Burkholderia kururiensis sp. nov., a trichloroethylene (TCE)-degrading bacterium isolated from an aquifer polluted with TCE

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A trichloroethylene (TCE)-degrading bacterium was isolated from an aquifer sample collected at a TCE-polluted site in Japan by enriching with phenol as sole carbon source. The isolate, designated strain KP23^T, was a Gram-negative, oval-shaped micro-organism. A phylogenetic study based on 16S rRNA gene sequences indicated that strain KP23^T should be placed in the genus *Burkholderia*. Cellular fatty acids of the strain were mainly composed of $C_{16:0}$, cyclopropanic acid $C_{17:0}$ and cyclopropanic acid $C_{19:0}$. Strain KP23^T also contained notable amounts of $C_{13:1}$ and $C_{17:1}$. The G+C content of total DNA was 64·8 mol%. Strain KP23^T oxidized various sugars and sugar alcohols as sole carbon source such as galactose, glucose, mannose, maltose, glycerol, inositol and mannitol. Comparisons of its phenotypic and genotypic characteristics with other known species belonging to the genus *Burkholderia* suggested that strain KP23^T represents a new species in the genus. The name *Burkholderia kururiensis* is proposed for this species, with strain KP23^T as the type strain (= JCM 10599^T).

Keywords: trichloroethylene degradation, *Burkholderia kururiensis* sp. nov., aquifer, 16S rRNA gene

INTRODUCTION

Trichloroethylene (TCE) has been used as a solvent in manufacturing to clean grease from machinery all over the world. Like many other chlorinated hydrocarbons, it has become one of the most abundant environmental pollutants because of its toxic properties and widespread occurrence in soil and groundwater in many countries. TCE removal by dumping or air stripping is restricted by legislation and thus attention is now being paid to biological degradation in soil and groundwater. In the past decade, research on the application of bacteria to the aerobic biodegradation of TCE has progressed and a wide variety of micro-organisms has been investigated for this purpose. Most of these bacteria belong to the *Proteobacteria* such as *Methylococcus capsulatus* (Stainthorpe *et al.*, 1990), *Methylosinus trichosporium* OB3b (Cardy *et al.*, 1991), *Methylocystis* sp. strain M (McDonald *et al.*, 1997), *Pseudomonas* sp. strain CF600 (Nordlund *et al.*, 1990), *Pseudomonas putida* strain H (Herrmann *et al.*, 1995), *Pseudomonas putida* F1 (Wackett & Householder, 1989), *Pseudomonas mendocina* (Yen *et al.*, 1991), *Burkholderia cepacia* G4 (Shields *et al.*, 1989), *Ralstonia pickettii* (objective synonym of *Pseudomonas pickettii*) (Byrne *et al.*, 1995) and *Ralstonia eutropha* (objective synonym of *Alcaligenes eutrophus*) (Kim *et al.*, 1996).

In these organisms, TCE degradation is catalysed by monoxygenases or dioxygenases which are induced by specific substrates relevant to the enzymes. The inducers include various aromatic and aliphatic hydrocarbons such as toluene (Nelson *et al.*, 1987, 1988), phenol (Folsom *et al.*, 1990; Harker & Kim, 1990), isopropylbenzene (Dabrock *et al.*, 1992), propane (Wackett *et al.*, 1989) and methane (Little *et al.*, 1988; Tsein *et al.*, 1989).

Recently, we isolated a novel TCE-degrading bacterium, designated strain KP23^T, from a site polluted

Abbreviations: FAME, fatty acid methyl ester; TCE, trichloroethylene; UQ, ubiquinone.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KP23^T is AB024310.

with this contaminant. The isolate was capable of substantial phenol degradation and TCE appeared to be co-metabolized with phenol. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain KP23^T belongs to the genus *Burkholderia* and that the strain is distant enough from any other known species in the genus to be classified as a new species.

In this paper, we detail the phenotypic and genotypic features of the isolate which distinguish it from authentic species in the genus *Burkholderia* and propose a new name for it, *Burkholderia kururiensis* sp. nov.

