

## *Burkholderia terrae* sp. nov., isolated from a forest soil

Hee-Chan Yang, Wan-Taek Im, Kwang Kyu Kim, Dong-Shan An and Sung-Taik Lee

Correspondence  
Sung-Taik Lee  
e\_stlee@kaist.ac.kr

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1, Guseong-dong, Yuseong-gu, Daejeon, 305-701, South Korea

A Gram-negative, slightly curved rod-shaped bacterium, designated strain KMY02<sup>T</sup>, was isolated from a forest soil in Daejeon, South Korea. On the basis of 16S rRNA gene sequence similarity, strain KMY02<sup>T</sup> was shown to belong to the family *Burkholderiaceae* of the *Betaproteobacteria*, and to be related most closely to *Burkholderia hospita* LMG 20598<sup>T</sup> (98.7%), *Burkholderia caribensis* LMG 18531<sup>T</sup> (98.0%) and *Burkholderia phymatum* LMG 21445<sup>T</sup> (97.4%). Its phylogenetic distance from all recognized species within the genus *Burkholderia* was less than 97%. Chemotaxonomic data [Q-8 as the major ubiquinone; C<sub>16:0</sub>, C<sub>17:0</sub> cyclo, summed feature 7 (C<sub>18:1ω7c/ω9t/ω12t</sub>) and C<sub>15:0</sub> as the major fatty acids] supported the affiliation of strain KMY02<sup>T</sup> to the genus *Burkholderia*. The results of DNA–DNA hybridization experiments and physiological and biochemical tests allowed genotypic and phenotypic differentiation of the strain from recognized *Burkholderia* species. Therefore, KMY02<sup>T</sup> (=KCTC 12388<sup>T</sup>=NBRC 100964<sup>T</sup>) represents the type strain of a novel species, for which the name *Burkholderia terrae* sp. nov. is proposed.

The genus *Burkholderia* was first described by Yabuuchi *et al.* (1992), and at the time of writing contains 38 species. These organisms have been isolated from diverse ecological niches, ranging from contaminated soils to the human respiratory tract (Coenye & Vandamme, 2003). During the characterization of bacteria isolated from a forest soil near the Korea Advanced Institute of Science and Technology (KAIST), strain KMY02<sup>T</sup> was cultivated on R2A agar at 30 °C, and was then subjected to a taxonomic investigation. The aim of this study was to determine the taxonomic position of the strain based on phenotypic, genotypic and chemotaxonomic characteristics and 16S rRNA gene sequence analysis. The results provide evidence that strain KMY02<sup>T</sup> represents a novel species within the genus *Burkholderia*.

Strain KMY02<sup>T</sup> was isolated from a broad-leaved forest soil collected near KAIST, Daejeon, South Korea. The forest soil was homogenized by using an Ace homogenizer (Nihonseiki Kaisha). The suspension was spread on R2A agar plates

(Difco) after being serially diluted with 50 mM phosphate buffer (pH 7.0). The plates were incubated at 30 °C for 2 weeks. Single colonies on the plates were purified by transferring them on to new plates and incubating again under the same conditions. The isolate was routinely cultured on R2A agar at 30 °C and maintained as a glycerol suspension (20%, w/v) at –70 °C.

Genomic DNA was extracted using a commercial kit (Solgent), and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to the methods described by Kim *et al.* (2005). Full sequences of the 16S rRNA gene were compiled using SeqMan software (DNASTAR). 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed by using the CLUSTAL\_X program (Thompson *et al.*, 1997), and gaps were edited in the BIOEDIT program (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1972) methods, using the MEGA3 program (Kumar *et al.*, 2004) and with bootstrap values based on 1000 replications (Felsenstein, 1985).

A nearly complete 16S rRNA gene sequence of strain KMY02<sup>T</sup> was obtained (1470 bp). Preliminary sequence comparison against 16S rRNA gene sequences deposited in

Published online ahead of print on 11 November 2005 as DOI 10.1099/ijs.0.63968-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KMY02<sup>T</sup> is AB201285.

A transmission electron micrograph of a cell of strain KMY02<sup>T</sup> and a table giving levels of DNA–DNA hybridization between this strain and the type strains of closely related *Burkholderia* species are available as supplementary material in IJSEM Online.

