Pseudoxanthomonas kalamensis sp. nov., a novel gammaproteobacterium isolated from Johnston Atoll, North Pacific Ocean

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An aerobic, mesophilic bacterium, strain JA40^T, was isolated from soil contaminated with polycyclic aromatic hydrocarbons and polychlorinated biphenyls collected from Johnston Atoll in the North Pacific Ocean. The strain formed yellow-pigmented colonies on heterotrophic media. The cells were Gram-negative, non-motile, non-sporulating rods. The strain reduced nitrite to nitrous oxide, the DNA G+C content was 64 mol% and the dominant fatty acids were 15 : 0 iso, 17 : 1 iso *cis*7 and 11 : 0 iso 3-OH. DNA sequencing of 1457 nt of the 16S rRNA gene established that JA40^T belongs in the genus *Pseudoxanthomonas* within the *Xanthomonadaceae* branch of the *Gammaproteobacteria*. Strain JA40^T can be differentiated from other mesophilic species in the genus on the basis of its physiological and biochemical characteristics and distinctive fatty acid profile. Thus strain JA40^T (=ATCC BAA-1031^T=CIP 108476^T) is the type strain of a novel species of the genus *Pseudoxanthomonas*, for which the name *Pseudoxanthomonas kalamensis* sp. nov. is proposed.

Johnston Atoll is located in the North Pacific Ocean (16° 45′ N, 169° 31′ W), 717 miles south-west of Honolulu, Hawaii. The atoll has been a designated wildlife refuge since 1926 and has been under the control of the US military since 1934. The site was used for high-altitude nuclear tests in the 1950s and 1960s, and, until late in 2000, as a storage and disposal site for chemical weapons. Operation of the chemical disposal facility has ceased and the decommissioning, dismantling and clean-up of the site was completed in 2003.

The genera *Stenotrophomonas*, *Xanthomonas* and *Pseudoxanthomonas* partly comprise the family *Xanthomonadaceae* in the *Gammaproteobacteria* (Finkmann *et al.*, 2000; Yang *et al.*, 2005). These genera share phenotypic traits (e.g. colony shape and colour, cell morphology, Gram reaction), the presence of branched-chain fatty acids and the presence of ubiquinones with eight isoprenoid units (Q-8). Members of the genus *Pseudoxanthomonas*, however, are differentiated from members of the genera *Xanthomonas* and *Stenotrophomonas* by their ability to reduce nitrite but not nitrate to N_2O and by the absence of the fatty acid $C_{13:0}$ iso 3-OH (Thierry *et al.*, 2004). Here we describe the isolation of strain JA40^T and its characterization as a novel species of the genus *Pseudoxanthomonas*.

Strain JA40^T was isolated from soil contaminated with polycyclic aromatic hydrocarbons and polychlorinated biphenyls from Johnston Atoll. The strain was originally cultivated from serial dilutions of soil spread-plated on marine agar 2216 (MA; Difco). Colonies that arose after 3–5 days incubation at 30 °C were streaked for isolation on MA. Strain purity was checked (through repeated transfers) by assessing the consistency of the colony characteristics, the cell morphology and the Gram reaction. Strain JA40^T was maintained on MA or in marine broth 2216 (MB; Difco). Stock cultures were stored at −80 °C in MB with 50 % (v/v) glycerol.

Unless noted, all characterizations of strain JA40^T were performed on cells grown for 5 days at 30 °C on MA. Motility was checked by means of the hanging drop method using light microscopy under a ×100 objective with oil immersion. The morphology of negatively stained cells (1 % uranyl acetate) was observed in an LEO 912 energy-filtering transmission electron microscope. Images were recorded as digital files with a Pro-scan frame transfer CCD camera. Cells prepared for transmission electron microscopy were grown for 4 days on tryptic soy agar (TSA; Difco) and resuspended in 0·85 % (w/v) sterile saline. For negative staining, 10 µl resuspended cells was placed on a 400-mesh Formvar-coated grid and stained with 1 % uranyl acetate (pH 4). Single colonies were tested for catalase with 3 % (v/v) H_2O_2 (Sigma) and for oxidase with tetramethyl-*p*-phenylenediamine on paper discs

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JA40^T is AY686710.

