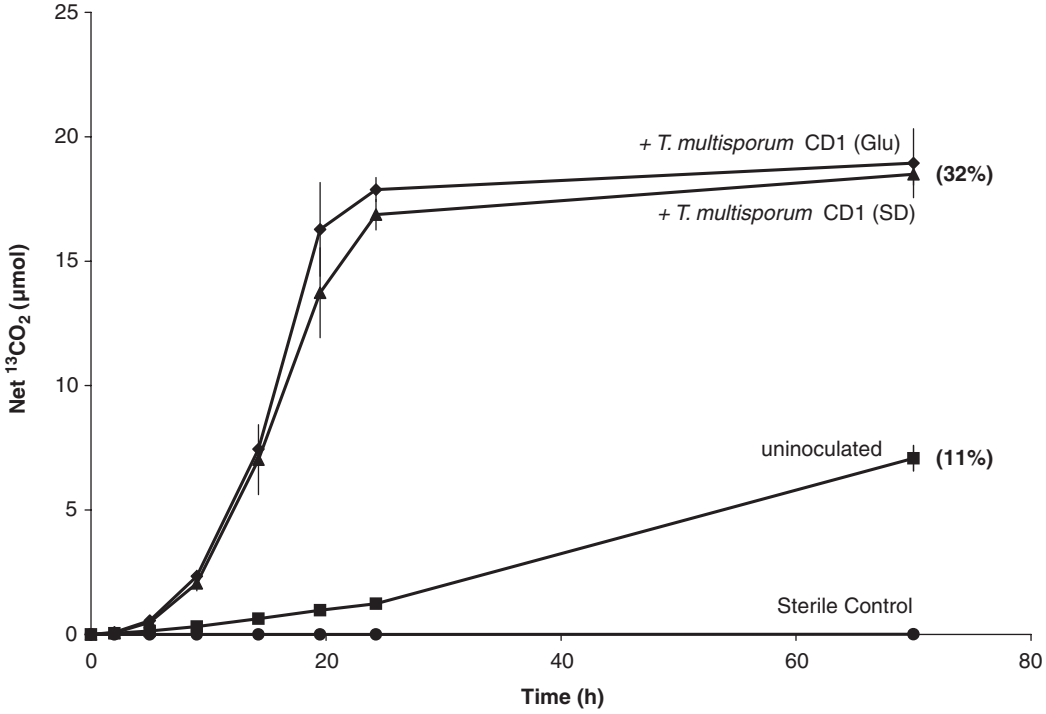

Figure 3 Evolution of $^{13}CO_2$ from [^{13}C]phenol added to soil (final soil concentration of 200 p.p.m.) in laboratory incubations with and without *Trichosporon multisporum* inoculation. Treatments included addition of $\sim$2 cm cells g^{-1} *T. multisporum* strain CD cells grown on 0.4% glucose (Glu) or 10% Sabouraud dextrose (SD) and uninoculated and autoclaved controls. Net $^{13}CO_2$ reflects total $^{13}CO_2$ minus background $^{13}CO_2$ (Padmanabhan *et al.*, 2003). The percentages in parentheses indicate proportions of the total added [^{13}C]carbon recovered as $^{13}CO_2$. Data points are the averages of the results from three replicate treatments. Error bars indicate standard deviations.

dendrogram revealed two distinct clades of *Trichosporon*. It may be noteworthy that isolate CD1 did not cluster with the dominant clade (41% of the library) of *Trichosporon* clones F2, F5 and F11. The ITS sequences of these clones were close (97.8%), but not identical to that of isolate CD1. Possible explanations for failing to cultivate representatives of the dominant clade include: (i) a relatively small number of isolates were sequenced (CD1 and CD2)—perhaps an isolate represented by clones F2, F5 and F11 was overlooked or (ii) organisms similar to clones F2, F5 and F11 were not able to flourish on the chosen agar medium.

To strengthen the case that *T. multisporum* strain CD1 was a key, primary phenol degrader in the field-based study, we implemented a laboratory-based study in which phenol mineralization was measured in site soil, both with and without added *T. multisporum* cells. We intentionally avoided physiologically biasing *T. multisporum* CD1 toward phenol metabolism by preparing the inocula from cultures grown on either 0.4% glucose or 10% Sabouraud dextrose. Results clearly showed that *T. multisporum* strain CD1 rapidly accelerated phenol mineralization in soil derived from its native habitat, thus strengthening our hypothesis that strain CD1's initial identification by SIP was the direct result of ^{13}C assimilation from ^{13}C-phenol. The data here provide new clues about *Trichosporon*'s nutritional niche in nature and are consistent with prior reports (for example, Middelhoven *et al.*, 2001) suggesting that *Trichosporon* actively participates in the mineralization of decaying plant material. In future studies, we plan to use *T. multisporum* in experiments aimed at exploring differential fitness traits between fungi and bacteria in the metabolism of carbon substrates in the soil habitat (de Boer *et al.*, 2005).

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