Microbacterium mitrae sp. nov., isolated from salted turban shell

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A novel bacterium (strain M4-8^T) belonging to the genus *Microbacterium* was isolated from salted turban shell, which is a traditional fermented food in Korea. Its morphology, physiology, biochemical features and 16S rRNA gene sequence were characterized. Cells of this strain were Gram-positive, non-motile, non-spore-forming rods that formed yellow-pigmented colonies. It grew in 0–8 % (w/v) NaCl and at 15–37 °C, with optimal growth occurring in 1 % (w/v) NaCl and at 30 °C. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain M4-8^T is associated with members of the genus *Microbacterium.* Within the phylogenetic tree, this novel strain shared a branching point with *Microbacterium hominis* IFO 15708^T (97.8 % similarity). The DNA G+C content was 71.3 mol% and DNA–DNA hybridization experiments showed a low level (<29 %) of DNA–DNA relatedness between M4-8^T and its closest relatives. The major fatty acids were iso-$C_{15:0}$ and anteiso-$C_{15:0}$ and the major cell-wall diamino acid was ornithine. Data obtained from DNA–DNA hybridization and chemotaxonomic phenotypic analysis support the conclusion that strain M4-8^T represents a novel species within the genus *Microbacterium*. The name *Microbacterium mitrae* sp. nov. is proposed, with M4-8^T (=KACC 21129^T =JCM 16363^T) as the type strain.

The genus *Microbacterium* was first described by Orla-Jensen (1919) and members of the genus can be isolated from a wide range of environments including soil, insects, human clinical specimens, marine environments and plants (Evtushenko & Takeuchi, 2006; Park *et al.*, 2008; Bakir *et al.*, 2008; Takeuchi & Hatano, 1998a, b; Lee *et al.*, 2006; Shivaji *et al.*, 2007; Collins & Bradbury, 1992). Members of the genus have also been found in the phyllospheres of sugar beet and spring wheat and as endophytes in sweetcorn and cotton (Thompson *et al.*, 1993; Legard *et al.*, 1994; McInroy & Kloepper, 1995).

In this study, a novel isolate (strain M4-8^T) belonging to the genus *Microbacterium* was isolated from salted turban shell, which is a traditional fermented food from Korea. The cultured cell biomass for cellular composition analysis and DNA extraction was collected from marine agar (MA; BBL) plates incubated at 30 °C for 2 days. The Gram reaction was performed using the non-staining method described by Buck (1982). Motility was examined by the wet-mount method and spore formation was analysed using the staining method of Schaeffer & Fulton (1933). Cell morphology was observed under a Nikon phase-contrast microscope at ×1000 magnification using cells grown for 2 days at 30 °C on MA plates. Growth at different temperatures (4, 15, 25, 30, 37 and 45 °C) and pH (3.0–13.0 at intervals of 1.0 pH unit) was assessed on marine broth (MB; BBL). NaCl tolerance was determined at 30 °C in MB prepared without NaCl and then supplemented with 0–10 (in increments of 1 %), 15 and 20 % (w/v) NaCl. Enzyme activity and utilization of various substrates as sole carbon sources were determined using API 20NE and API ZYM strips according to the manufacturer's instructions (bioMérieux). Casein and starch hydrolysis was tested as described by Smibert & Krieg (1994). Catalase activity was determined by observing bubble production in 3 % (v/v) hydrogen peroxide solution and oxidase activity was determined using an oxidase reagent (bioMérieux). Indole and H_2S formation were tested as described previously (Benson, 1994).

Strain M4-8^T was non-spore-forming, Gram-positive, rod-shaped and non-motile. After 2 days of incubation on MA at 30 °C, colonies were circular (0.5–2.0 mm in diameter), smooth, yellow and convex. Strain M4-8^T was able to grow at 15–37 °C, at pH 6.0–9.0 and in 0–8 % NaCl. Optimal growth conditions were 30 °C, pH 7.0 and 1 % NaCl. The strain was catalase-positive, oxidase-negative and able to hydrolyse casein but not starch. A detailed species description is presented below. Table 1 lists the characteristics that differentiate strain M4-8^T from related members of the genus *Microbacterium*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain M4-8^T is GQ351351.

Table 1. Differential characteristics of strain M4-8^T and the type strains of closely related species

Strains: 1, *Microbacterium mitrae* sp. nov. M4-8^T; 2, *M. hominis* IFO 15708^T; 3, *M. xylanilyticum* S3-E^T; 4, *M. flavescens* DSM 20643^T; 5, *M. trichothecenolyticum* IFO 15077^T. Data are from this study, except where marked. +, Positive; −, negative.