A transmission electron micrograph of strain JA40^T and a table comparing the fatty acid content of strain JA40^T with that of other *Pseudoxanthomonas* species are available as supplementary material in IJSEM Online.

(BBL). The growth temperature range was determined from 4 to 40 °C on MA. The salinity tolerance was measured by growth as a function of turbidity in tryptic soy broth (TSB) diluted to 50 % of the original concentration, supplemented with NaCl at final concentrations in the range 0·5–8 % (w/v). The OD_{650} was measured after 5 days incubation with shaking (130 r.p.m.) at 30 °C. The presence or absence of constitutive enzymes was checked using API ZYM (bioMérieux) and the profile of substrates used was obtained by using API 20NE (bioMérieux). Additionally, the oxidation of carbon substrates was determined with Biolog GN/GP microplates.

Genomic DNA was isolated from a 5-day culture in MB by using a phenol/chloroform extraction of pelleted cells (Marmur, 1961). A fragment of the 16S rRNA gene was amplified from the genomic DNA (100–200 ng μl^{-1}) by means of a PCR with *Ex-Taq* DNA polymerase (Takara Mirus Bio) and primers 27F and 1492R (Lane, 1991). The PCR product was purified using the Ultraclean PCR purification kit (Mo Bio Lab) and sequenced in both directions in an Applied Biosystems 377XL DNA sequencer. The 16S rRNA gene sequences were manually edited and assembled in SEQUENCHER and SEQMAN (Lasergene). Assembled sequences were compared with those in the public domain through a BLASTN search (Altschul *et al.*, 1997). The phylogenetic relationships between JA40^T and type strains in the genera *Pseudoxanthomonas*, *Stenotrophomonas*, *Xanthomonas* and *Xylella* were determined on the basis of comparative 16S rRNA gene sequence analysis. These analyses were performed with programs in the PHYLIP 3.63 package (Felsenstein, 2004). Evolutionary distances were calculated using the maximum-likelihood method with DNADIST. For bootstrap analyses based on 100 replicates, SEQBOOT, DNADIST and CONSENSE were used. Phylogenetic trees were constructed using DNAML with jumbled orders of the sequences and the neighbour-joining method (Saitou & Nei, 1987). Genomic DNA from strain JA40^T was hybridized with DNA from *Pseudoxanthomonas broegbernensis* DSM 12573^T by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), with modifications described by Huß *et al.* (1983), in a Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostat 6 × 6 multicell changer and temperature controller with an *in situ* temperature probe (Varian). The determination of the G + C content of strain JA40^T was also performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen using HPLC as described by Mesbah *et al.* (1989) and Tamaoka & Komagata (1984). Fatty acid methyl ester analysis was performed by MIDI Labs on whole cells grown on TSA incubated at 28 °C for 24 ± 2 h according to Sasser (1997). Quinones were extracted from fresh cells of strain JA40^T by using a mixture of chloroform and methanol (2 : 1, v/v) (Hiraishi, 1988). The extracts were filtered and evaporated to near dryness. The residue was redissolved in acetone prior to LC-MS analysis. An Agilent 1100 series liquid chromatograph equipped with a diode array detector and an MSD SL model mass spectrometer was used for the negative-mode electrospray analysis.

