Burkholderia oxyphila sp. nov., a bacterium isolated from acidic forest soil that catabolizes (+)-catechin and its putative aromatic derivatives

Yuichiro Otsuka,¹ Yuki Muramatsu,² Yasuyoshi Nakagawa,² Motoki Matsuda,³ Masaya Nakamura⁴ and Hitoshi Murata⁴

Correspondence

Hitoshi Murata

murmur@ffpri.affrc.go.jp

¹Department of Biomass Chemistry, Forestry and Forest Products Research Institute, Matsunosato 1, Tsukuba, Ibaraki 305-8687, Japan

²Resource Collection Division, NITE Biological Resource Centre Department of Biotechnology, National Institute of Technology and Evaluation, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba 292-0818, Japan

³Toyama Prefectural University, Kurokawa 5180, Imizu, Toyama 939-0398, Japan

⁴Department of Applied Microbiology and Mushroom Sciences, Forestry and Forest Products Research Institute, Matsunosato 1, Tsukuba, Ibaraki 305-8687, Japan

A novel bacterium, designated strain OX-01^T, was isolated from acidic soil, taxonomically investigated and identified as an agent that catabolizes (+)-catechin into taxifolin. Strain OX-01^T is a Gram-reaction-negative, aerobic, non-sporulating, non-motile and rod-shaped bacterium. 16S rRNA gene sequence analysis identified this strain as a member of the genus *Burkholderia* and occupying a phylogenetic position closest to, but clearly distinct from, *Burkholderia sacchari*. Strain OX-01^T does not have any *nif* genes, which are required for N₂-fixation, in its genome, a feature that is similar to *B. sacchari*, which lacks *nifH*, but is distinct from the N₂-fixing features of many other phylogenetically related taxa, such as *Burkholderia ferrariae*, *B. heleia*, *B. mimosarum*, *B. nodosa*, *B. silvatlantica*, *B. tropica* and *B. unamae*. Strain OX-01^T has the following chemotaxonomic characteristics: the major ubiquinone is Q-8, the DNA G + C content is 64 mol% and the major fatty acids are C_{16:0}, C_{17:0} cyclo and C_{18:1ω7c}. It also has a unique profile of carbohydrate utilization among other species of the genus *Burkholderia*. The strain cannot assimilate many pentoses, hexoses and oligosaccharides, whereas it can catabolize (+)-catechin and its putative aromatic derivatives, such as 4-hydroxy-3-methoxycinnamic acid, protocatechuic acid, *p*-hydroxybenzoic acid, *trans-p*-coumaric acid and vanillic acid. Based on its morphological, physiological and chemotaxonomic characteristics, together with DNA–DNA relatedness values and 16S rRNA gene sequence comparison data, we show that strain OX-01^T represents a novel species of the genus *Burkholderia*, for which the name *Burkholderia oxyphila* sp. nov. is proposed. The type strain is OX-01^T (=NBRC 105797^T = DSM 22550^T).

The genus *Burkholderia* comprises more than 50 species, many of which exist in association with soils, aquatic zones, fungi, plants and animals (Vandamme *et al.*, 2007) and confer great influence on terrestrial ecosystems and human society. Many of them are decomposers of biomass, biodegraders of toxic chemicals, pathogens of a variety of higher organisms, producers of anti-microbial compounds, endosymbionts and quasi-organelles of fungi, or plant growth promoters (Aizawa *et al.*, 2010; Aspray *et al.*, 2006; Coenye & Vandamme, 2003; Estrada-de los Santos *et al.*, 2001; Izumi *et al.*, 2006; Lehning *et al.*, 1997; Levy *et al.*,

2003; Lim *et al.*, 2003; Martínez-Aguilar *et al.*, 2008; Minerdi *et al.*, 2001; Seigle-Murandi *et al.*, 1996; Vandamme *et al.*, 2007).

We have recently isolated a strain, designated OX-01^T, that transforms (+)-catechin, a component of tannins, into taxifolin through a two-step oxidation process under the acidic conditions of a forest soil in Japan. This feature could be exploited for the biotransformation of components found in wood not suitable for lumber, especially in bark that contains a large amount of tannin, into raw materials, such as 2-pyrone-4,6-dicarboxylic acid (Otsuka *et al.*, 2006). In the present study, we carried out polyphasic taxonomic analysis on strain OX-01^T and found that this

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain OX-01^T is AB488693.

bacterium represents a novel species that is closely related to, but is independent of, *Burkholderia sacchari*.