Characteristic	1	2	3	4	5
Colony morphology	Smooth, yellow	Smooth, yellowish-white	Smooth, yellow	Smooth, yellow	Smooth, yellow
NaCl range (%) for growth	0–8	0–3	1–3	0–4	0–4
Motility	−	−	+	−	−
Growth at 37 °C	+	+	+	−	−
Oxidase activity	−	−	+	−	−
H_2S production	−	+	−	+	+
Voges–Proskauer test	−	+	+	−	−
Methyl red test	−	+	−	−	−
Nitrate reduction	−	−	+	−	+
Fermentation of D-glucose	−	−	−	+	−
Hydrolysis of:					
Casein	+	−	−	−	−
Starch	−	−	−	+	+
Enzyme activity:					
Esterase (C4)	+	−	−	−	+
Esterase lipase (C8)	+	−	−	−	+
Acid phosphatase	+	+	+	−	+
DNA G+C content (mol%)	71.3	70.9*	69.7†	66.9‡	69.0‡
Major cell-wall diamino acid§	Orn	Lys*	Orn†	Orn‡	Orn‡

*Data from Takeuchi & Hatano (1998b).
†Data from Kim *et al.* (2005).
‡Data from Yokota *et al.* (1993).
§Orn, Ornithine; Lys, lysine.

Fatty acid methyl esters were obtained from cells grown in MB medium for 2 days at 30 °C and analysed by GC-MS (Kuykendall *et al.*, 1988). For quantitative analysis of cellular fatty acid composition, a loop of cell mass was harvested after 2 days and cellular fatty acids were saponified, methylated and extracted according to the Sherlock Microbial Identification System (MIDI) as described by Sasser (1990). The amino acid composition of the cell-wall peptidoglycan was determined after hydrolysis with 6 M HCl at 100 °C for 16 h as described by Schleifer (1985). The predominant cellular fatty acids found in strain M4-8^T were iso-$C_{15:0}$ (55.0 %) and anteiso-$C_{15:0}$ (26.5 %). Minor fatty acids were iso-$C_{16:0}$ (7.5 %), iso-$C_{17:0}$ (6.1 %), anteiso-$C_{17:0}$ (2.4 %), iso-$C_{14:0}$ (2.3 %) and $C_{16:0}$ (0.3 %). No hydroxyl fatty acids were identified. The cellular fatty acid profile was consistent with those of other members of the genus *Microbacterium*. The cell-wall diamino acids were ornithine (major) and lysine.

Chromosomal DNA was extracted using a DNA extraction kit (iNtRON Biotechnology). The 16S rRNA gene sequence was PCR-amplified from chromosomal DNA using PCR Pre-Mix (Solgent) and two bacterial universal primers (Baker *et al.*, 2003). The PCR product was purified using a PCR purification kit (Qiagen) and sequencing was performed as previously described (Roh *et al.*, 2008). Almost full-length 16S rRNA gene sequences were assembled using SeqMan software (DNASTAR). The 16S rRNA gene sequence of the novel isolate was aligned with those of related taxa obtained from the NCBI database using the multiple sequence alignment program CLUSTAL X (1.8) (Thompson *et al.*, 1997). The phylogenetic relationship between representatives of the genus *Microbacterium* was determined using the MEGA3 software program (Kumar *et al.*, 2004). Distance matrices were determined using the method of Kimura (1980) and used to produce a dendrogram using the neighbour-joining (Saitou & Nei, 1987), minimum evolution (Rzhetsky & Nei, 1992) and maximum-parsimony (Kluge & Farris, 1969) methods. Bootstrap analysis evaluating the stability of the resulting trees was performed using a consensus tree based on 1000 randomly generated trees. DNA–DNA hybridization experiments were performed in triplicate using the fluorometric method of Ezaki *et al.* (1989). Hybridizations were determined at 37 °C, fluorescence values were quantified at 90 min using Synergy Mx (BioTek) and hybridization values were calculated as described previously (Chang *et al.*, 2008). Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain M4-8^T is related to members of the genus *Microbacterium*. Comparison of the 16S rRNA gene sequence of strain M4-8^T against those of known species of the genus *Microbacterium* using FASTA (EMBL/GenBank) showed that it had highest similarity with that of *Microbacterium*

hominis IFO 15708^T (97.8 %), followed by *Microbacterium xylanilyticum* S3-E^T (97.3 %), *Microbacterium flavescens* DSM 20643^T (97.1 %) and *Microbacterium trichothecenolyticum* IFO 15077^T (97.1 %). The clustering and phylogenetic tree drawn using the neighbour-joining, minimum-evolution and maximum-parsimony method is shown in Fig. 1. Phylogenetic trees based on 16S rRNA gene sequences showed a similar topology, regardless of the tree-making algorithm used (Fig. 1). DNA–DNA hybridization experiments were performed to determine the genomic relationship between the isolate and those strains with the greatest 16S rRNA gene sequence similarity ($\geqslant$97.0 %). DNA–DNA

