Cupriavidus pampae sp. nov., a novel herbicide-degrading bacterium isolated from agricultural soil

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A bacterial consortium able to degrade the herbicide 4-(2,4-dichlorophenoxy) butyric acid (2,4-DB) was obtained from an agricultural soil of the Argentinean Humid Pampa region which has a history of long-term herbicide use. Four bacterial strains were isolated from the consortium and identified as members of the genera *Cupriavidus*, *Labrys* and *Pseudomonas*. A polyphasic systematic analysis was carried out on strain CPDB6^T, the member of the 2,4-DB-degrading consortium able to degrade 2,4-DB as a sole carbon and energy source. The Gram-negative, rod-shaped, motile, non-sporulating, non-fermenting bacterium was shown to belong to the genus *Cupriavidus* on the basis of 16S rRNA gene sequence analyses. Strain CPDB6^T did not reduce nitrate, which differentiated it from the type species of the genus, *Cupriavidus necator*; it did not grow in 0.5–4.5 % NaCl, although most species of *Cupriavidus* are able to grow at NaCl concentrations as high as 1.5 %; and it was able to deamidate acetamide, which differentiated it from all other species of *Cupriavidus*. DNA–DNA hybridization data revealed low levels of genomic DNA similarity (less than 30 %) between strain CPDB6^T and the type strains of *Cupriavidus* species with validly published names. The major cellular fatty acids detected were *cis*-9-hexadecenoic (16 : 1ω7*c*) and hexadecanoic (16 : 0) acids. On the basis of phenotypic and genotypic characterizations, strain CPDB6^T was recognized as a representative of a novel species within the genus *Cupriavidus*. The name *Cupriavidus pampae* sp. nov. is proposed, with strain CPDB6^T (=CCUG 55948^T=CCM-A-29:1289^T) as the type strain.

Phenoxy herbicides are employed widely in agricultural fields throughout the world for controlling broad leaf weeds in alfalfa, maize, peanut, soybean and other important crops and pastures. The extensive use of these pesticides causes some degree of concern due to contamination of the soil and non-target sites, such as groundwater and surface water courses (US EPA, 1992; Johannesen & Aamand, 2003; EWG, 2006). The potential threat to the environment and human health, through possible carcinogenicity (classified as 2b; IARC, 2003) (Gosselin *et al.*, 1984; Schop *et al.*, 1990; Zahm *et al.*, 1990), as well as gastrointestinal, liver, reproductive and developmental toxicities, is well documented (Stevens & Sumner, 1991; Walker & Keith, 1992; Weed Science Society of America, 1994; HSDB, 2005).

The Argentinean Humid Pampa (AHP) is one of the most productive agricultural areas in South America, and phenoxy herbicides, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-(2,4-dichlorophenoxy) butyric acid (2,4-DB), are used frequently (INDEC, 2002). The dissipation of 2,4-D and 2,4-DB in high-humic-matter-containing soils from the AHP has been studied in microcosm systems (Merini *et al.*, 2007; Cuadrado *et al.*,

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-DB, 4-(2,4-dichlorophenoxy) butyric acid; 2,4-DCP, 2,4-dichlorophenol; AHP, Argentinean Humid Pampa; CC, Colón control; CPDB, Colón pasture 2,4-DB-treated; MPN, most-probable number.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain CPDB6^T, *C. basilensis* CCUG 49340^T, *C. laharis* CCUG 53908^T, *C. oxalaticus* CCUG 2086^T and *C. pinatubonensis* CCUG 53907^T are FN430567, FN597608, FN597609, FN597610 and FN597611, respectively.

Cellular fatty acid data for strain CPDB6^T and type strains of the most closely related species of the genus *Cupriavidus* are available with the online version of this paper.

