Gracilibacillus ureilyticus sp. nov., a halotolerant bacterium from a saline–alkaline soil

Ying-Yi Huo,[1] Xue-Wei Xu,[2,3] Heng-Lin Cui[4] and Min Wu[1]

Correspondence
Min Wu
wumin@zju.edu.cn

[1]College of Life Sciences, Zhejiang University, Hangzhou 310058, PR China

[2]Laboratory of Marine Ecosystem and Biogeochemistry, State Oceanic Administration, Hangzhou 310012, PR China

[3]Second Institute of Oceanography, State Oceanic Administration, Hangzhou 310012, PR China

[4]School of Food and Biological Engineering, Jiangsu University, Zhenjiang 212013, PR China

A Gram-stain-positive, halotolerant, neutrophilic, rod-shaped bacterium, strain MF38^T, was isolated from a saline–alkaline soil in China and subjected to a polyphasic taxonomic characterization. The isolate grew in the presence of 0–15 % (w/v) NaCl and at pH 6.5–8.5; optimum growth was observed with 3.0 % (w/v) NaCl and at pH 7.0. Chemotaxonomic analysis showed menaquinone MK-7 as the predominant respiratory quinone and anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$, $C_{17:0}$ and $C_{16:0}$ as major fatty acids. The genomic DNA G+C content was 35.3 mol%. 16S rRNA gene sequence similarities of strain MF38^T with type strains of described *Gracilibacillus* species ranged from 95.3 to 97.7 %. Strain MF38^T exhibited the closest phylogenetic affinity to the type strain of *Gracilibacillus dipsosauri*, with 97.7 % 16S rRNA gene sequence similarity. The DNA–DNA reassociation between strain MF38^T and *G. dipsosauri* DSM 11125^T was 45 %. On the basis of phenotypic and genotypic data, strain MF38^T represents a novel species of the genus *Gracilibacillus*, for which the name *Gracilibacillus ureilyticus* sp. nov. (type strain MF38^T =CGMCC 1.7727^T =JCM 15711^T) is proposed.

The genus *Gracilibacillus* was first proposed by Wainø *et al.* (1999) with the type species *Gracilibacillus halotolerans*, and *Bacillus dipsosauri* (Lawson *et al.*, 1996) was reclassified in the genus as *Gracilibacillus dipsosauri* at the same time. Seven further species, *Gracilibacillus orientalis* (Carrasco *et al.*, 2006), *G. boraciitolerans* (Ahmed *et al.*, 2007), *G. lacisalsi* (Jeon *et al.*, 2008), *G. halophilus* (Chen *et al.*, 2008a), *G. quinghaiensis* (Chen *et al.*, 2008b), *G. saliphilus* (Tang *et al.*, 2009) and *G. thailandensis* (Chamroensaksri *et al.*, 2010), have since been described. Most of them were isolated from saline lakes, the exceptions being *G. dipsosauri* (from a desert iguana; Lawson *et al.*, 1996), *G. boraciitolerans* (from a soil containing high levels of boron; Ahmed *et al.*, 2007) and *G. thailandensis* (from fermented fish; Chamroensaksri *et al.*, 2010). Here, we present the results of a polyphasic study describing a novel halotolerant *Gracilibacillus* strain isolated from a saline–alkaline soil in China.

The saline–alkaline soil sample was collected from Minfeng country located in Xinjiang Province, China, in December 2005. The sample contained some granulated salts and was alkaline (pH 8.5). Approximately 100 mg soil sample was incubated for 30 min in modified halophilic medium (HM) containing 10 % NaCl (w/v) without carbon source. The modified HM medium contained (per l distilled water): NaCl as indicated, 2.0 g KCl, 1.0 g $MgSO_4$, 0.36 g $CaCl_2.2H_2O$, 0.23 g NaBr, 0.06 g $NaHCO_3$, trace $FeCl_3$, 1.0 g yeast extract (Difco), 0.5 g peptone (Difco) and 0.1 g glucose (pH 7.5) (Ventosa *et al.*, 1982). The liquid was plated on modified HM agar plates, using a tenfold dilution series. After 3 days of incubation at 25 °C, a cream-coloured colony, designated MF38^T, was picked. The strain was purified by repeated restreaking; purity was confirmed by the uniformity of colony morphology.

