Clostridium nitrophenolicum sp. nov., a novel anaerobic *p*-nitrophenol-degrading bacterium, isolated from a subsurface soil sample

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An obligate anaerobic, mesophilic, motile and endospore-forming bacterium, designated 1D^T, was isolated from a subsurface soil sample. The young culture of strain 1D^T was Gram-positive and formed oval spores that were central in position. Based on the biochemical, chemotaxonomic and physiological data, strain 1D^T appears to be a member of the genus *Clostridium*. Strain 1D^T was found to be capable of degrading *p*-nitrophenol (pNP) at a concentration of 0.5 mM under anaerobic conditions as revealed by HPLC analysis. The major fatty acids were C_{16:0} (28.02 %), iso-C_{17:1} / anteiso B (23.05 %) and C_{14:0} (10.02 %). The major polar lipid content was diphosphatidylglycerol. Strain 1D^T showed highest 16S rRNA gene sequence similarity to *Clostridium aciditolerans* JW/YJL-B3^T (98.2 %) and similarity was less for *Clostridium scatologenes* ATCC 25775^T (95.1 %), *Clostridium drakei* SL1^T (95.0 %) and *Clostridium carboxidivorans* P7^T (95.0 %). Phylogenetic analysis showed that it formed a coherent cluster with the species belonging to cluster I of the genus *Clostridium*. The DNA G + C content was 35.5 mol%. DNA–DNA hybridization analysis indicated a mean value of 36.4 % between strain 1D^T and its closest relative *C. aciditolerans*. Several phenotypic differences from the closely related species were also revealed. On the basis of the polyphasic characteristics, strain 1D^T represents a novel species of the genus *Clostridium*, for which the name *Clostridium nitrophenolicum* sp. nov. is proposed. The type strain is 1D^T (=MTCC 7832^T=JCM 14030^T).

The genus *Clostridium* is one of the largest genera among prokaryotes that constitute anaerobic, Gram-positive and endospore-forming bacteria. They have been isolated from soil, sediment, decomposing biological material and the lower gut of mammals. Members of the genus *Clostridium* have diverse metabolic capabilities and are known to possess strong nitroreductase properties that have been extensively investigated with purified enzymes and cell cultures (Spain *et al.*, 2000). Attempts have been made to exploit this property for the remediation of soil contaminated with polynitroaromatics such as 2,4-dinitrophenol, 2,4,6-trinitrotoluene, 1,3,5-trinitrohexahydro-1,3,5-triazine etc. (Gorontzy *et al.*, 1993; Hughes *et al.*, 1998, 1999; Lewis *et al.*, 1996; Shin *et al.*, 1997). However, little is known about the *Clostridium* species that degrade mononitrophenols such as *p*-nitrophenol (pNP) and *m*-nitrophenol. To our knowledge, there is only a single report available on pNP

transformation by *Clostridium pasteurianum* and *Clostridium* sp. W1 under anaerobic conditions (Gorontzy *et al.*, 1993). In the present study, by using the polyphasic approach, we determined the taxonomic position of the bacterial strain 1D^T that is capable of degrading pNP under anaerobic conditions.

The bacterium 1D^T was isolated from a soil sample (red-sandy soil) in a pot that was obtained from the subsurface environment from a depth of about 3–4 m during an archaeological survey at Hyderabad, India located between longitude 78°30' east and latitude 17°23' north. The sample yielding strain 1D^T was isolated by performing standard plate-dilution technique on tryptone soy agar (TSA; Himedia), with 0.01 % of resazurin as an indicator, at 30 °C under anaerobic conditions. The isolate was maintained as a glycerol stock at –70 °C until further characterization. The reference strain *Clostridium aciditolerans* DSM 17425^T was obtained from DSMZ, Germany.

Strain 1D^T was found to be an obligate anaerobe and it formed a cream coloured colony. Cell morphology was observed under a phase-contrast microscope (Zeiss, Axiophot) using an oil-immersion objective (×100) to ascertain the shape. Presence of spore formation was

Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; pNP, *p*-nitrophenol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 1D^T is AM261414.

