Devosia yakushimensis sp. nov., isolated from root nodules of *Pueraria lobata* (Willd.) Ohwi

Vernans V. Bautista,¹ Rosario G. Monsalud² and Akira Yokota¹

Correspondence

Vernans V. Bautista

wbver@yahoo.com

¹Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-Ku, Tokyo 113-0032, Japan

²National Institute of Molecular Biology and Biotechnology, University of the Philippines – Los Baños, 4031 College, Laguna, Philippines

A Gram-negative, strictly aerobic bacterium, comprising non-endospore-forming motile rods (1.2–2.0 µm × 0.4–0.6 µm) with polar flagellae was isolated from root nodules of the leguminous plant *Pueraria lobata* (Willd.) Ohwi growing on the coast of Yakushima Island, Kagoshima Prefecture, Japan. The novel strain, designated Yak96B^T, grew at an optimum pH of 7.0 and an optimum temperature of 28 °C. Phylogenetic analysis based on the 16S rRNA gene sequences revealed that the new strain was closely related to *Devosia neptuniae* J1^T and *Devosia chinhatensis* IPL18^T, with sequence similarities of 98.1 % and 97.8 %, respectively. However, the DNA–DNA relatedness values of strain Yak96B^T with *D. neptuniae* LMG 21357^T and *D. chinhatensis* CCM 7426^T were 53.6 % and 34 %, respectively. The DNA G + C content of strain Yak96B^T was 65.3 mol%, the predominant isoprenoid quinone was Q10 (85 %) and the polar lipids were phosphatidylglycerol and diphosphatidylglycerol. The major fatty acids (>5 %) were 11-methyl C_{18:1}ω7c (35.0 %), C_{16:0} (22.4 %), C_{18:1}ω7c (21.8 %), C_{19:0} cyclo ω8c (6.8 %) and C_{18:0} (5.4 %). The infection/nodulation test was negative and *nifH* and *nodD* genes were not detected. Based on its chemotaxonomic and physiological characteristics, strain Yak96B^T represents a novel species of the genus *Devosia*, for which the name *Devosia yakushimensis* sp. nov. is proposed. The type strain is Yak96B^T (=KCTC 22147^T=NBRC 103855^T=LMG 24299^T).

Strain Yak96B^T was identified during a study to isolate nitrogen-fixing bacteria from leguminous plants thriving along the coast of Japan. The strain, probably belonging to the genus *Devosia*, was isolated along with strains belonging to the genus *Rhizobium* from a root nodule of the kudzu plant or Japanese arrowroot [*Pueraria lobata* (Willd.) Ohwi], which is native to and widely distributed in Japan. While the symbiotic relationship of *Pueraria* sp. with strains of the genera *Bradyrhizobium* and *Mesorhizobium* is well established (Abaidoo *et al.*, 2000; Kwon *et al.*, 2005; Germano *et al.*, 2006), to the authors' knowledge, few studies, if any, have described the association of strains of the genus *Devosia* with the legume *Pueraria*. In this study, the relationships between strain Yak96B^T and the *Rhizobium* spp. isolated and species of the genus *Pueraria* was not explored.

The genus *Devosia* (family Hyphomicrobiaceae) was created from the reclassification of *Pseudomonas riboflavina* as *Devosia riboflavina* (Nakagawa *et al.*, 1996). At the time of

writing, the genus *Devosia* comprised eight recognized species: *Devosia riboflavina* (Nakagawa *et al.*, 1996), *Devosia neptuniae* (Rivas *et al.*, 2003), *Devosia limi* (Vanparys *et al.*, 2005), *Devosia soli* (Yoo *et al.*, 2006), *Devosia insulae* (Yoon *et al.*, 2007), *Devosia subaequoris* (Lee, 2007), *Devosia chinhatensis* (Kumar *et al.*, 2008) and *Devosia geojensis* (Ryu *et al.*, 2008). Among these species, only *D. neptuniae* was isolated from an aquatic leguminous plant, *Neptunia natans*, and reported to possess the nitrogen-fixing (*nifH*) and the nodulating (*nodD*) symbiotic genes (Rivas *et al.*, 2003). In contrast, the remaining species of the genus *Devosia* have been isolated from non-plant sources and do not possess these symbiotic genes. In this study, a strain of the genus *Devosia* isolated from a root nodule of a leguminous plant is reported but it was found to be devoid of the aforementioned symbiotic genes.