The 16S rRNA gene was amplified and PCR products were cloned into pMD 19-T vector (TaKaRa) for sequencing (Xu *et al.*, 2007b). An almost-complete 16S rRNA gene sequence (1485 nt) was obtained and compared with closely related sequences of reference organisms from the FASTA and EzTaxon services (Chun *et al.*, 2007). Sequence data were aligned with CLUSTAL W 1.8 (Thompson *et al.*, 1994).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MF38^T is EU709020.

Tables comparing the taxonomic characteristics of strain MF38^T and type strains of related *Gracilibacillus* species and the cellular fatty acid compositions of strain MF38^T and *G. dipsosauri* DSM 11125^T, an electron micrograph of strain MF38^T and maximum-parsimony and maximum-likelihood 16S rRNA gene sequence-based phylogenetic trees are available as supplementary material with the online version of this paper.

Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods with the MEGA 4 program package (Tamura *et al.*, 2007) and the maximum-likelihood method (Felsenstein, 1981) with the TreePuzzle 5.2 program. Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980) for the neighbour-joining method.

Comparisons of 16S rRNA gene sequences showed that strain MF38^T should be positioned within the genus *Gracilibacillus*, related most closely to the type strain of *G. dipsosauri*, with 97.7 % similarity. Phylogenetic analysis based on 16S rRNA gene sequence showed that strain MF38^T had the closest phylogenetic affinity to the type strain of *G. dipsosauri*, with high levels of bootstrap support (Fig. 1). The topologies of phylogenetic trees built using the maximum-parsimony and maximum-likelihood algorithms also supported the notion that strain MF38^T formed a stable clade with *G. dipsosauri* (Supplementary Figs S1 and S2, available in IJSEM Online).

Growth at various NaCl concentrations (0, 0.5, 1.0, 2.0, 3.0, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 22.5 and 25.0 %, w/v) was investigated in trypticase soy broth (TSB; Difco) with 1 M KCl according to Lawson *et al.* (1996). The pH range for growth was determined at pH 5.0–10.0 (at intervals of 0.5 pH units) in TSB with 1 M KCl using the following buffers at 40 mM: MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5) and CAPSO (pH 9.0–10.0). The temperature range for growth was determined by incubation at 4, 10, 15, 20, 25, 30, 35, 37, 42, 45, 48 and 50 °C. Cell morphology and motility were examined by optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL). The NaCl concentration, pH and temperature for growth of strain MF38^T were 0–15 % (w/v), pH 6.5–8.5 and 10–45 °C. Cells of strain MF38^T were Gram-stain-positive, spore-forming rods and motile by means of peritrichous flagella (Supplementary Fig. S3).

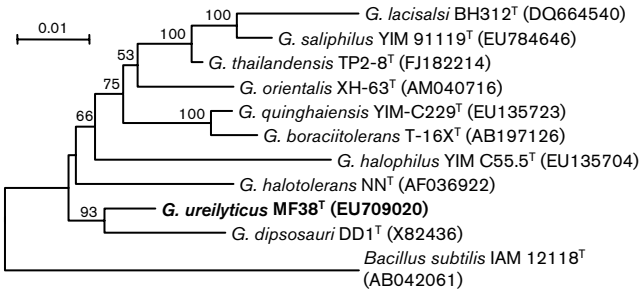

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain MF38^T and related taxa. Bootstrap values are based on 1000 replicates; values >50 % are shown. Bar, 0.01 substitutions per nucleotide position.

Single carbon source assimilation tests were performed using modified basal medium [l^{-1} distilled water: 1.0 g NH_4Cl, 0.044 g K_2HPO_4, 0.028 g $FeSO_4.7H_2O$, 500 ml artificial seawater, 50 ml Tris/HCl (1 M, pH 7.5)]. Artificial seawater contained (per l distilled water) 30.0 g NaCl, 24.6 g $MgSO_4.7H_2O$, 1.5 g KCl and 2.9 g $CaCl_2$. Filter-sterilized sugars (0.2 %), alcohols (0.2 %), organic acids (0.1 %) and amino acids (0.1 %) were separately added to the liquid medium. Acid production was investigated by using modified MOF medium [per l distilled water: 30.0 g NaCl, 2.5 g $MgCl_2.2H_2O$, 1.0 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.25 g $CaCl_2$, trace $FeSO_4$, 0.5 g $(NH_4)_2SO_4$, 1.0 g casitone (Difco), 0.1 g yeast extract (Difco), 0.5 g Tris base, 0.01 g phenol red (pH 7.5)] supplemented with 1 % sugars or alcohols (Leifson, 1963). Oxidase activity was determined by oxidation of 1 % *p*-aminodimethylaniline oxalate. Catalase activity was determined by bubble production in 3 % (v/v) H_2O_2 solution. Biochemical and nutritional tests were performed in the modified basal medium supplemented with 5 g yeast extract l^{-1} according to Mata *et al.* (2002). Additional enzyme activities and biochemical characteristics were determined using API 20E, API 20NE and API ZYM kits (bioMérieux); cells for inoculation of these API test strips were suspended in 3 % NaCl. API ZYM strips were read after 10 h and API 20NE and API 20E strips after 24 and 48 h. *G. dipsosauri* DSM 11125^T was used as control in the