Unless stated otherwise, strain OX-01^T was stored on a *Pseudomonas* agar F plate (Difco) at 4 °C or in 10% skimmed milk solution at -80 °C and was cultured aerobically in either LB broth or in minimal salts medium containing 0.2% glucose and 55 mM potassium phosphate (pH 6.8) at 28 °C for 24 h prior to the experiments (Miller, 1972). To confirm that the culture maintained its (+)-catechin-decomposing capacity, colonies were plated onto W-medium containing 0.2% (+)-catechin, pH 3.5 (Yano & Nishi, 1980). After culture and observation under a phase-contrast microscope, it was established that strain OX-01^T is a Gram-reaction-negative, non-sporulating, non-motile, rod-shaped bacterium 0.8–0.9 µm wide and 1.5–2.0 µm long. It grew both aerobically and anaerobically on LB and minimal glucose media and formed smooth, butter-like yellowish-white colonies on the LB plate. The optimal temperature for growth was 30 °C and the maximum temperature for growth was 40 °C. No growth was noted at temperatures above 42 °C or below 15 °C. Strain OX-01^T grew at pH 3.5–8.0.

The 16S rRNA gene was amplified from genomic DNA of strain OX-01^T by PCR using TaKaRa LA *Taq* polymerase (Takara) and the 16S rRNA gene universal primers 27f and 1492r (Johnston, 1994). The nucleotide sequence of the 1493 bp fragment was analysed with a BigDye terminator FS core kit version 3.1 and an ABI3100 autosequencer (Applied Biosystems). Primers used for sequencing are as follows: M13-21 and M13-R of the vector pCR2.1 and 5'-TGATCCAGCAATGCCGCGT-3' and 5'-GCCAATACTG-ACGCTCATG-3' targeting positions 356–374 and 712–730 of the insert DNA, respectively. The internal 1455 bp sequence was aligned with the corresponding sequences of species of the genus *Burkholderia* that had the highest sequence similarity to strain OX-01^T in the BLAST search. Sequences obtained were aligned with those of members of the genus *Burkholderia* by using CLUSTAL_X (Thompson *et al.*, 1997) and then modified manually by referring to the

16S rRNA secondary structure of *Escherichia coli* (Gutell *et al.*, 1994) and using the BioEdit sequence alignment editor (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Phylogenetic trees were reconstructed by the neighbour-joining method (Saitou & Nei, 1987) with calculated K_{nuc} values (Kimura, 1980). The topology of the tree was evaluated by bootstrap analysis (Felsenstein, 1985) with 1000 replicates. The phylogenetic tree based on the neighbour-joining method was consistent with that based on the maximum-parsimony method when carried out separately and showed that strain OX-01^T represents a novel species of the genus *Burkholderia* (Fig. 1).

To explore the biochemical properties of OX-01^T, the strain was precultured in LB medium at 30 °C for 24 h and examined using the API 20NE, API 50CH and API ZME systems by following the manufacturer's instructions (bioMérieux). Differential analysis between strain OX-01^T and phylogenetically related taxa was carried out three times; representative data are shown in Table 1.

Strain OX-01^T produces catalase but not oxidase. The following tests are positive: acid phosphatase, alkaline phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, urease and the assimilation of D-arabitol, adipic acid, *N*-acetyl-D-glucosamine, *n*-capric acid, fructose, gluconate, glucose, 2-ketogluconate, DL-malate, D-mannitol, D-mannose, phenylacetate, potassium gluconate, sodium citrate, sorbose and sorbitol. The following tests are negative: the production of acid and gas from glucose, oxidation and fermentation of glucose, indole production, nitrate reduction, hydrolysis of gelatin and aesculin, growth on MacConkey agar, *N*-acetyl-β-glucosaminidase, arginine dihydrolase, chymotrypsin, cystine arylamidase, cytochrome oxidase, C14 lipase, α-fucosidase, α- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, α-mannosidase, trypsin, valine arylamidase and the assimilation of adonitol, amygdalin, DL-arabinose, L-arabitol, arbutin, cellobiose, dulcitol, erythritol, aesculin, DL-fucose, galactose, gentiobiose, glycerol, glycogen, *myo*-inositol,

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences of strain OX-01^T and related taxa. The tree, based on the nearly complete sequence of the 16S rRNA gene, was created by using the neighbour-joining method. Numbers indicate percentages of bootstrap samplings, derived from 1000 replications. Bar, 0.01 substitutions per nucleotide site.