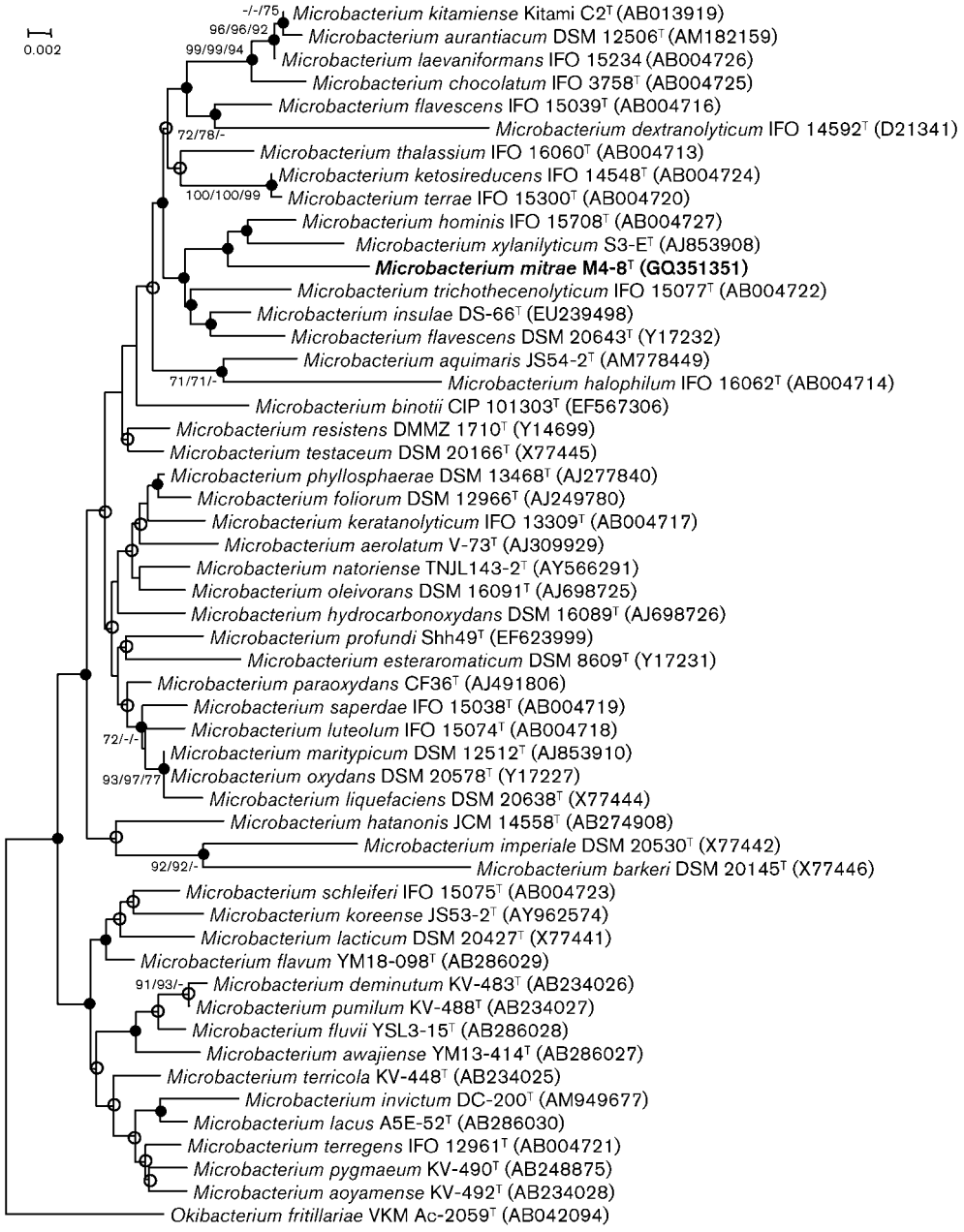

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences. The position of strain M4-8^T is shown with respect to other species of the genus *Microbacterium.* Phylogenetic trees were generated using the neighbour-joining and maximum-parsimony methods. GenBank accession numbers are shown in parentheses. Filled circles indicate generic branches that were also recovered using the neighbour-joining (NJ), minimum-evolution (ME) and maximum-parsimony (MP) algorithms. Open circles indicate that the corresponding node was also recovered in the trees generated by two algorithms (NJ and ME, ME and MP, or NJ and MP). Numbers at nodes indicate bootstrap values (>70 %) as calculated on the basis of NJ/ME/MP probabilities expressed as percentages of 1000 replications. Bar, 0.002 accumulated changes per nucleotide.