A table detailing the cellular fatty acid composition of strain 1D^T and *Clostridium aciditolerans* is available with the online version of this paper.

determined by staining with malachite green as described previously (Gerhardt *et al.*, 1994). Motility was confirmed by using the hanging-drop method immediately after taking out the culture from anaerobic conditions. Physiological tests such as growth at different temperatures, pH and salt concentrations were performed using basal TSA medium with L-cysteine hydrochloride (0.05 %) as a reducing agent. Strain 1D^T was found to grow between temperatures 20 and 45 °C with optimum growth at 30 °C. Biological buffers (pH 5.5–6.5 MES and 7.5–8.5 NaHCO₃/Na₂CO₃ system) were used for pH adjustment. The efficiency of strain 1D^T to degrade pNP was tested in tryptone soy broth (TSB) supplemented with 0.2–0.5 mM of pNP in serum, vials under anaerobic conditions. Analysis on quantification of pNP was done by using HPLC with a Waters 600 model equipped with a Waters 996 photodiode array detector operating at 315 nm. Separation was carried out with a Waters Spherisorb 5 µm C8 column and the mobile phase was 1 % acetic acid/methanol (solvent A) and 1 % acetic acid/water (solvent B). Compounds were eluted at a flow rate of 1.5 ml min⁻¹ by using a gradient starting with 35 % of solvent A followed by a gradual increase to 68 % (0–10 min).

For biochemical tests mentioned in Table 1 and species description, strain 1D^T was grown at 30 °C on TSA medium with L-cysteine hydrochloride (0.05 %) as a reducing agent and under anaerobic conditions. Catalase and oxidase tests were carried out as described by Cowan & Steel (1965). Indole test, Voges–Proskauer test, methyl red test, H₂S production and nitrate reduction were performed as described by Lanyi (1987). Casein, gelatin, starch and Tween 20 hydrolysis were determined as described by Smibert & Krieg (1994). Assimilation of various substrates by strain 1D^T was determined by using the Biolog system according to the manufacturer's instructions except that reinforced clostridial agar (RCA, Himedia) was used instead of the Biolog Universal agar with 5 % blood. Furthermore, the ability of strain 1D^T to produce acid from sugar was checked in medium containing (g 100 ml⁻¹) 0.01 KCl, 0.01 yeast extract, 0.025 K₂HPO₄, 0.025 MgSO₄, 0.2 (NH₄)₂SO₄, 0.01 bromocresol purple supplemented with 1.0 % of each filter-sterilized carbon compound. Fatty acid methyl esters (Sato & Murata, 1988) were extracted from cells grown on TSA with L-cysteine hydrochloride (0.05 %) at 30 °C under anaerobic conditions and analysed by Microbial Identification System (MIDI) as described by Pandey *et al.*

Table 1. Characteristics that differentiate strain 1D^T from related species of the genus *Clostridium*

Strains: 1, *C. nitrophenolicum* 1D^T; 2, *C. aciditolerans* JW/YJL-B3^T; 3, *C. drakei* SL1^T; 4, *C. scatologenes* ATCC 25775^T; 5, *C. carboxidivorans* P7^T; 6, *C. magnum* ATCC 49199^T. Data are from this and earlier studies (Lee *et al.*, 2007; Liou *et al.*, 2005; Küsel *et al.*, 2000; Schink, 1984). +, Positive; –, negative; NR, not reported.

