Salicola salis sp. nov., an extremely halophilic bacterium isolated from Ezzemoul sabkha in Algeria

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A novel, extremely halophilic bacterium was isolated from brine samples collected from Ezzemoul sabkha in north-east Algeria. Cells of this isolate, designated B2^T, were Gram-negative, rod-shaped and motile. Growth occurred between 10 and 25 % (w/v) NaCl and the isolate grew optimally at 15–20 % (w/v) NaCl. The pH range for growth was 6.0–9.0 with an optimum at pH 7.0–7.5. The predominant fatty acids were C_{16:0} and C_{18:1ω9c}. Other fatty acids present were C_{16:1ω9c}, C_{18:0} 10-methyl, C_{12:0} 3-OH, C_{10:0} and C_{12:0}. The G + C content of the genomic DNA was 56.0 mol%. 16S rRNA gene sequence analysis indicated that strain B2^T was closely related to *Salicola marasensis* in the *Gammaproteobacteria*. The level of 16S rRNA gene sequence similarity between strain B2^T and the type strain of *Salicola marasensis* was 99 %. DNA–DNA hybridization experiments between strain B2^T and *Salicola marasensis* indicated a level of relatedness of 52 %. The phenotypic characteristics of strain B2^T allowed its differentiation from recognized species of the genus *Salicola*. Strain B2^T was able to hydrolyse starch but not aesculin. It was unable to use carbohydrates and could not use citrate, pyruvate or succinate as sole carbon and energy sources. On the basis of the polyphasic data presented, strain B2^T is considered to represent a novel species of the genus *Salicola*, for which the name *Salicola salis* sp. nov. is proposed. The type strain is B2^T (=CECT 7106^T=LMG 23122^T).

Hypersaline environments, such as salt lakes and salterns, are found worldwide. Studies by Oren (1990a, b) using specific inhibitors have shown that all or nearly all of the heterotrophic activity in the saltern crystallizer ponds at Eilat, Israel, could be attributed to members of the *Archaea*, which are well adapted to this environment (Oren, 1994). However, the use of molecular biological approaches based on sequencing of 16S rRNA genes recovered from the environment and fluorescence *in situ* hybridization have indicated that hypersaline environments also harbour significant communities of *Bacteria* (Antón *et al.*, 1999, 2000; Eder *et al.*, 1999).

Among the *Bacteria*, most halophiles described to date are moderately rather than extremely halophilic and fall into established genera with non-halophilic representatives within the families *Halanaerobiaceae* (exclusively halophiles)

and *Halomonadaceae* (predominantly halophiles). However, there are a few halophilic taxa that resemble the haloarchaea in their salt requirement and tolerance: *Halorhodospira*, *Halovibrio*, *Halospina*, *Salicola* (*Gammaproteobacteria*), *Actinopolyspora halophila* (*Actinobacteria*) and *Salinibacter* (*Bacteroidetes*) (Antón *et al.*, 2002; Oren, 2002; Sorokin *et al.*, 2006; Maturrano *et al.*, 2006).

Algeria has numerous natural hypersaline lakes (sabkhas) located in the north and in the south of the country. These environments can be considered as athalassohaline because their hypersalinity derives from the dissolution of salts of continental origin. Sodium and chloride ions dominate this ecosystem. Recently, in the course of screening micro-organisms present in Ezzemoul sabkha, we isolated several archaeal and bacterial strains and characterized them taxonomically. One of the bacterial isolates, designated strain B2^T, an extremely halophilic, Gram-negative and rod-shaped bacterium, was subjected to further taxonomic study.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain B2^T is DQ129689.

Strain B2^T was isolated from brine samples collected from Ezzemoul sabkha, north-east Algeria. Samples were plated on hypersaline agar medium containing (per litre): 175 g NaCl, 20 g MgCl₂·6H₂O, 5 g K₂SO₄, 0.1 g CaCl₂·2H₂O and 5 g yeast extract. The pH of the medium was adjusted to 7.0 with NaOH. Incubation was at 37 °C.

