Classification of the biphenyl- and polychlorinated biphenyl-degrading strain LB400^T and relatives as *Burkholderia xenovorans* sp. nov.

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Strain LB400^T is the best-studied polychlorinated biphenyl (PCB) degrader. This organism has previously been allocated in the genus *Burkholderia*, since its 16S rRNA gene sequence shows 98·6 % sequence similarity to the type strains of *Burkholderia graminis* and *Burkholderia terricola*. A polyphasic study was undertaken to clarify the actual taxonomic position of this biotechnologically important organism and of two strains, one recovered from a blood culture vial and one from a coffee plant rhizosphere, both of which resembled strain LB400^T in their whole-cell protein patterns. DNA–DNA hybridization experiments revealed that the three strains represented a single novel species, for which the name *Burkholderia xenovorans* sp. nov. is proposed. Strains of this novel species can be differentiated phenotypically from nearly all other *Burkholderia* species by their inability to assimilate L-arabinose. The whole-cell fatty acid profile of *B. xenovorans* strains is consistent with their classification in the genus *Burkholderia*, with 18 : 1ω7*c*, 16 : 1ω7*c*, 16 : 0, 14 : 0 3OH, 16 : 0 3OH, 17 : 0 cyclo and 14 : 0 being the most abundant fatty acids. The G+C content of the species varies between 62·4 and 62·9 mol%. The type strain of *B. xenovorans* is LB400^T (=LMG 21463^T=CCUG 46959^T=NRRL B-18064^T).

The genus *Burkholderia* is a phylogenetically well-defined group of organisms, occupying very diverse ecological niches. The more than 30 currently described *Burkholderia* species comprise soil and rhizosphere bacteria as well as plant pathogens, human pathogens and human opportunistic pathogens (Coenye & Vandamme, 2003). Several *Burkholderia* strains have gained interest for their ability to degrade xenobiotic compounds, such as halogenated aromatics. One of the best-studied examples is *Burkholderia* sp. strain LB400^T. This strain co-metabolizes many polychlorinated biphenyl (PCB) congeners when grown on biphenyl (Gibson *et al.*, 1993). The pathways for degradation of PCBs by strain LB400^T have been extensively characterized at both the genetic and the molecular level (e.g. Erickson & Mondello, 1992; Hofer *et al.*, 1993) and have become a model system for the bacterial breakdown of these very persistent environmental contaminants (for a recent review on bacterial PCB degradation, see Furukawa, 2000).

Strain LB400^T (=LMG 21463^T=CCUG 46959^T=NRRL B-18064^T) was isolated from PCB-contaminated soil collected from a landfill in Moreau, New York, and originally identified as a *Pseudomonas* species (Bopp, 1986, 1989). It was referred to in more-recent scientific literature as *Burkholderia* sp. or *Burkholderia cepacia* (e.g. Bartels *et al.*, 1999; Kumamaru *et al.*, 1998; Seeger *et al.*, 1999). The allocation of strain LB400^T to the genus *Burkholderia* was confirmed in a recent taxonomic characterization by Fain & Haddock (2001). Furthermore, evidence provided by these authors excluded it from the *B. cepacia* complex. The actual species affiliation of strain LB400^T, however, remained unclear.

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Abbreviations: ARDRA, amplified rDNA restriction analysis; PCB, polychlorinated biphenyl.

BOX-PCR patterns and ribotype profiles are available as supplementary material in IJSEM Online.

In the course of a long-term study of the diversity of *B. cepacia*-like bacteria, two additional strains (CCUG 28445 and CAC-124) exhibited striking similarities with LB400^T in SDS-PAGE whole-cell protein patterns. This prompted the polyphasic taxonomic study described below. Strain CCUG 28445 (=LMG 16224) was retrieved in 1991 from a human blood culture specimen, containing blood of a 31-year-old woman in Göteborg, Sweden. Strain CAC-124 (=LMG 21720=CCUG 46958) is a coffee plant rhizosphere isolate from Coatepec, Veracruz State, Mexico (Estrada-de los Santos *et al.*, 2001). All three strains were grown aerobically on tryptic soy agar plates (Oxoid) at 28 °C.