2008). When evaluating 2,4-DB biodegradation, a soil with a long-term history of herbicide use exhibited higher degradation rates in comparison with pristine soils, and a most-probable number (MPN) of 1×10^5 2,4-DB-degrading bacteria per gram of soil after 14 days in a microcosm with an application of 500 p.p.m. 2,4-DB (Cuadrado *et al.*, 2008). Microcosm soils, with and without histories of herbicide use, were sampled to isolate micro-organisms responsible for the degradation of 2,4-DB. Two bacterial consortia able to degrade 2,4-DB were obtained and further characterized. Phenotypic and genotypic analyses revealed bacteria belonging to the genera *Labrys* and *Pseudomonas* in the 2,4-DB-degrading consortium obtained from the microcosm of pristine soil and strains belonging to the genera *Labrys*, *Pseudomonas* and *Cupriavidus* in the consortium from microcosms of long-term herbicide-treated soil. Strain CPDB6^T was a member of the 2,4-DB-degrading consortium, enriched from the chronic herbicide-exposed soil. Strain CPDB6^T was identified as belonging to the genus *Cupriavidus* by 16S rRNA gene sequencing.

The genus *Ralstonia* was proposed in 1995 to accommodate the misplaced species *Burkholderia pickettii*, *Burkholderia solanacearum* and *Alcaligenes eutrophus* (Yabuuchi *et al.*, 1995). The species classified in the genus *Ralstonia* were divided into species of *Ralstonia* (*sensu stricto*) and species of the novel genus *Wautersia*, with *Wautersia eutropha* as the type species (Vaneechoutte *et al.*, 2004). Subsequently, Vandamme & Coenye (2004) reported the genus name *Wautersia* to be a later synonym of *Cupriavidus* and proposed that all species of *Wautersia* be reclassified as *Cupriavidus*, with *Cupriavidus necator* as the type species. The genus *Cupriavidus* currently comprises 11 species derived from diverse ecological niches (Coenye *et al.*, 2003). Strains of *Cupriavidus* species have been isolated from environmental and human clinical sources. *C. necator* (Makkar & Casida, 1987), *Cupriavidus oxalaticus* (Sahin *et al.*, 2000), *Cupriavidus basilensis* (Steinle *et al.*, 1999), *Cupriavidus campinensis* (Goris *et al.*, 2001), *Cupriavidus metallidurans* (Goris *et al.*, 2001), *Cupriavidus pinatubonensis* and *Cupriavidus laharis* (Sato *et al.*, 2006) were isolated initially from environmental sources and several strains of the species have been recognized as potential agents for bioremediation of soil and water contaminated with heavy metals or chlorinated organic compounds (Steinle *et al.*, 1998; Goris *et al.*, 2001; Vandamme & Coenye, 2004). Other species, such as *Cupriavidus gilardii*, *Cupriavidus pauculus*, *Cupriavidus respiraculi* and *Cupriavidus taiwanensis*, have been isolated from both human clinical samples and environmental sources (Coenye *et al.*, 1999; Vandamme *et al.*, 1999; Chen *et al.*, 2001; Wauters *et al.*, 2001).

This report presents a systematic polyphasic analysis of strain CPDB6^T, a member of a 2,4-DB-degrading consortium, isolated from a herbicide-contaminated soil of the AHP region. Strain CPDB6^T (=CCUG 55948^T=CCM-A-29:1289^T) represents a novel species of the genus *Cupriavidus*, for which the name *Cupriavidus pampae* sp. nov. is proposed.

Soil samples were collected from agricultural fields located in the proximity of Colón City in the AHP region (33° 52′ 23.33″ S 61° 08′ 10.61″ W). The land was planted with a mixed pasture (alfalfa and other pasture species) and the soil, designated Colón Pasture 2,4-DB-treated (CPDB) soil, had a 20-year history of treatment with phenoxy herbicides (2,4-DB ester; 100 %; 0.5 l ha^{-1}). The control soil, Colón control (CC), was sampled from a wild pasture and had never received herbicide application.