The main objective of this study was to determine the exact taxonomic position of strain Yak96B^T based on a polyphasic taxonomic approach, which included phylogenetic analysis of the 16S rRNA gene sequences, DNA–DNA hybridization and chemotaxonomic, physiological and morphological characterizations.

Strain Yak96B^T was isolated from a root nodule of *Pueraria lobata* (Willd.) Ohwi collected from Yakushima Island,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Yak96B^T is AB361068.

Three additional phylogenetic trees showing the position of strain Yak96B^T in relation to recognized species of the genus *Devosia* and other members of the family Hyphomicrobiaceae are available with the online version of this paper.

Kagoshima Prefecture, Japan. Cells were isolated by serial dilution using yeast extract-mannitol agar (Vincent, 1970) supplemented with Congo red (15 mg l^{-1}) and Kabicidin (Nihon Seiyaku) as an antifungal agent. Plates were incubated at 28°C for 4 days. Cells that grew on this medium formed small colonies ranging from 1 to 2 mm in diameter. The colonies had an entire margin and were slightly convex, shiny and light pink in colour.

D. riboflavina LMG 2277^T, *D. neptuniae* LMG 21357^T, *D. limi* LMG 22951^T, *D. soli* KACC 11509^T, *D. insulae* KCTC 12821^T, *D. subaequoris* KCTC 12772^T, *D. chinhatensis* CCM 7426^T and *Devosia geojensis* BD-c194^T were used as reference strains. The reference strains and strain Yak96B^T were routinely cultured in tryptic soy agar (TSA) at 28°C for 72 h, with the exception of *D. insulae* KCTC 12821^T, which was grown at 25°C for 6 days, and *D. subaequoris* KCTC 12772^T, which was cultured on marine agar (MA) and incubated at 28°C for 72 h.

Cell morphology was determined by phase-contrast microscopy (BX60, Olympus) and transmission electron microscopy (TEM). The motility of the organism was assessed using a motility medium amended with tetrazolium chloride to provide contrast with the motility bands created by the organism. Flagellar staining (Heimbrook *et al.*, 1989) and negative staining using TEM were used to elucidate the presence of the polar flagellum (Fig. 1). The Gram reaction was determined using 3% potassium hydroxide (Gregersen's method) and the BD Gram Stain kit (Becton, Dickinson and Co.).

The temperature range for the growth of strain Yak96B^T was determined using TSA and cultures were incubated at

$4, 10, 17, 25, 28, 30, 37$ and 45°C . Tolerance to NaCl was assessed by varying the concentration of NaCl from 0–6% (w/v) at increments of 0.5% (w/v) on TSA. The pH range for growth was determined with filter-sterilized ($0.45 \mu\text{m}$, Advantec) tryptic soy broth (TSB) adjusted from pH 4.0 to pH 10.5 with 0.5 pH unit increments. Growth under anaerobic conditions was determined using an anaerobic chamber with AnaeroPack (Mitsubishi Gas Chemical Co., Inc.). Catalase and oxidase activities were determined using freshly prepared 3% hydrogen peroxide and cytochrome oxidase paper (Nissui Pharmaceutical Co. Ltd), respectively. The enzyme activity profiles of strain Yak96B^T and the reference strains were assessed using API ZYM (bioMérieux) kits incubated at 28°C for 7.5 h. The substrate utilization profiles were determined by inoculating aliquots of cells into API 50 CH (bioMérieux) strips and Biolog GN2 Microplates (Biolog) followed by incubation at 28°C for 72 h. The GN2 Microplates were read using a Biolog Micro Station (Biolog). The differential characteristics of strain Yak96B^T and type strains of members of the genus *Devosia* are presented in Table 1.