Table 1. Differentiating characteristics of strain MF38^T from its closest phylogenetic relative, *G. dipsosauri* DSM 11125^T

Unless indicated, data were obtained in this study under identical growth conditions. +, Positive; –, negative; W, weakly positive.

Characteristic	Strain MF38^T	*G. dipsosauri* DSM 11125^T
Hydrolysis of urea	+	–
Susceptibility to:		
Amoxicillin (10 μg)	+	–
Ampicillin (10 μg)	+	–
Bacitracin (0.04 IU)	+	–
Penicillin G (10 IU)	+	–
API ZYM tests		
α-Galactosidase	+	–
β-Galactosidase	+	W
β-Glucosidase	+	W
Fermentation/oxidation of:		
D-Glucose	–	+
D-Mannitol	–	+
L-Rhamnose	–	+
Sucrose	–	+
Melibiose	–	+
Amygdalin	–	+
L-Arabinose	–	+
DNA G+C content (mol%)	35.3	39.4*

*Data from Lawson *et al.* (1996).

tests. Detailed results are given in the species description and in Table 1 and Supplementary Table S1.

Fatty acid methyl esters obtained from cells grown on MA (Difco) for 2 days at 35 °C were analysed by using GC/MS (Kuykendall *et al.*, 1988). Isoprenoid quinones were analysed using reversed-phase HPLC as described previously (Komagata & Suzuki, 1987). Phospholipids and glycolipids were separated on silica gel plates (10 × 10 cm) by TLC and were analysed according to Xu *et al.* (2007a). The purified DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with calf intestine alkaline phosphatase; the G+C content of the resulting deoxyribonucleosides was determined by reversed-phase HPLC and calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) (Mesbah & Whitman, 1989). The major fatty acids of strain MF38^T were anteiso-$C_{15:0}$ (34.2 %), anteiso-$C_{17:0}$ (21.4 %), iso-$C_{15:0}$ (13.7 %), $C_{17:0}$ (7.0 %) and $C_{16:0}$ (6.2 %). This profile is similar to that of *G. dipsosauri* DSM 11125^T. Nevertheless, the absence of $C_{12:0}$, iso-$C_{13:0}$, iso-$C_{18:0}$, $C_{18:1}\omega 9c$, $C_{19:0}$ and anteiso-$C_{19:0}$ from the fatty acids is a distinct characteristic that differentiates strain MF38^T from *G. dipsosauri* DSM 11125^T (Supplementary Table S2).

Strain MF38^T exhibited the closest phylogenetic affinity and highest 16S rRNA gene sequence similarity to *G. dipsosauri* DSM 11125^T. DNA–DNA hybridizations were performed by the thermal denaturation and renaturation method of De Ley *et al.* (1970) as modified by Huß *et al.* (1983), using a Beckman DU 800 spectrophotometer. The level of DNA–DNA relatedness of 45 % between strain MF38^T and *G. dipsosauri* DSM 11125^T is significantly below the value of 70 % considered to be the threshold for the delineation of bacterial species (Wayne *et al.*, 1987). In addition, strain MF38^T could be differentiated from recognized species on the basis of some phenotypic characteristics, including spore shape, nitrate reduction, H_2S production, hydrolysis of substrates, susceptibility to antibiotics and enzyme activities (Supplementary Table S1). Strain MF38^T could also be distinguished from *G. dipsosauri* DSM 11125^T by several phenotypic characteristics, including hydrolysis of urea, α-galactosidase activity, susceptibility to bacitracin and penicillin G and fermentation of sugars (Table 1).

On the basis of the phylogenetic, genotypic, chemotaxonomic and phenotypic data, we propose to classify strain MF38^T as the type strain of a novel species within the genus *Gracilibacillus*, *Gracilibacillus ureilyticus* sp. nov.

Description of *Gracilibacillus ureilyticus* sp. nov.

