Alcanivorax balearicus sp. nov., isolated from Lake Martel

Raúl Rivas,^{1,2} Paula García-Fraile,¹ Alvaro Peix,³ Pedro F. Mateos,¹ Eustoquio Martínez-Molina¹ and Encarna Velázquez¹

Correspondence

Raúl Rivas
raul@wwwedu-micro.usal.es

¹Departamento de Microbiología y Genética, Universidad de Salamanca, Spain

²Laboratorium voor Microbiologie, Vakgroep Biochemie, Fysiologie en Microbiologie, Universiteit Gent KL, Ledeganckstraat 35, B-9000 Gent, Belgium

³Instituto de Recursos Naturales y Agrobiología, IRNASA-CSIC, Salamanca, Spain

A bacterial strain designated MACL04^T was isolated from Lake Martel, a subterranean saline lake in Mallorca (Spain). The complete 16S rRNA gene sequence of this strain showed nearly 100 % similarity to that of *Alcanivorax dieselolei* B-5^T. Despite this high similarity, strain MACL04^T showed phenotypic, chemotaxonomic and molecular differences with respect to *A. dieselolei*, indicating that strain MACL04^T represents a separate species. Cells of strain MACL04^T were motile by means of a single polar or subpolar flagellum and colonies formed on media containing 1 % (v/v) Tween 20 were opaque and mucoid, with blue–green iridescence. The generation time of strain MACL04^T in this medium was approximately half that of *A. dieselolei* B-5^T and strain MACL04^T did not produce lipases after incubation for 5 days. Strain MACL04^T did not require NaCl for growth and grew in the presence of up to 15 % (w/v) NaCl. The strain was able to use alkanes as a sole carbon source; however, glucose could also be used, albeit weakly, as a carbon source. Several amino acids and organic acids were used as carbon sources. Strain MACL04^T produced acid in media containing pyruvate as the sole carbon source. The major fatty acids were C_{19:0} cyclo ω8c and C_{16:0}. The fatty acid C_{16:1} ω8c, present in strain MACL04^T, was not detected in the recognized *Alcanivorax* species. The sequences of the large and short 16S–23S intergenic spacer regions showed similarities of 97.2 and 98.8 % (ungapped) with respect to *A. dieselolei* B-5^T. Partial sequences of *gyrB* and *alkB* genes showed 94.0 % similarity between strain MACL04^T and *A. dieselolei* B-5^T. The G + C content of strain MACL04^T was 62.8 mol%. The data from this polyphasic study indicate that strain MACL04^T represents a novel species of the genus *Alcanivorax*, for which the name *Alcanivorax balearicus* sp. nov. is proposed. The type strain is MACL04^T (=LMG 22508^T = CECT 5683^T).

The genus *Alcanivorax* currently comprises four species, *A. borkumensis*, *A. jadensis*, *A. venustensis* and *A. dieselolei*, all of which were isolated from marine environments (Yakimov

et al., 1998; Bruns & Berthe-Corti, 1999; Fernández-Martínez *et al.*, 2003; Liu & Shao, 2005). Here we characterize a strain, designated MACL04^T, which was isolated from water collected from Lake Martel, a subterranean saline lake in Mallorca (Balearic Islands), Spain. Strain MACL04^T is phylogenetically close to *A. dieselolei* B-5^T but, based on its phenotypic, chemotaxonomic and molecular characteristics, we propose that it should be considered to represent a novel species within the genus *Alcanivorax*.

Strain MACL04^T was isolated under aseptic conditions from water samples taken at a depth of 10 cm, during a bacterial biodiversity study of Lake Martel. Under sterile conditions, a 200 ml sample was filtered under vacuum through a membrane filter with a pore diameter of 45 µm (Millipore). The membrane was placed on a plate containing YED medium (0.5 % yeast extract, 0.7 % glucose and 2 % agar, w/v) supplemented with 1.5 % (w/v) NaCl and incubated at 28 °C.

Abbreviations: ITS, intergenic spacer; TP-RAPD, two-primers randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for strain MACL04^T are AY686709 (16S rRNA gene), EF091805 (small ITS), EF091804 (large ITS), AY683535 (partial *alkB* gene) and EF091802 (partial *gyrB* gene).