Microcosms of CPDB soil amended with 500 p.p.m. 2,4-DB exhibited an increase in 2,4-DB-degrading bacteria of five orders of magnitude in MPNs after 14 days (from non-detectable numbers at time 0 up to 2×10^5 MPN degrading bacteria per g dry soil) and 90 % degradation of the herbicide by day 28 (Cuadrado *et al.*, 2008). Samples (5 g) from microcosms of CPDB and CC soils were used as inocula for enrichment cultures in a mineral medium containing (l^{-1}): 0.5 g 2,4-DB as the sole source of carbon; 50 mg cycloheximide as inhibitor of fungal growth; 0.5 g K_2HPO_4; 0.5 g $(NH_4)_2SO_4$; 0.5 g $MgSO_4 . 7H_2O$; 15 mg $FeCl_3 . 6H_2O$; 11.4 mg $CaCl_2 . 2H_2O$; 0.16 mg $MnCl_2 . 4H_2O$; and 0.018 mg $ZnSO_4 . 7H_2O$. Cultures were incubated at 25 °C, with shaking at 200 r.p.m., and subcultured several times in the same medium, after confirmation of 2,4-DB dissipation by HPLC analysis.

A consortium obtained from soil with a history of herbicide application (CPDB) was able to grow in liquid culture with 2,4-DB as sole carbon source. Consortium CPDB exhibited 50 % removal of the herbicide (initial concentration of 350 p.p.m.) after 6 days. Consortium CPDB was characterized further by analysing growth kinetics and catabolic performance. Bacterial members of the CPDB consortium were isolated by subculture on 2,4-DB mineral medium and purification on R2A agar medium (Reasoner & Geldreich, 1985). Four bacterial strains were isolated from the CPDB soil consortium and characterized with respect to 2,4-DB and 2,4-D degradation in liquid culture, as well as phenotypically and genotypically. Strain CPDB6^T was selected for detailed characterization as a member of the CPDB consortium that was essential for herbicide degradation.

Strain CPDB6^T was cultured in liquid mineral medium with 2,4-DB or 2,4-D as the sole carbon source at a concentration range of 100–350 p.p.m. Cultivation was carried out in triplicate, at 30 °C, with shaking at 200 r.p.m., for 1 month, with controlled evaporation. Samples were taken periodically to measure bacterial biomass and the residual concentrations of the herbicides 2,4-DB or 2,4-D, as well as the appearance of the intermediate metabolite 2,4-dichlorophenol (2,4-DCP). Microbial biomass was estimated by spectrophotometric measurements of turbidity at 600 nm. Assessments of 2,4-DB and metabolites 2,4-D and 2,4-DCP were performed using previously optimized HPLC analyses (Merini *et al.*, 2008). Strain CPDB6^T, growing in mineral medium with 2,4-DB as the sole carbon source (350 p.p.m.), was able to degrade 22 % of the herbicide after 25 days (Fig. 1).

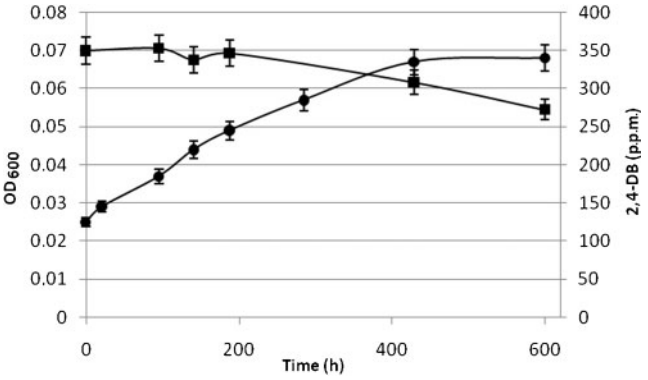

Fig. 1. Microbial growth and degradation of 2,4-DB herbicide by strain CPDB6^T in liquid culture. ●, OD at 600 nm; ■, 2,4-DB (p.p.m.). Bars, 1 SD (n=3).