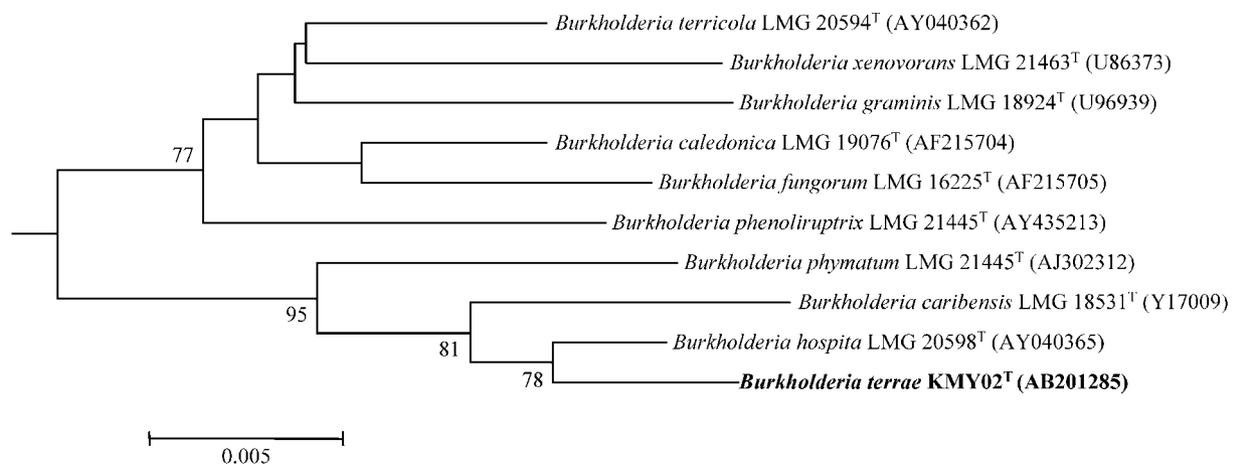
the GenBank database indicated that the isolate belonged to the family *Burkholderiaceae* of the *Betaproteobacteria*. On the basis of 16S rRNA gene sequence similarity, the closest cultured relatives to strain KMY02<sup>T</sup> were *Burkholderia hospita* LMG 20598<sup>T</sup> (98.7%), *Burkholderia caribensis* LMG 18531<sup>T</sup> (98.0%) and *Burkholderia phymatum* LMG 21445<sup>T</sup> (97.4%). This relationship between strain KMY02<sup>T</sup> and other members of the genus *Burkholderia* was also evident in the phylogenetic tree (Fig. 1). Strain KMY02<sup>T</sup> and the three type strains above formed a monophyletic clade with a high bootstrap value (95%), and this was supported by the neighbour-joining and maximum-parsimony algorithms.

The Gram reaction was performed by using the non-staining method described by Buck (1982). Cell morphology was examined by light microscopy (Nikon) and transmission electron microscopy (Carl Zeiss) after negative staining with 1% (w/v) phosphotungstic acid. Catalase and oxidase tests were performed by using the procedures outlined by Cappuccino & Sherman (2002). Substrate utilization as sole carbon source and several other physiological characteristics were determined with the API 32GN, API 20NE and API ZYM galleries according to the instructions of the manufacturer (bioMérieux). Tests for anaerobic growth were performed in a serum bottle containing R2A broth supplemented with thioglycolate (1 g l<sup>-1</sup>) and in which the upper air layer was substituted with nitrogen gas. Nitrate and nitrite reduction tests were performed in serum bottles containing R2A broth supplemented with KNO<sub>3</sub> (10 mM) and NaNO<sub>2</sub> (10 mM), respectively; reduction was monitored on an ion chromatograph (model 790 personal IC; Metrohm) equipped with a conductivity detector and anion exchange column (Metrosep Anion Supp 4; Metrohm). Nitrogen-fixing ability was determined by growth in 50 ml of a nitrogen-free medium (DSMZ medium no. 3) contained in

a 500 ml Erlenmeyer flask. The primer system PolF–PolR (Poly *et al.*, 2001) was used to amplify the *nifH* gene according to the methods described by Im *et al.* (2004). Degradation of DNA [using DNA agar (Difco) supplemented with 0.01% toluidine blue (Merck)], chitin, CM-cellulose, starch (Atlas, 1993), lipid (Kouker & Jaeger, 1987) and xylan (Ten *et al.*, 2004) was also investigated; reactions were read after 5 days. Growth at different temperatures and pH was assessed after 5 days incubation. Salt tolerance was tested on R2A medium supplemented with 1–10% (w/v) NaCl after 5 days incubation. Antibiotic-sensitivity tests were done using filter-paper discs containing the following: streptomycin (5, 10 and 15 µg ml<sup>-1</sup>), tetracycline (5, 10 and 15 µg ml<sup>-1</sup>), kanamycin (1.0, 1.5 and 2.0 mg ml<sup>-1</sup>) and ampicillin (20, 30 and 50 µg ml<sup>-1</sup>) (Sigma). Discs were placed on R2A plates spread with KMY02<sup>T</sup> culture and were then incubated at 30 °C for 5 days. All the phenotypic tests described above were performed in duplicate.