Characteristic	1	2	3	4	5	6
Gram reaction	+	–	–	–	+	–
Temperature optimum (°C)	30	35	30–37	37–40	38	15–45
pH range	6.5–8.0	3.8–8.9	4.6–7.8	4.6–8.0	4.4–7.5	6.0–7.5
Gelatin hydrolysis	+	+	–	NR	–	–
Casein hydrolysis	+	–	NR	NR	NR	NR
Esterase activity	–	+	NR	NR	NR	NR
Utilization of:						
Cellobiose	–	+	+	+	+	NR
Citrate	–	NR	NR	NR	NR	+
Glucose	–	+	+	+	+	+
Lactose	–	+	–	–	–	NR
Malate	–	NR	+	NR	–	+
Maltose	–	+	–	+	–	NR
Pyruvate	–	+	NR	+	NR	+
Mannose	–	+	+	+	+	NR
Raffinose	–	+	–	–	–	NR
Rhamnose	+	–	+	NR	NR	NR
Ribose	–	+	+	NR	+	NR
Sucrose	–	+	+	NR	+	+
Xylose	–	+	+	+	+	+
End products of glucose fermentation	Formate, acetate and pyruvate	Acetate, butyrate and ethanol	Acetate, butyrate, ethanol and butanol	Acetate and butyrate*	Acetate, butyrate, ethanol and butanol	Acetate and CO ₂
DNA G + C content (mol%)	35.5	30.8	32	31	31	29.1

*End products of fructose fermentation.

al. (2002). Peptidoglycan type was analysed according to Komagata & Suzuki (1987) and menaquinones were extracted and analysed as described by Minnikin *et al.* (1984). Polar lipids were analysed as described by Suresh *et al.* (2004). Fermentation end products of glucose were determined by GC with a flame-ionization detector (Shimadzu) as described by Mountfort & Rhodes (1991). DNA was isolated as described by Shivaji *et al.* (1989). The G+C content of genomic DNA was determined as described by Mesbah *et al.* (1989). The enzymically degraded nucleosides were separated by HPLC using a reverse-phase column (C18; Phenomenex); non-methylated λ DNA (49.85 mol% G+C) was used as the calibration reference. DNA–DNA hybridization was performed by the membrane filter method (Tourova & Antonov, 1987) as described by Shivaji *et al.* (1992).

Cells in young culture were Gram-positive rods, strictly anaerobic and motile. Other characteristics are described in Table 1 and the species description. They grew well in medium containing pNP at a concentration of 0.5 mM. HPLC analysis revealed that strain 1D^T is capable of degrading pNP and the complete degradation of pNP was observed within 24 h as there was no pNP spectrum observed through HPLC analysis. Neither intermediate(s) nor end product(s) was observed during the anaerobic degradation of pNP, suggesting complete degradation by strain 1D^T. However, further characterization of the biochemical steps involved in the degradation is the subject of future studies.

The 16S rRNA gene was amplified by PCR using primers 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') as described by Pandey *et al.* (2002). Amplified PCR product was purified using QIAquick PCR purification kit (Qiagen), sequenced using the Big Dye Terminator kit (Applied Biosystems) and

an ABI Prism model 3130×1 automatic DNA sequencer. The almost-complete 16S rRNA gene sequence (1461 bp continuous stretch) was obtained and used for the initial BLAST search. Closely related sequences were retrieved from the EMBL database. For alignment, CLUSTAL_X was used (Thompson *et al.*, 1997) and the results obtained were edited manually. Pair-wise evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1980). A neighbour-joining phylogenetic tree was constructed using TREECON (version 1.3b) (Van de Peer & De Wachter, 1997). The stability among the groupings of the phylogenetic tree was assessed based on 1000 replicates. *Sporacetigenium mesophilum* DSM 16796^T (AY682207) was used as the outgroup.

Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain 1D^T represents a novel species of the genus *Clostridium*. It exhibited highest similarity to *C. aciditolerans* JW/YJL-B3^T (98.2 %) followed by *Clostridium scatologenes* ATCC 25775^T (95.1 %), *Clostridium drakei* SL1^T (95.0 %), *Clostridium carboxidivorans* P7^T (95.0 %) and much lower similarity to other species of the genus with validly published names. Although *Clostridium magnum* ATCC 49199^T showed 95.3 % similarity in pair-wise sequence analysis, there was complete dissimilarity observed between nt 980 and 1022 (according to *Escherichia coli* number) that was not taken into account. The neighbour-joining phylogenetic analysis revealed that strain 1D^T formed a clade with the type strain of *C. aciditolerans*, with 100 % bootstrap value (Fig. 1), and formed a coherent cluster with other members of cluster I of the genus *Clostridium* (Collins *et al.*, 1994). Strain 1D^T showed highest similarity to *C. aciditolerans* at 16S rRNA gene sequence level; however, it exhibited only 36.4 % mean value of DNA–DNA hybridization at whole genome level, which is significantly below the recommended threshold