Filter paper discs containing specified amounts of antibiotics were laid on lawns of cells (approx. 1×10^5 c.f.u. ml^{-1} based on the McFarland Standard) on TSA and MA plates to determine the antibiotic susceptibilities of strain Yak96B^T and the reference strains. The antibiotics used in this study were (μg per disc unless otherwise stated): amikacin sulfate (30), ampicillin (10), carbenicillin disodium salt (100), cephalotin (30), chloramphenicol (30), ciprofloxacin (5), gentamicin sulfate (10), kanamycin (30), neomycin (30), oxacillin sodium monohydrate (30), polymyxin B sulfate (100 U), rifampicin (5), tetracycline hydrochloride (30) and vancomycin hydrochloride (30).

The isoprenoid quinones and DNA G+C content were determined by the HPLC method using a 5C-18AR Cosmosil packed column (Nacalai Tesque) as described by Collins & Jones (1981) and Mesbah *et al.* (1989), respectively. The major (85%) respiratory quinone type of strain Yak96B^T was Q10, which makes it similar to *D. riboflavina*, *D. neptuniae*, *D. limi*, *D. soli*, and *D. geojensis*, but not *D. insulae*, in which Q11 predominates (Yoon *et al.*, 2007). The DNA G+C content was 65.3 mol%, which was within the range of 59.5–66.2 mol% included in the emended description of the genus *Devosia* (Yoon *et al.*, 2007). The polar lipids were determined by two-dimensional TLC according to Minnikin *et al.* (1979). The polar lipids of strain Yak96B^T were composed of phosphatidylglycerol and diphosphatidylglycerol.

Cells that had been subcultured twice on TSA (Difco) at 28°C for 72 h were used for fatty acid extraction with the Sherlock Microbial Identification System (MIDI) and analysed in a 6890 Series GC System equipped with a 7860 Series Injector (Agilent). The fatty acid profile of strain Yak96B^T was very similar to those of the other type strains of the genus *Devosia*. However, significant differences in the amounts of individual fatty acids clearly showed that

Fig. 1. Negatively stained cell of strain Yak96B^T. Bar, 1 μm .

Table 1. Some characteristics of strain Yak96B^T and closely related species of the genus *Devosia*

Strain: 1, Yak96B^T; 2, *D. riboflavina* LMG 2277^T; 3, *D. neptuniae* LMG 21357^T; 4, *D. limi* 22951^T; 5, *D. soli* KACC 11509^T; 6, *D. insulae* KCTC 12821^T; 7, *D. subaequoris* KCTC 12772^T; 8, *D. chinhatensis* CCM 7426^T; 9, *D. geogensis* BD-c194^T. All strains were incubated at 28 °C except *D. insulae* KCTC 12821^T, which was incubated at 25 °C. All data were obtained from this study. The DNA G+C content was obtained from the respective authors of the descriptions of the type strains (except for *D. chinhatensis* CCM 7426^T). +, Positive; –, negative; w, weak; s, sensitive; R, resistant.