METHODS

Isolation and growth conditions. Strain KP23^T was isolated from an aquifer sample collected from a TCE-polluted site in Kururi, Chiba Prefecture, Japan (March, 1996). The geological and environmental properties of the aquifer were detailed by Hanada *et al.* (1998). The concentration of dissolved oxygen in the ground water was approximately 8 mg l^{-1}. The pH and temperature of the groundwater were 6·7 and 15–16 °C, respectively.

For enrichment from the environmental samples, BSM medium supplemented with phenol at a final concentration of 2 mM was used. The BSM medium (pH 7·0) contained the following components (l^{-1}): K_2HPO_4, 1·0 g; $MgSO_4 . 7H_2O$, 0·2 g; NaCl, 0·1 g; $CaCl_2 . H_2O$, 0·1 g; $(NH_4)_2SO_4$, 1·0 g; and $FeCl_3$, 0·02 g. The enrichment was shaken vigorously at 30 °C. LB (Luria–Bertani) medium supplemented with 1·5 % agar was used for isolation. PE medium (Hanada *et al.*, 1995) was used for physiological and morphological analyses of the isolate. The PE medium contained the following components (l^{-1}): sodium glutamate, 0·5 g; sodium succinate, 0·5 g; sodium acetate, 0·5 g; $(NH_4)_2SO_4$, 0·5 g; yeast extract (Difco), 0·5 g; Casamino acids (Difco), 0·5 g; KH_2PO_4, 0·38 g; K_2HPO_4, 0·39 g; vitamin mixture, 1 ml; and a basal salt solution, 5 ml. The vitamin mixture contained (per 100 ml) nicotinic acid, 100 mg; thiamin hydrochloride, 100 mg; biotin, 5 mg; *p*-aminobenzoic acid, 50 mg; vitamin B_{12}, 1 mg; calcium pantothenate, 50 mg; pyridoxine hydrochloride, 50 mg; and folic acid, 50 mg. The basal salt solution contained (l^{-1}) $FeSO_4 . 7H_2O$, 1·11 g; $MgSO_4 . 7H_2O$, 24·65 g; $CaCl_2 . 2H_2O$, 2·94 g; NaCl, 23·4 g; $MnSO_4 . 4H_2O$, 111 mg; $ZnSO_4 . 7H_2O$, 28·8 mg; $Co(NO_3)_2 . 6H_2O$, 29·2 mg; $CuSO_4 . 5H_2O$, 25·2 mg; $Na_2MoO_4 . 2H_2O$, 24·2 mg; H_3BO_4, 31·0 mg; and trisodium EDTA, 4·53 g. A simplified PE medium containing (l^{-1}) $(NH_4)_2SO_4$, 0·5 g; KH_2PO_4, 0·38 g; K_2HPO_4, 0·39 g; and basal salt solution, 5 ml was used for testing aromatic substrate utilization. The pH of the media was adjusted to 7·2 with NaOH. Liquid cultures of strain KP23^T were incubated at 37 °C with vigorous agitation.

Morphology. Gram staining was performed as described by Magee *et al.* (1975). Cell morphology was observed with a phase-contrast microscope (Olympus AX80T). For electron microscopy of ultra-thin sections, cells were embedded in Spurr medium (Kushida, 1980) after fixing with 2·5 % (v/v) glutaraldehyde and 1 % (v/v) osmium tetroxide. Photomicrographs of the sections were obtained with a Hitachi H-7000 transmission electron microscope operated at 75 kV.

Quinone analysis. Quinones were extracted with chloroform-methanol (2 : 1, v/v). The extract was purified with a Sep-Pak Plus column (Waters) and analysed by reverse-phase HPLC (Beckman System Gold with a Hewlett Packard Zorbox ODS column) for identification (Tamaoka *et al.*, 1983).

Physiological and biochemical characterization. TCE degradation was determined using the method described by Hanada *et al.* (1998). TCE degradation was assumed to follow pseudo-first-order kinetics with respect to substrate. The pseudo-first-order degradation rate constant k_1 (l g^{-1} h^{-1}) was measured as described by Speitel *et al.* (1993).