After 4 days of incubation, colonies of strain CPDB6^T growing on R2A agar medium were 3–4 mm in diameter, non-pigmented, darker in the centre with a translucent and undulating margin. Strain CPDB6^T grew well on R2A agar medium at 10, 22 and 30 °C, and showed good growth on blood agar medium (Columbia agar base plus 5 % defibrinated horse blood) at 30 °C. Strain CPDB6^T showed weak growth on blood agar at 37 °C and was not able to grow at 42 °C. It stained Gram-negative, exhibited rod-shaped, motile, non-sporulating, non-fermenting cells and was positive for catalase and oxidase. Strain CPDB6^T assimilated adipate, gluconate, malate, lactate and lactate + methionine. It was weakly positive for glucose and citrate assimilation and it was urease-negative. Strain CPDB6^T did not reduce nitrate, which differentiated it from *C. necator*, the type species of the genus. It did not grow in 0.5–4.5 % NaCl, although most species of *Cupriavidus* are able to grow at NaCl concentrations as high as 1.5 %. The ability of strain CPDB6^T to deamidate acetamide differentiated it from all other species of *Cupriavidus*. The phenotypic profile of strain CPDB6^T, in comparison with the type strains of all species of *Cupriavidus*, was determined using the tests listed in the NFX phenotyping worksheet for Gram-negative, aerobic, non-fermenting bacilli (www.ccug.se/default.cfm?navID=160), including the API 20NE and API ZYM test panels according to the instructions of the manufacturer (bioMérieux). Prior to analysis, strains were cultivated on the same medium (blood agar) under the same cultivation conditions (aerobic, 37 °C) and tests were carried out using standardized protocols; quality controls were done according to the recommendations of the Swedish Board for Accreditation and Conformity Assessment (SWEDAC). Differential phenotypic features of strain CPDB6^T and the type strains of all species of *Cupriavidus* with validly published names are shown in Table 1; results of all tests can be seen under the entry for strain CPDB6^T (=CCUG 55948^T) at the CCUG website (www.ccug.se).

Genomic DNA from strain CPDB6^T, as well as from reference strains used for comparisons, was extracted from cell biomass collected from agar medium, suspended in 100 µl TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) and 15 µl (0.05 U ml^{-1}) lysostaphin (Sigma), and incubated at 37 °C for 30 min. Proteinase K (Sigma) [10 µl (1.0 U µl^{-1})] was added and the suspension was incubated at 56 °C for 30 min. After a subsequent 10 min incubation at 95 °C, the suspension was centrifuged at 18 000 ***g*** for 10 min. The supernatant containing the bacterial DNA was separated and stored at −20 °C until use. The 16S rRNA gene was amplified from genomic DNA by PCR using primers 16F27 and 23R458 (Hernández *et al.*, 2008), hybridizing at positions 9–27 and 458–473 of the 16S and 23S rRNA gene sequence positions (*Escherichia coli* gene sequence nucleotide numbering), respectively. PCRs were carried out in duplicate 25 µl reaction volumes; PCR products from duplicate reactions were combined, purified (QIAquick PCR purification kit; Qiagen) and sequenced directly using the methods (BigDye Terminator v3.1 Cycle Sequencing kit and the Prism 3100-*Avant* Genetic Analyzer; Applied Biosystems) and oligonucleotide primers described previously (Hauben *et al.*, 1997). The nearly complete 16S rRNA gene sequence of strain CPDB6^T was determined (1504 nt positions; estimated 98.2 % of the complete gene). The 16S rRNA gene sequence of CPDB6^T was aligned and compared with sequences of the type strains of all species of the genus *Cupriavidus* with validly published names using the CLUSTAL_X software package (Thompson *et al.*, 1997). Reference sequences used for comparative analyses were obtained from GenBank/EMBL (www.ebi.ac.uk/embl/), except for those of *C. basilensis* CCUG 49340^T, *C. laharis* CCUG 53908^T, *C. oxalaticus* CCUG 2086^T and *C. pinatubonensis* CCUG 53907^T, which were determined in this study. A uniform sequence length of 1320 nt positions (corresponding to positions 62–1385 of the *E. coli* 16S rRNA gene sequence) was applied for determinations of sequence similarities, and phylogenetic relationships were calculated using the PHYLIP v. 3.5c (Felsenstein, 1989). The sequences of the type strains of *Ralstonia pickettii* (the type species of *Ralstonia*) and *Ralstonia solanacearum* were included in the analyses as an outgroup. Sequence similarities between the 16S rRNA gene sequence of strain CPDB6^T and those of the type strains of *Cupriavidus* species ranged from 98.3 % (*Cupriavidus respiraculi* AU3313^T) to a low similarity of 96.6 % (*C. laharis* CCUG 53908^T) (Table 2). These sequence similarity values are within the range of 16S rRNA gene sequence similarities observed between the type strains of the species of *Cupriavidus* (98.9 % between *C. pauculus* and *C. metallidurans* to 95.7 % between *C. metallidurans* and *C. laharis*). A comparison of the 16S rRNA gene sequence of CPDB6^T with those of type strains of the species of *Cupriavidus* indicated that strain CPDB6^T probably represents a novel species of the genus. Reconstructions of estimated phylogenetic relationships (Felsenstein, 1981) based on 16S rRNA gene sequence comparisons indicated that CPDB6^T was most closely

