RESEARCH LETTER

Burkholderia fungorum DBT1: a promising bacterial strain for bioremediation of PAHs-contaminated soils

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Abstract

An extensive taxonomic analysis of the bacterial strain *Burkholderia* sp. DBT1, previously isolated from an oil refinery wastewater drainage, is discussed here. This strain is capable of transforming dibenzothiophene through the 'destructive' oxidative pathway referred to as the Kodama pathway. *Burkholderia* DBT1 has also been proved to use fluorene, naphthalene and phenanthrene as carbon and energy sources, although growth on the first two compounds requires a preinduction step. This evidence suggests that the strain DBT1 exerts a versatile metabolism towards polycyclic aromatic hydrocarbons other than condensed thiophenes. Phylogenetic characterization using a polyphasic approach was carried out to clarify the actual taxonomic position of this strain, potentially exploitable in bioremediation. In particular, investigations were focused on the possible exclusion of *Burkholderia* sp. DBT1 from the *Burkholderia cepacia* complex. Analysis of the sequences of *16S*, *recA* and *gyrB* genes along with the DNA–DNA hybridization procedure indicated that the strain DBT1 belongs to the species *Burkholderia fungorum*, suggesting the proposal of the taxonomic denomination *B. fungorum* DBT1.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent an extended class of organic compounds containing two or more condensed aromatic rings. Their molecular stability and hydrophobicity are among the prominent factors that contribute to the persistence of these pollutants in the environment. Moreover, their low aqueous solubility and, consequently, their low bioavailability are the main obstacles to microbial degradation (Cerniglia, 1992). The presence of PAHs in environmental contexts depends on both natural processes (either biogenic or geochemical) and anthropogenic activities (Mueller *et al.*, 1996). Of the PAHs occurring in soils and groundwaters, about 0.04–5% w/w are sulphur heterocycles (Thompson, 1981), among which dibenzothiophene (DBT) represents the prevailing species.

Burkholderia sp. DBT1, which was first isolated from an oil refinery sewage drainage, has been proved to lead, within 3 days, to the nearly complete decay of DBT added to the growth substrate, through the so-called Kodama oxidative pathway (Di Gregorio *et al.*, 2004). A preliminary genomic study carried out on this strain revealed that the genes responsible for DBT transformation are actually harboured in two operons (p51 and pH1A) and show low similarity to both *nah*-like and *phn*-like genes (Di Gregorio *et al.*, 2004). However, recent *in situ* molecular investigations on soils contaminated by different PAHs have ascertained the presence of a sequence corresponding to a dioxygenase closely related to that found in *Burkholderia* DBT1 (Chadhain *et al.*, 2006; Sipilä *et al.*, 2006; Brennerova *et al.*, 2009). Thus, *Burkholderia* sp. DBT1 can be claimed to be a degrader of PAHs, often occurring along with condensed thiophenes in oil-contaminated sites; however, its taxonomic identity remains largely unknown.

The existence of *Burkholderia cepacia* strains causing life-threatening infections in humans with cystic fibrosis (Govan *et al.*, 1996) has led to the rejection of bacteria belonging to this genus as possible biological agents by the US Environmental Protection Agency (Davison, 2005). Furthermore, as *Burkholderia* sp. can be involved in food poisoning (Jiao *et al.*, 2003) or act as pathogens for plants and domesticated animals (Graves *et al.*, 1997; Brett *et al.*, 1998; Srinivasan *et al.*, 2001; Lee *et al.*, 2010), some concerns exist about the intentional release of potentially hazardous strains into the

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environment for biotechnological applications (Vandamme *et al.*, 1997; Parke & Gurian-Sherman, 2001). The present study aims to provide new insights into the phenotypic traits and the phylogenetic relationships of strain DBT1 for a proper taxonomic positioning within the genus *Burkholderia*.

Materials and methods

Bacterial strains

Burkholderia fungorum LMG 16225^T, *Burkholderia caledonica* LMG 19076^T, *Burkholderia graminis* LMG 18924^T and *B. cepacia* LMG 1222^T were purchased from the German Collection of Microorganisms and Cell Cultures [Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)]. *Burkholderia* sp. DBT1 was isolated from a drain collecting oil refinery discharges near Leghorn, Tuscany, Italy (Di Gregorio *et al.*, 2004).