Cell morphology of strain B2^T and flagellum type were examined by transmission and scanning electron microscopy. Samples were negatively stained with 2% (w/v) uranyl acetate (for 30 s) and after air-drying the grids were viewed in a model EM 902 transmission electron microscope (Zeiss). For ultrathin sections exponentially growing cells were harvested and fixed in cold 2.5% (w/v) glutaraldehyde (0.1% cacodylate buffer, pH 7.2). After 1 h of fixation, the cells were washed three times in cacodylate buffer and post-fixed for 1 h in 1% (w/v) osmium tetroxide with propanol and embedded in resin. Thin sections were prepared with an ultramicrotome and post-stained with uranyl acetate and lead citrate. Finally, the sections were examined in a Zeiss EM 902 transmission electron microscope. For scanning electron microscopy (Zeiss DSM950), samples were fixed in a solution containing 2.5% glutaraldehyde. After washing in 0.1 M sodium cacodylate for 4 h, the cells were dehydrated in a graded ethanol series (30, 50, 70, 90 and 100%) at room temperature. Specimens were dried and coated with gold and examined. Gram staining was performed by using acetic-acid-fixed samples as described by Dussault (1955).

The growth response to NaCl was examined in solid medium containing final concentrations of 0, 7.5, 10, 15, 20, 25 and 30% (w/v) NaCl. Tolerance to pH was tested in solid medium at pH 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. The temperature range for growth was tested by incubating cultures on agar plates at 30–50 °C. Anaerobic growth in the presence of nitrate (5 g l⁻¹) was determined and a control test without nitrate was included. Reduction of nitrate was detected by using sulfanilic acid and α -naphthylamine reagent (Smibert & Krieg, 1981). Formation of gas from nitrate was detected by using Durham tubes. Tests for formation of indole and hydrolysis of starch and aesculin were performed following the protocols of Gonzalez *et al.* (1978). Hydrolysis of gelatin and Tween 80 were tested as outlined by Gutiérrez & Gonzalez (1972). Catalase production was detected with 10% H₂O₂. The oxidase reaction was performed on filter paper moistened with a 1% (w/v) aqueous solution of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Formation of acids from carbohydrates and alcohols was tested in liquid media in which yeast extract was reduced to 0.1 g l⁻¹, amended with 0.5 g NH₄Cl l⁻¹ and 5 g of the substrate tested l⁻¹. The utilization of carbohydrates, alcohols or amino acids by strain B2^T was tested in medium in which yeast extract was reduced and amended with 0.5 g NH₄Cl l⁻¹, 0.05 g KH₂PO₄ l⁻¹, 1% of the respective carbohydrates, alcohols or sugars, or 0.01% of the respective amino acids and buffer

at pH 7.0. Determination of antibiotic susceptibility was performed by spreading bacterial suspensions on the standard growth agar medium plate and applying antibiotics discs.

Analysis of fatty acid methyl esters was carried out by the Analytical Service of the DSMZ (Braunschweig, Germany), using the MID/Hewlett Packard Microbial System Identification System (MIS), which relies upon high-resolution GC to obtain the fatty acid profile.

The 16S rRNA genes were amplified by PCR using two primers: 16F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and 16R1525 (5'-AAGGAGGTGWTCCARCC-3'). PCR reactions were carried out under the conditions described by Saiki *et al.* (1988). The PCR products were purified with Microcon-100 concentrator (Amicon) and sequenced directly using primers for the sequencing reactions previously described by Lane (1991) (16SF357: 5'-CTCCTACGGGAGGCAGCA-3'; 16SR519: 5'-GWATTACCGCGGCKGCTG-3'; 16SF945: 5'-GGGCCCCGACAAGCGGTGG-3'). Sequences were determined using an Applied Biosystems PRIZM TaqDyeDeoxy Terminator Cycle Sequencing kit and model 373A automatic DNA sequencer. Multiple sequence alignments were performed using CLUSTAL W 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed by the neighbour-joining method with the MEGA 3 program package (Kumar *et al.*, 2004).

Genomic DNA was isolated by using the method of Marmur (1961). G + C content was determined from the mid-point value (*T*_m) of the thermal denaturation profile (Marmur & Doty, 1962), with a Perkin Elmer UV-visible Lambda 3B spectrophotometer at 260 nm, programmed for temperature increases of 1.0 °C min⁻¹. *T*_m was determined by the graphic method described by Ferragut & Leclerc (1976), and the G + C content was calculated from this temperature by using the equation of Owen & Hill (1979). DNA–DNA hybridization experiments comparing strain B2^T with *Salicola marasensis* CECT 7107^T, *Halospina denitrificans* DSM 15505^T, *Halovibrio denitrificans* DSM 15503^T and [*Pseudomonas halophila*] DSM 3050^T were performed with the method described by Ziemke *et al.* (1998). DNA was double-labelled using DIG-11-dUTP and biotin-16-dUTP (Boehringer Mannheim). The labelling reaction was carried out using a Boehringer Mannheim Nick-Translation kit.

