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Brief report

Use of mycelia as paths for the isolation of contaminant-degrading bacteria from soil

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Summary

Mycelia of fungi and soil oomycetes have recently been found to act as effective paths boosting bacterial mobility and bioaccessibility of contaminants in vadose environments. In this study, we demonstrate that mycelia can be used for targeted separation and isolation of contaminant-degrading bacteria from soil. In a 'proof of concept' study we developed a novel approach to isolate bacteria from contaminated soil using mycelia of the soil oomycete *Pythium ultimum* as translocation networks for bacteria and the polycyclic aromatic hydrocarbon naphthalene (NAPH) as selective carbon source. NAPH-degrading bacterial isolates were affiliated with the genera *Xanthomonas, Rhodococcus* and *Pseudomonas*. Except for *Rhodococcus* the NAPH-degrading isolates exhibited significant motility as observed in standard swarming and swimming motility assays. All steps of the isolation procedures were followed by cultivation-independent terminal 16S rRNA gene terminal fragment length polymorphism (T-RFLP) analysis. Interestingly, a high similarity (63%) between both the cultivable NAPH-degrading migrant and the cultivable parent soil bacterial community profiles was observed. This suggests that mycelial networks generally confer mobility to native, contaminant-degrading soil bacteria. Targeted, mycelia-based dispersal hence may have high potential for the isolation of bacteria with biotechnologically useful properties.

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Introduction

As an adaptation to heterogeneous environments (Young and Crawford, 2004), soil fungi and other mycelial microorganisms (e.g. oomycetes) have developed a unique ramified growth form, which is sustained by polarized internal cellular organization to support apical growth (Glass *et al.*, 2004). Fungi represent about 75% of the subsurface microbial biomass (0.2–0.4 mg $g_{dry\ soil}^{-1}$) with the total length of their hyphae reaching up to 10^3–10^4 m per g of arable, pasture and forest topsoils respectively (Ritz and Young, 2004). In soil, fungi and bacteria often occupy shared microhabitats referred to as the bacterial–fungal interface (Johansson *et al.*, 2004). Their growing mycelia continuously form new interfaces and novel (hospitable or hostile) microhabitats for local soil bacteria (Warmink *et al.*, 2009). Mycelia may further modify the soil structure, enter soil pores (Wösten *et al.*, 1999) and release nutrients and/or carbon exudates (de Boer *et al.*, 2005). By connecting air-filled space between water-filled pores (Wessels, 1997) they also provide efficient dispersal networks ['fungal highways' (Kohlmeier *et al.*, 2005)] for otherwise immobilized bacteria and their catabolic capabilities (Wick *et al.*, 2007; Harms *et al.*, 2011). Mycelia-mediated bacterial dispersal appears to depend on various factors including synergistic and antagonistic interactions, bacterial mobility (Warmink and van Elsas, 2009), physicochemical surface properties of bacterial and mycelial surfaces (Kohlmeier *et al.*, 2005) or chemotactic movement along hyphae to chemoeffector hotspots (Furuno *et al.*, 2010). Such movement appears to facilitate the access to suitable microhabitats for growth (Nazir *et al.*, 2010) and/or efficient contaminant biodegradation (Banitz *et al.*, 2011). To date, mycelia-mediated transport of bacteria has only been reported for axenic cultures or defined microbial communities in sterile soil and never has been applied for the extraction and isolation of contaminant-degrading bacteria from soil. In a 'proof of concept study' we here present a novel approach for the isolation and enrichment of naphthalene (NAPH)-degrading bacteria from soil by taking advantage of bacterial dispersal along the mycelia of the oomycete *Pythium ultimum* (Maurhofer *et al.*, 1992). We thereby

