Taxonomic study of *Halorubrum distributum* and proposal of *Halorubrum terrestre* sp. nov.

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Halorubrum distributum (basonym, *Halobacterium distributum*) is an extremely halophilic, aerobic archaeon isolated from saline soils, which was described on the basis of phenotypic features of several strains. The designated type strain of the species (1m^T = VKM B-1733^T = JCM 9100^T) was shown recently to differ from the other strains. In this study, *Halorubrum distributum* isolates have been characterized with regard to phenotypic features, polar lipid content, comparison of 16S rRNA gene sequences and DNA–DNA hybridization. On the basis of these data, a novel species that includes the other isolates is proposed, with the name *Halorubrum terrestre* sp. nov. The type strain of this novel species is 4p^T (= VKM B-1739^T = JCM 10247^T). The DNA G + C content of this novel species is 64.2–64.9 mol% (64.4 mol% for the type strain).

In the course of a study of extremely halophilic micro-organisms from saline soils, Zvyagintseva & Tarasov (1987) proposed the species *Halobacterium distributus*, based on the features of four strains (designated 1m^T, 4p, 13 and 32). This name was validly published and corrected to *Halobacterium distributum* (Zvyagintseva & Tarasov, 1989). The strain designated as the type strain of this species is strain 1m^T, deposited as VKM B-1733^T (Zvyagintseva & Tarasov, 1989). Two almost-simultaneous studies (Kamekura & Dyll-Smith, 1995; McGenity & Grant, 1995) showed that most species that were previously assigned to the genus *Halobacterium* were not related to *Halobacterium salinarum*, the type species of this genus, and they were placed in the novel genus *Halorubrum* (McGenity & Grant, 1995). On the basis of 16S rRNA gene sequence comparison, *Halobacterium distributum* was also included in the genus *Halorubrum*, as *Halorubrum distributum* (Oren & Ventosa, 1996).

Zvyagintseva *et al.* (1996) showed that the features of strain 1m^T were different from those of the other three strains that were assigned to the species *Halorubrum distributum* and they proposed strain 4p (= VKM B-1739) as the new type strain of *Halorubrum distributum*. As this proposal was not in accordance with the rules of the International Code of Nomenclature of Bacteria (Lapage *et al.*, 1992), Oren

et al. (1997a) confirmed strain 1m^T (= VKM B-1733^T = JCM 9100^T) as the type strain of *Halorubrum distributum* and suggested that an exhaustive study should be carried out in order to 'describe and validate a new species of *Halorubrum* and include a full phenotypic characterization', and to propose (preferably) strain 4p (= VKM B-1739) as its type strain.

In the present paper, we study in detail the type strain of *Halorubrum distributum*, a species that has not been well characterized phenotypically, as well as the other strains that were previously assigned to this species; we describe these as a novel species, for which we propose the name *Halorubrum terrestre* sp. nov.

Strains used in this study are shown in Table 1. *Halorubrum distributum* VKM B-1733^T and the other four strains (VKM B-1739, VKM B-1916, VKM B-1954 and VKM B-2151) were isolated from saline soils (Zvyagintseva & Tarasov, 1987). Reference strains are type strains of species that belong to different halobacterial genera. They were cultured in a medium with a final concentration of approximately 25 % salts and 0.5 % yeast extract (Difco). The pH was adjusted to 7.5 with 1 M KOH (Torreblanca *et al.*, 1986). Incubation was carried out at 37 °C in an orbital shaker at 200 r.p.m. For phenotypic characterization, we followed the Minimal Standards for Description of New Taxa in the Order *Halobacteriales* (Oren *et al.*, 1997b). The tests performed are

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of *Halorubrum terrestre* strain VKM B-1739^T is AB090169.

Table 1. DNA G+C contents and levels of DNA–DNA relatedness between *Halorubrum distributum* VKM B-1733^T, strain VKM B-1739 and other related halobacterial strains

Hybridization of strains VKM B-1733^T and VKM B-1739 with the following strains was <20 %: *Halobacterium salinarum* DSM 3754^T, *Haloarcula vallismortis* ATCC 29715^T, *Haloarcula argentinensis* ATCC 700875^T, *Haloarcula hispanica* ATCC 33960^T, *Haloarcula japonica* JCM 7785^T, *Haloarcula marismortui* ATCC 43049^T, *Haloarcula quadrata* DSM 11927^T, *Halomicrobium mukohataei* DSM 12286^T, *Haloferax volcanii* NCMB 2012^T, *Haloferax denitrificans* DSM 4425^T, *Haloferax gibbonsii* ATCC 33959^T, *Haloferax mediterranei* ATCC 33500^T, *Haloferax lucentense* JCM 9276^T, *Halobaculum gomorrense* DSM 9297^T, *Haloterrigena turkmenica* JCM 9743, *Natrialba asiatica* JCM 9576^T and *Halococcus saccharolyticus* ATCC 49257^T.