Cells of strain B2^T were rods of approximately 0.4–0.6 × 0.9–1.3 µm and motile by means of polar flagella (Fig. 1). Cells stained Gram-negative. Colonies formed on standard agar medium were cream-beige, smooth, circular and convex, had entire margins, and were about 2–3 mm in diameter following 5 days cultivation at 37 °C.

Growth was observed at temperatures of 30–45 °C with optimum growth at 37 °C. Tests were not conducted at temperatures below 30 °C. The optimal pH for growth was 7.0–7.5; growth was observed at pH 6.0–9.0. Growth

occurred in the presence of NaCl concentrations ranging from 10 to 25 % (w/v) with an optimum observed at 15–20 % (w/v).

Growth was observed only with acetate. Glucose, lactose, sucrose, xylose, rhamnose, galactose, raffinose, arabinose, cellobiose, mannose, maltose, adonitol, dulcitol, mannitol, cellulose, salicin, malonate, succinate, pyruvate, citrate, lactate, fumarate, formate, benzoate and amino acids were not utilized. Acid was produced in the presence of glucose, rhamnose, galactose, arabinose and maltose. Nitrate was slightly reduced to nitrite. Strain B2^T hydrolysed gelatin, Tween 80 and starch but not aesculin. No indole was produced. The isolate exhibited catalase and oxidase activity. It was resistant to kanamycin, bacitracin and anisomycin but sensitive to penicillin G, chloramphenicol, ampicillin and streptomycin.

The fatty acid profiles of strain B2^T and several reference strains are given in Table 1. The major hydroxy fatty acid was C_{12:0} 3-OH. Note that members of the genera *Marinobacter* (Nguyen *et al.*, 1999), *Alcanivorax* (Yakimov *et al.*, 1998), *Saccharospirillum* (Labrenz *et al.*, 2003) and *Halomonas* (Yoon *et al.*, 2002) also produce C_{12:0} 3-OH. The fatty acids C_{10:0}, C_{12:0} and C_{12:0} 3-OH are present in *Salicola marasensis* (Maturrano *et al.*, 2006), [*P. halophila*] DSM 3050^T, *Halovibrio denitrificans* and *Halospina denitrificans* (Sorokin *et al.*, 2006), but at lower levels than found in strain B2^T. Additionally, strain B2^T contained C_{16:0} N alcohol (3.80 %). It also exhibited an abundance of ω 9c isomers of the fatty acids C_{16:1} and C_{18:1}, which are reported to predominate in *Marinobacter* (Gorshkova *et al.*, 2003), [*P. halophila*] DSM 3050^T, *Halospina denitrificans*, *Halovibrio denitrificans* (Sorokin *et al.*, 2006) and *Salicola marasensis* (Maturrano *et al.*, 2006). A greater proportion of C_{16:1} ω 9c and C_{16:0} but lesser proportion of C_{18:0} ω 9c and C_{18:0} 10-methyl was found in the fatty acid profile of strain B2^T compared with that of *Salicola marasensis*; a number of other differences in the fatty acid profiles were also found (Table 1). It is interesting to note that strain B2^T possessed C_{18:3} ω 6c (6, 9, 12), an unusual unsaturated fatty acid of bacterial species.

The 16S rRNA gene sequence of strain B2^T was determined by sequence analysis of PCR-amplified products. The sequence determined consisted of 1523 nt and was closely related to sequences of members of the *Gammaproteobacteria*. Strain B2^T displayed highest 16S rRNA gene sequence similarity to *Salicola marasensis* CECT 7107^T (99 %), [*P. halophila*] DSM 3050^T (96 %), *Halovibrio denitrificans* DSM 15503^T (96 %) and *Halospina denitrificans*

Fig. 1. Electron micrographs of cells of strain B2^T. (a) Negatively stained cells appear as rods with polar flagella. Bar, 0.25 μ m. (b) Transmission electron micrograph of an ultrathin section. Bar, 0.6 μ m. (c) Scanning electron micrograph. Bar, 1 μ m.