Table 1. Differential characteristics of strain OX-01^T and type strains of phylogenetically related species of the genus *Burkholderia*

Taxa: 1, strain OX-01^T; 2, *B. sacchari* DSM 17165^T (data from Brämer *et al.*, 2001); 3, *B. unamae* DSM 17197^T (Caballero-Mellado *et al.*, 2004); 4, *B. tropica* DSM 15359^T (Reis *et al.*, 2004); 5, *B. ferrariae* NBRC 106233^T (Valverde *et al.*, 2006); 6, *B. silvatlantica* NBRC 106337^T (Perin *et al.*, 2006); 7, *B. mimosarum* NBRC 106338^T (Chen *et al.*, 2006); 8, *B. nodosa* BCRC 17575^T (Chen *et al.*, 2007); 9, *B. heleiia* NBRC 101817^T (Aizawa *et al.*, 2010). +, Good growth; –, no growth; v, variable; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9
Assimilation of:									
Adonitol	–	+	+	+	+	+	–	+	+
D-Arabinose	–	+	+	+	–	+	+	+	+
L-Arabinose	–	+	+	+	+	+	+	+	+
Cellobiose	–	–	+	+	+	+	–	+	–
Dulcitol	–	–	–	–	+	–	–	–	–
D-Fucose	–	+	–	+	–	–	+	+	+
L-Fucose	–	+	+	+	+	+	+	+	+
Erythritol	–	–	–	–	–	+	–	+	+
Galactose	–	+	+	+	+	+	+	+	+
Gentiobiose	–	–	–	+	–	–	–	–	–
Glycerol	–	+	+	+	+	+	+	+	+
Inositol	+	+	+	+	+	+	+	+	+
Lactose	–	–	–	v	–	–	–	–	–
D-Lyxose	–	+	–	v	–	–	–	+	–
Raffinose	–	+	–	–	–	–	–	–	–
Rhamnose	–	–	+	+	–	+	–	+	+
Ribose	–	–	–	+	+	+	–	+	+
Salicin	–	–	–	v	–	–	–	–	–
Sorbose	+	–	–	–	–	–	–	–	–
Sorbitol	+	+	+	+	–	+	+	+	+
Sucrose	–	+	–	–	–	+	–	–	–
D-Tagatose	–	–	–	–	+	–	–	–	–
Trehalose	–	–	+	v	+	–	–	+	+
D-Xylose	–	+	+	+	+	+	–	+	+
Major fatty acid content (%)									
C _{14:0}	4.0	1.1	4.3	4.2	5.8	4.7	6.1	3.4	4.4
C _{16:0}	18.2	15.4	16.0	19.7	15.4	22.7	19.7	20.3	21.2
C _{16:0} 3-OH	4.0	3.5	3.6	4.1	5.5	4.0	ND	4.3	4.0
C _{17:0} cyclo	18.4	11.6	7.6	15.9	9.3	21.3	13.5	2.9	12.4
C _{18:1} ω7c	34.6	41.7	38.1	29.7	28.1	18.8	33.3	37.6	29.5
C _{19:0} cyclo ω8c	2.7	1.2	3.2	8.0	2.4	15.0	2.0	0.6	3.7
Summed feature 2*	4.8	4.3	4.3	4.7	7.0	4.7	5.5	5.3	4.7
Summed feature 3†	6.4	14.7	16.0	7.3	15.4	3.3	12.6	20.8	11.8

*Comprises C_{14:0} 3-OH and/or iso-C_{16:1} I.

†Comprises iso-C_{15:0} 2-OH and/or C_{16:1}ω7c.

inulin, 5-ketogluconate, lactose, D-lyxose, maltose, methyl α-D-glucoside, methyl α-D-mannoside, melezitose, melibiose, methyl β-D-xyloside, raffinose, rhamnose, ribose, salicin, starch, sucrose, D-tagatose, trehalose, turanose, xylitol and DL-xylose.