Table 1. Phenotypic features differentiating strain CPDB6^T and the type strains of the species of the genus *Cupriavidus*

Strains: 1, strain CPDB6^T; 2, *C. necator* CCUG 52238^T; 3, *C. basilensis* CCUG 49340^T; 4, *C. campinensis* CCUG 44526^T; 5, *C. gilardii* CCUG 38401^T; 6, *C. laharis* CCUG 53908^T; 7, *C. metallidurans* CCUG 13724^T; 8, *C. oxalaticus* CCUG 2086^T; 9, *C. pauculus* CCUG 12507^T; 10, *C. pinatubonensis* CCUG 53907^T; 11, *C. respiraculi* CCUG 46809^T; 12, *C. taiwanensis* CCUG 44338^T. −, Negative; (+), weakly positive; +, positive; ND, not determined. Complete results of all phenotypic tests done on all strains can be found under the respective strain entry at www.ccug.se.

Phenotypic test	1	2	3	4	5	6	7	8	9	10	11	12
Reaction to penicillin*	R	S	I	R	R	S	R	I	R	S	R	R
Motility (at 30 °C)	+	−	−	−	ND	−	+	+	+	−	+	+
Reduction of:												
Nitrate	−	+	−	+	−	+	+	+	−	+	−	−
Nitrite	−	−	−	−	−	+	+	+	−	−	−	−
Acetamide deamidation	+	−	−	−	−	−	−	−	−	−	−	−
Growth at/in:												
42 °C, NA	−	−	−	(+)	+	−	(+)	+	+	−	+	+
0.5 % NaCl	−	+	+	+	(+)	+	+	+	+	−	+	+
1.5 % NaCl	−	(+)	(+)	(+)	(+)	−	−	+	+	−	(+)	(+)
Assimilation of:												
Adipate	+	−	+	+	−	+	+	+	+	+	+	(+)
Arginine	−	−	−	−	−	−	−	+	−	−	−	−
Caprate	−	(+)	+	+	(+)	−	+	+	+	−	+	+
Citrate	(+)	(+)	(+)	−	−	+	+	+	+	+	−	+
Norleucine	−	(+)	−	−	−	+	−	+	−	−	(+)	(+)
Phenylacetate	−	(+)	+	(+)	−	+	+	+	(+)	+	−	+
Acid production from:												
Adipate	+	−	+	+	−	+	+	+	+	+	+	(+)
L-Arabinose	−	(+)	−	−	−	−	−	−	−	−	−	−
D-Gluconate	+	+	+	+	+	+	+	+	−	+	+	−
D-Glucose	(+)	(+)	−	−	−	−	−	−	−	−	(+)	−
Phenylacetate	−	+	+	(+)	−	+	(+)	+	−	+	−	+
Enzyme activity:												
Acid phosphatase	−	+	+	+	−	+	+	+	+	+	−	+
Alkaline phosphatase	−	+	+	+	(+)	+	+	+	+	+	−	+
Cysteine arylamidase	−	−	−	−	−	−	−	−	+	−	−	−
Ester lipase (C8)	−	+	−	(+)	(+)	−	+	+	+	(+)	−	(+)
Esterase (C4)	−	+	−	(+)	(+)	(+)	+	+	+	+	(+)	(+)
Lipase (C14)	−	−	−	−	−	−	−	−	+	−	−	−
Phosphoamidase	−	+	+	−	−	+	(+)	−	+	+	−	+
Urease	−	−	−	−	−	−	−	−	+	−	−	−