Denitrification reactions were investigated using strain JA40^T grown in Hungate tubes (Bellco) in TSB supplemented with either 10 mM Na($^{15}NO_3^-$) or 10 mM Na($^{15}NO_2^-$) (Isotec). The medium was pre-reduced by purging the hermetically sealed tubes with oxygen-free nitrogen gas prior to sterilization. Inoculated tubes were incubated for 7 days at 30 °C without shaking. Reduction of nitrate was determined by the traditional colorimetric method of adding sulfanilic acid, *N,N*-dimethyl-1-naphthylamine and zinc powder (as required) to the culture medium after incubation (Smibert & Krieg, 1994). Additionally, gas samples from the headspace were collected and analysed by using a GC-MS method similar to the one described by Finkmann *et al.* (2000). GC-MS analysis was performed with a CP 3800 gas chromatograph interfaced with a 1200 quadrupole mass spectrometer (Varian). The injector temperature was kept at 100 °C, the GC oven at 50 °C, the GC-MS transfer line at 200 °C and the source at 250 °C. The GC-MS was performed using apparatus in electron-impact mode at 70 eV; full scans were acquired and the molecular ions for ^{15}NO, 31^+, $^{14,15}N_2O$, 45^+, and $^{15,15}N_2O$, 46^+, were monitored.

The cells of strain JA40^T are single, straight rods 0·5 × ~1·0–1·25 μm in size. The strain grows slowly on MA at 28, 30 and 37 °C, but there is no growth at 4 or 40 °C. Colonies are small (1–1·5 mm), shiny, pale yellow in colour and circular after 4–5 days on MA at 30 and 37 °C. On TSA, however, colonies are darker yellow and circular, but still small (1–1·5 mm). The strain grows at salinities in the range 0·5–3 % (w/v) NaCl in 50 % TSB with optimal growth in the presence of 2 % (w/v) NaCl. Strain JA40^T is non-sporulating and non-motile. Transmission electron microscopy revealed the absence of flagella (see Supplementary Fig. S1 in IJSEM Online). All other *Pseudoxanthomonas* spp. have a single polar flagellum, with the exception of *Pseudoxanthomonas koreensis* KCTC 12208^T and the thermophile *Pseudoxanthomonas taiwanensis* ATCC BAA-404^T (Theirry *et al.*, 2004; Chen *et al.*, 2002; Yang *et al.*, 2005). The constitutive enzyme activities expressed in API ZYM tests included alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucosidase and *N*-acetyl-β-glucosamidase. Comparisons of the physiological characteristics of JA40^T with those of related *Pseudoxanthomonas* species, based on API 20NE tests, are listed in Table 1. Like all other *Pseudoxanthomonas* species, except *Pseudoxanthomonas taiwanensis* (for which there are no data), strain JA40^T is unable to assimilate caprate, adipate or phenylacetate.

The fatty acids comprising more than 5 % of the total in JA40^T are as follows: 11 : 0 iso (6·87 %), 11 : 0 iso 3-OH (8·32 %), 15 : 0 iso (40·02 %), 16 : 0 iso (6·85 %), 17 : 0 iso (6·68 %) and 17 : 1 iso *cis*7 (9·04 %). Smaller amounts of

Table 1. Characteristics, based on API 20NE tests, that differentiate strain JA40^T from other *Pseudoxanthomonas* species

Strains: 1, *Pseudoxanthomonas kalamensis* sp. nov. JA40^T; 2, *Pseudoxanthomonas broegbernensis* DSM 12573^T (Thierry *et al.*, 2004); 3, *Pseudoxanthomonas mexicana* DSM 15133^T (Thierry *et al.*, 2004); 4, *Pseudoxanthomonas japonensis* JCM 11525^T (Thierry *et al.*, 2004); 5, *Pseudoxanthomonas taiwanensis* ATCC BAA-404^T (Chen *et al.*, 2002); 6, *Pseudoxanthomonas koreensis* KCTC 11208^T (Yang *et al.*, 2005); 7, *Pseudoxanthomonas daejeonensis* KCTC 12207^T (Yang *et al.*, 2005); 8, *Pseudoxanthomonas kaohsiungensis* LMG 22530^T (Chang *et al.*, 2005). ND, No data available.

