Tolumonas osonensis sp. nov., isolated from anoxic freshwater sediment, and emended description of the genus *Tolumonas*

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A polyphasic taxonomic study was performed on a strain of an unknown Gram-negative, non-motile, saccharolytic, facultatively anaerobic bacterium, strain OCF 7^T, isolated from anoxic freshwater sediment. The strain grew optimally at 22 °C and pH 7.5, and was able to grow under strictly anaerobic conditions. Major fermentation products from glucose metabolism were formate, acetate, ethanol and lactate. Comparative 16S rRNA gene sequence analysis indicated that strain OCF 7^T was phylogenetically related to the type strain of *Tolumonas auensis* (97.2 % similarity) within the family *Aeromonadaceae* of the *Gammaproteobacteria*. However, OCF 7^T did not produce toluene from phenylacetate, phenylalanine, phenoxyacetate, phenylsuccinate or phenylbutyrate in the presence of glucose. Phenol was not produced from tyrosine or phenoxyacetate in the presence of glucose. Dominant fatty acids of this micro-organism included C_{16:0}, C_{18:1ω7c} and C_{16:1ω7c} (and/or iso-C_{15:0} 2-OH). Major polar lipids were phosphatidylglycerol and phosphatidylethanolamine, and the respiratory quinone was menaquinone MK-8. The genomic DNA G + C content of strain OCF 7^T was 52.1 mol%. Based on phylogenetic and phenotypic evidence, strain OCF 7^T should be classified as a representative of a novel species of *Tolumonas*, for which the name *Tolumonas osonensis* sp. nov. is proposed; the type strain is OCF 7^T (=DSM 22975^T =ATCC BAA-1908^T). An emended description of the genus *Tolumonas* is also given.

Within the *Gammaproteobacteria*, the family *Aeromonadaceae* comprises five genera: *Aeromonas*, *Oceanimonas*, *Oceanisphaera*, *Tolumonas* and *Zobellella* (Lin & Shieh, 2006; Martin-Carnahan & Joseph, 2005a; Romanenko *et al.*, 2003). However, inclusion of the genus *Tolumonas* (represented solely by *Tolumonas auensis*) in this family has been questioned due to the fact that it is oxidase-negative and has a DNA G + C content of 49 mol% (Martin-Carnahan & Joseph, 2005a). *T. auensis* is also distinctive in its ability to transform substituted aromatic compounds (e.g. phenylacetate, phenylalanine, phenyllactate or phenylpyruvate) into toluene in the presence of a usable carbon source (Fischer-Romero *et al.*, 1996). *T. auensis* will also transform tyrosine into phenol in the presence of a primary substrate. While the microbial production of phenol from tyrosine has been known for decades (Berthelot, 1917; Chen & Levin, 1975; Elsden *et al.*, 1976), to our knowledge, *T. auensis* is the only bacterium to co-metabolize aromatic compounds into toluene. In this study, isolation of a Gram-negative,

non-motile, facultatively anaerobic bacterium cultivated from anoxic freshwater sediment is described. Based on data from a polyphasic study incorporating both phylogenetic and phenotypic methods, it is proposed that the unknown bacterium represents a novel species of *Tolumonas*.

Anoxic freshwater sediment from Oso Creek, Corpus Christi, Texas, USA (27° 43' N 97° 32' W) was collected and incubated at 37 °C in anaerobic enrichment medium (pH 7.3) containing (l⁻¹): 10 ml mineral solution (Tanner, 2007), 10 ml vitamin solution (Tanner, 2007), 10 ml trace metal solution (Tanner, 2007), 10 g TES (Sigma-Aldrich), 0.05 g yeast extract (Difco) and 5.0 g NaHCO₃. The medium was reduced with cysteine sulfide (Tanner, 2007), fructose (0.5 %) was added as substrate and the gas phase was N₂:CO₂ (80:20). Dilution of the resulting bacterial enrichment and isolation using agar roll tubes (Hungate, 1969) were performed and the isolate was propagated on the same medium except that yeast extract concentration was increased to 0.5 g l⁻¹. The unknown organism recovered from anoxic freshwater sediment was a Gram-negative, non-motile rod that appeared singly, in pairs or occasionally in short chains. Growth was observed between 15 and 37 °C, with the optimum growth temperature being 22 °C; no growth was observed at 4 or 40 °C. All further incubations were at 22 °C unless otherwise noted. Growth occurred in

Abbreviation: FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain OCF 7^T is GU370947.