Cells of strain KMY02<sup>T</sup> were Gram-negative, slightly curved rods, and were motile by means of a single polar flagellum (see Supplementary Fig. S1 in IJSEM Online). Nitrogen fixation determined by growth in nitrogen-free medium and a *nifH* gene was positive. Physiological characteristics of strain KMY02<sup>T</sup> are summarized in the species description, and comparison of selective characteristics with those of the type strains of its most closely related species is given in Table 1.

Quinones were extracted from cells grown on a nutrient broth (Difco) and analysed as described by Komagata & Suzuki (1987) by using reversed-phase HPLC. Cellular fatty acids were analysed in organisms grown on trypticase soy agar (TSA; Difco) for 2 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System



**Fig. 1.** Neighbour-joining tree showing the phylogenetic positions of KMY02<sup>T</sup> and other related taxa based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) greater than 70% are shown at branch points. Bar, 0.005 substitutions per nucleotide position.

**Table 1.** Differential phenotypic characteristics of strain KMY02<sup>T</sup> and phylogenetically closely related *Burkholderia* species

Strains: 1, KMY02<sup>T</sup>; 2, *B. hospita* LMG 20598<sup>T</sup>; 3, *B. phymatum* LMG 21445<sup>T</sup>; 4, *B. caribensis* LMG 18531<sup>T</sup>. +, Positive reaction; -, negative reaction. The following features were present in all strains investigated: motility; growth at 30 °C and in the presence of 0.5–1.5 % NaCl; growth on D-glucose, D-gluconate, D-mannose, D-mannitol, D-ribose, D-sorbitol, 3-hydroxybutyrate, inositol, L-alanine, L-arabinose, L-fucose, L-proline, N-acetylglucosamine, phenylacetate and rhamnose; positive for oxidase, alkaline and acid phosphatase, arginine dihydrolase,  $\beta$ -galactosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and urease. The following features were absent in all strains investigated: growth in the presence of 3.0, 4.5 or 6.0 % NaCl; production of indole; nitrite reduction; acidification of D-glucose; hydrolysis of aesculin; assimilation of acetate, adipate, citrate, D-melibiose, 4-hydroxybenzoate, glycogen, malonate, maltose, salicin, amylase, chitinase, cellulase, DNase, protease, xylanase, valine arylamidase, cystine arylamidase, lipase C14, trypsin,  $\alpha$ -chymotrypsin,  $\alpha$ -galactosidase,  $\alpha$ -glucosidase,  $\beta$ -glucosidase,  $\beta$ -glucuronidase, N-acetyl- $\beta$ -glucosaminidase,  $\alpha$ -mannosidase and  $\alpha$ -fucosidase.

Characteristic	1	2	3	4
Nitrate reduction	-	+	-	+
Catalase	+	+	-	+
Growth at 42 °C	-	+	-	-
Antibiotic resistance				
Kanamycin (1 mg ml <sup>-1</sup> )	-	+	-	-
Streptomycin (5 $\mu$ g ml <sup>-1</sup> )	-	+	-	+
Assimilation of:				
Caprate	+	+	+	-
L-Malate	+	+	+	-
Propionate	-	-	-	+
Valerate	-	+	+	-
Histidine	+	+	+	-
2-Ketogluconate	+	+	+	-
Sucrose	-	+	-	-
Itaconate	-	-	+	-
Suberate	+	-	-	-
DL-Lactate	+	-	+	+
5-Ketogluconate	+	-	-	-
3-Hydroxybenzoate	-	-	+	-
L-Serine	+	+	+	-
API ZYM results				
Esterase (C4)	-	-	-	+
Esterase lipase (C8)	+	-	-	-
Mean DNA G+C content (mol%)	62	62	61	61

(MIDI). The fatty acids analysed by GC (Hewlett Packard 6890) were identified using the Microbial Identification software package (Sasser, 1990).