Oxidase activity was determined by monitoring the oxidation of tetramethyl-*p*-phenylenediamine on a filter paper, and catalase activity was determined by adding cells to a 3 % hydrogen peroxide solution (Smibert & Krieg, 1994). Gelatin liquefaction was tested at 25 °C by the method described by Yabuuchi *et al.* (1992). Carbon source oxidation was determined with a BIOLOG GN system. Results were read automatically with a spectrophotometer after 7 and 24 h incubation at 37 °C. All tests were run in triplicate. Aromatic substrate utilization was tested by supplementing aromatic substrates to the simplified PE medium at a final concentration of 2 mM.

Fatty acid methyl ester (FAME) analysis. FAME analysis, which is based on the conversion of fatty acids to methyl esters by mild acidic methanolysis, followed by gas chromatography analysis, was performed at the Microbial Analysis Laboratory of Microcheck Inc. (Northfield, USA) using the methods described by Welch (1991) and Sasser & Wichman (1991).

DNA base composition. Total DNA of strain KP23^T was extracted according to the procedure of Saito & Miura (1963). It was digested with P1 nuclease using a Yamasa GC kit (Yamasa Shoyu). The G+C content was measured by HPLC (Kamagata & Mikami, 1991).

16S rRNA gene sequence and phylogenetic analysis. For determination of the 16S rRNA gene (rDNA) sequence of strain KP23^T, cells were lysed by the method of Hiraishi (1992). The 16S rDNA fragment was amplified by PCR (Hiraishi *et al.*, 1994) using the universal primers forward: 5′-AGAGTTTGATCATGGCTCGA-3′ (positions 8–27 of the *Escherichia coli* 16S rRNA gene) and reverse: 5′-GGCTACCTTGTTACGACTT-3′ (positions 1510–1492). The PCR product was directly sequenced on an ABI 377 DNA sequencer using a dRhodamine Dye Terminator Cycle Sequencing kit (Applied Biosystems).

The 16S rDNA sequence was aligned with reference sequences by using the CLUSTAL W program, version 1.5 (Thompson *et al.*, 1994). A phylogenetic tree was constructed from the evolutionary distance matrix calculated by the neighbour-joining method (Saitou & Nei, 1987). The neighbour-joining analysis was performed with the MEGA program (Kumar *et al.*, 1993).

RESULTS

TCE degradation by strain KP23^T

Strain KP23^T was isolated from an aquifer sample collected at a TCE-polluted site using 2 mM phenol as sole carbon source. This isolate showed TCE degradation activity. Its TCE transformation capacity (*Tc*), which is the maximum mass of TCE that can be transformed per unit mass of cells, was 54·4 µg (g dry

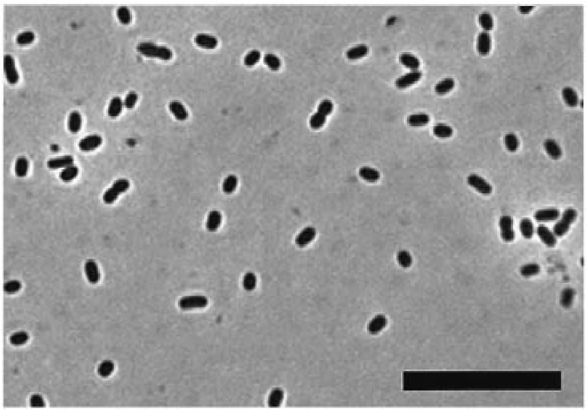

Fig. 1. Phase-contrast photomicrograph of strain KP23^T cells aerobically grown in PE medium at 37 °C. Bar, 10 μm.