Figures showing the colony morphologies of strain MACL04^T and *Alcanivorax dieselolei* B-5^T grown on media containing Tween 20, an electron micrograph of a cell of strain MACL04^T grown on nutrient agar, and TP-RAPD profiles and phylogenetic trees based on 16S–23S ITS and partial *alkB* and *gyrB* gene sequences and a table detailing the fatty acid profiles of strain MACL04^T and recognized species of the genus *Alcanivorax* are available as supplementary material with the online version of this paper.

Cells of strain MACL04^T formed white–cream-coloured colonies on nutrient agar medium supplemented with 1.5 % NaCl. Optimum growth was observed on TPA medium (1 % v/v Tween 20, 1 % w/v peptone, 0.5 % w/v NaCl, 0.01 % w/v CaCl₂ and 1.5 % w/v agar). On this medium, colonies of strain MACL04^T were opaque and showed blue–green iridescence, whereas colonies of *A. dieselolei* B-5^T were transparent and glistening, without coloured iridescence (see Supplementary Fig. S1 available in IJSEM Online). Gram staining was carried out by using the procedure described by Doetsch (1981). Cells were gently suspended in sterile water, stained with 0.2 % uranyl acetate and examined at 80 kV with a Zeiss EM 209 transmission electron microscope (Peix *et al.*, 2003). Cells of strain MACL04^T were Gram-negative, short rod-shaped (1.1–1.3 × 0.6–0.8 µm) and motile by means of a polar or subpolar flagellum (see Supplementary Fig. S2 available in IJSEM Online), whereas cells of *A. dieselolei* showed several lophotrichous flagella (see Table 1). The generation times of strain MACL04^T and *A. dieselolei* B-5^T were compared using turbidimetry in liquid TPA medium (Koch, 1981). The results showed that *A. dieselolei* B-5^T had a generation time of approximately 5 h, whereas the generation time for strain MACL04^T was approximately 2.5 h.

For sequencing of the 16S rRNA gene, DNA extraction was carried out as described previously (Rivas *et al.*, 2001). Amplification of the gene and sequencing were performed according to methods described previously (Rivas *et al.*, 2002b). An almost complete 16S rRNA gene sequence was obtained and compared with sequences deposited in databases. The sequences were aligned using CLUSTAL_X

software (Thompson *et al.*, 1997) and distances were calculated according to the Kimura two-parameter method (Kimura, 1980). Phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987) and bootstrap analysis was performed, based on 1000 resamplings. The MEGA2 package (Kumar *et al.*, 2001) was used for all analyses. Comparison with sequences held in GenBank indicated that strain MACL04^T was very closely related to *A. dieselolei* (Figure 1). The 16S rRNA gene sequence of strain MACL04^T showed nearly 100 % similarity to that of *A. dieselolei* B-5^T. Despite this fact, taking into account the morphological and growth behaviour differences between strain MACL04^T and *A. dieselolei* B-5^T, we performed a polyphasic study to establish the taxonomic status of strain MACL04^T.

Two-primers randomly amplified polymorphic DNA (TP-RAPD) patterns were analysed as described previously (Rivas *et al.*, 2002a), using the primer pair 879F (5'-GCCTGGGGAGTACGGCCGCA-3') and 1522R (5'-AAGGAGGTGATCCANCCRCA-3'), which correspond to *Escherichia coli* positions 879–898 and 1509–1522, respectively. According to our previous results, strains showing the same pattern belong to the same species and different species display different TP-RAPD patterns (Rivas *et al.*, 2001, 2004). Moreover, from results obtained previously, TP-RAPD patterns are not strain dependent (Rivas *et al.*, 2001) and in some cases the profiles can be slightly different in subspecies of the same species (Rivas *et al.*, 2002a). Therefore, we analysed the TP-RAPD pattern of strain MACL04^T and compared it with those of the type strains of recognized species of the genus *Alcanivorax* (see

Table 1. Differential phenotypic characteristics among *Alcanivorax balearicus* sp. nov. strain MACL04^T and recognized species of the genus *Alcanivorax*

Taxa: 1, strain MACL04^T; 2, *A. dieselolei*; 3, *A. borkumensis*; 4, *A. jadensis*; 5, *A. venustensis*. Data from Yakimov *et al.* (1998), Bruns & Berthe-Corti (1999), Fernández-Martínez *et al.* (2003), Liu & Shao (2005) and this study. +, Positive, –, negative; w, weak; v, variable; ND, no data.