Chemicals

DBT, naphthalene, fluorene and phenanthrene were purchased from Sigma-Aldrich (Milan, Italy). All the compounds were analytical grade. They were dissolved in *N-N*-dimethylformamide (Sigma-Aldrich) before addition to the bacterial cultures.

Microbiological techniques

Growth tests with PAHs

All the growth tests were carried out in 100-mL Erlenmeyer flasks containing 50 mL of minimal defined medium (DM; Frassinetti *et al.*, 1998), supplemented with different organic compounds (naphthalene, phenanthrene, fluorene and DBT, at a final concentration of 100 mg L^{-1}) as the sole carbon source, and finally incubated at 27 °C on an orbital shaker (200 r.p.m.). Each flask was inoculated with aliquots from stationary-phase cultures of the *Burkholderia* sp. DBT1 strain until a final OD of 0.01 was reached. Culture samples collected at different times during the experiment were monitored for microbial growth by measuring the $OD_{600\,nm}$. Afterwards, cells were pelleted by centrifugation (10 000 ***g*** for 10 min) and absorbance of the resulting solutions was checked at 600 nm to evaluate the possible contribution of coloured metabolites to the final OD.

Growth tests starting from both nonpretreated and pretreated cells were arranged. In tests with nonpretreated cells, a preinoculum of the DBT1 was obtained in YMB medium (0.5 g L^{-1} K_2HPO_4; 0.1 g L^{-1} $MgSO_4 \cdot 7H_2O$; 0.1 g L^{-1} NaCl; 0.4 g L^{-1} yeast extract; 10 g L^{-1} mannitol) after 48 h of incubation. Conversely, in tests with pretreated cells, a preinoculum of DBT1 was grown in DM supplied with DBT or phenanthrene (500 mg L^{-1}) for 72 h to induce the PAH-degrading genes. The cells were then collected by centrifugation (5000 ***g*** for 5 min at 4 °C) and washed twice with physiological solution (NaCl 0.9%).

DBT1 reactivity to PAHs

Tests were performed on YMA media (YMB added to 1.5% bacteriological agar). Naphthalene, phenanthrene, fluorene and DBT were supplied as a vapour by incubating Petri dishes containing PAH crystals placed in their base. Plates were then incubated at 27 °C and colonies were picked and restreaked on fresh media every week for a month.

DNA extraction and PCR amplification of 16S rRNA, *recA* and *gyrB* gene sequences

Total DNA for PCR amplification was prepared as follows: overnight bacterial cultures were pelleted and resuspended in 567 μL TE buffer, 3 μL of 10% sodium dodecyl sulphate and 3 μL of 20 mg mL^{-1} proteinase K and incubated for 1 h at 37 °C. A 100-μL aliquot of 5 M NaCl and 80 μL CTAB/NaCl solution were then added and incubated again for 10 min at 65 °C. Samples were extracted with an equal volume of phenol/chloroform/isoamyl alcohol mixture. DNA was obtained after precipitation with 0.6 volumes of isopropanol and finally resuspended in 50 μL TE buffer.

All PCR reactions were carried out in 25 μL of total volume containing 0.8 μM of each primer, 0.4 mM of dNTPs, 1 U of GoTaq^{TM} DNA polymerase (Promega, Milan, Italy) and 5 μL of 5 × PCR buffer. The gene encoding for 16S rRNA gene (1500 bp) was amplified using *FD1* and *rp2* primers (Weisburg *et al.*, 1991). PCR conditions were as follows: 95 °C for 5 min, then 30 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min, with a final extension step at 72 °C for 5 min. A specific *B. fungorum recA* PCR-amplification assay was performed using the primers FunF and FunR as described by Chan *et al.* (2003). PCR amplification for an 869-bp ORF *recA* was carried out according to Payne *et al.* (2005), while *gyrB* amplification was performed as described by Ait Tayeb *et al.* (2008).