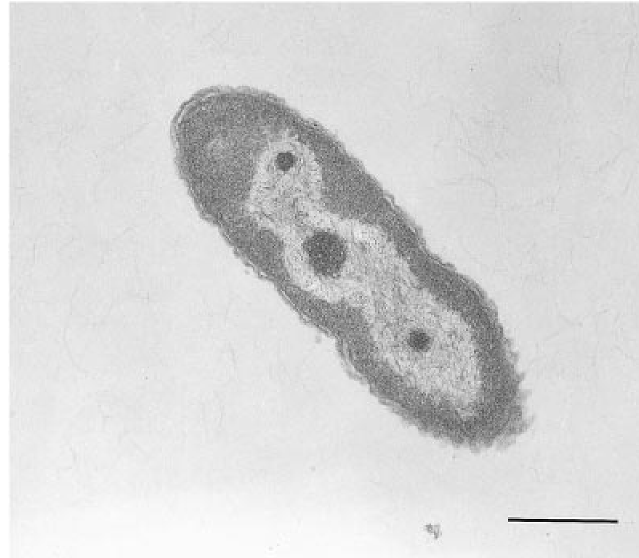

Fig. 2. Transmission electron micrograph of strain KP23^T cells showing the typical cell wall structure of Gram-negative bacteria. Bar, 0·5 μm.

cell wt)$^{-1}$. The pseudo-first-order degradation rate constant, k_1, was 12·8 l g^{-1} h^{-1}.

Aromatic substrate utilization and induction of TCE degradation were investigated. The following 28 substrates were tested: phenol, benzene, fluorobenzene, *p*-cresol, toluene, chlorobenzene, nitrobenzene, *m*-chlorotoluene, *o*-nitrotoluene, *p*-nitrotoluene, *m*-nitrotoluene, *m*-dichlorobenzene, *o*-dichlorobenzene, *p*-dichlorobenzene, naphthalene, *m*-chlorophenol, *o*-chlorophenol, *p*-chlorophenol, *m*-cresol, *o*-cresol, 2-fluorophenol, 2,5-dichlorophenol, 2,4,6-trichlorophenol, *m*-nitrophenol, indigo, indene, indan and xylene. Of the substrates tested, phenol, benzene, fluorobenzene and *p*-cresol were utilized as sole carbon source, and only phenol induced TCE degradation.

Morphology

Colonies of strain KP23^T on PE medium plates were light yellow to grey. No diffusible pigment was produced. Under the microscope, the cells appeared as ovoids to short rods 1·2–1·5 μm in length and 1·0 μm in width (Fig. 1). Cells were non-motile. Spores were not observed. Gram-staining was negative. Electron microscopy demonstrated that the cells of strain KP23^T possessed a typical Gram-negative cell wall structure and no invaginations of intracytoplasmic membranes (Fig. 2).

Physiological and biochemical characteristics

Strain KP23^T grew between 15 and 42 °C with an optimum temperature of 37 °C, whilst no growth was detected at 10 or 45 °C within 10 d incubation. The pH range for growth was between 6·0 and 7·8, with the optimum pH at 7·2. The doubling time was approximately 1 h under optimum growth conditions in liquid PE medium.

A comparison of carbon source oxidation of strain KP23^T with type strains of 16 species in the genus *Burkholderia* is summarized in Table 1. Strain KP23^T was able to oxidize various sugars and sugar alcohols such as arabinose, galactose, glucose, fructose, fucose, lactulose, maltose, mannose, psicose, rhamnose, adonitol, arabitol, glycerol, inositol, mannitol, sorbitol and xylitol. In addition to the nutritional profile shown in Table 1, strain KP23^T was also able to oxidize the following substrates: acetate, citrate, formate, galacturonate, lactate, propionate, succinate, alanine, asparagine, aspartate, glutamate, glycine, histidine, leucine, phenylalanine, proline, serine, threonine, inosine and 2,3-butanediol. The following were not oxidized: cellobiose, lactose, melibiose, raffinose, sucrose, trehalose, malonate, dextrin, thymidine, uridine, glucose 1-phosphate and glucose 6-phosphate.

Neither starch hydrolysis nor gelatin liquefaction was observed, but Tween 80 and glycogen were hydrolysed. Catalase and oxidase were produced.

Quinone and cellular fatty acid components

The major respiratory quinone of strain KP23^T was ubiquinone (UQ)-8 (96·1 % of total quinones). Small amounts of UQ-7 (3·4 %) and UQ-9 (0·5 %) were also detected.