Characteristic	1	2	3	4	5
Motility/flagella arrangement	+ /Polar or subpolar flagellum	+ /Lophotrichous flagella	–	–	+ /Polar flagella
Ionic requirement	–	Na ⁺	Complex	Na ⁺	Complex
Growth at 17 % NaCl	–	w*	–	–	+
Growth at 45 °C	–	+	–	+	+
Hydrolysis of Tween 20 after 4 days incubation	–*	+	ND	ND	ND
Utilization of carbohydrates:					
Glucose	w	–	–	–	–
L-Arabinose	w	–	–	ND	–
Utilization of organic acids:					
Acetate	w	+	+	+	+
Lactate	–	+	ND	ND	v
Pyruvate	+ (acid pH)	+ (alkaline pH)	+	+	+
Propionate	w	–*	+	ND	+

*Data from this study.

Fig. 1. Phylogenetic tree obtained using the neighbour-joining method based on nearly complete 16S rRNA gene sequences of strain MACL04^T and recognized species of the genus *Alcanivorax*. The significance of each branch is indicated by percentage bootstrap values calculated for 1000 subsets. Bar, 1 substitution per 100 nucleotides. The sequence of *Pseudomonas fluorescens* IAM 12022 was used as an outgroup.

Supplementary Fig. S3 available in IJSEM Online). The TP-RAPD profiles of all these strains were different and the differences observed between *A. dieselolei* B-5^T and strain MACL04^T indicated that they could belong to different species.

The 16S–23S intergenic spacer (ITS) region was amplified and sequenced as described by Liu & Shao (2005). PCR amplification of the ITS region yielded two products, as occurs for all *Alcanivorax* species. Phylogenetic analysis based on the ITS sequences showed similar groupings to those obtained using the 16S rRNA gene and confirmed the close phylogenetic relationship between strain MACL04^T and *A. dieselolei* (see Supplementary Fig. S4 available in IJSEM Online). The sequences of the large and short ITS regions showed 97.2 and 98.8 % similarities (ungapped) with respect to *A. dieselolei* B-5^T. As the genus *Alcanivorax* currently comprises only four species that are well separated phylogenetically, we could not come to any conclusions about the similarity among strains from different species. Nevertheless, the similarity values between strain MACL04^T and *A. dieselolei* B-5^T were similar to those found in different species of *Pseudomonas* that are phylogenetically closely related on the basis of 16S rRNA gene sequences. For example, the ITS regions of *Pseudomonas graminis* and *Pseudomonas lutea* (GenBank accession nos DQ023307 and EF091800, respectively) exhibit nearly 99 % similarity.

A fragment of the *alkB* gene was amplified and sequenced using the primers ALKBF (5'-TCAATACMGGDCAYGAG-3') and ALKBR (5'-TAGTTSACSAYCTCCAGC-3'), which were designed in this study based on the conserved regions of the codifying genes for alkane hydrolase sequences from *Alcanivorax* species. The PCR conditions used were: pre-heating at 95 °C for 9 min; 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. Strain MACL04^T showed 94 % similarity with respect to *A. dieselolei* B-5^T. Phylogenetic analysis based on the *alkB* gene yielded results similar to those obtained in the 16S and ITS analyses, showing that strain MACL04^T and *A. dieselolei* B-5^T form a group together with *A. venustensis* that is phylogenetically separate from the other species of the genus *Alcanivorax* (see Supplementary Fig. S5 available in IJSEM Online).

Partial sequences of the *gyrB* gene were obtained for strain MACL04^T and *A. dieselolei* B-5^T, by using primers designed on the basis of conserved sequences of a hypervariable zone of this gene in *Alcanivorax* and other phylogenetically related species sequenced previously by Okamoto *et al.* (2004): GYRBUNIVF (5'-GGBCTBCAYGGBGTRGG-3') and GYRBUNIVR (5'-CCC GCWGARTCACCTCC-3'). The PCR conditions used were the same as those for the amplification of the *alkB* gene described above, except that the annealing temperature used was 52 °C. Strain MACL04^T showed 94 % similarity with respect to strain *A. dieselolei* B-5^T. Despite the fact that this value was higher than those obtained among other species of *Alcanivorax*, they are distant from a phylogenetic point of view and, as was observed in the case of the ITS region, similarity limits among the closely related species of this genus could not be established (see Supplementary Fig. S6 available in IJSEM Online). However, in the related genus *Pseudomonas*, similarity values greater than 95 % for *gyrB* have been reported for several species, such as *Pseudomonas kilonensis*, *Pseudomonas brassicacearum* and *Pseudomonas thivervalensis* (Cladera *et al.*, 2006).