Two supplementary tables are available with the online version of this paper.

the absence of vitamins and yeast extract. Cells growing on glucose with a N₂ headspace were used for pH and temperature experiments. For pH experiments, the following Good's buffers (Sigma-Aldrich) were used (30 g l⁻¹): HOMOPIES (pH 5.0 and 5.5), MES (pH 6.0 and 6.5), TES (pH 7.0 and 7.5), TAPS (pH 8.0 and 8.5), CAPSO (pH 9.0) and CAPS (pH 10.0 and 10.5). The isolate grew at pH 5.5–8.5, with optimum growth at pH 7.5; no growth was observed at pH 5.0 or 9.0. The isolate was rod-shaped (2.4–3.6 µm long and 0.9 µm wide) and non-motile, similar to *T. auensis* (Fischer-Romero *et al.*, 1996). The Gram reaction was examined using the Hucker method (Beveridge *et al.*, 2007) and a KOH lysis test (Tindall *et al.*, 2007). An overnight culture of strain OCF 7^T stained Gram-negative and was positive for lysis in the KOH test. Nutritional requirements and substrate use were examined as described previously (Liou *et al.*, 2005). Positives were scored by an increase in optical density and serial passage compared with negative controls. The isolate was characterized biochemically by using a combination of conventional tests as described previously (Tindall *et al.*, 2007). Cells grown aerobically on tryptic soy agar (TSA; Difco) at 22 °C were screened using the Biolog GN2 MicroPlate. Cells grown anaerobically on tryptic soy broth (TSB) at 22 °C were screened using the API Rapid ID 32 A and API ZYM test systems (bioMérieux) according to the manufacturer's instructions. Acids and alcohols were quantified as described previously (Allen *et al.*, 2008). Fatty acid methyl ester (FAME) analysis was performed on overnight cultures grown on TSA at 22 °C by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, as described previously (Miller, 1982; Sasser, 1990). Polar lipid and respiratory quinone analysis was carried out by the Identification Service of the DSMZ and Dr Brian J. Tindall (DSMZ).

The ability to transform substituted aromatic compounds to toluene or phenol was examined under anaerobic conditions using the basal medium described above and was tested in both the presence and absence of glucose (0.5 %). Aromatic substrates were amended from sterile, anoxic stock bottles to an initial concentration of 2 mM and included phenylacetate, phenylalanine, phenoxyacetate, phenylsuccinate, phenylbutyrate and tyrosine (Sigma). A series of tubes in which various amounts of phenylacetate (50, 100, 200, 400 and 600 µM) were added in the presence of glucose (0.5 %) was also examined to look at concentration effects. Toluene was determined by headspace analysis with a Hewlett Packard 5890 series II GC equipped with a flame-ionization detector and a DB-1 capillary column (J&W Scientific), operated isothermally at 150 °C. Phenol was measured by HPLC using a Prevail C₁₈ reversed-phase column (Alltech) with a mobile phase of 25 mM KH₂PO₄/CH₃CN (50:50) at a flow rate of 0.9 ml min⁻¹.

When grown anaerobically, strain OCF 7^T and *T. auensis* DSM 9187^T can be differentiated by their abilities to use arabinose, fumarate, lactose and pyruvate and to hydrolyse urea (Table 1; see species description for full list of

Table 1. Characteristics useful for distinguishing between strain OCF 7^T and *T. auensis* DSM 9187^T when grown anaerobically

Data from this study and Fischer-Romero *et al.* (1996).