Total DNA for determination of the G+C content was extracted from cells grown on a nutrient agar plate (Difco) using the method described by Ausubel *et al.* (1995). RNA

in the DNA solution was removed by incubation with a mixture of RNase A and T1 (each at 20 units ml<sup>-1</sup>) at 30 °C for 1 h. The G+C content of the total DNA was analysed as described by Mesbah *et al.* (1989) using reversed-phase HPLC. DNA-DNA hybridization was performed fluorometrically according to the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and micro-dilution wells.

Ubiquinone Q-8 was detected as the predominant quinone system in strain KMY02<sup>T</sup>; this is also the case in all other species of the genus *Burkholderia*. The cellular fatty acid profile of strain KMY02<sup>T</sup> included C<sub>16:0</sub> (28.1%), C<sub>17:0</sub> cyclo (23.4%), summed feature 7 (C<sub>18:1 $\omega$ 7cl $\omega$ 9t $\omega$ 12t</sub>, 11.8%) and C<sub>15:0</sub> (7.5%). Significant differences were found between strain KMY02<sup>T</sup> and the other *Burkholderia* species investigated with regard to the fatty acid profile; the proportion of hydroxyl fatty acids is lower in strain KMY02<sup>T</sup> and it has C<sub>15:0</sub> as a major component (Table 2).

The DNA G+C content of strain KMY02<sup>T</sup> is 62 mol%, a value similar to those of members of the genus *Burkholderia* (Table 1). The level of DNA-DNA relatedness between

**Table 2.** Cellular fatty acid content (%) of strain KMY02<sup>T</sup> and phylogenetically closely related *Burkholderia* species

Strains: 1, KMY02<sup>T</sup>; 2, *B. hospita* LMG 20598<sup>T</sup>; 3, *B. caribensis* LMG 18531<sup>T</sup>; 4, *B. phymatum* LMG 21445<sup>T</sup>. Fatty acids that account for less than 0.5 % of the total are not shown.

Fatty acid	1	2	3	4
<b>Saturated</b>				
C <sub>12:0</sub>	3.0			1.7
C <sub>14:0</sub>	4.7	4.8	5.9	3.7
C <sub>15:0</sub>	7.5			
C <sub>16:0</sub>	28.1	18.5	16.0	18.4
C <sub>17:0</sub>	1.8			
C <sub>18:0</sub>	1.5			
<b>Branched</b>				
iso-C <sub>17:0</sub> 3-OH			1.2	
<b>Hydroxy</b>				
C <sub>14:0</sub> 2-OH	1.6			
C <sub>16:0</sub> 2-OH		1.8	5.6	3.2
C <sub>16:1</sub> 2-OH		1.5	2.9	5.8
C <sub>16:0</sub> 3-OH		4.8	8.9	4.8
C <sub>18:1</sub> 2-OH			1.2	
<b>Cyclo</b>				
C <sub>17:0</sub> cyclo	23.4	10.4	24.9	23.7
C <sub>19:0</sub> cyclo $\omega$ 8c	6.3	3.5	15.1	7.0
<b>Summed features*</b>				
2: C <sub>14:0</sub> 3-OH/ iso-C <sub>16:1</sub> I	6.7	6.9	11.7	5.1
3: C <sub>16:1<math>\omega</math>7cl</sub> iso-C <sub>15:0</sub> 2-OH	3.3	17.8	2.3	7.2
7: C <sub>18:1<math>\omega</math>7cl<math>\omega</math>9t<math>\omega</math>12t</sub>	11.8	30.0	4.2	19.4

\*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system.

strain KMY02<sup>T</sup> and the type strains of *B. hospita*, *B. caribensis* and *B. phymatum* was 28, 36 and 22 %, respectively (see Supplementary Table S1 in IJSEM Online). Levels of DNA–DNA hybridization were determined to be less than 70 %, which is the threshold used to delineate a genomic species (Stackebrandt & Goebel, 1994). The results therefore support the designation of strain KMY02<sup>T</sup> as representing a separate, previously unrecognized species.

On the basis of its morphological, physiological and chemotaxonomic characteristics, together with data from 16S rRNA gene sequence comparisons, strain KMY02<sup>T</sup> should be placed in the genus *Burkholderia* as a novel species, for which the name *Burkholderia terrae* sp. nov. is proposed.

### Description of *Burkholderia terrae* sp. nov.

*Burkholderia terrae* (ter'rae. L. gen. n. *terrae* of the earth).