Characteristic	1	2	3	4	5	6	7	8	9
Assimilation of:									
N-Acetylglucosamine	–	+	–	–	–	–	–	w	+
D-Glucose	+	+	+	+	–	–	–	+	+
L-Arabinose	+	+	+	+	–	–	–	+	–
D-Mannose	+	+	+	+	–	–	w	+	+
D-Mannitol	+	+	+	–	–	–	w	+	+
Maltose	+	+	+	+	–	–	w	+	+
Enzyme activities									
Acid phosphatase	+	+	+	+	+	+	–	+	+
Cystine arylamidase	–	–	–	–	–	–	–	w	–
Esterase (C4)	w	w	–	+	+	–	+	+	+
Esterase lipase (C8)	w	+	+	+	+	w	+	+	+
α-Fucosidase	–	+	+	–	–	w	–	–	–
α-Galactosidase	+	+	+	–	+	–	–	+	+
β-Galactosidase	+	+	+	–	+	+	+	+	+
α-Glucosidase	+	+	–	+	+	w	–	+	–
β-Glucosidase	+	+	+	+	+	+	w	+	+
Lipase (C14)	–	–	–	w	–	–	–	–	–
α-Mannosidase	+	+	+	–	+	–	–	–	–
Naphthol-AS-BI-phosphohydrolase	w	–	w	–	–	w	+	–	w
Trypsin	+	+	–	–	–	–	+	+	+
Valine arylamidase	–	–	–	+	+	–	–	+	w
Antibiotic susceptibility									
Chloramphenicol	S	S	S	S	S	S	S	S	R
Gentamicin	R	S	R	S	S	R	R	R	R
Kanamycin	R	R	R	S	R	R	R	R	R
Neomycin	S	S	S	S	S	R	R	R	S
Oxacillin	R	S	S	S	S	S	R	S	S
Tetracycline	S	S	S	S	S	R	S	S	S
Vancomycin	S	S	S	S	S	R	S	S	S
DNA G+C content (mol%)	65.3	61.4	62	61.9	59.5	66.2	59.1	63.5	60.8

strain Yak96B^T represents a novel species of the genus *Devosia* (Table 2). The predominant fatty acids (>5%) were 11-methyl C_{18:1}ω7c (35.0%), C_{16:0} (22.4%), C_{18:1}ω7c (21.8%), C_{19:0} cyclo ω8c (6.8%) and C_{18:0} (5.4%). Minor fatty acids, namely C_{13:0} iso 3-OH, C_{17:0} iso, C_{18:1}ω9c, and ECL 18.814, which have been found in other species of the genus *Devosia*, were not detected in strain Yak96B^T.

The DNA template for PCR amplification of the 16S rRNA gene was prepared according to the method of Hiraishi (1992). The gene was amplified by PCR primers 8F and 1510R (Brosius *et al.*, 1978). The amplified PCR products were cleaned using PEG 6000 and 70% ethanol. The refined PCR products were directly sequenced with the use of a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) using the conserved 16S rRNA gene primers indicated by Kurahashi & Yokota (2002). BigDye PCR

products were cleaned with CleanSEQ (Agencourt) and sequenced in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

The sequences were edited and aligned in BioEdit v7.0.5.3 (Hall, 1999) and compared with the sequences obtained from GenBank and the DNA Data Bank of Japan (DDBJ). Multiple alignments of the sequences were carried out in CLUSTAL_X v1.83 (Thompson *et al.*, 1997). Alignment gaps and ambiguous bases were not taken into consideration when the bases of 16S rRNA gene nucleotides were compared. The phylogenetic and molecular evolutionary analyses were determined using MEGA software v4 (Tamura *et al.*, 2007). Phylogenetic trees were constructed using the neighbour-joining method (Saitou & Nei, 1987) utilizing the Kimura two-parameter model (Kimura, 1983), Jukes–Cantor (Jukes & Cantor, 1969), maximum-composite likelihood and maximum-parsimony algorithms (see

Table 2. Fatty acid profiles of strain Yak96B^T and the type strains of the genus *Devosia*

Strains: 1, Yak96B^T; 2, *D. riboflavina* LMG 2277^T; 3, *D. neptuniae* LMG 21357^T; 4, *D. limi* LMG 22951^T; 5, *D. soli* KACC 11509^T; 6, *D. insulae* KCTC 12821^T; 7, *D. subaequoris* KCTC 12772^T; 8, *D. chinhatensis* CCM 7426^T; 9, *D. geogensis* BD-c194^T. Strains 1–5 and 8–9 were grown with TSA at 28 °C for 72 h. Strain 6 was grown with TSA at 25 °C for 6 days and strain 7 was grown on MA at 28 °C for 72 h. –, Not detected.