The main fatty acids in the cells of strain KP23^T were cyclopropanic acid ($C_{19:0}$; 25·7 % of total fatty acid methyl esters), $C_{16:0}$ (22·6 %) and cyclopropanic acid ($C_{17:0}$; 16·6 %). Strain KP23^T also contained $C_{14:0}$ (7·5 %), $C_{18:1}$ (7·5 %), $C_{16:1}$ (6·8 %), 2-OH $C_{16:0}$ (4·5 %), 3-OH $C_{16:0}$ (3·7 %), $C_{13:1}$ (3·3 %), $C_{17:1}$ (1·8 %) and a trace amount of 3-OH $C_{14:0}$.

Table 1. Characteristics of carbon source oxidation of strain KP23^T as opposed to type strains of *Burkholderia* species

Strains: 1, strain KP23^T; 2, *B. phenazinium*; 3, *B. glathei*; 4, *B. cepacia*; 5, *B. pyrrocinia*; 6, *B. vietnamiensis*; 7, *B. glumae*; 8, *B. plantarii*; 9, *B. gladioli*; 10, *B. caryophylli*; 11, *B. andropogonis*; 12, *B. plantarii* (formerly *B. vandii*; Coenye *et al*., 1999); 13, *B. gladioli* (formerly *B. cocovenenans*; Coenye *et al*., 1999); 14, *B. mallei*; 15, *B. pseudomallei*; 16, *B. graminis*; and 17, *B. caribensis*. The results for type strains of *Burkholderia* are those of Viallard *et al*. (1998) and Achouak *et al*. (1999). +, Positive; –, negative. Galactose, gluconate, glucose, glycerol, inositol, mannitol and mannose were oxidized by all strains.

Oxidation of:	Strains																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Adonitol	+	+	+	+	+	−	+	−	+	+	+	−	−	−	−	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+
Arabitol	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+
Cellobiose	−	−	−	+	+	+	−	−	−	−	−	+	−	−	−	+	−
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+
Fucose	+	+	+	+	+	+	+	+	+	+	−	+	+	−	+	+	+
Lactose	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	+	+
Maltose	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Melibiose	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Raffinose	−	−	−	−	−	+	+	−	−	+	−	−	−	−	−	+	−
Rhamnose	+	+	+	−	−	−	−	+	−	+	−	−	−	−	−	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+
Sucrose	−	+	−	+	+	+	−	−	−	+	−	−	−	+	+	+	−
Trehalose	−	−	−	+	+	+	+	+	+	+	−	+	+	+	−	+	+
Xylitol	+	+	+	+	+	−	−	−	−	+	−	−	+	−	−	+	+

Phylogenetic analysis based on 16S rRNA sequence

A sequence (1461 nucleotides) of the 16S rRNA gene of strain KP23^T was determined. A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) (Fig. 3). The sequence of *Neisseria gonorrhoeae*, which belongs to the β-subclass of the *Proteobacteria*, was used to root the tree. Strain KP23^T was placed as a member of the genus *Burkholderia*. It was closely related to *Burkholderia caribensis* (sequence similarity 95·9 %), *Burkholderia graminis* (94·9 %) and *Burkholderia phenazinium* (94·9 %).

The G+C content of the total DNA of strain KP23^T was 64·8 mol %.

DISCUSSION

Strain KP23^T, which showed TCE-degrading activity, was isolated from an aquifer polluted with TCE by enriching with phenol as sole carbon source. The TCE degrading activity was comparable with other known TCE-degraders. Of the substrates tested, only phenol induced TCE-degrading activity, suggesting that degradation is catalysed by phenol hydroxylase. Phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain KP23^T is a member of the β-*Proteobacteria* and is distant from the other bacteria known as TCE degraders in this subclass, i.e. *Burkholderia cepacia* strain G4, *Burkholderia* sp. strain KP24, *Burkholderia* sp. strain MBIC3837 and a few degraders belonging to the genus *Ralstonia* (Fig. 3).