Cells are Gram-negative, slightly curved rods, 1.6–2.0 µm long by 0.6–0.8 µm wide, motile by means of a single polar flagellum. Colonies grown on R2A are circular, convex and cream-coloured. Temperature range for growth is 25–30 °C; no growth occurs at 42 °C. Growth occurs in the absence of NaCl and in the presence of 1.5 % (w/v) NaCl, but not above 3.0 % (w/v) NaCl. Nitrate is not reduced. Nitrogen fixation is positive. Catalase, oxidase, arginine dihydrolase, urease and β-galactosidase activities are positive. Tryptophanase and β-glucosidase activities are negative. Positive for assimilation of mannose, gluconate, caprate, phenylacetate, mannitol, D-glucose, L-fucose, D-sorbitol, L-arabinose, malate, histidine, 2-ketogluconate, 3-hydroxybutyrate, L-proline, rhamnose, N-acetylglucosamine, D-ribose, inositol, substrate, DL-lactate, L-alanine, 5-ketogluconate and L-serine. Negative for assimilation of adipate propionate, valerate, sucrose, itaconate and 3-hydroxybenzoate. Positive for alkaline phosphatase, esterase lipase C8, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase; negative for esterase C4, lipase C14, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Resistant to 20 µg ampicillin ml<sup>-1</sup> and 5 µg tetracycline ml<sup>-1</sup> but susceptible to 1 mg kanamycin ml<sup>-1</sup> and 5 µg streptomycin ml<sup>-1</sup>. Predominant ubiquinone is Q-8. The major fatty acids are C<sub>16:0</sub>, C<sub>17:0</sub> cyclo, summed feature 7 (C<sub>18:1ω7clω9t/ω12t</sub>) and C<sub>15:0</sub>. The G + C content of the genomic DNA is 62 mol%.

The type strain is KMY02<sup>T</sup> (=KCTC 12388<sup>T</sup>=NBRC 100964<sup>T</sup>).

### Acknowledgements

This work was supported by the Eco-Technopia-21, Ministry of Environment and the 21C Frontier Microbial Genomics and Application Center Program, Ministry of Science & Technology (grant MG05-0101-4-0), South Korea.

### References

- Atlas, R. M. (1993). *Handbook of Microbiological Media*. Edited by L. C. Parks. Boca Raton, FL: CRC Press.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (editors) (1995). *Short Protocols in Molecular Biology: a Compendium of Methods from Current Protocols in Molecular Biology*, 3rd edn. New York: Wiley.
- Buck, J. D. (1982). Nonstaining (KOH) method for determination of Gram reactions of marine bacteria. *Appl Environ Microbiol* **44**, 992–993.
- Cappuccino, J. G. & Sherman, N. (2002). *Microbiology: a Laboratory Manual*, 6th edn. Menlo Park, CA: Benjamin Cummings.
- Coenye, T. & Vandamme, P. (2003). Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environ Microbiol* **5**, 719–729.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in micro-dilution 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.
- Fitch, W. M. (1972). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.
- Hall, T. A. (1999). BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Im, W.-T., Bae, H.-S., Yokota, A. & Lee, S. T. (2004). *Herbaspirillum chlorophenolicum* sp. nov., a 4-chlorophenol-degrading bacterium. *Int J Syst Evol Microbiol* **54**, 851–855.
- Kim, M. K., Im, W.-T., Ohta, H., Lee, M. & Lee, S.-T. (2005). *Sphingopyxis granulii* sp. nov., a β-glucosidase producing bacterium in the family Sphingomonadaceae in α-4 subclass of the Proteobacteria. *J Microbiol* **43**, 111–116.
- Kimura, M. (1983). *The Neutral Theory of Molecular Evolution*. Cambridge: Cambridge University Press.
- Komagata, K. & Suzuki, K. I. (1987). Lipid and cell wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161–207.
- Kouker, G. & Jaeger, K.-E. (1987). Specific and sensitive plate assay for bacterial lipase. *Appl Environ Microbiol* **53**, 211–213.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Poly, F., Monrozier, L. J. & Bally, R. (2001). Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Res Microbiol* **152**, 95–103.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sasser, M. (1990). *Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids*. MIDI Technical Note 101. Newark, DE: MIDI.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Ten, L. N., Im, W.-T., Kim, M.-K., Kang, M.-S. & Lee, S.-T. (2004). Development of a plate technique for screening of

polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates. *J Microbiol Methods* **56**, 375–382.

**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.

**Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. & Arakawa, M. (1992).** Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol Immunol* **36**, 1251–1275.