Table 1. Fatty acid composition of strain B2^T in comparison with closely related species

Taxa: 1, strain B2^T; 2, *Salicola marasensis* CECT 7107^T (data from Maturrano *et al.*, 2006); 3, *Halovibrio denitrificans* DSM 15503^T (Sorokin *et al.*, 2006); 4, *Halospina denitrificans* DSM 15505^T (Sorokin *et al.*, 2006); 5, [*Pseudomonas halophila*] DSM 3050^T (Sorokin *et al.*, 2006). Values are percentages of the total fatty acids. —, Not detected.

Fatty acid	1	2	3	4	5
C _{10:0}	5.23	3.26	1.92	1.62	2.09
C _{12:0}	2.30	1.53	—	1.17	0.65
C _{12:0} 3-OH	7.73	5.77	3.35	3.36	3.61
C _{14:1}	—	—	0.84	1.23	1.51
C _{14:1} 3-OH	—	—	—	1.06	—
Summed feature*	4.51	—	—	—	—
C _{16:0} N alcohol	3.80	—	—	—	—
C _{16:1} ω ₉ c	7.45	2.26	3.20	—	4.79
C _{16:1} ω ₇ c	—	—	5.22	—	—
C _{16:1} ω ₅ c	—	3.14	—	—	—
C _{16:1}	—	—	—	1.47	0.45
C _{16:0}	25.70	15.81	15.40	7.98	15.36
Unidentified	—	—	8.02	6.82	8.10
C _{18:3} ω ₆ c	1.66	—	—	—	—
C _{18:1} ω ₉ c	25.79	33.41	46.56	65.52	55.23
C _{18:1} ω ₇ c	0.21	—	10.18	—	0.91
C _{18:0}	3.33	8.50	3.32	5.45	3.13
C _{18:0} 10-methyl	7.91	21.55	2.01	—	3.46

*Summed feature comprises iso-C_{16:1} and/or C_{14:0} 3-OH.

DSM 15505^T (95%). A tree depicting the phylogenetic relationships of strain B2^T and its closest relatives is shown in Fig. 2. The G+C content of the genomic DNA of strain B2^T was 56.0 mol% as determined by the thermal denaturation method. This is somewhat lower than the value reported for *Salicola marasensis* (Table 2). DNA–DNA hybridization experiments between strain B2^T and *Salicola marasensis* CECT 7107^T, *Halospina denitrificans* DSM 15505^T, *Halovibrio denitrificans* DSM 15503^T and [*P. halophila*] DSM 3050^T revealed levels of relatedness of 52, 40, 43 and 51 %, respectively.

Based on analysis of its 16S rRNA gene sequence, strain B2^T was shown to be affiliated with members of the *Gammaproteobacteria*. Its closest relative was *Salicola marasensis* (Fig. 2). The fatty acid profile of strain B2^T was similar to that of *Salicola marasensis*, based on the fact that C_{16:0} and C_{18:1}ω₉c comprised the dominant components. However, detailed examination of the fatty acid profiles indicated that strain B2^T could be distinguished from *Salicola marasensis* (see Table 1). Other differences between the two organisms were also found, including the pH range for growth, and the ability to hydrolyse some substrates and to use carbohydrates and other organic compounds. The level of DNA–DNA hybridization is low

Fig. 2. Unrooted neighbour-joining tree showing the phylogenetic relationship between strain B2^T and closely related species of *Bacteria* based on 16S rRNA gene sequences. The 16S rRNA gene sequence of *Bacillus subtilis* ATCC 6633 was used as an outgroup (not shown). Numbers at branch points indicate the level of bootstrap support, based on 100 replications. Bar, 0.1 substitutions per nucleotide position.

enough to classify strain B2^T as representing a novel species in the genus *Salicola*, for which we propose the name *Salicola salis* sp. nov.

Description of *Salicola salis* sp. nov.

Salicola salis [sa'lis. L. gen. n. *salis* (poet.) of salt water, of brine].