The sequence analysis suggested that the new isolate belongs to the genus *Burkholderia*. Several phenotypic characteristics of the strain supported this assignment. The dominant respiratory quinone of the isolate was UQ-8, like other members of this genus (Urakami *et al*., 1994). FAME analysis of strain KP23^T showed the presence of 3-OH C16:0, which was a characteristic feature of the genus *Burkholderia* (Viallard *et al*., 1998). The main cellular fatty acids in the strain were similar to those of representative species in the genus *Burkholderia* (Yabuchi *et al*., 1992, 1995). Strain KP23^T was able to oxidize galactose, glucose, mannose, glycerol, inositol, mannitol and sorbitol. This oxidation profile resembles those of all species belonging to the genus *Burkholderia*. The total DNA G+C content of the isolate (64·8 mol %) was also within the range of *Burkholderia* species (Yabuchi *et al*., 1992; Urakami *et al*., 1994).

However, the similarities of the 16S rRNA gene sequence of strain KP23^T with those of all *Burkholderia* species were less than 96 %. The sequence similarities to closely related species, *B. caribensis*, *B. graminis* and *B. phenazinium*, were 95·9, 94·9 and 94·9 %, respectively. Such low sequence similarities suggest that the isolate can be assigned to a novel species of the genus. Our phenotypic analysis of strain KP23^T also revealed