Characteristic	OCF 7 ^T	<i>T. auensis</i> DSM 9187 ^T
pH range (optimum)	5.5–8.5 (7.5)	6.0–7.5 (7.2)
Utilization of:		
Arabinose	–	+
Fumarate	+	–
Lactose	+	–
Pyruvate	+	–
Hydrolysis of urea	+	–
Major products from glucose*	F, A, E, L	F, A, E
DNA G + C content (mol%)	52	49

*A, Acetate; E, ethanol; F, formate; L, lactate.

substrates). Major end products from glucose for strain OCF 7^T were formate, acetate, ethanol and lactate (Table 1). In addition, trace amounts of pyruvate were also produced. *T. auensis* DSM 9187^T produces acetate, ethanol and formate as end products of peptone-yeast extract-glucose metabolism. However, the greatest difference between *T. auensis* DSM 9187^T and strain OCF 7^T was the ability to form toluene from substituted aromatic compounds in the presence of other substrates (on which the genus name was based). *T. auensis* DSM 9187^T can produce toluene from phenylacetate, phenylalanine, phenyllactate and phenylpyruvate (and phenol from tyrosine) in the presence of a usable carbon source (Fischer-Romero *et al.*, 1996). Strain OCF 7^T did not anaerobically transform phenylacetate, phenylalanine or similarly related compounds, phenylbutyrate, phenylsuccinate or phenoxyacetate, in the presence of glucose even after 70 days of incubation (data not shown). Furthermore, strain OCF 7^T did not transform tyrosine or phenoxyacetate to phenol. Toluene and phenol were not produced in substrate-unamended controls either. Although overnight growth of strain OCF 7^T on glucose was unaffected by the starting concentration of the aromatic compounds, a series of tubes was prepared to evaluate the effect of concentration. Again, in the presence of glucose, toluene was not detected after 70 days of incubation when the starting concentration of phenylacetate was 50–600 µM. Other differences between *T. auensis* DSM 9187^T and strain OCF 7^T were observed when aerobically grown cells were tested with the 24 h Biolog GN2 MicroPlate. Of the 95 substrates tested for catabolism in the Biolog plate, only four substrates were positive for both micro-organisms: glucose, fructose, galactose and arabinose. The other substrates exhibited opposite reactions or were not utilized by either micro-organism (see Supplementary Table S1, available in IJSEM Online).

Whole-cell fatty acid analysis of strain OCF 7^T revealed C_{12:0} (4.2 %), C_{14:0} (4.9 %), C_{16:0} (22.3 %), C_{18:1ω7c}

(19.0 %), a summed feature of $C_{16:1}\omega 7c$ and/or iso- $C_{15:0}$ 2-OH (43.3 %) and a summed feature of $C_{14:0}$ 3-OH and/or iso- $C_{16:1}$ I (4.5 %) as the primary fatty acids. Strain OCF 7^T was also grown aerobically on TSA and on the basal toluene production medium of Fischer-Romero *et al.* (1996). Cellular fatty acid composition of the isolate was stable when grown on different media and under different conditions (Supplementary Table S2). This FAME analysis agrees well with that of *T. auensis*, the major fatty acids of which were reported to be $C_{12:0}$, $C_{14:0}$, $C_{14:0}$ 3-OH, $C_{16:1}\omega 7c$, $C_{16:0}$ and $C_{18:1}\omega 7c$ (Fischer-Romero *et al.*, 1996). The summed feature $C_{16:1}\omega 7c$ and/or iso- $C_{15:0}$ 2-OH is probably the single fatty acid $C_{16:1}\omega 7c$, reported as the dominant fatty acid in the phylogenetically close neighbours of strain OCF 7^T, *Oceanimonas doudoroffii* (42 %) (Brown *et al.*, 2001) and *Oceanisphaera litoralis* (41 %) (Romanenko *et al.*, 2003). Although *Oceanisphaera donghaensis* (46.2 %) (Park *et al.*, 2006) and *Aeromonas sharmiana* (29.3 %) (Saha & Chakrabarti, 2006) also present the summed feature $C_{16:1}\omega 7c$ and/or iso- $C_{15:0}$ 2-OH as their dominant fatty acid, it should be noted that 2-hydroxy fatty acids have not been detected in members of any closely related genera, namely *Tolumonas*, *Aeromonas*, *Vibrio*, *Haemophilus*, *Actinobacillus*, *Pasteurella*, *Succinivibrio*, *Succinimonas*, *Anaerospirillum* or *Ruminobacter* (Fischer-Romero & Tindall, 2005).