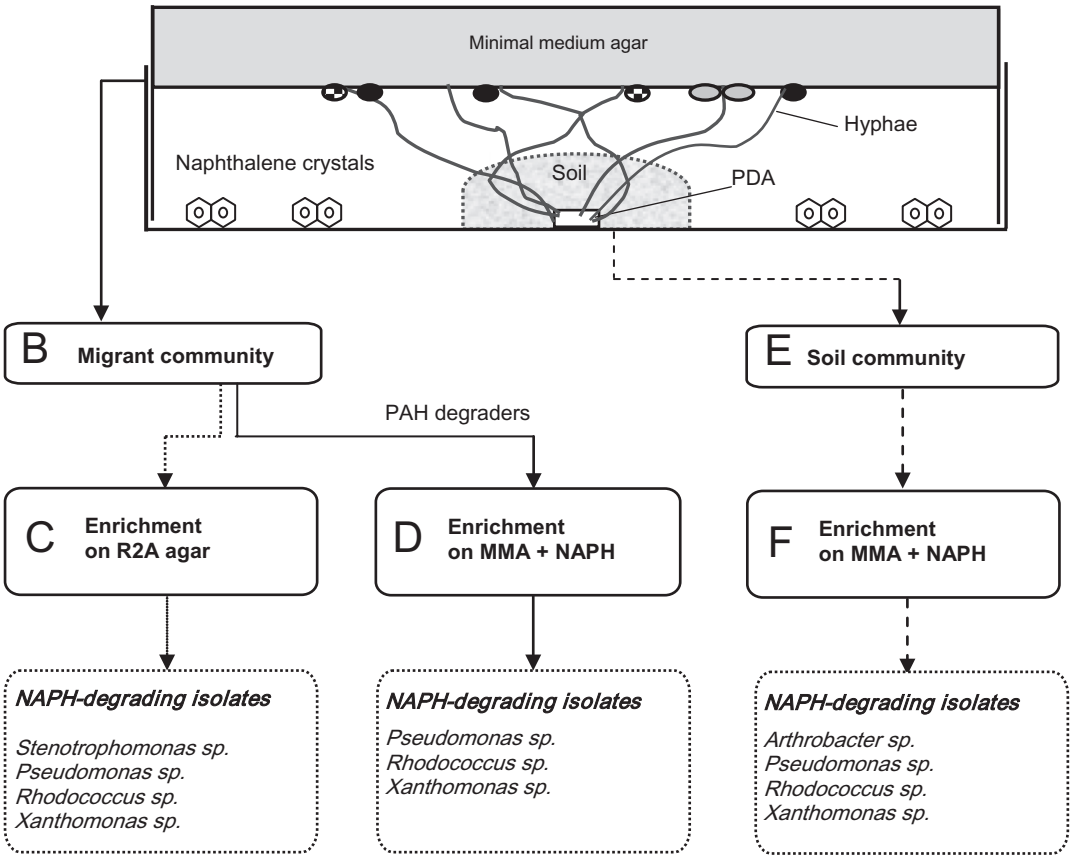

Fig. 1. Schematic diagram of the work flow of the procedure used in this study. The sketch of the reversed minimal medium agar (MMA) plate depicts the microcosm employing mycelia as paths for the separation and enrichment of NAPH-degrading bacteria from soil: a potato dextrose agar (PDA) piece inoculated with filamentous *P. ultimum* was positioned in the lid of an upside-down placed minimal medium agar (MMA) plate and covered with 1.5 g of NAPH-contaminated urban soil (containing the bacterial community **A** prior to addition to the microcosm) allowing for > 0.5 cm headspace to the agar. The oomycete was subsequently allowed to penetrate the headspace and to serve as path for the movement of bacteria to the MMA. Air-borne NAPH emanating from solid NAPH deposited at > 1 cm distance to the soil served as carbon source. Capital letters indicate the sample label of the T-RFLP community analysis of bacterial 16S rRNA genes; i.e. enrichment **B** denominates the wash-off of the migrant community from the agar surface 5 days after first contact of the hyphae with the agar, which gave rise to isolated colonies on MMA/NAPH (enrichment **D**) and R2A agar (enrichment **C**). Simultaneously, bacteria from soil overlying the *P. ultimum*-inoculated patch (community **E**) were enriched on MMA/NAPH (enrichment **F**). Please refer to *Supporting information* for detailed description of the microcosms, the cultivation, sample handling techniques, the T-RFLP analysis and the identification and phylogenetic characterization of the bacterial strains.

propose the use of mycelia-bound bacterial dispersal as a selection criterion for the targeted isolation and channelling of specialized bacteria to suitable growth environments.