Note that OX-01^T had a unique profile of carbon source assimilation among species of the genus *Burkholderia*. OX-01^T could not assimilate many pentoses, hexoses or oligosaccharides. On the other hand, this strain could catabolize various aromatic compounds as sole carbon sources, such as (+)-catechin, vanillic acid, protocatechuic

acid, *p*-hydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid (ferulic acid) and *trans*-*p*-coumaric acid.

Cellular fatty acids of all strains were extracted and analysed using the Sherlock Microbial Identification System version 5.0 (MIDI system) and standard MIS Library TSBA40. Briefly, samples of bacteria were cultured on LB agar at 25 °C for 24 h and subjected to GC analysis of fatty acid methyl esters by following the protocol recommended by the manufacturer. Differential fatty acid profiles of OX-01^T and phylogenetically related species of the genus *Burkholderia* are given in Table 1; the

profile of strain OX-01^T, including components comprising >1 % of the total, is as follows: C_{14:0} (4.0 %), C_{16:0} (18.2 %), C_{16:0} 2-OH (2.5 %), C_{16:0} 3-OH (4.0 %), C_{16:1} 2-OH (2.0 %), C_{17:0} cyclo (18.4 %), C_{18:1} ω7c (34.6 %), C_{19:0} cyclo ω8c (2.7 %), C_{18:1} 2-OH (1.6 %), summed feature 2 (C_{14:0} 3-OH/iso-C_{16:1} I; 4.8 %) and summed feature 3 (iso-C_{15:0} 2-OH/C_{16:1} ω7c; 6.4 %). While the fatty acid composition of strain OX-01^T is similar to that of other species of the genus *Burkholderia*, the entire profile does not match that of any bacterial species in the database.

The quinone system was analysed by using the standard protocol of Nishijima *et al.* (1997). Briefly, ubiquinones were extracted with chloroform/methanol (2:1) from dried bacterial cells that had been aerobically cultured in trypticase soy broth at 30 °C for 48 h. The extracts, dissolved in acetone, were analysed by using the Waters 600 HPLC system equipped with an Inersil ODS-3 (4.6 × 150 mm; GL Science) column. The analysis showed that the major ubiquinone in strain OX-01^T is Q-8.

Total DNA was isolated from strain OX-01^T, *B. sacchari* DSM 17165^T (Brämer *et al.*, 2001), *Burkholderia unamae* DSM 17197^T (Caballero-Mellado *et al.*, 2004), *Burkholderia mimosarum* NBRC 106338^T (Chen *et al.*, 2006), *Burkholderia tropica* DSM 15359^T (Reis *et al.*, 2004), *Burkholderia heleaia* NBRC 101817^T (Aizawa *et al.*, 2010), *Burkholderia ferrariae* NBRC 106233^T (Valverde *et al.*, 2006), *Burkholderia silvatlantica* NBRC 106337^T (Perin *et al.*, 2006) and *Burkholderia nodosa* BCRC 17575^T (Chen *et al.*, 2007) by using the standard protocol of Staskawicz *et al.* (1984) for pseudomonads. Samples were purified by cesium chloride/ethidium bromide density-gradient ultracentrifugation. To remove ethidium bromide and cesium chloride, the DNA band isolated was dissolved in 2-propanol saturated with NaCl and dialysed repeatedly against TE buffer. The DNA G+C content of strain OX-01^T, based on a standard method described by Takagi *et al.* (1993), was estimated to be 64 mol%. The 16S rRNA gene sequence similarity between OX-01^T and *B. sacchari* DSM 17165^T, *B. unamae* DSM 17197^T, *B. mimosarum* NBRC 106338^T, *B. tropica* DSM 15359^T, *B. heleaia* NBRC 101817^T, *B. ferrariae* NBRC 106233^T, *B. silvatlantica* NBRC 106337^T and *B. nodosa* BCRC 17575^T was 98.8, 98.0, 97.7, 97.5, 97.5, 97.3, 97.0 and 96.8 %, respectively.