Source of unlabelled DNA	G+C content (mol%)	Relatedness (%) with ³ H-labelled DNA from	
		VKM B-1733 ^T	VKM B-1739
<i>Halorubrum distributum</i> VKM B-1733 ^T	63.9	100	55
Strain VKM B-1739	64.4	63	100
Strain VKM B-1916	64.9	60	98
Strain VKM B-1954	64.6	65	95
Strain VKM B-2151	64.2	25	99
<i>Halorubrum saccharovororum</i> ATCC 29252 ^T	64.3*	32	38
<i>Halorubrum coriense</i> JCM 9275 ^T	66.3*	31	18
<i>Halorubrum lacusprofundi</i> DSM 5036 ^T	65.3*	45	5
<i>Halorubrum sodomense</i> ATCC 33755 ^T	66.0*	20	21
<i>Halorubrum trapanicum</i> NCIMB 13488 ^T	64.3	33	6

*Data from Grant *et al.* (2001).

included in the species description. The methodology used has been described previously (Torreblanca *et al.*, 1986). Unless otherwise indicated, tests were carried out in media that contained 25 % salts, at pH 7.5 and incubated at 37 °C.

Polar lipids were extracted with chloroform/methanol as described previously (Kamekura, 1993). One-dimensional TLC was done by using Merck HPTLC 10 × 20 cm plates, silica gel 60 (code 1.05641) or Aldrich 20 × 20 cm plates (code Z12271-8) in a solvent system that contained chloroform, methanol, acetic acid and water (85:22.5:10:4, v/v) (Torreblanca *et al.*, 1986). Glycolipids were detected as purple spots by spraying with 0.5 % α -naphthol in methanol/water (1:1) and then with sulfuric acid/ethanol (1:1), followed by slight heating at 160 °C. Other lipids were detected as brown spots after prolonged heating.

For extraction of genomic DNA and determination of DNA G+C content, cells were harvested, washed and suspended in 0.15 M NaCl/0.1 M EDTA buffer (pH 8.0). Lysis was accomplished at 60 °C for 10 min by adding SDS at a final concentration of 2 % (w/v). DNA was extracted and purified by the method of Marmur (1961). Purity was assessed from A_{260}/A_{280} and A_{230}/A_{260} absorbance ratios (Johnson, 1994). DNA G+C content was determined from the mid-point value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962), which was obtained by using a Perkin-Elmer UV-Vis 551S spectrophotometer at

260 nm. This instrument was programmed for temperature increases of 1.0 °C min⁻¹. The T_m was determined by a 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), in 0.1 × SSC buffer (0.15 M NaCl buffered with 0.015 M trisodium citrate, pH 7.0). The T_m value of reference DNA from *Escherichia coli* NCTC 9001^T was taken as 74.6 °C in 0.1 × SSC (Owen & Pitcher, 1985).

DNA was labelled by the multiprime system with a commercial kit with deoxy(1',2',5'-³H)cytidine 5'-triphosphate (Amersham Biosciences). The mean specific activity obtained with this procedure was 8.4×10^6 c.p.m. (mg DNA)⁻¹. Labelled DNA was denatured before hybridization by heating at 100 °C for 5 min and then placed on ice. DNA–DNA hybridization studies were performed by the competition procedure of the membrane method, as described by Johnson (1994). Competitor DNA was sonicated (B. Braun Melsungen) at 50 W for two intervals of 15 s. Membrane filters (HAHY; Millipore) that contained reference DNA (25 µg cm⁻²) were placed in 5 ml screw cap vials that contained labelled, sheared, denatured DNA and denatured, sheared competitor DNA. The ratio of the concentrations of competitor to labelled DNA was at least 150:1. The final volume and concentration were adjusted to 140 µl, 2 × SSC and 30 % formamide. The hybridization temperature was 57 °C, which is within the limits of validity for the filter method (De Ley

& Tijtgat, 1970). Vials were shaken slightly for 18 h in a water bath; these procedures were done in triplicate. After hybridization, filters were washed in $2 \times$ SSC at the optimal renaturation temperature (58 °C). Radioactivity bound to the filters was measured in a liquid scintillation counter (Beckman) and relatedness (%) was calculated according to Johnson (1994). At least two independent determinations were carried out for each experiment; reported results are mean values.

16S rRNA genes were amplified by PCR with a primer set [5'-ATTCCGGTTGATCCTGCCGG-3' (positions 6–25, according to *E. coli* numbering) and 5'-AGGAGGTGATCC-AGCCGAG-3' (positions 1540–1521)] by using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen). PCR products were cloned into the *Sma*I site of vector pUC119 and sequencing was carried out with a Beckman Coulter capillary DNA sequencer SEQ2000XL. 16S rDNA sequences were obtained from databases and aligned according to the Ribosome Database Project II. Tree reconstructions were performed by using the neighbour-joining and Kimura's two-parameter calculation methods.