The almost-complete (1466 bases) 16S rRNA gene sequence of strain LB400^T was determined previously by P. C. K. Lau and H. Bergeron (unpublished data) and deposited in the EMBL sequence database under accession number U86373. This sequence was compared with those of other *Burkholderia* species using the BioNumerics software package version 3.0 (Applied Maths). A phylogenetic tree was constructed based on the neighbour-joining method, with bootstrap values based on 1000 resamplings (Fig. 1). As observed by Fain & Haddock (2001), strain LB400^T clustered within the '*Burkholderia graminis* group'. Besides *B. graminis*, this group contains *Burkholderia phenazinium*, *Burkholderia caribensis*, several recently described species and a number of partially characterized *Burkholderia* isolates (Fig. 1). Strain LB400^T showed the highest 16S rRNA gene sequence similarity (98·6 %) to the type strains of *B. graminis* and *Burkholderia terricola* and to *Burkholderia* sp. strain N3P2. The latter strain was isolated from soil contaminated with polycyclic aromatic hydrocarbons (Mueller *et al.*, 1997).

To clarify taxonomic relationships at the species level, DNA was prepared, the DNA base composition was analysed and DNA–DNA hybridization experiments were performed as described previously (Goris *et al.*, 2001). DNA–DNA hybridization experiments were done in microplates (Willems *et al.*, 2001) at a hybridization temperature of 55 °C. DNA–DNA reassociation experiments were performed with strains LB400^T, CCUG 28445, CAC-124 and with the type strains of the closest-related *Burkholderia* species, as evidenced by 16S rRNA gene sequence data (Table 1). DNA–DNA hybridization values between the former three strains were above 70 %, indicating a relationship at the species level (Stackebrandt *et al.*, 2002; Wayne *et al.*, 1987). In contrast, low to intermediate hybridization values (mean of reciprocal values $\leqslant$35 %) were obtained in hybridizations of strain LB400^T with type strains of other *Burkholderia* species. The G+C values (mol%) of *Burkholderia* type strains and DNA–DNA hybridization values (Table 1) were very similar to those reported previously (Achouak *et al.*, 1999; Coenye *et al.*, 2001; Goris *et al.*, 2002; Viallard *et al.*, 1998).

Whole-cell protein electrophoresis, repetitive-element PCR fingerprinting, ribotying and amplified rDNA restriction analysis (ARDRA) were performed to verify their discriminatory power for strains LB400^T, CCUG 28445, CAC-124 and their closest phylogenetic neighbours. Whole-cell protein analysis by SDS-PAGE was performed as described by Pot *et al.* (1994), with cultivation conditions and analysis