Fatty acid	1	2	3	4	5	6	7	8	9
C _{10:0} 3-OH	0.6	1.2	0.4	1.1	1.1	—	—	—	—
C _{13:0} iso 3-OH	—	—	—	—	—	1.1	—	—	—
C _{16:0}	22.4	18.7	20.6	11.8	18.1	14.1	8.7	12.6	17.4
C _{17:0} cyclo	0.4	0.6	2.4	0.5	—	—	0.2	—	—
C _{17:0} iso	—	—	—	—	—	13.4	—	—	—
C _{17:0}	2.0	—	0.3	0.5	—	2.2	0.8	3.2	3.0
C _{18:1} ω9c	—	—	—	—	—	1.9	—	—	—
C _{18:1} ω7c	21.8	47.2	24.5	33.8	55.4	17.1	57.7	55.4	26.3
C _{18:0} 3-OH	2.9	—	2.9	3.2	2.4	—	2.5	2.2	—
C _{18:0}	5.4	3.6	4.6	10.8	2.5	4.6	4.8	3.5	11.3
11-methyl C _{18:1} ω7c	35.0	24.8	31.8	30.5	17.3	31.0	20.9	19.8	30.6
C _{19:0} cyclo ω8c	6.8	1.5	8.8	2.7	0.4	1.7	1.0	—	3.7
ECL 14.959	1.2	—	1.2	1.2	—	—	0.8	—	—
ECL 18.814	—	—	—	—	—	11.0	—	—	7.4
Summed feature 3*	0.6	2.2	1.8	2.2	1.5	—	2.3	1.1	—

*Summed feature 3 comprised C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

Supplementary Figs S1, S2 and S3 in IJSEM Online). Evolutionary distances were computed using the Kimura two-parameter model and bootstrap values based on 1000 replications were selected in the program. Phylogenetic analysis based on 16S rRNA gene sequences (1356 bp) indicated that strain Yak96B^T was positioned within the genus *Devosia* (Fig. 2). It was closely related to *D. neptuniae* J1^T and *D. chinhatensis* IPL18^T with sequence similarities of 98.1 % and 97.8 %, respectively. The novel strain showed ≤97 % gene sequence similarities when compared with the other recognized species of the genus *Devosia*.

DNA–DNA hybridization was determined by using photobiotin-labelled probes in a polystyrene 96-well plate (NuncMaxisorp) as described by Ezaki *et al.* (1989). The hybridization was performed with five replications and the hybridization temperature was 55 °C. The mean DNA–DNA relatedness values of strain Yak96B^T to *D. neptuniae* LMG 21357^T and *D. chinhatensis* CCM 7426^T were 53.6 % and 34 %, respectively, which were lower than the 70 % value considered as the threshold for the delineation of genomic species (Stackebrandt & Goebel, 1994). These hybridization values clearly revealed that strain Yak96B^T represents a separate and a novel species of the genus *Devosia*.

The presence of the nitrogen-fixing (*nifH*) and nodulation (*nodD*) genes was tested using the primers and PCR conditions described by Rivas *et al.* (2002). The DNA templates of strain Yak96B^T, *D. neptuniae* LMG 21357^T and *Rhizobium leguminosarum* IAM 12609^T (as positive controls) for PCRs were prepared using the method of Marmur (1961), the Sarkosyl method (Rivas *et al.*, 2002)

and the boiling method followed by plasmid DNA extraction with a QuickLyse Miniprep kit (Qiagen). The control bacterial strains showed specific bands for both *nifH* and *nodD* genes, but no bands were observed for strain Yak96B^T. An infection assay was conducted according to the method of Somasegaran & Hoben (1994) using *Macropodium atropurpureum* (common name Siratro) as the host plant to determine the nodulation ability of the test strains. Strain Yak96B^T failed to nodulate with *M. atropurpureum*.