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1461 bases) showing the relationship between strain 1D^T and other related species of the genus *Clostridium*. Bootstrap values greater than 50 % are given at the nodes. Bar, 0.01 substitutions per site.

value of less than 70 % for the delineation of a new species (Wayne *et al.*, 1987). There were several phenotypic properties as well as end products of glucose fermentation to distinguish strain 1D^T from *C. aciditolerans* (Table 1). The fatty acid analysis of strains 1D^T and *C. aciditolerans* indicated that they had C_{16:0}, iso-C_{17:1}I/anteiso B and C_{14:0} as major components; however, fatty acids iso-C_{13:0}, C_{13:0}, iso-C_{15:0}, anteiso-C_{15:0}, C_{15:1}ω8c, C_{15:1}ω6c and iso-C_{18:1} H were present in *C. aciditolerans* in significant quantities but absent in strain 1D^T (see Supplementary Table S1 available in IJSEM Online). Presence of C_{16:0} as a major fatty acid, which is also reported in other species (Wilde *et al.*, 1997; Broda *et al.*, 2000a, b; Spring *et al.*, 2003), further affiliates strain 1D^T to the genus *Clostridium*. The polar lipid analysis revealed the presence of diphosphatidylglycerol (DPG), as a major lipid and other lipids include phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and two unknown phospholipids. Based on these data presented, strain 1D^T represents a novel species in the genus *Clostridium*, for which the name *Clostridium nitrophenolicum* sp. nov. is proposed.

Description of *Clostridium nitrophenolicum* sp. nov.

Clostridium nitrophenolicum (ni.tro.phen.o'li.cum. N.L. n. *nitrophenol*; L. suff. *-icus -a -um* suffix used with the sense of belonging to; N.L. neut. adj. *nitrophenolicum* referring to the substrate nitrophenol that can be utilized by the species).

Cells are rod shaped measuring 3.5–5.0 µm in length and 0.6–0.9 µm in width and strictly anaerobic. They form spores that are oval and central in position. Colonies grown on TSA are circular, smooth and convex with wavy margin. Growth occurs between pH 6.5 and 8.0 and no growth is observed below pH 6.5 and above 8.0. Growth occurs between 20 and 45 °C and does not grow at 15 or 50 °C. Optimum growth is observed at pH 7.2 and temperature 30 °C. Growth occurs between 0 and 1 % NaCl and does not grow with 2.0 % NaCl. Strain 1D^T is negative for catalase and oxidase, and positive for indole and methyl red test. Starch and urea are not hydrolysed. Does not reduce nitrate to nitrite and is negative for Voges–Proskauer test, citrate utilization and H₂S production. Using Biolog MicroPlates (AN), the strain showed a positive reaction for the assimilation of D-fructose, L-fructose, D-galactose, D-galacturonic acid, palatinose and L-rhamnose and a negative reaction for acetic acid, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, adonitol, L-alaninamide, L-alanine, L-alanyl L-glutamine, L-alanyl L-histidine, L-alanyl L-threonine, amygdalin, D-arabitol, arbutin, L-asparagine, D-cellobiose, α-cyclodextrin, β-cyclodextrin, dextrin, dulcitol, i-erythritol, formic acid, D-gluconic acid, α-D-glucose 1-phosphate, α-D-glucose 6-phosphate, L-glutamic acid, L-glutamine, glycerol, glyoxylic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, inosine, myo-inositol, itaconic acid, α-ketobutyric acid, β-ketobutyric acid, lactic acid, lactulose, malic acid, maltose, maltotriose, D-mannitol,