As with *T. auensis*, the major polar lipids for strain OCF 7^T were determined to be phosphatidylglycerol and phosphatidylethanolamine. The presence of phospholipid and aminophospholipid was also detected in strain OCF 7^T. However, the respiratory quinones of strain OCF 7^T differed from those of *T. auensis*. The respiratory quinone of strain OCF 7^T grown on TSB (22 °C) under both aerobic and anaerobic conditions was menaquinone MK-8 (100 %), whereas respiratory quinones of *T. auensis* were Q-8 and MK-8 (Fischer-Romero *et al.*, 1996). Like *T. auensis*, strain OCF 7^T was catalase-positive and oxidase-negative.

The ability to reduce nitrate (but not denitrify) is a common characteristic among members of the family *Aeromonadaceae* (Brown *et al.*, 2001; Martin-Carnahan & Joseph, 2005a; Martin-Carnahan & Joseph, 2005b; Park *et al.*, 2006; Romanenko *et al.*, 2003). However, strain OCF 7^T did not reduce nitrate (the result for nitrate reduction by *T. auensis* is not known).

DNA G + C content was determined by HPLC (Mesbah *et al.*, 1989) using a Prevail C_{18} reversed-phase column (Alltech) at room temperature with a mobile phase of 25 mM KH_2PO_4 : CH_3CN (96:4), pH 2.5. The genomic DNA G + C content was 52.1 ± 0.1 mol%. The 16S rRNA gene of strain OCF 7^T was amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG) and 1492R (5'-GGCTACCTGTTACGACTT) (numbers of primers refer to positions relative to the *Escherichia coli* 16S rRNA gene) (Madrid *et al.*, 2001). Amplified products were treated with ExoSAP-IT (USB) and sequenced directly with primers directed towards conserved positions of the 16S rRNA gene. Sequencing reactions were performed using the

BigDye Terminator cycle sequencing kit, version 3.1 (Applied Biosystems), as described by the manufacturer and were analysed using an Applied Biosystems 3730 DNA analyser. The closest known relatives of the isolate were determined by performing database searches using the program FASTA (Lipman & Pearson, 1985). These sequences and those of other related strains were retrieved from GenBank and aligned with the newly determined sequence using the program SEQtools (Rasmussen, 2002). The resulting multiple sequence alignment was corrected manually using the program GeneDoc (Nicholas *et al.*, 1997) and a phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) with the programs SEQtools and TreeView (Page, 1996). Phylogenetic analysis was performed on 1320 shared nucleotides using the neighbour-joining and maximum-parsimony algorithms; the stability of the groupings was estimated by bootstrap analysis (1000 replications) using the same programs. Sequence searches of GenBank and Ribosomal Database Project libraries revealed that the unknown organism was a member of the family *Aeromonadaceae* in the *Gammaproteobacteria*. It is evident from treeing analysis that strain OCF 7^T is specifically related to the type strain of *T. auensis*. A pairwise comparison revealed a 2.8 % sequence divergence based on the almost full-length 16S rRNA gene sequences (1516 nt). A phylogenetic tree, constructed by the neighbour-joining method, depicting the phylogenetic position of OCF 7^T is shown in Fig. 1. All major branching nodes were confirmed by maximum-parsimony (not shown). Other phylogenetic neighbours of strain OCF 7^T all had <95 % 16S rRNA gene sequence similarity, specifically the type strains of *A. sharmiana* (94.3 %), *Aeromonas hydrophila* (93.0 %), *Oceanimonas doudoroffii* (91.3 %) and *Oceanisphaera litoralis* (90.6 %).