Characteristic	1	2	3	4	5	6	7	8
Motility and flagellation	−	+	+	+	−	−	+	+
Optimum growth temp. (°C)	30–37	30	30–37	30–37	50	30	30	35
Growth at 50 °C	−	−	−	−	+	−	−	−
Catalase	+	+	+	−	+	+	+	−
β-Galactosidase	−	+	−	+	+	−	+	+
β-Glucosidase	+	−	+	+	+	−	+	+
Gelatin hydrolysis (protease)	−	−	+	+	−	−	−	−
Urease	−	−	−	−	ND	+	−	−
Arginine dihydrolase	−	−	−	−	ND	+	−	−
Carbon-substrate assimilation								
D-Glucose	+	+	+	+	+	−	+	+
L-Arabinose	+	+	−	−	−	−	+	−
D-Mannose	−	+	+	−	−	−	−	−
D-Mannitol	−	−	−	−	ND	−	−	−
N-Acetylglucosamine	+	+	+	+	ND	−	+	+
D-Maltose	+	+	+	+	−	−	+	+
Malate	−	−	−	+	−	−	−	−
Citrate	−	+	−	−	ND	−	−	−
DNA G+C content (mol%)	64	66·5±0·8	67·8±2	65·5±1	69·9–70·1	69·5	68·7	60·1

15 : 0 anteiso (4·61 %), 16 : 0 (2·92 %), 16 : 1 *cis*10 alcohol (1·92 %), 16 : 1 *cis*5 (1·21 %) and 17 : 0 anteiso (1·84 %) are present. The fatty acids that distinguish strain JA40^T from other *Pseudoxanthomonas* species are listed in Supplementary Table S1 (available in IJSEM Online) and include 12 : 0 iso 3-OH, 15 : 1 *cis*9, 16 : 1 *cis*5 and 16 : 1 *cis*9 alcohol, and larger amounts of 11 : 0 iso and 11 : 0 iso 3-OH.

The GC-MS analysis confirmed that strain JA40^T reduced NO_2^- but not NO_3^-, under the conditions tested. Upon reduction of NO_2^-, supplied as $Na(^{15}NO_2^-)$, the only product found was $^{15,15}N_2O$ as the ion 46^+. It was not determined in this study whether nitrous oxide was the sole and final product of the nitrite reduction. Similar results have been observed for all other *Pseudoxanthomonas* species, except *Pseudoxanthomonas koreensis* KCTC 12208^T and *Pseudoxanthomonas daejeonensis* KCTC 12207^T. On the basis of 16S rRNA gene nucleotide sequences, the closest relatives of strain JA40^T are *Pseudoxanthomonas* species (Fig. 1). In fact, strain JA40^T shares 95·5 % sequence identity with *Pseudoxanthomonas broegbernensis* DSM 12573^T, 96·1 % with *Pseudoxanthomonas japonensis* CIP 107388^T, 96·1 % with *Pseudoxanthomonas daejeonensis* KCTC 12207^T, 95·7 % with *Pseudoxanthomonas koreensis* KCTC 12208^T and 96·3 % with *Pseudoxanthomonas mexicana* ATCC 700999^T (in each case determined over more than 1400 nt). The level of DNA–DNA reassociation between genomic DNA from strain JA40^T and that from the *Pseudoxanthomonas* type species, *Pseudoxanthomonas broegbernensis* DSM 12573^T, was 40·4 %, which is sufficient to distinguish JA40^T from *Pseudoxanthomonas broegbernensis* according to the recommendation of the ad hoc committee on bacterial systematics (Wayne *et al.*, 1987). The G+C content for strain JA40^T is 64 mol%. On the basis of the morphological characteristics, 16S rRNA gene sequence, physiology, unique fatty acid composition and denitrification reaction of strain JA40^T, it represents the type strain of a novel species of the genus *Pseudoxanthomonas*, for which we propose the name *Pseudoxanthomonas kalamensis* sp. nov.

Description of *Pseudoxanthomonas kalamensis* sp. nov.

Pseudoxanthomonas kalamensis (ka.lam.en′sis. N.L. fem. adj. *kalamensis* of Kalama Island, referring to the Hawaiian name for Johnston Atoll, where the organism was isolated).