Cloning, sequencing and phylogenetic analysis

PCR products were transformed in *Escherichia coli* Xl1-blue using the Promega pGEM-T vector system according to the manufacturer's instructions, sequenced on both strands and finally searched for homology using the BLASTN database (Altschul *et al.*, 1997).

The sequences were initially aligned using the multiple alignment program CLUSTAL_X 1.83 (Thompson *et al.*, 1997). A phylogenetic tree was constructed based on the neighbour-joining method using the MEGA version 4.0 software package (Kumar *et al.*, 2008). Bootstrap analysis was performed on the basis of 1000 bootstrap replications.

DNA–DNA hybridization

DNA–DNA hybridization was performed by DSMZ (Braunschweig, Germany) through spectroscopic analysis in 2 × SSC+5% formamide at 70 °C. DNA was isolated using a French pressure cell press (Thermo Spectronic, Rochester, NY) and purified by chromatography on hydroxyapatite (Cashion *et al.*, 1977). The analytical protocol was according to De Ley *et al.* (1970) as modified by Huss *et al.* (1983), using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian, Palo Alto, CA).

API 20NE

Testing with the API 20NE system was performed following the manufacturer's specifications (bioMérieux Italia, Bagno a Ripoli, Italy). Substrate assimilations were checked after 24 and 48 h.

Results and discussion

Phenotypic characteristics

Growth tests in the presence of different PAHs

Growth tests carried out in the presence of different PAHs demonstrated that *Burkholderia* sp. DBT1 is able to grow on both phenanthrene and DBT as the sole sources of carbon and energy, although the growth on this latter substrate proceeds with a lower yield (Fig. 1). Moreover, DBT1 is also capable of utilizing naphthalene and fluorene provided after a 3-day induction on phenanthrene (Fig. 1) or DBT (data not shown).

When strain DBT1 was grown on YMA plates added with crystals of different PAHs, a change in the colour of the colonies was detected. Briefly, DBT1 colonies became red in the presence of DBT, yellow when treated with fluorene and orange/pink and weakly yellow when phenanthrene and naphthalene were added to Petri dishes, respectively (Fig. 2). This change in colour may be attributed to PAH cleavage. In particular, DBT1 colonies became red when treated with DBT, owing to the transformation of DBT to oxidized intermediates (Kodama *et al.*, 1970, 1973). When fluorene crystals were added to Petri dishes, DBT1 colonies acquired a yellow colour, as already observed by Casellas *et al.* (1997) and Seo *et al.* (2009). On the other hand, when grown in the presence of phenanthrene, the strain DBT1 produced an orange/pink pigment. This phenotype has also been reported in *Alcaligenes faecalis* AFK2, which degrades phenanthrene via *o*-phthalate by a protocatechuate pathway (Kiyohara *et al.*, 1982). Finally, with the addition of naphthalene crystals, DBT1 colonies became weakly yellow,