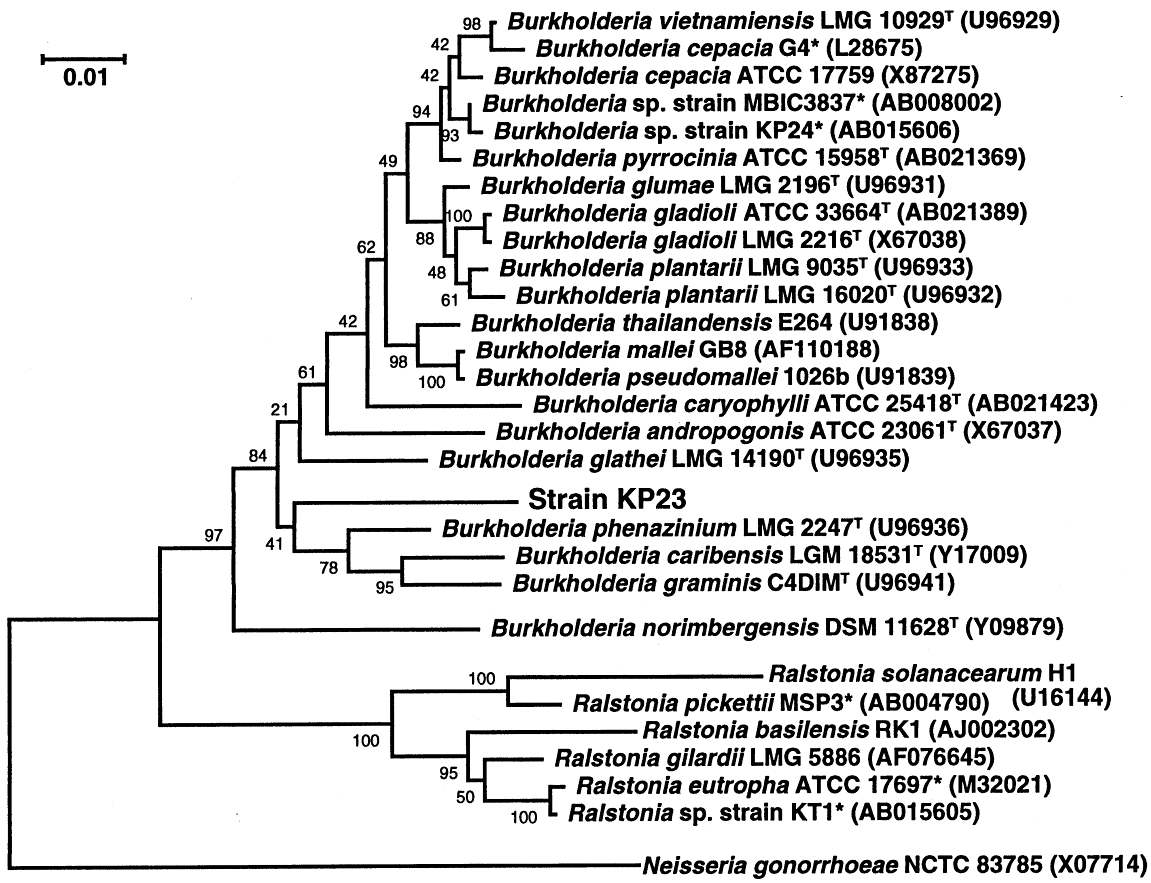

Fig. 3. Phylogenetic tree showing the relationship between strain KP23^T and related species in the β-subclass of the *Proteobacteria* based on 16S rDNA sequences. The bar represents 1 nucleotide substitution per 100 nucleotides. Bootstrap probabilities (Kumar *et al.*, 1993) are indicated at the branch points. Asterisks indicate strains or species which are known to be TCE degraders. The GenBank accession number for each reference strain is shown in parentheses.

that there are several sufficient differences between the isolate and existing members of the genus *Burkholderia* to assign it to a new species: (1) cells of strain KP23^T grown on PE medium are ovoids to short rods and morphologically different from known *Burkholderia* species, which are typical rod-shaped micro-organisms (Palleroni, 1984); (2) no motility is observed in strain KP23^T whilst all *Burkholderia* species (except *Burkholderia mallei*) are motile by means of one or several flagella; (3) strain KP23^T contains $C_{13:1}$ and $C_{17:1}$, which have not been detected in any other *Burkholderia* species; (4) the isolate grows optimally at 37 °C, whilst the optimum growth temperatures for almost all of the other strains belonging to the genus are in the range 28 to 30 °C.

Analysis of carbon source oxidation by the BIOLOG system revealed that the isolate was able to oxidize maltose. This is a remarkable nutritional feature of the isolate because all the other members of the genus *Burkholderia*, except for *B. caribensis*, lack the ability to oxidize this substrate. *B. caribensis* is one of the closest relatives based on 16S rRNA gene sequences. These two bacteria are, however, clearly distinguishable by their ability to oxidize lactose and trehalose: strain KP23^T does not oxidize these sugars but *B. caribensis* does.

On the basis of above phenotypic and phylogenetic analyses, strain KP23^T should be classified as a new species in the genus *Burkholderia*, and here we propose the name *Burkholderia kururiensis* sp. nov for the isolate.

Description of *Burkholderia kururiensis* sp. nov.

Burkholderia kururiensis (ku.ru.ri.en′sis. M.L. adj. *kururiensis* referring to Kururi, Chiba Prefecture, Japan, where the strain was isolated).

Cells are Gram-negative, non-motile, ovoids to short rods (1·0 μm in width and 1·2–1·5 μm in length), and occur singly or in pairs. Growth occurs between 15 and 42 °C with the optimum at 37 °C. The pH range is 6·0–7·8. Optimum growth occurs at pH 7·2. The doubling time is approximately 1 h under optimum growth conditions. Oxidase and catalase are produced. No hydrolysis of starch and gelatin is observed, but glycogen and Tween 80 are hydrolysed. The following are oxidized: arabinose, fructose, fucose, galactose, glucose, lactulose, maltose, mannose, psicose, rhamnose, adonitol, arabitol, glycerol, inositol, mannitol, sorbitol, xylitol, *N*-acetylgalactosamine, acetate, citrate, formate, galacturonate, gluconate, lactate, propionate, alanine, asparagine, aspartate, glutamate, glycine, histidine, leucine, phenylalanine, proline, serine, threonine, inosine, 2,3-butanediol, benzene, *p*-cresol, fluorobenzene and phenol. The following are not oxidized: cellobiose, lactose, melibiose, raffinose, sucrose, trehalose, dextrin, malonate, uridine, thymidine, glucose 1-phosphate and glucose 6-phosphate. UQ-8 is the dominant respiratory quinone. Main cellular fatty acids are $C_{16:0}$, cyclopropanic acid $C_{17:0}$, cyclopropanic acid $C_{19:0}$, $C_{16:1}$ and $C_{18:1}$. $C_{13:1}$ and $C_{17:1}$ are also present. The G+C content is 64·8 mol%. The organism was isolated from an aquifer polluted with TCE in Kururi, Chiba Prefecture, Japan and shows degradation activity for this contaminant when cells are grown in the presence of phenol. The type strain is KP23^T (= JCM 10599^T).

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