While there is no precise correlation between the level of 16S rRNA gene sequence divergence and species delineation, studies by Stackebrandt & Ebers (2006) demonstrated that sequence similarity values between novel species could be as high as almost 99 % without loss of resolution and recommended mandatory DNA–DNA hybridization only for values above 98.7–99.0 % sequence similarity. Although tree topology and sequence divergence values (2.8 %) showed that the unidentified rod-shaped organism warrants classification within a novel species, its genus assignment is somewhat problematic. A significant characteristic of *T. auensis* is its ability to transform phenylacetate, phenylalanine and substituted aromatic acids into toluene, but strain OCF 7^T did not exhibit this activity. However, FAME analysis, biochemical and polar lipid data are all compatible with the genus *Tolumonas*. From a practical standpoint, the proliferation of taxa based on just a few morphological, biochemical or even chemotaxonomic criteria should be avoided and robust taxonomic units retained where possible. While debate has occurred on the validity of describing novel genera and species based on a single strain, it is important to name novel taxa based on thorough polyphasic studies to allow other members of the scientific community to identify these bacteria as other

Fig. 1. Unrooted tree showing the phylogenetic relationships of strain OCF 7^T (*Tolumonas osonensis* sp. nov.) and closely related microorganisms within the *Gammaproteobacteria*. Circles indicate generic branches that were present in both phylogenetic consensus trees generated by the neighbour-joining and maximum-parsimony algorithms. Numbers at nodes indicate bootstrap values, expressed as percentages of 1000 replications, calculated by neighbour-joining/maximum-parsimony probabilities; only values above 90% are shown. Bar, 1 substitution per 100 nucleotide positions.

strains of them are recovered. Thus, based on the presented phylogenetic and phenotypic evidence, it is proposed that the unidentified isolate recovered from anoxic freshwater creek sediment be assigned to the genus *Tolumonas*, within the novel species *Tolumonas osonensis* sp. nov. In addition to its unique 16S rRNA gene sequence, strain OCF 7^T can be distinguished from *T. auensis* by characteristics shown in Table 1 and Supplementary Table S1.

Emended description of the genus *Tolumonas* Fischer-Romero *et al.* 1996

The phenotypic description of *Tolumonas* is essentially the same as that in *Bergey's Manual of Systematic Bacteriology* (Fischer-Romero & Tindall, 2005) based on that of Fischer-Romero *et al.* (1996) with the following changes. May or may not produce toluene from phenylacetate or phenylalanine in the presence of another carbon source. May or may not produce phenol from tyrosine in the presence of another carbon source. May or may not utilize arabinose, fumarate, lactose or pyruvate. May or may not have the ability to hydrolyse urea. End products from glucose are acetate, ethanol, formate and/or lactate. Grows between pH 5.5 and 8.5. DNA G+C content is 49–52 mol%.

Description of *Tolumonas osonensis* sp. nov.

Tolumonas osonensis (o.so.nen'sis. N.L. fem. adj. *osonensis* pertaining to Oso Creek, Corpus Christi, Texas, USA, where the type strain was isolated).

Cells are non-motile, Gram-negative rods (2.4–3.6 µm long and 0.9 µm wide) and are found singly, in pairs or occasionally in short chains. Facultative anaerobe. Grows at 15–37 °C (optimum, 22 °C) and pH 5.5–8.5 (optimum, pH 7.5). Vitamins or yeast extract are not required for growth. Glucose, fructose, cellobiose, melezitose, maltose, lactose, galactose, sucrose, ribose, raffinose, melibiose, trehalose, mannose, turanose, glycogen, mannitol, sorbitol, amygdalin, pyruvate, fumarate, salicin, aesculin, inulin, *N*-acetyl-D-glucosamine and yeast extract are used as growth substrates. Arabinose, xylose, glycerol, starch, inositol, adonitol, malate, glycolate, choline, betaine, trimethylamine,