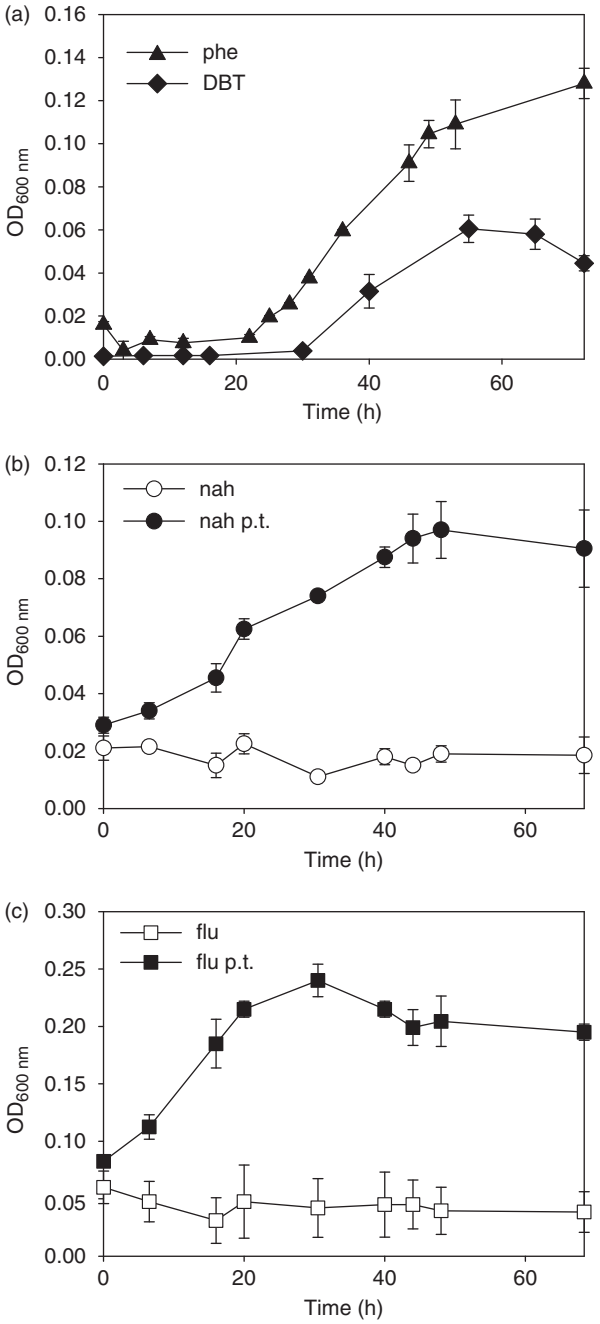

Fig. 1. Time courses of microbial growth by *Burkholderia* sp. DBT1 in the presence of 100 mg L^{-1} of (a) phenanthrene (phe) or DBT, (b) naphthalene (nah) and (c) fluorene (flu) with or without preinduction (p.t.) with phenanthrene. Each curve shows means based on the results of three experiments.

as already observed in a *Pseudomonas* strain (Kiyohara & Nagao, 1977).

These results suggest that the strain DBT1 may rely on a broad substrate specificity towards different PAHs. Interestingly, enzymes for the degradation of naphthalene and

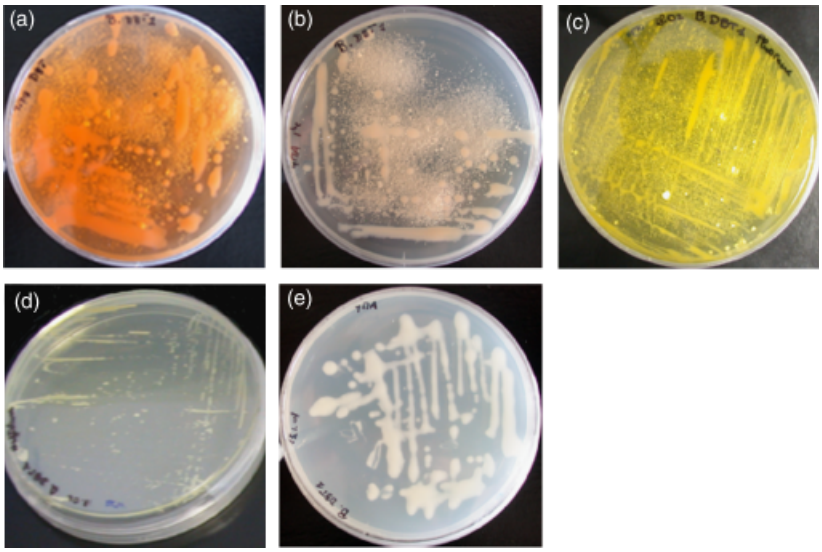

Fig. 2. *Burkholderia* DBT1 grown on YMA supplied with (a) DBT, (b) phenanthrene, (c) fluorene and (d) naphthalene crystals amended on the Petri dish bottom. (e) *Burkholderia* DBT1 grown on YMA without PAH crystals (negative control).

fluorene can be induced by either phenanthrene or DBT. This indicates that these compounds, chiefly phenathrene, may act as major substrates for *Burkholderia* sp. DBT1.