mannose, D-melibiose, L-methionine, methyl β-D-galactoside, methyl α-D-glucoside, methyl β-D-glucoside, 3-methyl-D-glucose, L-phenylalanine, propionic acid, pyruvic acid, D-raffinose, L-rhamnose, D-saccharic acid, salicin, L-serine, D-sorbitol, stachylose, succinic acid, sucrose, m-tartaric acid, L-threonine, thymidine, trehalose, turanose, uridine, urocanic acid and L-valine. It produced acid from glucose, dulcitol, fructose, galactose, maltose, mannose, sucrose and trehalose, but not from adonitol, arabinose, cellobiose, inositol, mannitol, melibiose, salicin or sorbitol. Fermentation end products from glucose include acetate, formate and pyruvate. Menaquinone present is MK-7 and the cell wall amino acid is meso-diaminopimelic acid. Polar lipids present are DPG, PG, PE and two unknown phospholipids (UKP1 and UKP2). The major cellular fatty acids are (%); C_{16:0} (28.02), iso-C_{17:1}I/anteiso B (23.05), C_{16:1}ω7c/iso-C_{15:0} 2-OH (10.82) and C_{14:0} (10.02). The DNA G + C content is 35.5 mol%.

The type strain, 1D^T (=MTCC 7832^T=JCM 14030^T), was isolated from a subsurface soil sample from a depth of about 3–4 m.

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References

- Broda, D. M., Saul, D. J., Lawson, P. A., Bell, R. G. & Musgrave, D. R. (2000a). *Clostridium gasigenes* sp. nov., a psychrophile causing spoilage of vacuum-packed meat. *Int J Syst Evol Microbiol* **50**, 107–118.
- Broda, D. M., Saul, D. J., Bell, R. G. & Musgrave, D. R. (2000b). *Clostridium algidixylanolyticum* sp. nov., a psychrotolerant, xylan-degrading, spore-forming bacterium. *Int J Syst Evol Microbiol* **50**, 623–631.
- Collins, M. D., Lawson, P. A., Willems, A., Cardoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.
- Cowan, S. T. & Steel, K. J. (1965). *Manual for the Identification of Medical Bacteria*. London: Cambridge University Press.
- Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (editors) (1994). *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.
- Gorontzy, T., Kuver, J. & Blotevogel, K. H. (1993). Microbial transformation of nitroaromatic compounds under anaerobic conditions. *J Gen Microbiol* **139**, 1331–1336.
- Hughes, J. B., Wang, C. W., Bhadra, R., Richardson, A., Bennett, G. N. & Rudolph, F. (1998). Reduction of 2,4,6-trinitrotoluene by *Clostridium acetobutylicum* through hydroxylamino-nitrotoluene intermediates. *Environ Toxicol Chem* **17**, 343–348.
- Hughes, J. B., Chuan, Y. W. & Chunlong, Z. (1999). Anaerobic biotransformation of 2,4-dinitrotoluene and 2,6-dinitrotoluene by