Description of *Devosia yakushimensis* sp. nov.

Devosia yakushimensis (ya.ku.shi.men'sis. N.L. fem. adj. *yakushimensis* pertaining to Yakushima, from where the organism was isolated).

Cells are obligately aerobic, Gram-negative, motile rods of 1.2–2.0 µm long and 0.4–0.6 µm wide that are non-endospore-forming and possess a polar flagellum. Colonies on TSA are circular with an entire margin (1–2 mm in diameter) and are convex, shiny and beige in colour after 2 days of incubation at 28 °C. Does not require NaCl for growth, but tolerates up to 2.5 % (w/v) NaCl. The pH range for growth is narrow at pH 5.5–7.0; good growth occurs at pH 7.0. The major (85 %) respiratory quinone is ubiquinone 10 (Q10). Polar lipids consist of phosphatidylglycerol and diphosphatidylglycerol. The predominant fatty acids (>5 %) are 11-methyl C_{18:1}ω7c, C_{16:0}, C_{18:1}ω7c, C_{19:0} cyclo ω8c and C_{18:0}. Positive in tests for the assimilation of D- and L-arabinose, D-ribose, D- and L-xylose, D-galactose, D-glucose, D-mannose, L-rhamnose, aesculin, salicin, cellobiose, maltose, D-lactose (bovine

Fig. 2. Neighbour-joining (Saitou & Nei, 1987) phylogenetic tree using the Kimura two-parameter model (Kimura, 1983), based on 16S rRNA gene sequences showing the taxonomic position of strain Yak96B^T with respect to recognized species of the genus *Devosia* and some members of the family *Hyphomicrobiaceae*. Filled circles indicate generic branches that were also recovered by the Jukes–Cantor model (Jukes & Cantor, 1969) and maximum-composite likelihood algorithms. Bootstrap values >50% are shown as percentages of 1000 replications. GenBank accession numbers are given in parentheses. The 16S rRNA sequence for *S. paucimobilis* was obtained from the NITE-NBRC website. Bar, 1 substitution per 100 nucleotides.

origin), D-melibiose, gentiobiose, D-lyxose, D-tagatose, D- and L-fucose, potassium 5-ketogluconate, D-fructose, L-fucose, *myo*-inositol, raffinose, D-sorbitol, trehalose and D-gluconic acid. Produces urease, catalase, oxidase, alkaline phosphatase, leucine arylamidase, trypsin, acid phosphatase, α - and β -galactosidase, α - and β -glucosidase, *N*-acetyl- β -glucosaminidase and α -mannosidase, but not lipase (C14), valine arylamidase, cystine arylamidase, α -chymotrypsin, β -glucuronidase or α -fucosidase. Weak enzyme activity for esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase. Susceptible to amikacin, ampicillin, carbenicillin, cephalotin, chloramphenicol, neomycin, rifampicin, tetracycline and vancomycin, but resistant to ciprofloxacin, gentamicin, kanamycin, oxacillin and polymyxin B.

The type strain, Yak96B^T (=KCTC 22147^T=NBRC 103855^T=LMG 24299^T), was isolated from a root nodule of the kudzu plant [*Pueraria lobata* (Willd.) Ohwi] thriving on the coast of Yakushima Island, Kagoshima Prefecture, Japan. The DNA G+C content of the type strain is 65.3 mol%.

Acknowledgements

The authors are deeply grateful to the Salt Science Research Foundation for the generous research funds, to the Ministry of Education, Culture, Sports, Science and Technology of Japan for the scholarship grant to the senior author, to Professor Karen A. Salandanan for valuable suggestions, and to the BCM/LMG, CCM and Dr Che Ok Jeon for generously providing the strains of *D. neptuniae* LMG 21357^T, *Devosia chinhatensis* CCM 7426^T and *D. geojensis* BD-c194^T, respectively.

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