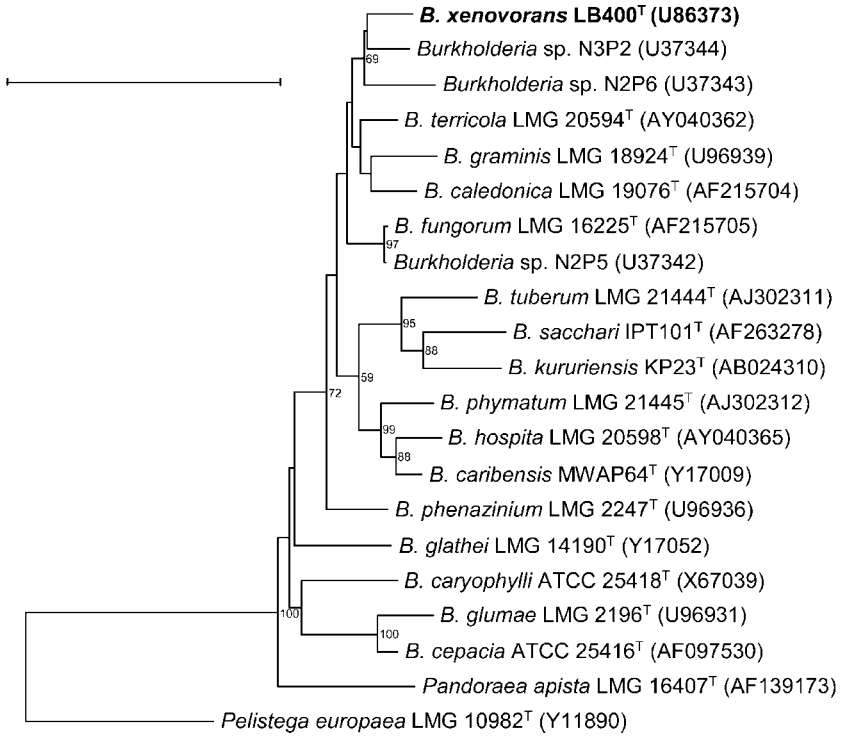

Fig. 1. The phylogenetic position of *B. xenovorans* sp. nov. as revealed by 16S rRNA gene sequence comparisons. The bar represents 5 % sequence divergence. Bootstrap values were calculated based on 1000 resamplings.

Table 1. DNA–DNA hybridization values and DNA nucleotide composition of *B. xenovorans* strains and closest relatives

Strain	G+C (mol%)	1	2	3	4	5	6	7	8	9	10
1. *B. xenovorans* LB400^T	62·6	100	76	83	12	33	29	13	23	22	15
2. *B. xenovorans* CCUG 28445	62·5	69	100	86							
3. *B. xenovorans* CAC-124	62·9	83	94	100							
4. *B. hospita* LMG 20598^T	62·0	18			100	14	16	55	11	15	12
5. *B. terricola* LMG 20594^T	63·9	37			15	100	34	15	25	28	19
6. *B. graminis* LMG 18924^T	62·8	41			22	39	100	21	25	48	24
7. *B. caribensis* LMG 18531^T	62·4	24			65	20	21	100	16	20	17
8. *B. fungorum* LMG 16225^T	61·8	39			15	36	26	14	100	24	24
9. *B. caledonica* LMG 19076^T	62·0	35			17	36	42	15	21	100	20
10. *B. phenazinium* LMG 2247^T	62·5	18			11	21	16	11	16	15	100

parameters as reported by Coenye *et al.* (2001). Repetitive-element PCR fingerprinting with the BOXA1R primer was performed using the protocol of Rademaker & De Bruijn (1997), with some minor modifications (Goris *et al.*, 2002). For ribotyping, a Southern blot of total *Eco*RI DNA digests was hybridized with a *Hin*dIII–*Hin*dIII 700 bp internal fragment from *Escherichia coli rrnB* 16S rRNA genes cloned in pKK3535 (Brosius *et al.*, 1981). ARDRA and analysis of *nifHDK* hybridization patterns were performed as described previously (Estrada-de los Santos *et al.*, 2001). Strains CCUG 28445, CAC-124 and LB400^T showed striking similarities in their whole-cell protein and ARDRA profiles (data not shown), ribotypes (autoradiogram provided as supplementary material in IJSEM Online) and BOX-PCR patterns (see supplementary material in IJSEM Online). In addition, each of these profile types was useful to distinguish the three strains from their nearest phylogenetic neighbours. The BOX-PCR patterns showed minor differences in DNA fragments at positions corresponding to 1200, 970 and 790 bp (see supplementary material in IJSEM Online). Likewise, the ARDRA profile obtained with enzyme *Hha*I from strain CCUG 28445 was slightly different from strains LB400^T and CAC-124 (data not shown). These differences indicated that the strains represent different genetic clones.