Analysis of the fatty acid composition was performed as described by Zimmermann *et al.* (1998) using the culture conditions recommended by Yakimov *et al.* (1998). This analysis was performed at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) as was also the case for *A. dieselolei*. The cellular fatty acid pattern of strain MACL04^T comprised 17 different fatty acids (see Supplementary Table S1 available in IJSEM Online). The major fatty acid of strain MACL04^T was C_{19:0} cyclo ω8c (29.33 %). The fatty acids C_{16:0} (24.40 %) and C_{18:1ω7c} (14.68 %) were also present in high amounts. Other fatty acids were present in smaller amounts, including C_{16:1ω7c} (7.90 %), C_{12:0} 3-OH (5.84 %), C_{12:0} (5.46 %), C_{10:0} (2.56 %), C_{16:1ω8c} (2.10 %), C_{17:0} (1.49 %), C_{17:0} cyclo (1.45 %) and C_{12:0} 2-OH (1.38 %). Fatty acids C_{11:0}, C_{18:0}, 11-methyl C_{18:1ω7c}, C_{20:2ω6,9c}, an unidentified fatty acid with an equivalent chain-length value of 12.484 and an unidentified fatty acid with an equivalent chain-length value of 11.799 were present in amounts of less than 1 %. This profile has several differences with respect to that of *A. dieselolei* (Liu & Shao, 2005). The major fatty acid of strain MACL04^T, C_{19:0} cyclo ω8c, was not the main fatty acid in *A.*

dieselolei. Such a large amount of C_{19:0} cyclo ω 8c has not been reported previously for *Alcanivorax* species. The fatty acid C_{18:1} ω 7c was one of the two major fatty acids in *A. dieselolei*, but not in strain MACL04^T. The fatty acid C_{16:1} ω 8c present in strain MACL04^T was not detected in *A. dieselolei* or in the other *Alcanivorax* species. Quantitative differences were detected for C_{16:0}, C_{16:1} ω 7c and C_{12:0} 3-OH between strain MACL04^T and *A. dieselolei* B-5^T.

DNA for base composition analysis was prepared according to Chun & Goodfellow (1995). The G+C content of the DNA was determined using the thermal denaturation method (Mandel & Marmur, 1968). The DNA G+C content of strain MACL04^T was 62.8 mol%. DNA–DNA hybridization was carried out using the method of Ezaki *et al.* (1989), following the recommendations of Willems *et al.* (2001). The results obtained showed 67% ($\pm$ 1%, SD of three determinations) DNA–DNA relatedness between strain MACL04^T and *A. dieselolei* B-5^T.

The phenotypic characterization was performed as described by Yakimov *et al.* (1998), Bruns & Berthe-Corti (1999) and Liu & Shao (2005). In all the tests, *A. dieselolei* B-5^T was used as a reference. Utilization of carbon sources was tested using SM1 medium (Yakimov *et al.*, 1998), supplemented with 1.5% (w/v) NaCl and 1% (w/v) of the various carbon sources (Table 1). The pH indicator Bromothymol blue (0.05%, w/v) was included in the media. Assimilation of n-alkanes (decane, tetradecane, hexadecane and eicosane) was tested as described by Bruns & Berthe-Corti (1999). The physiological and biochemical characterizations were completed using API 20NE strips (bioMérieux), according to the manufacturer's instructions. The medium supplied with the API 20NE test strips was supplemented with 1.5% (w/v) NaCl before use. The temperature range for growth was determined by incubating cultures in YED medium supplemented with 1.5% (w/v) NaCl at 4–45 °C. The pH range was determined using YED medium, with final pH values of 4.0–10.0. Salt tolerance was studied using nutrient agar medium (Difco, Becton-Dickinson) containing 0–20% (w/v) NaCl. Antibiotic susceptibility tests were performed as described by Liu & Shao (2005).