Biochemical tests

API 20NE tests were carried out on the following strains: *Burkholderia* sp. DBT1, *B. fungorum* LMG 16225^T and *B. cepacia* LMG 1222^T. *Burkholderia fungorum* and *B. cepacia* were compared with DBT1 as they represent, respectively, the closest phylogenetically related species and the most representative species listed in the *Burkholderia cepacia* complex (Bcc), whose members are often responsible for opportunistic human infections (Govan *et al.*, 1993). Furthermore, sometimes, *B. fungorum* isolates can be misidentified as Bcc organisms (Coenye *et al.*, 2001, 2002). Strains DBT1, LMG 16225^T and LMG 1222^T were capable of utilizing D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, gluconate, malate, citrate and phenylacetate. None of the strains considered was positive for indole production, arginine dihydrolase, glucose acidification, urease activity or maltose assimilation. In fact, strain DBT1 showed almost the same biochemical traits as both *B. fungorum* and *B. cepacia* type strains (Table 1). Nevertheless, the findings on LMG 1222^T were consistent with previous studies (Fain & Haddock, 2001). On the other hand, LMG 16625^T is listed as positive for the assimilation of caprate and adipate in Coenye *et al.* (2001).

Table 1. Carbon source utilization and biochemical tests that differentiate strain DBT1 from *Burkholderia cepacia* LMG 1222^T and *Burkholderia fungorum* LMG 16225^T

Tests	*B. cepacia* LMG 1222^T	*B. fungorum* LMG 16225^T	*Burkholderia* sp. DBT1
Nitrate reduction	−	+	+
Aesculin hydrolysis	+	−	+
Caprate assimilation	+	−	−
Adipate assimilation	+	−	+

−, Negative; +, positive.

Phylogenetic analysis

Sequence analysis of 16S rRNA, *recA* and *gyrB genes*

A 1493-bp fragment of DBT1 16S rRNA gene was sequenced and nucleotide BLAST (NCBI) analysis was performed. Thereafter, multiple alignment and evolutionary distances were calculated with 16S rRNA genes of related and nonrelated taxa in order to construct a phylogenetic tree based on the neighbour-joining algorithm (Fig. 3). The 16S rRNA gene sequence of strain DBT1 was closely related (99.7–100% similarity) to those of different strains of *B. fungorum*. *Burkholderia fungorum* strains LMG 16225^T and LMG 16307 were isolated from the white-rot fungus *Phanerochaete chrysosporium* and cerebrospinal fluid, respectively (Coenye *et al.*, 2001). Strain N2P5 was isolated from a PAH-contaminated soil (Mueller *et al.*, 1997; Coenye *et al.*, 2001) and might have useful degradative properties similar to DBT1. *Burkholderia phytofirmans* LMG 22487^T was ranked as the second most closely related bacterial species to DBT1, with a 98.9% similarity. Good similarities of 16S rRNA gene sequences were also found between DBT1 and *B. caledonica* LMG 19076^T (98.5%), *Burkholderia megapolitana* LMG 23650^T (98.4%) and *Burkholderia phenazinium* LMG 2247^T (98.4%). Still significant similarities to DBT1 were shown by *Burkholderia phenoliruptrix* LMG 21445^T, *Burkholderia xenovorans* LMG 21463^T, *Burkholderia terricola* LMG 20594^T, *B. graminis* LMG 18924^T and *Burkholderia caryophylli* LMG 2155^T in the range 97.9–97.3%. Finally, the similarities