Gracilibacillus ureilyticus (u.re.i.ly′ti.cus. N.L. n. *urea* urea; N.L. adj. *lyticus* able to dissolve; N.L. masc. adj. *ureilyticus* urea-dissolving).

Cells are Gram-stain-positive, spore-forming, motile rods, 0.7–1.0 μm wide and 1.5–4.5 μm long. Colonies on MA are 1–2 mm in diameter, rough, slightly elevated and cream-coloured with irregular edges after 2 days at 37 °C. Growth occurs at NaCl concentrations of 0–15 % (w/v), with optimum growth at 3.0 %, and at pH 6.5–8.5 and 10–45 °C (optimum growth at pH 7.0 and 35–37 °C). Oxidase- and catalase-positive. Aesculin, gelatin, starch, Tween 20 and urea are hydrolysed. Casein, DNA, Tweens 40, 60 and 80 and tyrosine are not hydrolysed. Arginine dihydrolase, indole production, lysine and ornithine carboxylases, citrate utilization, tryptophan deaminase and fermentation of amygdalin, L-arabinose, D-glucose, inositol, D-mannitol, melibiose, L-rhamnose, D-sorbitol and sucrose are negative. Voges–Proskauer and *o*-nitrophenyl-β-D-galactopyranosidase tests are positive. Nitrate is reduced to nitrite. H_2S is not produced. The following substrates are utilized for growth: L-arabinose, cellobiose, D-galactose, gluconate, glucose, lactose, maltose, D-mannitol, D-mannose, raffinose, L-rhamnose, D-salicin, starch, sucrose, trehalose and D-xylose. The following compounds are not utilized as sole carbon and energy sources: acetate, L-alanine, L-arginine, L-asparagine, L-aspartate, citrate, L-cysteine, ethanol, formate, fumarate, L-glutamate, L-glutamine, glycine, L-histidine, isoleucine, lactate, L-lysine, malate, malonate, L-methionine, L-ornithine, propionate, pyruvate, ribitol, L-serine, D-sorbitol, L-sorbose, succinate and L-valine. Acid is produced from L-arabinose, cellobiose, D-fructose, D-galactose, glucose, lactose, maltose, D-mannitol, raffinose, L-rhamnose, D-salicin, starch, sucrose, trehalose and D-xylose, but not from ethanol, ribitol, D-sorbitol or L-sorbose. In the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), α- and β-galactosidase and α- and β-glucosidase activities are present, whereas acid phosphatase, α-chymotrypsin, *N*-acetyl-β-glucosaminidase, cystine arylamidase, β-fucosidase, β-glucuronidase, leucine arylamidase, lipase (C14), α-mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase activities are absent. Susceptible to discs containing (μg unless otherwise stated) amoxicillin (10), ampicillin (10), bacitracin (0.04 IU), carbenicillin (100), cefotaxime (30), cefoxitin (30), chloramphenicol (30), erythromycin (15), kanamycin (30), neomycin (30), nitrofurantoin (300), novobiocin (30), penicillin G (10 IU), rifampicin (5), tetracycline (10) and tobramycin (10), but not susceptible to nystatin (100) or streptomycin (10). The predominant menaquinone is MK-7. The major polar lipids include diphosphatidylglycerol, phosphatidylglycerol, three unidentified phospholipids and three glycolipids. The major fatty acids (>5 %) include anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$, $C_{17:0}$ and $C_{16:0}$. The DNA G+C content of the type strain is 35.3 mol%.

The type strain, MF38^T (=CGMCC 1.7727^T =JCM 15711^T), was isolated from a saline–alkaline soil sample from Minfeng county, Xinjiang, China.

Acknowledgements

We thank Jean Euzéby for his help with the specific etymology and nomenclature. This work was supported by grants from the Ministry

of Science and Technology of China (973 Program, 2004CB719604-3; 863 Program, 2007AA021305) and the Key Project of Zhejiang Science and Technology (2006C13053).

References

Ahmed, I., Yokota, A. & Fujiwara, T. (2007). *Gracilibacillus boraciitolerans* sp. nov., a highly boron-tolerant and moderately halotolerant bacterium isolated from soil. *Int J Syst Evol Microbiol* **57**, 796–802.

Carrasco, I. J., Márquez, M. C., Yanfen, X., Ma, Y., Cowan, D. A., Jones, B. E., Grant, W. D. & Ventosa, A. (2006). *Gracilibacillus orientalis* sp. nov., a novel moderately halophilic bacterium isolated from a salt lake in Inner Mongolia, China. *Int J Syst Evol Microbiol* **56**, 599–604.