Results and discussion

Isolation and enrichment of naphthalene-degrading bacteria

This study was motivated by earlier observations that mycelial growth of soil microorganisms enables the dispersal of defined bacterial populations in air-filled porous media (Leben, 1984; Kohlmeier *et al.*, 2005; Warmink and van Elsas, 2009) suggesting the idea of using 'fungal highways' (Kohlmeier *et al.*, 2005), i.e. mycelia as paths for the separation and isolation of contaminant-degrading bacteria. Figure 1 depicts the experimental set-up and the work flow for the separation and enrichment of NAPH-degrading bacteria by migration along the dense mycelial network of *P. ultimum* (Furuno *et al.*, 2010): bacterial suspensions obtained from the mineral medium agar (MMA) positioned opposite to the soil 5 days after first contact with the hyphae (enrichment B) gave rise to isolated colonies on MMA/NAPH (enrichment D) and R2A-agar (enrichment C). Simultaneously, bacteria from soil overlying the *P. ultimum*-inoculated patch were isolated on MMA/NAPH (enrichment F). Amplified 16S ribosomal DNA restriction analysis (ARDRA) of the 57 isolates picked (based on visually different morphotypes) revealed

five distinct operational taxonomic units (OTUs). Three of them were found in both the soil-community and the migrant communities. 16S rRNA gene sequence analysis of several representatives of each OTU identified the five OTUs (sequence homology within one OTU: 98–100% similarity) as *Arthrobacter* sp. ($n = 3$ colonies), *Pseudomonas* sp. ($n = 20$), *Stenotrophomonas* sp. ($n = 2$), *Rhodococcus* sp. ($n = 18$) and *Xanthomonas* sp. ($n = 14$) (Table 1). Except for *Arthrobacter* sp., all OTUs were found in the migrant communities. NAPH-degrading isolates were further tested for their ability to utilize selected polycyclic aromatic hydrocarbons (PAHs) (i.e. phenanthrene, fluorene, pyrene and anthracene). Except for *Stenotrophomonas* sp. and *Xanthomonas* sp., the isolates grew on most of the PAHs tested (Table 1). The mycelia-based discrimination seems to be driven by the inherent motility of the bacteria. A recent study for instance revealed that mycelia allow for chemotactic movement of PAH-degrading bacteria to substrate hotspots even in water-unsaturated systems (Furuno *et al*., 2010). Such studies support the relevance of mycelial networks for successful colonization of new microhabitats in soil (Wick *et al*., 2007; Nazir *et al*., 2010) especially in vadose environments. The results of this study suggest that a majority of the cultivable NAPH-degrading bacterial consortium (Table 1) may have been capable of using the hyphal network for dispersal. Both enrichments shared three out of the five NAPH-degrading isolates (*Pseudomonas* sp., *Rhodococcus* sp. and *Xanthomonas* sp.). In contrast, *Arthrobacter* sp. and *Stenotrophomonas* sp. were detected only in enrichment F or C, respectively (Fig. 1). Most isolates exhibited either swarming or swimming motility on standard agar plate assays with average colony diameters $\leq$ of motile soil bacterium *Pseudomonas putida* PpG7 (NAH7) but $\geq$ of poorly motile *Mycobacterium frederiksbergense* LB501T (Table 1). It is remarkable that the poorly motile *Arthrobacter* sp. appears to be fully retained in the soil, whereas the likewise poorly motile *Rhodococcus* sp. was found to move along the hydrophilic (Smits *et al*., 2003) hyphae of *P. ultimum*. Due to the suspected effect of physicochemical cell surface properties of the bacteria on mycelia-mediated bacterial transport (Kohlmeier *et al*., 2005), the water contact angles (θ_w) and ζ-potentials of the migrating bacteria were further measured as descriptors for the hydrophobicity and charge of the isolates' cell surfaces. No significant differences, however, of θ_w were observed with all strains being moderately hydrophobic [θ_W of 30°–70° (Table 1)] according to the classification by others (Bastiaens *et al*., 2000). The ζ-potentials of the isolates ranged from –3 to –46 mV and exhibited no significant difference between isolates derived from enrichments C, D (migrant communities) and F (soil bacterial community) as did the results from motility tests of the same strains.