- Clostridium acetobutylicum*: a pathway through dihydroxylamino intermediates. *Environ Sci Technol* **33**, 1065–1070.
- 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.
- Komagata, K. & Suzuki, K. (1987).** Lipid and cell wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161–207.
- Küsel, K., Dorsch, T., Acker, G., Stackebrandt, E. & Drake, H. L. (2000).** *Clostridium scatologenes* strain SL1 isolated as an acetogenic bacterium from acidic sediments. *Int J Syst Evol Microbiol* **50**, 537–546.
- Lanyi, B. (1987).** Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* **19**, 1–67.
- Lee, Y.-J., Romanek, C. S. & Wiegel, J. (2007).** *Clostridium aciditolerans* sp. nov., an acid tolerant spore-forming anaerobic bacterium from constructed wetland sediment. *Int J Syst Evol Microbiol* **57**, 311–315.
- Lewis, T. A., Goszcynski, S., Crawford, R. L., Korus, R. A. & Admassu, W. (1996).** Products of anaerobic 2,4,6-trinitrotoluene (TNT) transformation by *Clostridium bifermentans*. *Appl Environ Microbiol* **62**, 4669–4674.
- Liou, J. S.-C., 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.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1998).** Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A. & Parlett, J. H. (1984).** An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* **2**, 233–241.
- Mountfort, D. O. & Rhodes, L. L. (1991).** Anaerobic growth and fermentation characteristics of *Paecilomyces lilacinus* isolated from mullet gut. *Appl Environ Microbiol* **57**, 1963–1968.
- Pandey, K. K., Mayilraj, S. & Chakrabarti, T. (2002).** *Pseudomonas indica* sp. nov., a novel butane-utilizing species. *Int J Syst Evol Microbiol* **52**, 1559–1567.
- Sato, N. S. & Murata, N. (1988).** Membrane lipids. In *Methods in Enzymology*, vol. 167, pp. 251–259. Edited by L. Packer & A. N. Glazer. New York: Academic Press.
- Schink, B. (1984).** *Clostridium magnum* sp. nov., a non-autotrophic homoacetogenic bacterium. *Arch Microbiol* **137**, 250–255.
- Shin, C. Y., Lewis, T. A. & Crawford, D. L. (1997).** In *In Situ and On-Site Bioremediation*, vol. 2, pp. 23–29. Edited by B. C. Alleman & A. Leeson. Columbus: Battelle Press.
- Shivaji, S., Rao, N. S., Saisree, L., Reddy, G. S. N., Kumar, G. S. & Bhargava, P. M. (1989).** Isolates of *Arthrobacter* from the soils of Schirmacher Oasis, Antarctica. *Polar Biol* **10**, 225–229.
- Shivaji, S., Ray, M. K., Rao, N. S., Saisree, L., Jagannadham, M. V., Seshu Kumar, G., Reddy, G. S. N. & Bhargava, P. M. (1992).** *Sphingobacterium antarcticus* sp. nov., a psychrotrophic bacterium from the soils of Schirmacher Oasis, Antarctica. *Int J Syst Bacteriol* **42**, 102–106.
- Smibert, R. M. & Krieg, N. R. (1994).** Phenotypic characterization: In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt. Washington, DC: American Society for Microbiology.
- Spain, J. C., Hughes, J. B. & Knackmuss, H.-J. (2000).** *Biodegradation of Nitroaromatic Compounds and Explosives*. Boca Raton, FL: CRC Press LLC.
- Spring, S., Merkhoffer, B., Weiss, N., Kroppenstedt, R. M., Hippe, H. & Stackebrandt, E. (2003).** Characterization of novel psychrophilic clostridia from an Antarctic microbial mat: description of *Clostridium frigoris* sp. nov., *Clostridium lacusfruxellense* sp. nov., *Clostridium bowmanii* sp. nov. and *Clostridium psychrophilum* sp. nov. and reclassification of *Clostridium laramiense* as *Clostridium estertheticum* subsp. *laramiense* subsp. nov. *Int J Syst Evol Microbiol* **53**, 1019–1029.
- Suresh, K., Reddy, G. S. N., Sengupta, S. & Shivaji, S. (2004).** *Deinococcus indicus* sp. nov., an arsenic-resistant bacterium from an aquifer in West Bengal, India. *Int J Syst Evol Microbiol* **54**, 457–461.
- 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.
- Tourova, T. P. & Antonov, A. S. (1987).** Identification of micro-organisms by rapid DNA–DNA hybridization. *Methods Microbiol* **19**, 333–355.
- Van de Peer, Y. & De Wachter, R. (1997).** Construction of evolutionary distance trees with TREECON for windows: accounting for variation in nucleotide substitution rate among sites. *Comput Appl Biosci* **13**, 227–230.
- 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.
- Wilde, E., Collins, M. D. & Hippe, H. (1997).** *Clostridium pascui* sp. nov., a new glutamate-fermenting sporeformer from a pasture in Pakistan. *Int J Syst Evol Microbiol* **47**, 164–170.