Classical phenotypic tests were performed as described previously (Vandamme *et al.*, 1993). API 20 NE and API ZYM (bioMérieux) microtest galleries were utilized according to the protocol supplied by the manufacturer. Several additional characteristics were determined. Prior to the acetylene reduction activity (ARA) assays (Mascarua-Esparza *et al.*, 1988), strains were grown in nitrogen-free semi-solid BMGM medium (Estrada-de los Santos *et al.*, 2001) for 3 days at 29 °C. The utilization of xenobiotic compounds was examined in a minimal medium (K1), which has been used previously to study degradation of PCBs by bacteria including strain LB400^T (Maltseva *et al.*, 1999; Zaitsev & Karasevich, 1985). Growth on naphthalene, toluene and phenol was assessed on K1 agar plates, while growth on benzoate was tested in liquid K1 medium. Growth on biphenyl was evaluated both on K1 agar plates with biphenyl provided as a vapour from solid particles in the lid of the Petri dish and in liquid K1 medium containing 5 mM biphenyl. Plates and liquid medium were incubated for up to 21 days at 30 °C. Degradation of PCBs was evaluated using the resting-cell assay as described by Bedard *et al.* (1986). Prior to the resting-cell assay, cells were grown in liquid medium containing 5 mM biphenyl, 5 mM benzoate or both. For fatty acid methyl ester (FAME) analysis, cells were grown for 24 h on tryptic soy agar plates (Oxoid) at 28 °C and FAMEs were extracted, prepared, separated and identified using the Microbial Identification System (Microbial ID) as reported previously (Vauterin *et al.*, 1991).

Phenotypic traits useful for the differentiation of strains LB400^T, CCUG 28445 and CAC-124 from closely related *Burkholderia* species are summarized in Table 2. Remarkably, the three strains differed from nearly all *Burkholderia* strains in their inability to assimilate L-arabinose. All three showed ARA and the presence of *nifHDK* genes was confirmed (data not shown). The sizes of hybridization bands

Table 2. Phenotypic tests useful for the differentiation of *B. xenovorans* sp. nov. from its closest relatives and from the type species of the genus *Burkholderia*, *B. cepacia*

Taxa: 1, *B. xenovorans*; 2, *B. hospita*; 3, *B. terricola* (data in columns 2 and 3 from Goris *et al.*, 2002); 4, *B. graminis*; 5, *B. caribensis*; 6, *B. fungorum*; 7, *B. caledonica* (columns 4–7, Coenye *et al.*, 2001); 8, *B. tuberum*; 9, *B. phymatum* (columns 8 and 9, Vandamme *et al.*, 2002); 10, *B. phenazinium* (Coenye *et al.*, 2001); 11, *B. cepacia* (nine *B. cepacia* strains were examined, including the type strain). All tests were performed under identical experimental conditions. +, Phenotypic trait present; −, phenotypic trait absent; V, strain-dependent reaction; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
L-Arabinose assimilation	−	+	+	+	+	V	+	+	+	+	+
Citrate assimilation	+	V	V	+	+	+	−	−	−	−	+
Sucrose assimilation	−	−	+	+	−	−	V	ND	ND	+	+
Growth in O–F with D-xylose	+	−	−	V	−	V	+	ND	ND	V	V
β-Galactosidase activity	−	+	+	−	+	−	−	+	+	−	V

corresponding to *nifHDK* genes were identical in the three strains. Previously, ARA assays revealed that strain CAC-124 was capable of fixing N_2 with benzoate as the single carbon source (Estrada-de los Santos *et al.*, 2001), and this ability with this carbon source was also observed for strains LB400^T and CCUG 28445 (data not shown). Furthermore, the three strains were able to grow on benzoate, with doubling times of approximately 2·5 h. None of the strains grew on naphthalene, toluene or phenol. LB400^T was the only strain that grew on biphenyl. PCB degradation was tested for cells grown on K1 medium containing both benzoate and biphenyl and was observed only for strain LB400^T. We can therefore conclude that, although strains CAC-124 and CCUG 28445 are highly related to strain LB400^T, they do not share the unique biodegrading capacities of this strain.