formate, acetate, propionate, butyrate, lactate, succinate, methanol, ethanol, n-propanol, n-butanol, peptone, tryptone, Casamino acids, H₂/CO₂ and CO/CO₂ are not utilized. Urea is hydrolysed, but cellulose, gelatin, chitin and starch are not. Nitrate and sulfate are not reduced. End products from glucose are formate, acetate, ethanol and lactate. Toluene is not formed from phenylacetate, phenylalanine, phenylsuccinate, phenylbutyrate or phenoxyacetate in the presence of glucose. Phenol is not formed from tyrosine or phenoxyacetate. Under oxic conditions, catabolizes the following organic substrates when analysed with the 24 h Biolog GN2 MicroPlate: *N*-acetyl-D-glucosamine, L-arabinose, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, methyl β-D-glucoside, D-psicose, raffinose, D-sorbitol, sucrose, trehalose and turanose. Using the API Rapid ID 32A kit, positive reactions are observed for α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, arginine arylamidase, leucine arylamidase (weak), alanine arylamidase, glycine arylamidase and serine arylamidase. Furthermore, mannose and raffinose are fermented. Activity is not detected for urease, arginine dihydrolase, β-galactosidase-6-phosphate, α-arabinosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, glutamic acid decarboxylase, α-fucosidase, alkaline phosphatase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, histidine arylamidase and glutamyl glutamic acid arylamidase. In addition, indole is not produced and nitrate is not reduced. Using the API ZYM kit, positive enzyme activities are high for β-galactosidase and β-glucosidase, moderate for esterase (C4), leucine arylamidase, α-galactosidase and α-glucosidase and weak for alkaline phosphatase, esterase lipase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase. Lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are not detected. The major long-chain fatty acids are C_{16:0}, C_{18:1ω7c} and C_{16:1ω7c} (and/or iso-C_{15:0} 2-OH). Catalase-positive and oxidase-negative when grown aerobically. The respiratory quinone under both aerobic and anaerobic conditions is menaquinone

MK-8. Major polar lipids are phosphatidylethanolamine and phosphatidylglycerol.

The type strain is OCF 7^T (=DSM 22975^T =ATCC BAA-1908^T), isolated from anoxic sediment of a freshwater creek. The DNA G + C content of the type strain is 52 mol%.