*Penicillin sensitivity: R, <10 mm zone; I, 11–20 mm zone; S, >20 mm zone.

related to *C. gilardii*, *C. pauculus*, *C. respiraculi* and *C. metallidurans* (Fig. 2). Alternative analyses of the sequence data were carried out using different algorithms (DNA neighbour-joining, DNA parsimony and DNA Fitch–Margoliash) and all analyses supported the phylogenetic position of CPDB6^T within the genus *Cupriavidus*. The reconstructions of phylogenetic relationships by all algorithms used indicated that the genus *Cupriavidus* comprises at least three phylogenetic 'lineages': 'lineage' 1, including species related to *C. necator* (the type species of the genus); 'lineage' 2, including CPDB6^T and other species related to *C. metallidurans*; and 'lineage' 3, including *C. campinensis* (Fig. 2). Comparisons of the 16S rRNA gene sequence of CPDB6^T with sequences deposited in the public databases using the FASTA-nucleotide matching tool (Pearson & Lipman, 1988) further suggested that strain CPDB6^T represents a bacterium that may not have been recognized or isolated previously. The most similar 16S rRNA gene sequences of organisms included within the GenBank/EMBL Prokaryote and the GenBank/EMBL Environmental databases comprised sequences from bacterial isolates and sequences from environmental samples (i.e. cloned DNA), respectively, and showed similarities of less than 99.0 % to that of strain CPDB6^T.

As the similarities between the 16S rRNA gene sequences of strain CPDB6^T and the type strains of most species of *Cupriavidus* were greater than 97.0 % (Stackebrandt & Goebel, 1994; Table 2), DNA–DNA hybridizations were performed in duplicate using a non-radioactive method, as

Table 2. 16S rRNA gene sequence similarities and genomic DNA–DNA similarities between *Cupriavidus* sp. CPDB6^T and the type strains of species of *Cupriavidus*

Pooled standard deviations of all hybridization experiments were between 0.4 and 1.8.

Cupriavidus spp. strains	16S rRNA gene sequence similarities with CPDB6^T	DNA–DNA hybridization values with:	
		CPDB6^T	*C. metallidurans* CCUG 13724^T
1. CPDB6^T CCUG 55948^T	100	100	18
2. *C. necator* CCUG 52238^T	97.8	13	12
3. *C. basilensis* CCUG 49340^T	97.3	15	20
4. *C. campinensis* CCUG 44526^T	97.6	29	41
5. *C. gilardii* CCUG 38401^T	98.1	10	11
6. *C. laharis* CCUG 53908^T	96.6	16	23
7. *C. metallidurans* CCUG 13724^T	97.7	15	100
8. *C. oxalaticus* CCUG 2086^T	97.8	20	25
9. *C. pauculus* CCUG 12507^T	98.2	14	24
10. *C. pinatubonensis* CCUG 53907^T	97.2	15	31
11. *C. respiraculi* CCUG 46809^T	98.3	12	14
12. *C. taiwanensis* CCUG 44338^T	97.5	15	27

described previously in detail (Urdiain *et al.*, 2008), with genomic DNAs isolated and purified according to the method of Marmur (1961). The DNAs of strain CPDB6^T and *C. metallidurans* CCUG 13724^T were labelled with DIG-11-dUTP and Biotin-16-dUTP using a Nick Translation kit (Boehringer). Each labelled DNA was