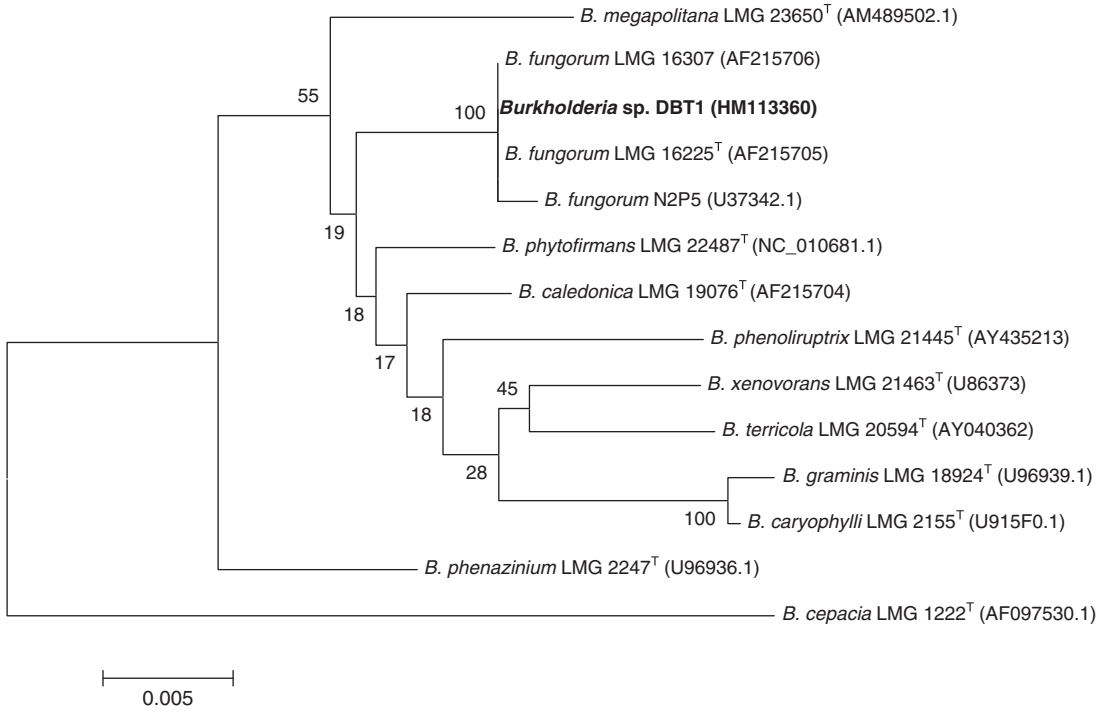

Fig. 3. Neighbour-joining phylogenetic tree based on the sequence of the 16S rRNA gene of *Burkholderia* sp. DBT1 and related strains. Bootstrap values are given at branch nodes and are based on 1000 replicates. The scale bar indicates 0.005 substitutions per nucleotide position.

between DBT1 and the other *Burkholderia* sp. considered in this study were $< 97.0\%$. In particular, 16S rRNA gene phylogeny shows that DBT1 and *B. cepacia* (94.9% similarity) are not related species.

Although the analysis of the 16S rRNA gene sequence represents a basic step in the taxonomic characterization of bacterial genera (Vandamme *et al.*, 1996), often, it is not adequate to solve uncertainties in comparisons of closely related species (Ash *et al.*, 1991; Fox *et al.*, 1992). In the present study, an 869-bp portion of the *recA* gene sequence from *Burkholderia* sp. DBT1 was amplified by PCR and sequenced. Related *recA* sequences were aligned and a phylogenetic tree was constructed (Fig. 4). The similarity of *recA* gene sequences between strain DBT1 and single *B. fungorum* strains ranged from 99.4% to 99.1%. On the other hand, the similarity for the same sequence to *B. phytofirmans* LMG 22487^T, *B. xenovorans* LMG 21463^T, *B. caledonica* LMG 19076^T and *B. graminis* LMG 18924^T declined to 95.5%, 93.9%, 92.0% and 91.4%, respectively.

In the last few years, species-specific primers, namely FunF and FunR, have been designed for *recA*-based PCR assays targeted for *B. fungorum* (Chan *et al.*, 2003). These primers were used to assign *Burkholderia* sp. DBT1 incontrovertibly to the *B. fungorum* species. PCR assays carried out with genomic DNA obtained from *B. cepacia* LMG 1222^T, *B. caledonica* LMG 19076^T and *B. graminis* LMG 18924^T were used as negative controls, and the test carried out with DNA from *B. fungorum* LMG 16225^T was taken as a positive control. An amplicon of 330 bp was obtained through PCR analysis of DNAs from either *B. fungorum* LMG 16225^T or strain DBT1. Afterwards, the amplicons were purified and sequenced to confirm the identity of the fragments with the correct sequence of the *recA* gene. No amplification products were generated with DNA from the other *Burkholderia* strains tested (Fig. 5).