Development of microbial communities was followed by cultivation-independent 16S rRNA gene-based T-RFLP analysis of DNA isolated from soil and the total bacterial colonies on agar surface in contact with the mycelia during the experiment (Fig. 1). Multivariate redundancy analysis (RDA) explained 70% of the variability of the T-RFLP fingerprints (Fig. 2) and revealed considerable variations between soil prior exposure to *P. ultimum* (A), soil penetrated by *P. ultimum* (E) and the bacteria mobilized by *P. ultimum* (B). Not surprisingly, the similarities ($P < 0.05$) between A and B (22%), A and E (29%), as well as B and E (29%) were low. Higher similarities, however, were found between communities directly derived from each other by dilution and plating, namely 36% between B and C (different C-substrate) and 58% between B and D (identical C-substrate). Interestingly, a high similarity (63%) between both the cultivable NAPH-degrading migrant (B) and the cultivable parent soil bacterial community profiles (F) was observed. This supports the hypothesis that mycelial networks confer mobility to a majority of the NAPH-degrading soil bacteria and likewise demonstrates that previously predicted mycelia-bound dispersal of axenic bacteria also holds true for native, soil-bound bacteria.

At this time, generalizations about the selectivity of the dispersal are difficult to make; mycelia-mediated bacterial translocation is multifactorious and may include active flagellar or non-flagellar migration in aqueous or mucoid films surrounding the hyphae of fungi and other mycelia-forming microorganisms (Wong and Griffin, 1976; Kohlmeier *et al*., 2005; Warmink and van Elsas, 2009) and the likely passive transport after adhesion to the apical zone of growing hyphae (Kohlmeier *et al*., 2005). Warmink and van Elsas (2009) even found that fungus-mediated dispersal may depend on bacterial community interactions as solely 4 out of 10 species migrated as single species along the mycelia of a saprotrophic fungus. Bacterial migration in the hyphosphere may further be driven by other factors including random bacterial dispersal, metabolic interactions and biofilm formation on the hyphae (Frey-Klett *et al*., 2007), chemotactic (Furuno *et al*., 2010) and infotactic (Vergassola *et al*., 2007) dispersal. Chemotactic swimming towards hyphae may already preselect certain bacteria by controlling the access to mycelial networks in soil as their metabolites may be available to bacterial commensals in the hyphosphere (Wick *et al*., 2010). Furthermore, due to the likely bioaccumulation of organic chemicals in the hyphal cell membranes, mycelia may act as an effective three-dimensional bio-sorbent network for hydrophobic organic compounds and thus allow for easy chemotaxis-driven access of the heterogeneously distributed contaminant to degrading bacteria in the hyphosphere.

Table 1. Origin and characterization of bacterial isolates obtained from enrichment on NAPH or R2A as described in Fig. 1.

Isolates			*Motility*[a 24h]		*Surface properties*[b]		*PAH degradation*[c]				
Origin	Next related culture in NCBI	Accession No.	Swarming (cm)	Swimming (cm)	Zeta potential (mV)	Contact angle (degree)	NAPH	PHEN	FLUO	ANTH	PYRE
C	*Stenotrophomonas* sp.	GU586312	0.3 ± 0.0	0.3 ± 0.0	–3 ± 1	37 ± 1	No	No	No	No	No
C	*Xanthomonas* sp.	EU373342	2.1 ± 0.0	4.5 ± 0.9	–4 ± 0	33 ± 2	Yes	No	No	No	No
C	*Pseudomonas* sp.	GU391489	0.9 ± 0.0	2.2 ± 0.3	–28 ± 1	43 ± 2	Yes	Yes	Yes	Yes	Yes
C	*Rhodococcus opacus*	AF095715	0.3 ± 0.1	0.1 ± 0.0	–29 ± 2	67 ± 4	Yes	Yes	Yes	Yes	Yes
D	*Pseudomonas* sp.	EF062805	1.6 ± 0.1	2.1 ± 0.2	–4 ± 1	42 ± 3	Yes	Yes	Yes	Yes	Yes
D	*Pseudomonas* sp.	GU391489	*Vide infra*	*Vide infra*	–46 ± 2	40 ± 2	Yes	Yes	Yes	Yes	Yes
D	*Pseudomonas* sp.	EF062805	1.9 ± 1.1	0.6 ± 0.2	–18 ± 1	39 ± 1	Yes	Yes	Yes	Yes	Yes
D	*Rhodococcus opacus*	FJ768000	0.6 ± 0.2	0.4 ± 0.1	–29 ± 2	67 ± 4	Yes	Yes	Yes	Yes	Yes
D	*Xanthomonas* sp.	EU373342	1.1 ± 0.1	1.7 ± 0.1	–10 ± 1	43 ± 2	Yes	No	No	No	No
F	*Arthrobacter* sp.	DQ158001	0.6 ± 0.3	0.1 ± 0	–24 ± 2	41 ± 2	Yes	Yes	Yes	Yes	Yes
F	*Rhodococcus opacus*	FJ768000	1.4 ± 0.7	1.0 ± 0.4	–28 ± 1	48 ± 1	Yes	Yes	Yes	Yes	Yes
	Reference strains										
–	*M. frederiksbergense* LB501T[d]		0.1 ± 0.0	0.2 ± 0.0	–41 ± 5	107 ± 3	No	No	No	Yes	No
–	*P. putida* PpG7 (NAH7)[e]		2.0 ± 1.1	4.1 ± 0.3	–35 ± 3	41 ± 2.5	Yes	Yes	No	No	No