Strains VKM B-1739, VKM B-1916, VKM B-1954 and VKM B-2151 are pleomorphic, motile, Gram-negative and able to grow in a wide range of salinities (15–30 % salt; optimal growth at 25 %). They lyse in distilled water. Colonies on solid media are orange-red-pigmented. Results of phenotypic tests and nutritional features of these strains are included in the species description. The polar lipid composition of these four strains are $C_{20}C_{20}$ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and a sulfated diglycosyl diether, which has been shown to be a distinctive feature of species of the genus *Halorubrum* (McGenity & Grant, 2001).

The DNA G + C content of the four strains studied ranged from 64.2 to 64.9 mol% (Table 1). This result is within the range of G + C contents reported for the genus *Halorubrum* (McGenity & Grant, 2001). The DNA G + C content of *Halorubrum distributum* VKM B-1733^T was 63.9 mol%, a very similar value to that reported previously (63.6 mol%) (McGenity & Grant, 2001). Results of DNA–DNA hybridization experiments are shown in Table 1. High levels of

relatedness were found between strain VKM B-1739 and the other three related strains (95–100 %); however, levels of DNA relatedness with the type strains of species of the genus *Halorubrum*, as well as other genera of the *Halobacteriaceae*, were <70 %, which is the value that is currently accepted for the delineation of prokaryotic species (Stackebrandt & Goebel, 1994).

The 16S rRNA gene sequence of strain VKM B-1739 was most closely related to those of *Halorubrum coriense* (97.1 % similarity) and *Halorubrum distributum* (97.0 % similarity). Fig. 1 shows a phylogenetic tree, in which the relationships of strain VKM B-1739 and other related halobacteria are seen. The 19 signature bases that are specific for the genus *Halorubrum* or are shared by only one other genus (Grant *et al.*, 2001; Kamekura *et al.*, 2004) were completely conserved.

Our results show that strains VKM B-1739, VKM B-1916, VKM B-1954 and VKM B-2151 are significantly different from other previously described species of the genus *Halorubrum*; on the basis of DNA–DNA hybridization data, as well as 16S rRNA gene sequence comparison, we propose to place these strains in a novel species, with the name *Halorubrum terrestre* sp. nov.

Description of *Halorubrum terrestre* sp. nov.

Halorubrum terrestre (ter.res'tre. L. neut. adj. *terrestre* of the soil, from which the strains were isolated).

Cells are pleomorphic, flat and disc-shaped, 1.0–1.5 × 1.5–2.5 µm in size. Motile. Gas vacuoles are not produced. Colonies are orange-red. Growth occurs in media that contain 15–30 % NaCl, with optimum growth at 25 % NaCl. Growth occurs between 28 and 50 °C (optimum, 37–45 °C) and pH 5–9 (optimum, 7.5). Chemo-organotrophic. Aerobic. Oxidase- and catalase-positive. Acid is produced from glycerol, but not from arabinose, fructose, galactose, glucose, lactose, maltose, sucrose or trehalose. Nitrate is not reduced to nitrite. Indole is not produced from tryptophan. Voges–Proskauer test is negative. Starch, gelatin and casein are not hydrolysed. H₂S is not produced. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are not produced. The following compounds are not

Fig. 1. Phylogenetic tree of strain VKM B-1739 and other related halobacterial species based on their 16S rRNA gene sequences (GenBank accession numbers are shown in parentheses). Numbers on branches represent confidence levels from 1000 replicate bootstrap resamplings; values >70 % are shown. Bar, 0.02 expected changes per site.

used as sole carbon and energy sources: arabinose, cellobiose, aesculin, fructose, fucose, gluconolactone, glucose, glucosamine, inulin, mannose, melibiose, raffinose, rhamnose, ribose, sucrose, trehalose, xylose, adonitol, dulcitol, erythritol, ethanol, glycerol, mannitol, *meso*-inositol, propanol, sorbitol, α -aminovalerate, butyrate, caprylate, citrate, fumarate, glutamate, glycerate, 2-oxoglutarate, malate, malonate, oxalate, propionate, saccharate and tartrate. The following compounds are not used as sole carbon, nitrogen or energy sources: L-alanine, L-arginine, L-asparagine, betaine, creatine, L-glutamine, glycine, L-histidine, L-lysine, L-methionine, L-ornithine, L-proline, putrescine, sarcosine, L-serine, L-threonine and L-valine. Susceptible to anisomycin, bacitracin and novobiocin; resistant to ampicillin, chloramphenicol, kanamycin, nalidixic acid, penicillin G, polymyxin, streptomycin and tetracycline. Polar lipids are C₂₀C₂₀ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and a sulfated diglycosyl diether. DNA G+C content is 64.2–64.9 mol% (*T_m* method).

Type strain is 4p^T (=VKM B-1739^T=JCM 10247^T). DNA G+C content of this strain is 64.4 mol% (*T_m* method). Isolated from saline soils.

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