Aerobic. Yellow colonies form on heterotrophic media after 4–5 days (e.g. TSA, MA). Cells are Gram-negative, single, straight rods 1·0–1·25 μm in length and 0·5 μm in width. Non-motile. Mesophilic, with an optimum temperature range of 30–37 °C. Cytochrome oxidase- and catalase-positive. Negative for β-galactosidase (ONPG hydrolysis) and positive for β-glucosidase (aesculin hydrolysis). The spectra of substrates utilized is presented in Table 1. Additionally, strain JA40^T oxidizes diverse carbon sources available in Biolog GN and GP, namely α-cyclodextrin, β-cyclodextrin, dextrin,

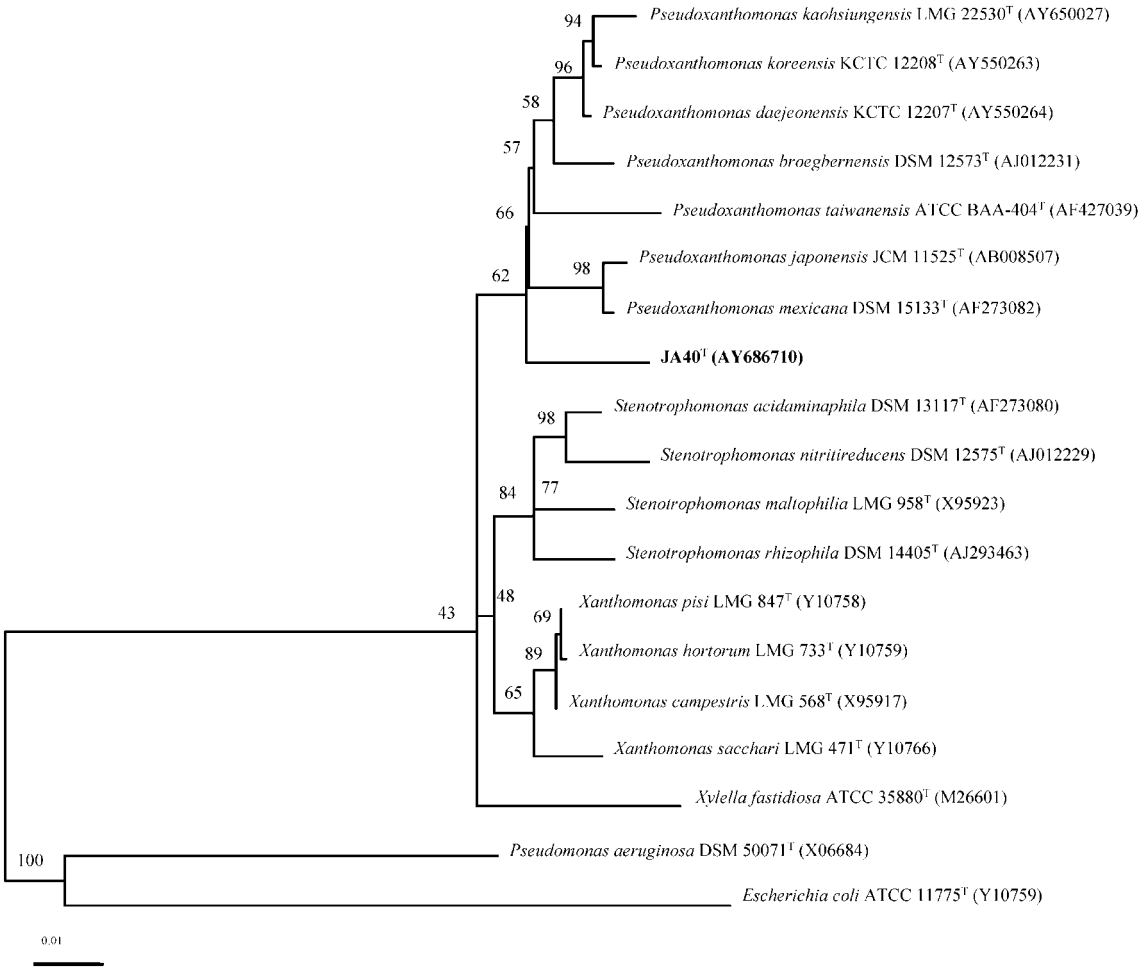

Fig. 1. Neighbour-joining tree, based on nearly complete 16S rRNA gene sequences, showing relationships between strain JA40^T and members of the *Xanthomonadaceae*. Numbers at the nodes are bootstrap percentages based on maximum-likelihood analyses of 100 resampled datasets. *Escherichia coli* ATCC 11775^T and *Pseudomonas aeruginosa* DSM 50071^T were used as outgroups. Bar, 0·01 nucleotide substitutions per site.