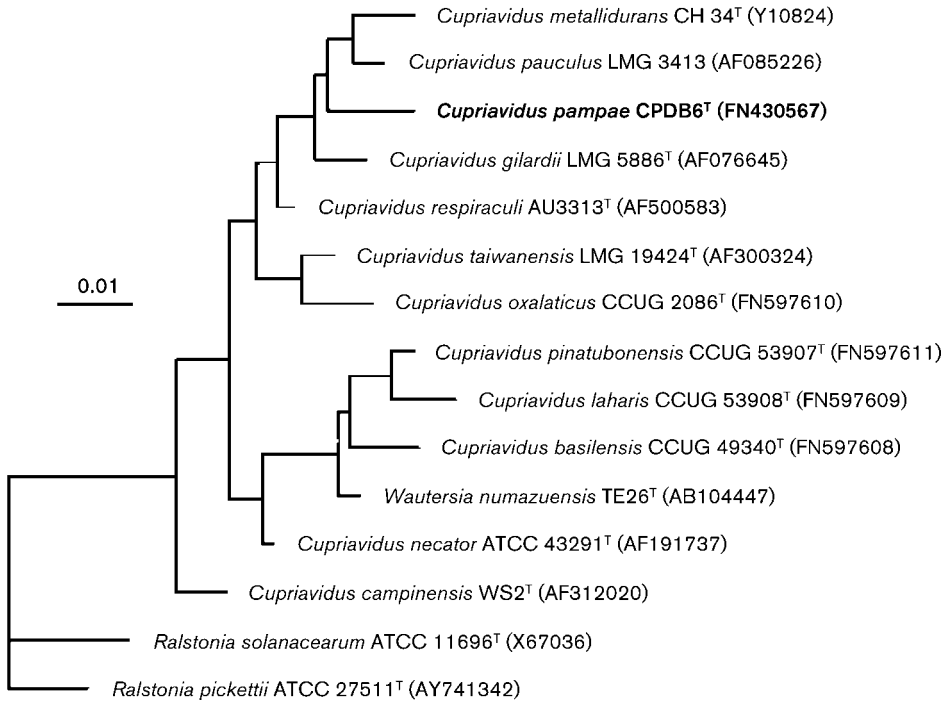

Fig. 2. Dendrogram of estimated phylogenetic relationships between strain *Cupriavidus* sp. CPDB6^T and the species of *Cupriavidus* based on 16S rRNA gene sequence comparisons of 1320 aligned nucleotide positions. The dendrogram was constructed using the DNA maximum-likelihood method (Felsenstein, 1981). Tree topology was visualized using the program TreeView (Page, 1996). Bootstrap values greater than 500, based on 1000 replications, are indicated at branching nodes. Bar, 0.01 nt substitutions per site.

hybridized against itself as well as against the DNAs of the type strains of all species of *Cupriavidus*. The genomic DNA–DNA hybridization values between strain CPDB6^T and all *Cupriavidus* species were, in all cases, less than 30 % (Table 2), thus confirming that strain CPDB6^T could be delineated as a novel species of *Cupriavidus*.

Cellular fatty acid methyl ester analyses were performed using GC and a standardized protocol similar to that of the MIDI Sherlock MIS system (http://www.ccug.se/pages/CFA_method_2008.pdf). Prior to cellular fatty acid extraction, strains were grown and harvested under the same conditions using blood agar as the cultivation medium. Cellular fatty acids were identified and quantified and the relative amount of each fatty acid in a strain was expressed as a percentage of the total fatty acids in the profile of that strain and presented in comparison with the type strain of the type species of the genus *Cupriavidus* (*C. necator* CCUG 52238^T) and with the type strains of the most closely related species (*C. respiraculi* CCUG 46809^T, *C. pauculus* CCUG 12507^T and *C. gilardii* CCUG 38401^T) (Supplementary Table S1, available in IJSEM Online). The major cellular fatty acids detected in strain CPDB6^T were hexadecanoic (16 : 0) and *cis*-9-hexadecenoic (16 : 1ω7*c*) acids, comprising 25 and 30 %, respectively, of the total summed cellular fatty acids, as indicated in Supplementary Table S1. The relative amount of tetradecanoic acid (14 : 0) could be used to differentiate strain CPDB6^T from related *Cupriavidus* species. Strain CPDB6^T was also distinguishable from *C. respiraculi*, *C. gilardii* and *C. pauculus*, the closest related species according to 16S rRNA gene sequence comparative analyses, in the relative amounts of summed *cis*-11-octadecenoic acid (18 : 1ω7*c*/9*t*/12*t*) (*C. respiraculi* and *C. gilardii*) and the amounts of *cis*-9-hexadecenoic acid (16 : 1ω7*c*) and Δ-*cis*-9,10-methylene-hexadecanoic acid (17 : 0 cyclo) (*C. pauculus*). The presence of the minor fatty acid iso-C16 : 0 in strain CPDB6^T was an additional differential characteristic.