The phenotypic characteristics of strain MACL04^T are given in Table 1. Unlike other species of the genus *Alcanivorax*, strain MACL04^T did not require NaCl for growth. Strain MACL04^T was unable to grow at 45 °C, whereas *A. dieselolei* can grow at this temperature. Utilization of glucose and L-arabinose by strain MACL04^T was weakly positive, whereas *A. dieselolei* was unable to use carbohydrates as was reported previously by Liu & Shao (2005). Hydrolysis of Tween 20 was negative after 4 days incubation for strain MACL04^T, whereas at this time *A. dieselolei* B-5^T produced abundant lipases (tweenases). Strain MACL04^T differed in the use of acetate, lactate and propionate as sole carbon sources. Use of pyruvate was positive in both strain MACL04^T and *A. dieselolei* B-5^T; however, acid pH was produced by strain

MACL04^T, whereas alkaline pH was produced by *A. dieselolei* B-5^T.

The phenotypic differences together with differences observed in the chemotaxonomic and molecular analyses support the classification of strain MACL04^T as representing a novel species of the genus *Alcanivorax*, for which the name *Alcanivorax balearicus* sp. nov. is proposed.

Description of *Alcanivorax balearicus* sp. nov.

Alcanivorax balearicus (ba.le.a'ri.cus. L. masc. adj. *balearicus* of the Balearic Islands, where the organism was isolated).

Gram-negative, aerobic, rod-shaped cells. Motile by means of a polar or subpolar flagellum. Colonies on nutrient agar supplemented with 1.5% (w/v) NaCl are circular, smooth and white–cream-coloured; colonies are opaque and mucoid with blue–green iridescence on media containing Tween 20, and usually 2–4 mm in diameter within 5 days at 28 °C. Oxidase- and catalase-positive. Urease-negative. Phylogenetically related to members of the family *Alcanivoracaceae*. Halotolerant; ionic supplements are not required for growth. Major fatty acids are C_{19:0} cyclo ω 8c, C_{16:0} and C_{18:1} ω 7c; fatty acids C_{16:1} ω 7c, C_{12:0} 3-OH, C_{12:0}, C_{10:0}, C_{16:1} ω 8c, C_{17:0}, C_{17:0} cyclo, C_{12:0} 2-OH, C_{18:0}, 11-methyl C_{18:1} ω 7c, C_{20:2} ω 6,9c and C_{11:0} are present in smaller amounts. Growth occurs in NaCl at concentrations up to 15% (w/v), although NaCl is not required for growth. Temperature range for growth is 4–37 °C (optimal growth occurs at 28 °C). pH range for growth is 5.5–9 (optimal growth occurs at pH 7). Negative for production of lipase in media containing Tween 20 after 4 days incubation at 28 °C; weak and slow production is detected after incubation for 1 week. Aesculin, casein, gelatin and starch are not hydrolysed and arginine dihydrolase and β -galactosidase activities and indole production and nitrate reduction are negative. Can grow using glucose and other carbohydrates as a carbon source. Decane, tetradecane, hexadecane, eicosane, adipate, caproate, citrate, malate, mannitol, phenylacetate, glutarate, quinate and pyruvate can be used as sole carbon sources. Weak growth occurs with acetate, propionate, glucuronate and L-arabinose. Does not grow with lactate, N-acetylglucosamine, gentiobiose, maltose, D-mannose, xylose or sucrose. Sensitive to neomycin and polymyxin B and resistant to cefuroxime, ampicillin, penicillin, erythromycin and ciprofloxacin. The DNA G+C content of the type strain is 62.8 mol%.

The type strain, MACL04^T (= LMG 22508^T = CECT 5683^T), was isolated from water from Lake Martel, a subterranean saline lake in the Balearic Islands, Spain.

Acknowledgements

This work was supported by the CAICYT-DGES and the JCyL (Spanish Government). We are grateful to the DSMZ staff for chemotaxonomic analyses. R.R. acknowledges a PhD fellowship from Ministerio de Educación y Ciencia.