Moreover, a 432-bp portion of the *gyrB* gene was amplified by PCR starting from the genomic DNAs of *B. cepacia* LMG 1222^T, *B. fungorum* LMG 16225^T and *Burkholderia* DBT1. The amplicons were then cloned and sequenced. In this case, the degree of similarity of DBT1 to LMG 16225^T and LMG 1222^T was 98.2% and 86.5%, respectively. The *gyrB* sequence of DBT1 was compared through the available DNA sequence databases using the BLAST interface (NCBI). The following similarities were found: 94.0% to *B. xenovorans* LMG 21463^T (GenBank accession no. CP000270), 93.7% to *B. phytofirmans* LMG 22487^T (GenBank accession no. CP001052) and 91.1% to *B. graminis* LMG 18924^T (GenBank accession no. EU024212).

Strain DBT1, within the phylogenetic trees based on the comparison of both 16S rRNA and *recA* gene sequences, forms a well-substantiated clade with *B. fungorum* strains. Moreover, *gyrB* gene sequence similarity scoring also

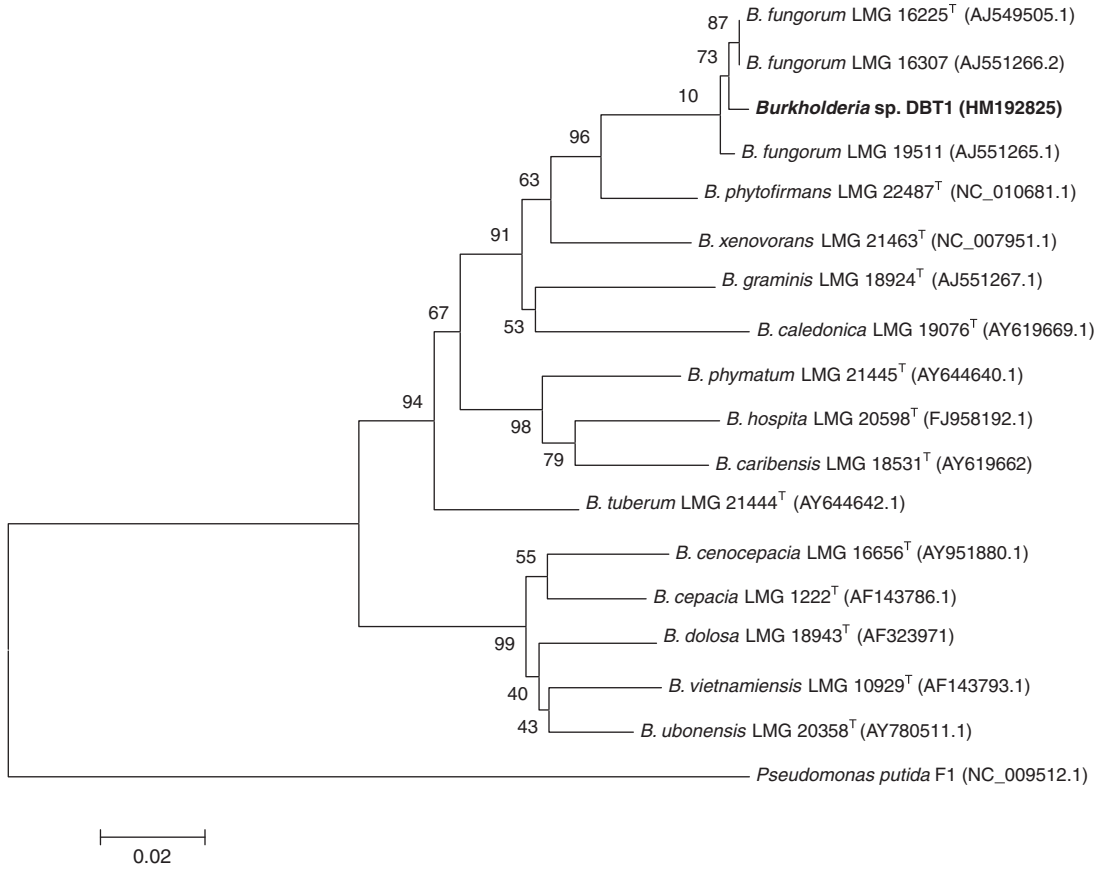

Fig. 4. Neighbour-joining phylogenetic tree based on the sequence of the *recA* gene of *Burkholderia* sp. DBT1 and related strains. Bootstrap values are given at branch nodes and are based on 1000 replicates. The scale bar indicates 0.02 substitutions per nucleotide position. *Pseudomonas putida* F1 was used as the outgroup.

indicates that DBT1 closely fits strains of the species *B. fungorum*, although databases are poor in bacterial *gyrB* sequence information.

DNA–DNA hybridization

Clusters of bacteria sharing almost identical 16S rRNA gene sequences have sometimes been identified. However, their DNAs hybridize at significantly lower than 70%. In these cases, the microorganisms represented distinct species (Fox *et al.*, 1992; Tønjum *et al.*, 1998). Therefore, to clarify conclusively the taxonomic affiliation of strain DBT1, DNA–DNA hybridization was performed against *B. fungorum* LMG 16225^T. A complementation of $78.2 \pm 2.9\%$ demonstrated that *Burkholderia* DBT1 belongs to the species *B. fungorum* according to the definition of bacterial species by Wayne *et al.* (1987).