Based on genotypic and phenotypic analyses, it is concluded that strain CPDB6^T represents a novel species within the genus *Cupriavidus*. The name *Cupriavidus pampae* sp. nov. is proposed.

Description of *Cupriavidus pampae* sp. nov.

Cupriavidus pampae [pam′pa.e. N.L. n. *pampa* (from Quechuan noun *pampa*) pampa; N.L. gen. n. *pampae* of pampa, the grassland plains of temperate South America, especially Argentina, where the soil samples were obtained from which the type strain of the species was isolated].

Colonies on R2A agar are 3–4 mm in diameter, non-pigmented, circular and darker in the centre, with a translucent and undulating margin. Cells are aerobic, Gram-negative, non-fermenting, non-sporulating, motile rods. Growth on R2A agar medium occurs at 10–30 °C. On blood agar medium, good growth is observed at 30 °C; weak growth is observed at 37 °C. Reactions for oxidase and catalase are positive, whereas urease, esterase (C4), lipase, acid phosphatase, β-galactosidase and β-glucosidase are negative. No nitrate reduction, indole formation, glucose fermentation or aesculin and arginine dihydrolase activities are observed. D-Glucose, D-gluconate, caprate, adipate, L-malate, citrate and lactate are assimilated. Trehalose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, sucrose and phenylacetate are not assimilated.

The type strain is CPDB6^T (=CCUG 55948^T=CCM-A-29:1289^T), isolated from soils chronically exposed to phenoxy herbicides in the AHP region, Argentina.

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Supplementary Table S1. Profiles of per cent cell fatty acids (CFAs) of *Cupriavidus* sp. CPDB6^T (CCUG 55948^T) and the type strains of the most closely related species of the genus *Cupriavidus*

Strains were cultivated on blood agar medium, aerobic, 30 °C. CFAs were not included in the profiles if they did not comprise more than 1% of total CFAs for any species. Summed features: $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$; and $C_{18:1}$ ω7c and/or $C_{18:1}$ ω9t and/or $C_{18:1}$ ω12t.

Fatty acid	***Cupriavidus* sp. CPDB6^T**	***C. necator* CCUG 52238^T**	***C. respiraculi* CCUG 46809^T**	***C. pauculus* CCUG 12507^T**	***C. gilardii* CCUG 38401^T**
C14:0	8.0	2.4	4.8	5.1	5.2
C14:0 2OH	3.2	2.2	0.3	–	–
C14:0 3OH/ 6:1 ISO I	8.3	6.8	6.2	6.1	6.8
C16:0	25.0	28.0	22.5	21.9	21.8
C16:0 2OH	–	–	1.0	2.6	2.2
C16:0 ISO	1.9	–	0.5	2.2	–
C16:1 2OH	–	–	–	2.2	–
C16:1 ω7c	30.3	35.4	33.6	20.3	27.3
C17:0 CYCLO	10.4	3.1	6.8	16.9	8.4
C18:1 ω7c/9t/12t	12.8	21.0	21.4	16.1	24.6
C18:1 2OH	–	0.7	1.6	1.4	–
C19:0 CYCLO ω8c	–	–	–	2.9	1.7
Unidentified	–	–	0.6	1.1	0.4
Total summed CFAs	100	100	100	100	100