Cells are Gram-negative rods (0.4–0.6 × 0.9–1.3 μm), and are motile. Colonies are cream–beige, smooth, circular and convex with entire margins. Oxidase- and catalase-positive. Growth occurs at NaCl concentrations of 10–25 % (w/v) with an optimum between 15 and 20 % (w/v) NaCl. Temperature range for growth is 30–45 °C (optimum 37 °C). Grows at pH 6.0–9.0 (optimum about pH 7.0–7.5). Tween 80 is hydrolysed but indole is not produced. Nitrate is weakly reduced. Sugars and amino acids are not utilized. Grows on acetate. Hydrolyses starch and gelatin. Aesculin is not hydrolysed. Cells are resistant to kanamycin, bacitracin and anisomycin but susceptible to chloramphenicol and penicillin G. The major fatty acids are C_{10:0}, C_{12:0}, C_{12:0} 3-OH, iso-C_{16:1} and/or C_{14:0} 3-OH, C_{16:0} N alcohol, C_{16:1}ω₉c, C_{16:0}, C_{18:3}ω₆c, C_{18:1}ω₉c, C_{18:0} and C_{18:0} 10-methyl; other fatty acids are present in minor proportions. The G+C content of the DNA is 56.0 mol% (*T*_m).

The type strain, B2^T (=CECT 7106^T = LMG 23122^T), was isolated from brines of Ezzemoul sabkha (Algeria).

Table 2. Phenotypic characteristics of strain B2^T and closely related extremely halophilic bacteria

Taxa: 1, strain B2^T; 2, *Salicola marasensis* 7Sm5^T (data from Maturrano *et al.*, 2006); 3, *Halovibrio denitrificans* HGD 3^T (Sorokin *et al.*, 2006); 4, *Halospina denitrificans* HGD 1-3^T (Sorokin *et al.*, 2006); 5, [*Pseudomonas halophila*] DSM 3050^T (Sorokin *et al.*, 2006; Ventosa *et al.*, 1998). +, Positive; –, negative; NR, not reported.

Characteristic	1	2	3	4	5
Cell morphology	Rods	Rods	Short spirilla	Long, flexible rods	Vibrios or short spirilla
Flagella	Polar	Polar	Polar	None	Polar
Catalase	+	+	NR	NR	NR
Oxidase	+	+	NR	NR	+
NaCl range (M)	1.7–4.3	1.7–5.2	2.0–5.0	2.0–5.0	1.5–4.5
Optimal growth salinity (M)	2.5–3.5	2.5	2.0–2.5	2.5–3.0	NR
Temperature range (°C)	30–45	NR	NR	NR	15–37
Optimal growth temperature (°C)	37	35	NR	NR	NR
pH range	6.0–9.0	6.0–8.0	6.7–8.5	6.7–8.5	6.7–8.5
Hydrolysis of:					
Gelatin	+	+	NR	NR	–
Starch	+	–	NR	NR	–
Aesculin	–	+	NR	NR	+
Tween 80	+	NR	NR	NR	NR
Growth on:					
Acetate	+	+	+	+	+
Citrate	–	+	–	–	–
Pyruvate	–	+	–	+	–
Succinate	–	+	+	+	–
Carbohydrate utilization	–	+	–	–	–
Nitrate reduction	Slightly	–	+	+	+
DNA G+C content (mol%)	56.0	61.6–61.9	61.7	60.1	61.6