References

- Bruns, A. & Berthe-Corti, L. (1999). *Fundibacter jadensis* gen. nov., sp. nov., a new slightly halophilic bacterium, isolated from intertidal sediment. *Int J Syst Bacteriol* **49**, 441–448.
- Chun, J. & Goodfellow, M. (1995). A phylogenetic analysis of the genus *Nocardia* with 16S rRNA gene sequences. *Int J Syst Bacteriol* **45**, 240–245.
- Cladera, A. M., Sepúlveda-Torres, L. del C., Valens-Vadell, M., Meyer, J.-M., Lalucat, J. & García-Valdés, E. (2006). A detailed phenotypic and genotypic description of *Pseudomonas* strain OX1. *Syst Appl Microbiol* **29**, 422–430.
- Doetsch, R. N. (1981). Determinative methods of light microscopy. In *Manual of Methods for General Bacteriology*, pp. 21–33. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington: American Society for Microbiology.
- 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.
- Fernández-Martínez, J., Pujalte, M. J., García-Martínez, J., Mata, M., Garay, E. & Rodríguez-Valera, F. (2003). Description of *Alcanivorax venustensis* sp. nov. and reclassification of *Fundibacter jadensis* DSM 12178^T (Bruns and Berthe-Corti 1999) as *Alcanivorax jadensis* comb. nov., members of the emended genus *Alcanivorax*. *Int J Syst Evol Microbiol* **53**, 331–338.
- 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.
- Koch, A. L. (1981). Growth measurement. In *Manual of Methods for General Bacteriology*, pp. 21–33. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington: American Society for Microbiology.
- Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics* **17**, 1244–1245.
- Liu, C. & Shao, Z. (2005). *Alcanivorax dieselolei* sp. nov., a novel alkane-degrading bacterium isolated from sea water and deep-sea sediment. *Int J Syst Evol Microbiol* **55**, 1181–1186.
- Mandel, M. & Marmur, J. (1968). Use of ultraviolet absorbance temperature profile for determining the guanine plus cytosine content of DNA. *Methods Enzymol* **12B**, 195–206.
- Okamoto, T., Maruyama, A., Imura, S., Takeyama, H. & Naganuma, T. (2004). Comparative phylogenetic analyses of *Halomonas variabilis* and related organisms based on 16S rRNA, *gyrB* and *ectBC* gene sequences. *Syst Appl Microbiol* **27**, 323–333.
- Peix, A., Rivas, R., Mateos, P. F., Martínez-Molina, E., Rodríguez-Barrueco, C. & Velázquez, E. (2003). *Pseudomonas rhizosphaerae* sp. nov., a novel species that actively solubilizes phosphate *in vitro*. *Int J Syst Evol Microbiol* **53**, 2067–2072.
- Rivas, R., Velázquez, E., Valverde, A., Mateos, P. F. & Martínez-Molina, E. (2001). A two primers random amplified polymorphic DNA procedure to obtain polymerase chain reaction fingerprints of bacterial species. *Electrophoresis* **22**, 1086–1089.
- Rivas, R., Velázquez, E., Palomo, J. L., Mateos, P., García-Benavides, P. & Martínez-Molina, E. (2002a). Rapid identification of *Clavibacter michiganensis* subspecies *sepedonicus* using two primers random amplified polymorphic DNA (TP-RAPD) fingerprints. *Eur J Plant Pathol* **108**, 179–184.
- Rivas, R., Velázquez, E., Willems, A., Vizcaino, N., Subba-Rao, N. S., Mateos, P. F., Gillis, M., Dazzo, F. B. & Martínez-Molina, E. (2002b). A new species of *Devosia* that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume *Neptunia natans* (L.f.) Druce. *Appl Environ Microbiol* **68**, 5217–5222.
- Rivas, R., Abril, A., Trujillo, M. E. & Velázquez, E. (2004). *Sphingomonas phyllosphaerae* sp. nov., from the phyllosphere of *Acacia caven* in Argentina. *Int J Syst Evol Microbiol* **54**, 2147–2150.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- 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 Acid Res* **25**, 4876–4882.
- Willems, A., Doignon-Bourcier, F., Goris, J., Coopman, R., de Lajudie, P., De Vos, P. & Gillis, M. (2001). DNA–DNA hybridization study of *Bradyrhizobium* strains. *Int J Syst Evol Microbiol* **51**, 1315–1322.
- Yakimov, M. M., Golyshin, P. N., Lang, S., Moore, E. R. B., Abraham, W.-R., Lünsdorf, H. & Timmis, K. N. (1998). *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. *Int J Syst Bacteriol* **48**, 339–348.
- Zimmermann, O., Spröer, C., Kroppenstedt, R. M., Fuchs, E., Köchel, H. G. & Funke, G. (1998). *Corynebacterium thomsseni* sp. nov., a *Corynebacterium* with *N*-acetyl- β -glucosaminidase activity from human clinical specimens. *Int J Syst Bacteriol* **48**, 489–494.