glycogen, mannan, Tween 80, amygdalin, arbutin, D-cellobiose, D-fructose, gentiobiose, α-D-glucose, *myo*-inositol, maltose, maltotriose, D-mannose, D-melezitose, methyl α-D-galactoside, methyl α-D-glucoside, methyl *β*-D-glucoside, D-raffinose, salicin, sucrose, D-trehalose, turanose, *β*-hydroxybutyric acid, D-malic acid, succinic acid monomethyl ester, succinamic acid, *N*-acetyl-L-glutamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-glutamic acid, glycyl L-glutamic acid, L-serine, D-fructose 6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate and D-lactic acid methyl ester. The DNA G+C content is 64 mol%. The predominant fatty acids are 15:0 iso, 17:1 iso *cis*7 and 11:0 iso 3-OH. Nitrite is reduced, with the production of nitrous oxide as a main product; nitrate is not reduced.

The type strain, JA40^T (=ATCC BAA-1031^T=CIP 108476^T), was isolated from soil contaminated with polycyclic aromatic hydrocarbons and polychlorinated biphenyls collected from Johnston Atoll in the North Pacific Ocean.

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References

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.

Chang, J.-S., Chou, C.-L., Lin, G.-H., Sheu, S.-Y. & Chen, W.-M. (2005). *Pseudoxanthomonas kaohsiungensis*, sp. nov., a novel bacterium isolated from oil-polluted site produces extracellular surface activity. *Syst Appl Microbiol* **28**, 137–144.

Chen, M.-Y., Tsay, S.-S., Chen, K.-Y., Shi, Y.-C., Lin, Y.-T. & Lin, G.-H. (2002). *Pseudoxanthomonas taiwanensis* sp. nov., a novel thermophilic, N_2O-producing species isolated from hot springs. *Int J Syst Evol Microbiol* **52**, 2155–2161.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

Felsenstein, J. (2004). PHYLIP (phylogeny inference package), version 3.63. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.

Finkmann, W., Altendorf, K., Stackebrandt, E. & Lipski, A. (2000). Characterization of N_2O-producing *Xanthomonas*-like isolates from biofilters as *Stenotrophomonas nitritireducens* sp. nov., *Luteimonas mephitis* gen. nov., sp. nov. and *Pseudoxanthomonas broegbernensis* gen. nov., sp. nov. *Int J Syst Evol Microbiol* **50**, 273–282.

Hiraishi, A. (1988). Respiratory quinoine profiles as tools for identifying different bacterial populations in activated sludge. *J Gen Appl Microbiol* **34**, 39–56.

Huß, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrant & M. Goodfellow. Chichester: Wiley.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

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.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sasser, M. (1997). *Identification of Bacteria by Gas Chromatography of Cellular Fatty Acids*. MIDI Technical Note 101. Newark, DE: MIDI.

Smibert, R. M. & Krieg, W. 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.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Thierry, S., Macarie, H., Iizuka, T. & 9 other authors (2004). *Pseudoxanthomonas mexicana* sp. nov. and *Pseudoxanthomonas japonensis* sp. nov., isolated from diverse environments, and emended descriptions of the genus *Pseudoxanthomonas* Finkmann *et al.* 2000 and of its type species. *Int J Syst Evol Microbiol* **54**, 2245–2255.

Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 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.

Yang, D.-C., Im, W.-T., Kim, M.-T. & Lee, S.-T. (2005). *Pseudoxanthomonas koreensis* sp. nov. and *Pseudoxanthomonas daejeonensis* sp. nov. *Int J Syst Evol Microbiol* **55**, 787–791.