relatedness ranged from 3.2–28.8 % with its closest relatives: *M. hominis* IFO 15708^T (17.0 %), *M. xylanilyticum* S3-E^T (15.9 %), *M. flavescens* DSM 20643^T (3.2 %) and *M. trichothecenolyticum* IFO 15077^T (28.8 %). The level of 16S rRNA gene sequence similarity coupled with low DNA–DNA relatedness values below the 70 % threshold (Wayne *et al.*, 1987) indicate that strain M4-8^T represents a distinct genospecies.

Data for growth of strain M4-8^T and its closely related phylogenetic neighbours at various NaCl concentrations show that strain M4-8^T has more NaCl tolerance than the four most closely related species. Casein hydrolysis can also be used to differentiate between strain M4-8^T and its closest relatives (Table 1). Data from 16S rRNA gene sequence analysis, DNA–DNA relatedness experiments, and physiological and biochemical tests highlighted genotypic and phenotypic differences between strain M4-8^T and other species of the genus *Microbacterium*. Therefore, it is concluded that strain M4-8^T (=KACC 21129^T =JCM 16363^T) represents a novel species of the genus *Microbacterium*, for which the name *Microbacterium mitrae* sp. nov. is proposed.

Description of *Microbacterium mitrae* sp. nov.

Microbacterium mitrae [mi′trae. L. n. *mitra* a headband, coif, turban of the Asiatics, also a zoological genus name (*Mitra*); L. gen. n. *mitrae* of *Mitra* sp.].

Cells are Gram-positive, non-motile, non-spore-forming and rod-shaped, forming yellow-pigmented, round colonies with a diameter of 0.5–2.0 mm after incubation for 2 days on MB agar plates at 30 °C. Growth occurs with 0–8 % NaCl, at 15–37 °C and at pH 6.0–9.0, with optimal growth occurring in 1 % (w/v) NaCl, at 30 °C and at pH 7.0. Does not reduce nitrate to nitrite and does not produce indole. Catalase-positive and oxidase-negative. Casein is hydrolysed, but starch is not. H_2S is not formed. Results from the API 20NE system show that the strain hydrolyses aesculin ferric citrate, gelatin and 4-nitrophenyl-β-D-galactopyranoside, but not D-glucose, L-arginine, urea, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate or phenylacetic acid. Assays using the API ZYM system are positive for esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase, but negative for lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, β-galactosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The predominant fatty acids are iso-$C_{15:0}$ and anteiso-$C_{15:0}$. Minor fatty acids are iso-$C_{16:0}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$, iso-$C_{14:0}$ and $C_{16:0}$. The major cell-wall diamino acid is ornithine.

The type strain is M4-8^T (=KACC 21129^T =JCM 16363^T), isolated from salted turban shell, which is a traditional fermented food in Korea. The genomic DNA G+C content of the type strain is 71.3 mol%.

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References

Baker, G. C., Smith, J. J. & Cowan, D. A. (2003). Review and re-analysis of domain-specific 16S primers. *J Microbiol Methods* **55**, 541–555.

Bakir, M. A., Kudo, T. & Benno, Y. (2008). *Microbacterium hatanonis* sp. nov., isolated as a contaminant of hairspray. *Int J Syst Evol Microbiol* **58**, 654–658.

Benson, H. J. (1994). *Microbiological Applications: a Laboratory Manual in General Microbiology*, 6th edn. Dubuque, IA: Wm. C. Brown.

Buck, J. D. (1982). Nonstaining (KOH) method for determination of Gram reactions of marine bacteria. *Appl Environ Microbiol* **44**, 992–993.

Chang, H.-W., Nam, Y.-D., Jung, M. Y., Kim, K.-H., Roh, S. W., Kim, M.-S., Jeon, C. O., Yoon, J.-H. & Bae, J.-W. (2008). Statistical superiority of genome-probing microarrays as genomic DNA-DNA hybridization in revealing the bacterial phylogenetic relationship compared to conventional methods. *J Microbiol Methods* **75**, 523–530.

Collins, M. D. & Bradbury, J. F. (1992). The genera *Agromyces*, *Aureobacterium*, *Clavibacter*, *Curtobacterium* and *Microbacterium*. In *The Prokaryotes*, 2nd edn, pp. 1355–1368. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.