a. Motility was scored as the diameter of bacterial displacement on swarm plates after incubation for 24 h at 20°C. Data represent the means ± standard deviation from six readings of three replicate plates per treatment.
b. Growth in liquid culture on NAPH except for *Stenotrophomonas* sp. and *M. frederiksbergense* LB501T with growth on R2A.
c. Growth on MMA in presence of solid PAH: NAPH = naphthalene; PHEN = phenanthrene; FLUO = fluoranthene; ANTH = anthracene; PYRE = pyrene. Bacterial growth was tested on minimal medium agar (MMA) plates with 15 mg of the corresponding solid PAH spread onto the agar surface. Growth and utilization of PAH as carbon source was considered to take place after formation of clearly visible colonies after 17 days of incubation, otherwise not found if the strains were plated on MMA in the absence of a PAH source.
d. Bastiaens and colleagues (2000).
e. Dunn and Gunsalus (1973).
BLAST sequence identity to next hit of all isolates was ≥ 98%. Partial sequences were deposited at GenBank (NCBI) under the Accession Nos HM623661, HM623662, HM623664, HM623666–623673. *Mycobacterium frederiksbergense* LB501T and *P. putida* PpG7 (NAH7) are listed as reference strains of known motility and previously described ability to disperse along fungal mycelia (Kohlmeier *et al*., 2005; Wick *et al*., 2007). Surface motility of the strains was tested the following standard assay described previously (Baehler *et al*., 2006). Motility was scored by mean diameter of colonies after 24 h of inoculation. The zeta potential ζ, as an indirect measure of cell surface charge, was calculated from the electrophoretic mobility according to the method of Helmholtz and Smoluchowski, as presented by Hiementz (Hiementz, 1986). The electrophoretic mobility of bacterial suspensions in 10 mM KNO_3 at pH 6.2 was determined in a Doppler electrophoretic light scattering analyser at 100 V as described elsewhere (van Loosdrecht *et al*., 1987). Cell surface hydrophobicity was derived by the water contact angles (θ_w) using an automated goniometric analysis system as described earlier (Kohlmeier *et al*., 2005).