Eventually, DNA–DNA hybridization confirmed the affiliation of strain DBT1 to the *B. fungorum* species. Thus, on the basis of these evidences, *Burkholderia* DBT1 can be ascribed to *B. fungorum*, excluding any relationship with Bcc representatives.

Conclusion

Burkholderia DBT1, a bacterial strain isolated from oil refinery drainage, has been shown to be capable of degrading DBT in liquid culture oxidatively, through the Kodama pathway, within 3 days of incubation (Di Gregorio *et al.*, 2004). Because DBT behaves as a recalcitrant compound and tends to bioaccumulate throughout the food chains, the isolation and characterization of bacterial strains able to degrade condensed thiophenes, using them as the sole source of carbon and energy, can result in applications in bioremediation protocols. Nevertheless, for the harmless exploitation of *Burkholderia* DBT1 in environmental biotechnology, a probative exclusion of this strain from the *B. cepacia* complex is a prerequisite.

The versatile metabolism of DBT1 towards PAHs such as naphthalene, phenanthrene and fluorene shown in the present study is an encouraging trait for the possible use of

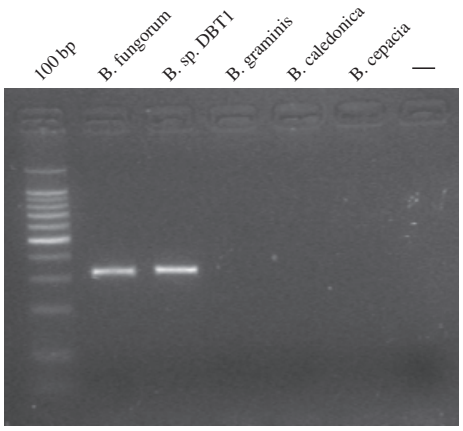

Fig. 5. *recA* PCR-amplification assay using species-specific primers for *Burkholderia fungorum*. Lanes from left to right contain a 100 bp molecular marker (BioLabs) and, respectively, PCR amplicons obtained using DNA templates from *B. fungorum* LMG 16225^T, *Burkholderia* sp. DBT1, *Burkholderia graminis* LMG 18924^T, *Burkholderia caledonica* LMG 19076^T, *Burkholderia cepacia* LMG 1222^T and negative control (PCR carried out without a template).

this strain in the clean-up of contaminated sites. Moreover, the taxonomic details gained in this study attribute the strain DBT1 to the species *fungorum*, excluding any possible association of this isolate to the Bcc.

Acknowledgements

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