Evtushenko, L. I. & Takeuchi, M. (2006). The family *Microbacteriaceae*. In *The Prokaryotes: a Handbook on the Biology of Bacteria*, 3rd edn, vol. 3, pp. 1020–1098. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. New York: Springer.

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.

Kim, K. K., Park, H. Y., Park, W., Kim, I. S. & Lee, S.-T. (2005). *Microbacterium xylanilyticum* sp. nov., a xylan-degrading bacterium isolated from a biofilm. *Int J Syst Evol Microbiol* **55**, 2075–2079.

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.

Kluge, A. G. & Farris, F. S. (1969). Quantitative phyletics and the evolution of anurans. *Syst Zool* **18**, 1–32.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

Kuykendall, L. D., Roy, M. A., O'Neill, J. J. & Devine, T. E. (1988). Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. *Int J Syst Bacteriol* **38**, 358–361.

Lee, J.-S., Lee, K. C. & Park, Y.-H. (2006). *Microbacterium koreense* sp. nov., from sea water in the South Sea of Korea. *Int J Syst Evol Microbiol* **56**, 423–427.

Legard, D. E., McQuilken, M. P., Whipps, J. M., Fenlon, J. S., Fermor, T. R., Thompson, I. P., Bailey, M. J. & Lynch, J. M. (1994). Studies of seasonal changes in the microbial populations on the phyllosphere of spring wheat as a prelude to the release of a genetically modified microorganism. *Agric Ecosyst Environ* **50**, 87–101.

McInroy, J. A. & Kloepper, J. W. (1995). Survey of indigenous bacterial endophytes from cotton and sweet corn. *Plant Soil* **173**, 337–342.

Orla-Jensen, S. (1919). *The Lactic Acid Bacteria*. Copenhagen: Høst and Son.

Park, M.-J., Kim, M. K., Kim, H.-B., Im, W.-T., Yi, T.-H., Kim, S.-Y., Soung, N.-K. & Yang, D.-C. (2008). *Microbacterium ginsengisoli* sp. nov., a β-glucosidase producing bacterium isolated from soil of a ginseng field. *Int J Syst Evol Microbiol* **58**, 429–433.

Roh, S. W., Sung, Y., Nam, Y.-D., Chang, H.-W., Kim, K.-H., Yoon, J.-H., Jeon, C. O., Oh, H.-M. & Bae, J.-W. (2008). *Arthrobacter soli* sp. nov., a novel bacterium isolated from wastewater reservoir sediment. *J Microbiol* **46**, 40–44.

Rzhetsky, A. & Nei, M. (1992). A simple method for estimating and testing minimum-evolution trees. *Mol Biol Evol* **9**, 945–967.

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 Inc.

Schaeffer, A. B. & Fulton, M. D. (1933). A simplified method of staining endospores. *Science* **77**, 194.

Schleifer, K. H. (1985). Analysis of the chemical composition and primary structure of murein. *Methods Microbiol* **18**, 123–156.

Shivaji, S., Bhadra, B., Rao, R. S., Chaturvedi, P., Pindi, P. K. & Raghukumar, C. (2007). *Microbacterium indicum* sp. nov., isolated from a deep-sea sediment sample from the Chagos Trench, Indian Ocean. *Int J Syst Evol Microbiol* **57**, 1819–1822.

Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Takeuchi, M. & Hatano, K. (1998a). Union of the genera *Microbacterium* Orla-Jensen and *Aureobacterium* Collins *et al.* in a redefined genus *Microbacterium*. *Int J Syst Bacteriol* **48**, 739–747.

Takeuchi, M. & Hatano, K. (1998b). Proposal of six new species in the genus *Microbacterium* and transfer of *Flavobacterium marinotypicum* ZoBell and Upham to the genus *Microbacterium* as *Microbacterium maritypicum* comb. nov. *Int J Syst Bacteriol* **48**, 973–982.

Thompson, I. P., Bailey, M. J., Fenlon, J. S., Fermor, T. R., Lilley, A. K., Lynch, J. M., McCormack, P. J., McQuilken, M. P., Purdy, K. J. & other authors (1993). Quantitative and qualitative seasonal changes in the microbial community from the phyllosphere of sugar beet (*Beta vulgaris*). *Plant Soil* **150**, 177–191.

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.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Yokota, A., Takeuchi, M., Sakane, T. & Weiss, N. (1993). Proposal of six new species in the genus *Aureobacterium* and transfer of *Flavobacterium esteraromaticum* Omelianski to the genus *Aureobacterium* as *Aureobacterium esteraromaticum* comb. nov. *Int J Syst Bacteriol* **43**, 555–564.