DNA–DNA reassociation levels between strain OX-01^T and type strains of phylogenetically related species of the genus *Burkholderia* were determined by hybridization in the presence of 50 % formamide at 50 °C followed by quantification of hybridization signals with a streptavidin-biotinylated peroxidase detection system (Ezaki *et al.*, 1989). DNA–DNA relatedness values between strain OX-01^T and proximal taxa *B. sacchari* DSM 17165^T, *B. unamae* DSM 17197^T, *B. mimosarum* NBRC 106338^T, *B. tropica* DSM 15359^T, *B. heleaia* NBRC 101817^T, *B. ferrariae* NBRC 106233^T, *B. silvatlantica* NBRC 106337^T and *B. nodosa* BCRC 17575^T were 46, 25, 21, 22, 13, 27, 37 and

24 %, respectively, indicating that strain OX-01^T does not belong to any known species of *Burkholderia*.

The whole genome of strain OX-01^T was sequenced by using the Illumina GAII 50 bp pairwise analysis system and data were assembled and annotated using Illumina Pipeline software version 1.3. Genomic information of *Burkholderia phytofirmans* PsJN^T, *Burkholderia xenovorans* LB400^T and *Burkholderia multivorans* ATCC 17616^T, the species that showed the closest matches in BLAST searches (Otsuka *et al.*, unpublished), was used as a reference. The total read length was ~510 Mbp, which should cover the genome of strain OX-01^T over 50 times if its genome size is equal to the average genome size of other species of the genus *Burkholderia*, especially that of closely related species (6.5–8.6 Mbp; Martínez-Aguilar *et al.*, 2008). BLAST analysis showed that the genome of strain OX-01^T does not contain *nifH*, a gene that is required for N₂-fixation. This feature is shared by *B. sacchari* but is distinct from the phylogenetically proximal taxa *B. ferrariae*, *B. heleaia*, *B. mimosarum*, *B. nodosa*, *B. silvatlantica*, *B. tropica* and *B. unamae* (Aizawa *et al.*, 2010; Chen *et al.*, 2006, 2007; Martínez-Aguilar *et al.*, 2008). In fact, strain OX-01^T does not have any *nif* genes in its genome.

These genomic properties underline the results of the biochemical, physiological and chemotaxonomic tests described above, which, when taken together, suggest that strain OX-01^T represents a novel species of the genus *Burkholderia*, for which the name *Burkholderia oxyphila* sp. nov. is proposed.

Description of *Burkholderia oxyphila* sp. nov.

Burkholderia oxyphila (ox.y.phi'la. Gr. adj. *oxys* sharp, acidic; Gr. fem. adj. *phila* loving; N.L. fem. adj. *oxyphila* loving an acidic habitat).

Cells are Gram-reaction-negative, both aerobic and anaerobic, non-sporulating, non-motile and rod-shaped, 0.8–0.9 µm wide and 1.5–2.0 µm long. Colonies are smooth, butter-like and yellowish-white when grown on LB medium. Growth occurs at 15–40 °C (optimum 30 °C) in LB broth. The pH range for sustaining growth is pH 3.5–8.0. Catabolizes the following non-humic aromatics: (+)-catechin (pH 3.5), vanillic acid (pH 6.9), protocatechuic acid (pH 6.9), *p*-hydroxybenzoic acid (pH 6.9), 4-hydroxy-3-methoxycinnamic acid (pH 6.9) and *trans-p*-coumaric acid (pH 6.9). Catalase is produced but oxidase is not. Tests for the following attributes are positive: alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and urease activities and assimilation of glucose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, potassium gluconate, *n*-capric acid, adipic acid, DL-malate, sodium citrate, phenylacetate, fructose, sorbose, sorbitol, D-arabitol, gluconate and 2-ketogluconate. Tests for the following attributes are negative: production of acid and gas from glucose, oxidation and fermentation of glucose, indole production, arginine dihydrolase, nitrate reduction,

hydrolysis of gelatin and aesculin, β -galactosidase, growth on MacConkey agar, cytochrome oxidase and assimilation of DL-arabinose, maltose, glycerol, erythritol, ribose, DL-xylose, adonitol, methyl β -D-xyloside, galactose, rhamnose, dulcitol, *myo*-inositol, methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, arbutin, aesculin, salicin, cellobiose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, DL-fucose, L-arabitol and 5-ketoglucuronate. The major ubiquinone is Q-8. The major fatty acids are C_{16:0}, C_{17:0} cyclo and C_{18:1 ω 7c}. The DNA G+C content is 64 mol%. No *nif* genes exist in the genome.