Chamroensaksri, N., Tanasupawat, S., Akaracharanya, A., Visessanguan, W., Kudo, T. & Itoh, T. (2010). *Gracilibacillus thailandensis* sp. nov., from fermented fish (*pla-ra*) in Thailand. *Int J Syst Evol Microbiol* **60**, 944–948.

Chen, Y.-G., Cui, X.-L., Zhang, Y.-Q., Li, W.-J., Wang, Y.-X., Xu, L.-H., Peng, Q., Wen, M.-L. & Jiang, C.-L. (2008a). *Gracilibacillus halophilus* sp. nov., a moderately halophilic bacterium isolated from saline soil. *Int J Syst Evol Microbiol* **58**, 2403–2408.

Chen, Y.-G., Cui, X.-L., Zhang, Y.-Q., Li, W.-J., Wang, Y.-X., Xu, L.-H., Peng, Q., Wen, M.-L. & Jiang, C.-L. (2008b). *Gracilibacillus quinghaiensis* sp. nov., isolated from salt-lake sediment in Qaidam Basin, north-west China. *Syst Appl Microbiol* **31**, 183–189.

Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y.-W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259–2261.

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. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.

Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.

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.

Jeon, C. O., Lim, J.-M., Jang, H. H., Park, D.-J., Xu, L.-H., Jiang, C.-L. & Kim, C.-J. (2008). *Gracilibacillus lacisalsi* sp. nov., a halophilic Gram-positive bacterium from a salt lake in China. *Int J Syst Evol Microbiol* **58**, 2282–2286.

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.

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.

Lawson, P. A., Deutch, C. E. & Collins, M. D. (1996). Phylogenetic characterization of a novel salt-tolerant *Bacillus* species: description of *Bacillus dipsosauri* sp. nov. *J Appl Bacteriol* **81**, 109–112.

Leifson, E. (1963). Determination of carbohydrate metabolism of marine bacteria. *J Bacteriol* **85**, 1183–1184.

Mata, J. A., Martínez-Cánovas, J., Quesada, E. & Béjar, V. (2002). A detailed phenotypic characterisation of the type strains of *Halomonas* species. *Syst Appl Microbiol* **25**, 360–375.

Mesbah, M. & Whitman, W. B. (1989). Measurement of deoxyguanosine/thymidine ratios in complex mixtures by high-performance liquid chromatography for determination of the mole percentage guanine + cytosine of DNA. *J Chromatogr* **479**, 297–306.

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

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Tang, S.-K., Wang, Y., Lou, K., Mao, P.-H., Jin, X., Jiang, C.-L., Xu, L.-H. & Li, W.-J. (2009). *Gracilibacillus saliphilus* sp. nov., a moderately halophilic bacterium isolated from a salt lake. *Int J Syst Evol Microbiol* **59**, 1620–1624.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Ventosa, A., Quesada, E., Rodriguez-Valera, F., Ruiz-Berraquero, F. & Ramos-Cormenzana, A. (1982). Numerical taxonomy of moderately halophilic Gram-negative rods. *J Gen Microbiol* **128**, 1959–1968.

Wainø, M., Tindall, B. J., Schumann, P. & Ingvorsen, K. (1999). *Gracilibacillus* gen. nov., with description of *Gracilibacillus halotolerans* gen. nov., sp. nov.; transfer of *Bacillus dipsosauri* to *Gracilibacillus dipsosauri* comb. nov., and *Bacillus salexigens* to the genus *Salibacillus* gen. nov., as *Salibacillus salexigens* comb. nov. *Int J Syst Bacteriol* **49**, 821–831.

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.

Xu, X.-W., Wu, Y.-H., Wang, C.-S., Oren, A., Zhou, P.-J. & Wu, M. (2007a). *Haloferax larsenii* sp. nov., an extremely halophilic archaeon from a solar saltern. *Int J Syst Evol Microbiol* **57**, 717–720.

Xu, X.-W., Wu, Y.-H., Zhou, Z., Wang, C.-S., Zhou, Y.-G., Zhang, H.-B., Wang, Y. & Wu, M. (2007b). *Halomonas saccharevitans* sp. nov., *Halomonas arcis* sp. nov. and *Halomonas subterranea* sp. nov., halophilic bacteria isolated from hypersaline environments of China. *Int J Syst Evol Microbiol* **57**, 1619–1624.