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References

- Antón, J., Llobet-Brossa, E., Rodríguez-Valera, F. & Amann, R. (1999). Fluorescence *in situ* hybridization analysis of the prokaryotic community inhabiting crystallizer ponds. *Environ Microbiol* **1**, 517–523.
- Antón, J., Roselló-Mora, R., Rodríguez-Valera, F. & Amann, R. (2000). Extremely halophilic *Bacteria* in crystallizer ponds from solar salterns. *Appl Environ Microbiol* **66**, 3052–3057.
- Antón, J., Oren, A., Benlloch, S., Rodríguez-Valera, F., Amann, R. & Roselló-Mora, R. (2002). *Salinibacter ruber* gen. nov., sp. nov., a novel, extremely halophilic member of the *Bacteria* from saltern crystallizer ponds. *Int J Syst Evol Microbiol* **52**, 485–491.
- Dussault, H. P. (1955). An improved technique for staining red halophilic bacteria. *J Bacteriol* **70**, 484–485.
- Eder, W., Ludwig, W. & Huber, R. (1999). Novel 16S rRNA gene sequences retrieved from highly saline brine sediments of Kebrat Deep, Red Sea. *Arch Microbiol* **172**, 213–218.
- Ferragut, C. & Leclerc, H. (1976). Etude comparative des méthodes de détermination du T_m de l'ADN bactérien. *Ann Microbiol* **127A**, 223–235 (in French).
- Gonzalez, C., Gutiérrez, C. & Ramirez, C. (1978). *Halobacterium vallismortis* sp. nov., an amyolytic and carbohydrate-metabolising extremely halophilic bacterium. *Can J Microbiol* **24**, 710–715.
- Gorshkova, N. M., Ivanova, E. P., Sergeev, A. F., Zhukova, N. V., Alexeeva, Y., Wright, J. P., Nicolau, D. V., Mikhailov, V. V. & Christen, R. (2003). *Marinobacter excellens* sp. nov., isolated from sediments of the Sea of Japan. *Int J Syst Evol Microbiol* **53**, 2073–2078.
- Gutiérrez, C. & Gonzalez, C. (1972). Methods for simultaneous detection of proteinase and esterase in extremely halophilic bacteria. *Appl Microbiol* **24**, 516–517.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA 3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Labrenz, M., Lawson, P. A., Tindall, B. J., Collins, M. D. & Hirsch, P. (2003). *Saccharospirillum impatiens* gen. nov., sp. nov., a novel γ -*Proteobacterium* isolated from hypersaline Ekho Lake (East Antarctica). *Int J Syst Evol Microbiol* **53**, 653–660.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–216.
- Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.
- Maturrano, L., Valens-Vadell, M., Roselló-Mora, R. & Antón, J. (2006). *Salicola marasensis* gen. nov., sp. nov., an extremely

halophilic bacterium isolated from the Maras solar salterns in Peru. *Int J Syst Evol Microbiol* **56**, 1685–1691.

Nguyen, B. H., Denner, E. B. M., Dang, T. C. H., Wanner, G. & Stan-Lotter, H. (1999). *Marinobacter aquaeolei* sp. nov., a halophilic bacterium isolated from a Vietnamese oil-producing well. *Int J Syst Bacteriol* **49**, 367–375.

Oren, A. (1990a). Estimation of the contribution of halobacteria to the bacterial biomass and activity in solar salterns by the use of bile salts. *FEMS Microbiol Ecol* **73**, 41–48.

Oren, A. (1990b). The use of protein synthesis inhibitors in the estimation of the contribution of halophilic archaeobacteria to bacterial activity in hypersaline environments. *FEMS Microbiol Ecol* **73**, 187–192.

Oren, A. (1994). The ecology of the extremely halophilic archaea. *FEMS Microbiol Rev* **13**, 415–440.

Oren, A. (2002). Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. *J Ind Microbiol Biotechnol* **28**, 56–63.

Owen, R. J. & Hill, L. R. (1979). The estimation of base compositions, base pairing and genome size of bacterial deoxyribonucleic acids. In *Identification Methods for Microbiologists*, pp. 277–296. Edited by F. A. Skinner & D. W. Lovelock. London: Academic Press.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–491.

Smibert, R. M. & Krieg, N. R. (1981). General characterization. In *Manual of Methods for General Bacteriology*, pp. 409–443. Edited by

P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. D. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.

Sorokin, D. Y., Tourova, T. P., Galinski, E. A., Belloch, C. & Tindall, B. J. (2006). Extremely halophilic denitrifying bacteria from hypersaline inland lakes, *Halovibrio denitrificans* sp. nov., and *Halospina denitrificans* gen. nov., sp. nov., and evidence that the genus name *Halovibrio* Fendrich 1989 with the type species *H. variabilis* should be associated with DSM 3050. *Int J Syst Evol Microbiol* **56**, 379–388.

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., Nieto, J. & Oren, A. (1998). Biology of moderately halophilic aerobic bacteria. *Microbiol Mol Biol Rev* **62**, 504–544.

Yakimov, M. M., Golyshin, P. N., Lang, S., Moore, E. R. B., Abraham, W.-R., Lünsdorf, H. & Timmis, K. N. (1998). *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. *Int J Syst Bacteriol* **48**, 339–348.

Yoon, J.-H., Lee, K.-C., Kho, Y. H., Kang, K. H., Kim, C.-J. & Park, Y.-H. (2002). *Halomonas alimentaria* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. *Int J Syst Evol Microbiol* **52**, 123–130.

Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998). Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.