The data presented clearly demonstrate that strains LB400^T, CCUG 28445 and CAC-124 represent a single novel *Burkholderia* species, *Burkholderia xenovorans* sp. nov., which can be differentiated from its nearest phylogenetic neighbours by a range of phenotypic, chemotaxonomic and genotypic tests.

Description of *Burkholderia xenovorans* sp. nov.

Burkholderia xenovorans [xe.no′vo.rans. Gr. adj. *xenos* foreign; L. part. pres. *vorans* devouring, digesting; N.L. part. adj. *xenovorans* digesting foreign (xenobiotic) compounds].

Cells are Gram-negative, motile, non-sporulating, straight rods (1–2 μm long and 0·5 μm wide). The strains grow on nutrient agar at 28 °C, but not at 42 °C. No growth is observed on *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (cetrimide) or on 10 % (w/v) lactose. The strains do not grow in the presence of acetamide or in the presence of 3·0, 4·5 or 6·0 % (w/v) NaCl. Growth in the presence of 0·5 or 1·5 % (w/v) NaCl is strain-dependent (negative for the type strain). All strains grow on blood agar at 30 °C and on Drigalski agar, while growth on blood agar at 37 °C is strain-dependent (negative for the type strain). Haemolysis of horse blood is not observed. Liquefaction of gelatin or hydrolysis of aesculin is not observed. Tween 80 is hydrolysed. No production of acid or H_2S in triple-sugar-iron agar, no indole or pigment production. Acetylene is reduced. Nitrate and nitrite reduction is strain-dependent (negative for the type strain). In O–F medium, D-glucose, D-fructose and D-xylose are oxidized, but not maltose or adonitol. D-Glucose is not fermented. Assimilation of D-glucose, DL-norleucine, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, D-gluconate, caprate, adipate, L-malate, citrate, phenyl acetate, DL-lactate and DL-lactate with methionine, but not of trehalose, L-arabinose, maltose or sucrose. Assimilation of L-arginine is strain-dependent (positive for the type strain). Catalase, oxidase, alkaline and acid phosphatase, esterase C4, ester lipase C8, leucine arylamidase and phosphoamidase activity is present. Amylase, DNase, lipase C14, tryptophanase, lysine decarboxylase, ornithine decarboxylase, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α- and β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase and arginine dihydrolase activity are not detected. Activities of urease, valine arylamidase and cysteine arylamidase are strain-dependent (enzyme activities are, respectively, absent, present and present in the type strain). The whole-cell fatty acid profile comprises 14 : 0 (4·7 %), 14 : 0 3OH (8·5 %), 16 : 1ω7*c* (19·1 %), 16 : 0 (18·2 %), 17 : 0 cyclo (5·1 %), 16 : 1 2OH (2·2 %), 16 : 0 2OH (2·2 %), 16 : 0 3OH (7·1 %), 18 : 1ω7*c* (27·3 %), 18 : 0 (0·5 %), 19 : 0 cyclo ω8*c* (3·6 %) and 18 : 1 2OH (0·9 %) as major components [summed feature 2 (comprising 14 : 0 3OH, 16 : 1 iso I, an unidentified fatty acid with equivalent chain-length value of 10·928 or 12 : 0 ALDE or any combination of these fatty acids) and summed feature 3 (comprising 16 : 1ω7*c* or 15 iso 2OH or both) are mentioned above as 14 : 0 3OH and 16 : 1ω7*c*, respectively, as these fatty acid have been reported in *Burkholderia* species (Stead, 1992)]. The G+C content varies between 62·4 and 62·9 mol%.

The type strain (LB400^T = LMG 21463^T = CCUG 46959^T = NRRL B-18064^T) was isolated from a PCB-contaminated soil collected from a landfill in Moreau, New York. The G+C content of the type strain is 62·6 mol%.

Currently, whole-genome sequence analysis of strain LB400^T is in progress (http://genome.jgi-psf.org/draft_microbes/burfu/burfu.home.html).

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