The type strain, OX-01^T (=NBRC 105797^T =DSM 22550^T), was isolated from acidic forest soil from Japan.

Acknowledgements

This work was supported by a Grant-in-Aid for Young Scientists (Start-up) from KAKENHI (19880035), a Grant-in-Aid for Young Scientists (B) from KAKENHI (21780169) and by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan.

References

- Aizawa, T., Ve, N. B., Nakajima, M. & Sunairi, M. (2010). *Burkholderia helea* sp. nov., a nitrogen-fixing bacterium isolated from an aquatic plant, *Eleocharis dulcis*, that grows in highly acidic swamps in actual acid sulfate soil areas of Vietnam. *Int J Syst Evol Microbiol* **60**, 1152–1157.
- Aspray, T. J., Frey-Klett, P., Jones, J. E., Whipps, J. M., Barbaye, J. & Bending, G. D. (2006). Mycorrhization helper bacteria: a case of specificity for altering ectomycorrhiza architecture but not ectomycorrhiza formation. *Mycorrhiza* **16**, 533–541.
- Brämer, C. O., Vandamme, P., da Silva, L. F., Gomez, J. G. C. & Steinbuchel, A. (2001). *Burkholderia sacchari* sp. nov., a polyhydroxyalkanoate-accumulating bacterium isolated from soil of a sugar-cane plantation in Brazil. *Int J Syst Evol Microbiol* **51**, 1709–1713.
- Caballero-Mellado, J., Martínez-Aguilar, L., Paredes-Valdez, G. & Estrada-de los Santos, P. (2004). *Burkholderia unamae* sp. nov., an N₂-fixing rhizospheric and endophytic species. *Int J Syst Evol Microbiol* **54**, 1165–1172.
- Chen, W.-M., James, E. K., Coenye, T., Chou, J.-H., Barrios, E., de Faria, S. M., Elliott, G. N., Sheu, S.-Y., Sprent, J. I. & Vandamme, P. (2006). *Burkholderia mimosarum* sp. nov., isolated from root nodules of *Mimosa* spp. from Taiwan and South America. *Int J Syst Evol Microbiol* **56**, 1847–1851.
- Chen, W.-M., de Faria, S. M., James, E. K., Elliott, G. N., Lin, K.-Y., Chou, J.-H., Sheu, S.-Y., Cnockaert, M., Sprent, J. I. & Vandamme, P. (2007). *Burkholderia nodosa* sp. nov., isolated from root nodules of the woody Brazilian legumes *Mimosa bimucronata* and *Mimosa scabrella*. *Int J Syst Evol Microbiol* **57**, 1055–1059.
- Coenye, T. & Vandamme, P. (2003). Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environ Microbiol* **5**, 719–729.
- Estrada-de los Santos, P., Bustillos-Cristales, R. & Caballero-Mellado, J. (2001). *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. *Appl Environ Microbiol* **67**, 2790–2798.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Gutell, R. R., Larsen, N. & Woese, C. R. (1994). Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol Rev* **58**, 10–26.
- Izumi, H., Anderson, I. C., Alexander, I. J., Killham, K. & Moore, E. R. (2006). Endobacteria in some ectomycorrhiza of Scots pine (*Pinus sylvestris*). *FEMS Microbiol Ecol* **56**, 34–43.
- Johnston, J. L. (1994). Similarity analysis of rRNAs. In *Methods for General and Molecular Bacteriology*, pp. 683–700. Edited by P. Gerhardt, R. G. E. Murray & W. A. Wood. Washington, DC: American Society for Microbiology.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Lehning, A., Fock, U., Wittich, R.-M., Timmis, K. N. & Pieper, D. H. (1997). Metabolism of chlorotoluenes by *Burkholderia* sp. strain PS12 and toluene dioxygenase of *Pseudomonas putida* F1: evidence for monooxygenation by toluene and chlorobenzene dioxygenases. *Appl Environ Microbiol* **63**, 1974–1979.
- Levy, A., Chang, B. J., Abbott, L. K., Kuo, J., Harnett, G. & Inglis, T. J. J. (2003). Invasion of spores of the arbuscular mycorrhizal fungus *Gigaspora decipiens* by *Burkholderia* spp. *Appl Environ Microbiol* **69**, 6250–6256.
- Lim, Y. W., Baik, K. S., Han, S. K., Kim, S. B. & Bae, K. S. (2003). *Burkholderia sordidicola* sp. nov., isolated from the white-rot fungus *Phanerochaete sordida*. *Int J Syst Evol Microbiol* **53**, 1631–1636.
- Martínez-Aguilar, L., Díaz, R., Peña-Cabriales, J. J., Estrada-de los Santos, P., Dunn, M. F. & Caballero-Mellado, J. (2008). Multichromosomal genome structure and confirmation of diazotrophy in novel plant-associated *Burkholderia* species. *Appl Environ Microbiol* **74**, 4574–4579.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Minerdi, D., Fani, R., Gallo, R., Boarino, A. & Bonfante, P. (2001). Nitrogen fixation genes in an endosymbiotic *Burkholderia* strain. *Appl Environ Microbiol* **67**, 725–732.
- Nishijima, M., Araki-Sakai, M. & Sano, H. (1997). Identification of isoprenoid quinones by frit-FAB liquid chromatography-mass spectrometry for the chemotaxonomy of microorganisms. *J Microbiol Methods* **28**, 113–122.
- Otsuka, Y., Nakamura, M., Shigehara, K., Sugimura, K., Masai, E., Ohara, S. & Katayama, Y. (2006). Efficient production of 2-pyrone 4,6-dicarboxylic acid as a novel polymer-based material from protocatechuate by microbial function. *Appl Microbiol Biotechnol* **71**, 608–614.
- Perin, L., Martínez-Aguilar, L., Paredes-Valdez, G., Baldani, J. I., Estrada-de los Santos, P., Reis, V. M. & Caballero-Mellado, J. (2006). *Burkholderia silvatlantica* sp. nov., a diazotrophic bacterium associated with sugar cane and maize. *Int J Syst Evol Microbiol* **56**, 1931–1937.
- Reis, V. M., Estrada-de los Santos, P., Tenorio-Salgado, S., Vogel, J., Stoffels, M., Guyon, S., Mavingui, P., Baldani, V. L. D., Schmid, M. & other authors (2004). *Burkholderia tropica* sp. nov., a novel nitrogen-fixing, plant-associated bacterium. *Int J Syst Evol Microbiol* **54**, 2155–2162.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