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References

- Allen, T. D., Kraus, P. F., Lawson, P. A., Drake, G. R., Balkwill, D. L. & Tanner, R. S. (2008). *Desulfovibrio carbinolophilus* sp. nov., a benzyl alcohol-oxidizing, sulfate-reducing bacterium isolated from a gas condensate-contaminated aquifer. *Int J Syst Evol Microbiol* **58**, 1313–1317.
- Berthelot, A. (1917). Production of phenol by bacteria. *C R Acad Sci* **164**, 196–199.
- Beveridge, T. J., Lawrence, J. R. & Murray, R. G. E. (2007). Sampling and staining for light microscopy. In *Methods for General and Molecular Microbiology*, pp. 19–33. Edited by C. A. Reddy, T. J. Beveridge, J. A. Breznak, G. A. Marzluf, T. M. Schmidt & L. R. Snyder. Washington, DC: American Society for Microbiology.
- Brown, G. R., Sutcliffe, I. C. & Cummings, S. P. (2001). Reclassification of [*Pseudomonas*] *doudoroffii* (Baumann *et al.* 1983) into the genus *Oceanomonas* gen. nov. as *Oceanomonas doudoroffii* comb. nov., and description of a phenol-degrading bacterium from estuarine water as *Oceanomonas baumannii* sp. nov. *Int J Syst Evol Microbiol* **51**, 67–72.
- Chen, T. C. & Levin, R. E. (1975). Isolation of *Aeromonas* sp. ATCC 29063, a phenol-producing organism, from fresh haddock. *Appl Microbiol* **30**, 120–122.
- Elsden, S. R., Hilton, M. G. & Waller, J. M. (1976). The end products of the metabolism of aromatic amino acids by clostridia. *Arch Microbiol* **107**, 283–288.
- Fischer-Romero, C. & Tindall, B. J. (2005). Genus incertae sedis III. *Tolumonas* Fischer-Romero, Tindall and Jüttner 1996, 187^{VP}. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2B, pp. 579–581. Edited by G. M. Garrity, D. J. Brenner, N. R. Krieg & J. T. Staley. New York: Springer.
- Fischer-Romero, C., Tindall, B. J. & Jüttner, F. (1996). *Tolumonas auensis* gen. nov., sp. nov., a toluene-producing bacterium from anoxic sediments of a freshwater lake. *Int J Syst Bacteriol* **46**, 183–188.
- Hungate, R. E. (1969). A roll tube method for cultivation of strict anaerobes. *Methods Microbiol* **3B**, 117–132.
- Lin, Y.-T. & Shieh, W. Y. (2006). *Zobellella denitrificans* gen. nov., sp. nov. and *Zobellella taiwanensis* sp. nov., denitrifying bacteria capable of fermentative metabolism. *Int J Syst Evol Microbiol* **56**, 1209–1215.
- Liou, J. S., Balkwill, D. L., Drake, G. R. & Tanner, R. S. (2005). *Clostridium carboxidivorans* sp. nov., a solvent-producing clostridium isolated from an agricultural settling lagoon, and reclassification of the acetogen *Clostridium scatologenes* strain SL1 as *Clostridium drakei* sp. nov. *Int J Syst Evol Microbiol* **55**, 2085–2091.
- Lipman, D. J. & Pearson, W. R. (1985). Rapid and sensitive protein similarity searches. *Science* **227**, 1435–1441.
- Madrid, V. M., Taylor, G. T., Scranton, M. I. & Chistoserdov, A. Y. (2001). Phylogenetic diversity of bacterial and archaeal communities in the anoxic zone of the Cariaco Basin. *Appl Environ Microbiol* **67**, 1663–1674.
- Martin-Carnahan, A. & Joseph, S. W. (2005a). Family I. *Aeromonadaceae* Colwell, MacDonell and De Ley 1986, 474^{VP}. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2B, p. 556. Edited by G. M. Garrity, D. J. Brenner, N. R. Krieg & J. T. Staley. New York: Springer.
- Martin-Carnahan, A. & Joseph, S. W. (2005b). Genus I. *Aeromonas* Stanier 1943, 213^{AL}. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2B, pp. 557–578. Edited by G. M. Garrity, D. J. Brenner, N. R. Krieg & J. T. Staley. New York: Springer.
- 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.
- Miller, L. T. (1982). Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. *J Clin Microbiol* **16**, 584–586.
- Nicholas, K. B., Nicholas, H. B., Jr & Deerfield, D. W., II (1997). GeneDoc: analysis and visualization of genetic variation. *EMBnet News* **4** (2), 1–4. <http://www.nrbcs.org/gfx/genedoc/ebinet.htm>
- Page, R. D. M. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.
- Park, S.-J., Kang, C.-H., Nam, Y.-D., Bae, J.-W., Park, Y.-H., Quan, Z.-X., Moon, D.-S., Kim, H.-J., Roh, D.-H. & Rhee, S.-K. (2006). *Oceanisphaera donghaensis* sp. nov., a halophilic bacterium from the East Sea, Korea. *Int J Syst Evol Microbiol* **56**, 895–898.
- Rasmussen, S. W. (2002). SEQtools, a software package for analysis of nucleotide and protein sequences. <http://www.seqtools.dk/>
- Romanenko, L. A., Schumann, P., Zhukova, N. V., Rohde, M., Mikhailov, V. V. & Stackebrandt, E. (2003). *Oceanisphaera litoralensis* gen. nov., sp. nov., a novel halophilic bacterium from marine bottom sediments. *Int J Syst Evol Microbiol* **53**, 1885–1888.
- Saha, P. & Chakrabarti, T. (2006). *Aeromonas sharmana* sp. nov., isolated from a warm spring. *Int J Syst Evol Microbiol* **56**, 1905–1909.
- 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.
- Stackebrandt, E. & Ebers, J. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* **33**, 152–155.
- Tanner, R. S. (2007). Cultivation of bacteria and fungi. In *Manual of Environmental Microbiology*, 3rd edn, pp. 69–78. Edited by C. J. Hurst, R. L. Crawford, A. L. Mills, J. L. Garland, L. D. Stetzenbach & D. A. Lipson. Washington, DC: American Society for Microbiology.
- Tindall, B. J., Sikorski, J., Smibert, R. A. & Krieg, N. R. (2007). Phenotypic characterization and the principles of comparative systematics. In *Methods for General and Molecular Microbiology*, pp. 330–393. Edited by C. A. Reddy, T. J. Beveridge, J. A. Breznak, G. A. Marzluf, T. M. Schmidt & L. R. Snyder. Washington, DC: American Society for Microbiology.