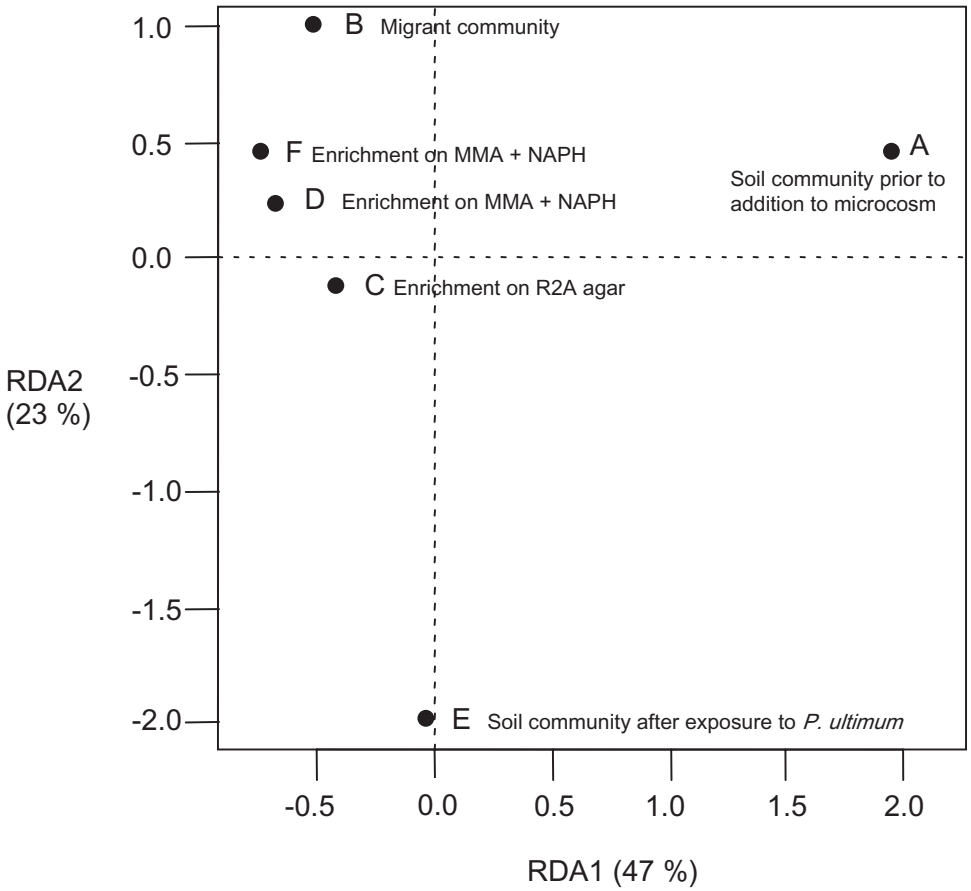

Fig. 2. Redundancy analysis (RDA; Shepard analysis factor: 0.091) depicting the qualitative similarity measure of 16S rRNA gene T-RFLP profiles of bacterial communities during separation and enrichment steps as schematized in Fig. 1. Normalization of the T-RFLP profiles and generation of a matrix with relative peak abundance for statistical analysis were performed as described in Wu and colleagues (2009) using the statistical software R-2.10 (R Development Core Team, 2009). Functions for identifying the 'true' peaks and binning the different fragment lengths are available at http://www.webpages.uidaho.edu/~joyce/Lab%20page/TRFLP-STATS.html (Abdo *et al.*, 2006). Differences in T-RFLP patterns were calculated by application of a RDA. The RDA combines the spatiotemporal changes of T-RFLP fingerprints and was conducted using R- 2.10 (R Development Core Team, 2009). The two axes represent 70% of the variance of the data.

Potential for biotechnological applications

This study therefore has been designed to deliver the 'proof of principle' that mycelia facilitate bacterial movement in non-sterilized soil and that this phenomenon may be used for the isolation of contaminant-degrading bacteria. All isolates obtained by our approach belong to genera previously known to biodegrade PAHs (e.g. Efroymson and Alexander, 1991; Uz *et al.*, 2000; Polek *et al.*, 2002; Molina *et al.*, 2009). Although no new genera were isolated by the enrichment procedures used in this study, there is much potential to tailor mycelia-based enrichment set-ups for the isolation of bacterial strains with desired specific properties. Possible variations include (i) the choice of the mycelial network (e.g. phylum, physicochemical surface properties, PAH-degradation capacity or other ecological traits of the hyphal microorganism), (ii) the time allowed for migration to separate bacteria of variable motility (i.e. allowing for a chromatographic effect in the dispersal of soil bacteria), and (iii) meaningful positioning of chemoattractants or chemorepellents. Beside its potential value for the isolation of novel degrader organisms, this research may lead to a better understanding of the interactions between mycelia (of, for example, fungi) and bacteria. It can thus result in improved remediation approaches for soils polluted with PAHs or other hydrophobic organic contaminants.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Detailed information on experimental procedures on: Organisms and culture conditions; microcosm for the separation of bacteria after migration along mycelia; isolation of NAPH degraders from soil; identification and phylogenetic characterization of bacterial strains; T-RFLP analysis of the enriched microbial communities; determination of physicochemical cell surface properties; and bacterial motility assays.

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