- Seigle-Murandi, F., Guiraud, P., Croizé, J., Falsen, E. & Eriksson, K.-E. L. (1996).** Bacteria are omnipresent on *Phanerochaete chrysosporium* Burdsall. *Appl Environ Microbiol* **62**, 2477–2481.
- Staskawicz, B. J., Dahlbeck, D. & Keen, N. T. (1984).** Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on *Glycine max* (L.) Merr. *Proc Natl Acad Sci U S A* **81**, 6024–6028.
- Takagi, H., Shida, O., Kadowaki, K., Komagata, K. & Udaka, S. (1993).** Characterization of *Bacillus brevis* with descriptions of *Bacillus migulanus* sp. nov., *Bacillus choshinensis* sp. nov., *Bacillus parabrevis* sp. nov., and *Bacillus galactophilus* sp. nov. *Int J Syst Bacteriol* **43**, 221–231.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Valverde, A., Delvasto, P., Peix, A., Velázquez, E., Santa-Regina, I., Ballester, A., Rodríguez-Barrueco, C., García-Balboa, C. & Igual, J. M. (2006).** *Burkholderia ferrariae* sp. nov., isolated from an iron ore in Brazil. *Int J Syst Evol Microbiol* **56**, 2421–2425.
- Vandamme, P., Govan, J. R. W. & LiPuma, J. J. (2007).** Diversity and role of *Burkholderia* spp. In *Burkholderia: Molecular Microbiology and Genomics*, pp. 1–28. Edited by T. Coenye & P. Vandamme. Wymondham, UK: Horizon Bioscience.
- Yano, K. & Nishi, T. (1980).** pKJ, a naturally occurring conjugative plasmid coding for toluene degradation and resistance to streptomycin and sulfonamides. *J Bacteriol* **143**, 552–560.