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ORIGINAL PAPER

Halobellus rufus sp. nov., an extremely halophilic archaeon isolated from non-purified solar salt

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Abstract A halophilic archaeon, designed strain CBA1103^T, was isolated from non-purified solar salt. The cells of strain CBA1103^T were observed to be Gram-stain negative and pleomorphic, and the colonies appear red. Strain CBA1103^T was observed to grow between 20 and 55 °C (optimum 37 °C), and in NaCl concentrations of 10–30 % (w/v) (optimum 15 %) with 0–0.5 M $MgSO_4 \cdot 7H_2O$ (optimum 0.1 M) and at pH 6.0–9.0 (optimum pH 7.0). Additionally, the cells lyse in distilled water. The major polar lipids of strain CBA1103^T are phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and two glycolipids chromatographically identical to sulfated mannosyl glucosyl diether and manosyl glucosyl diether. Strain CBA1103^T is shown to belong to the *Halobellus* genus and exhibits similarity to related taxa; the 16S rRNA gene sequence similarity between strain CBA1103^T and *Halobellus rarus* 18362^T, *Hbs. limi* 16811^T, *Hbs. litoreus* JCM 17118^T, *Hbs. inordinatus* YC20^T, *Hbs. clavatus* TNN18^T and *Hbs. salinus* CSW2.24.4^T is 97.3, 96.5, 96.5, 94.5, 94.5 and 93.7 %, respectively. The RNA polymerase subunit B gene sequence of strain CBA1103^T shows 93.7 % similarity with the sequence of *Hbs. litoreus* JCM 17118^T; the similarity is lower with sequences from the type strains of other species of *Halobellus*. The genomic DNA G+C

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content of strain CBA1103^T was determined to be 67.0 mol% a value which is in the range of the genomic DNA G+C content of members of the genus *Halobellus* (61.5–69.2 mol%). These results suggest that strain CBA1103^T should be considered to represent a new taxon for which the name *Halobellus rufus* sp. nov. is proposed, with the type strain CBA1103^T (=CECT 8423^T =JCM 19434^T).

Keywords Haloarchaea · *Halobellus rufus* · Solar salt · Polyphasic taxonomy

Introduction

Extremely halophilic archaea, which are members of the family *Halobacteriaceae*, have been detected in various saline environments, such as soda lakes, salt lakes, and solar salterns. High salt concentrations are harmful to most cells, but haloarchaeal cells have adapted to hypersaline environments (Grant 2004). These archaea require at least 1.5 M NaCl for growth and grow optimally in NaCl concentrations of 2.6 M or higher (Ventosa et al. 1998). The family *Halobacteriaceae* is currently classified into 40 genera. Of these, the genus *Halobellus* was described by Cui et al. (2011). Presently, the genus comprises six species, *Halobellus clavatus* TNN18^T (Cui et al. 2011), *Hbs. limi* TBN53^T, *Hbs. salinus* CSW2.24.4^T (Cui et al. 2012), *Hbs. inordinatus* YC20^T (Qiu et al. 2013), *Hbs. rarus* YC21^T (Zhang et al. 2013) and *Hbs. litoreus* GX31^T (Zhao et al. 2013). Five species of the genus *Halobellus* (*Hbs. clavatus*, *Hbs. limi*, *Hbs. salinus, Hbs. litoreus*, *Hbs. inodinatus*) were isolated from solar salterns (Cui et al. 2011, 2012; Qiu et al. 2013; Zhang et al. 2013), whereas one species (*Hbs. rarus*) was isolated from an inland salt lake (Zhang et al. 2013). Members of the genus *Halobellus* are characteristically pleomorphic rod-shaped, aerobic, heterotrophic, Gram-stain negative and lyse in distilled water. The growth conditions of members in the genus *Halobellus* are as follows: with 5–30 % (w/v) NaCl, at 20–55 °C and pH of 5.5–9.5 (Cui et al. 2011; Zhang et al. 2013; Zhao et al. 2013).

In this paper, we describe a novel strain CBA1103^T, which was isolated from non-purified solar salt from a solar saltern in the Republic of Korea and conclude from physiological and biochemical analyses that it represents a novel halophilic species of the genus *Halobellus*, for which the name *Halobellus rufus* sp. nov. is proposed.

Materials and methods

Archaeal strain and culture conditions

Strain CBA1103^T was isolated from a sample of non-purified solid salt from a solar saltern (E126°06′16″, N34°35′22″) in the Republic of Korea. The solar salt was serially diluted and spread onto DSM medium no. 372 (M372). This medium contained the following components (l^{-1}): 5 g casamino acids, 5 g yeast extract, 1 g sodium glutamate, 3 g trisodium citrate, 2 g KCl, 20 g $MgSO_4 \cdot 7H_2O$, 36 mg $FeCl_2 \cdot 4H_2O$, 0.36 mg $MnCl_2 \cdot 4H_2O$, 200 g NaCl and 20 g agar. The medium was adjusted to pH 7.0–7.2 using 1 N NaOH. The plates were incubated at 37 °C for 1 month. To obtain a pure culture, colonies were successively restreaked at least three times onto the same medium. The reference strains used were *Hbs. rarus* JCM 18362^T, *Hbs. limi* JCM 16811^T and *Hbs. litoreus* JCM 17118^T. These reference strains were obtained from the Japan Collection of Microorganisms (JCM) and grown under comparable conditions.

Morphological, physiological, and biochemical characterisation

The phenotypic characteristics of strain CBA1103^T were determined according to the proposed minimal standards for describing extremely halophilic archaea (Oren et al. 1997). All the tests were performed in triplicate unless stated otherwise. The optimal conditions for growth were determined using M372 medium, and varying the concentrations of NaCl (0–30 % [w/v] at 5 % increments), and incubation temperature (5, 10, 15, 20, 25, 30, 35, 37, 40, 45, 50, 55 and 60 °C in increments of 5 °C). To measure growth at different pH levels, the pH of the M372 was adjusted to pH 5.0–11.0 with increments of 1.0 unit by using the following buffers: 10 mM MES for pH 5.0 and 6.0; 10 mM bis-Tris propane for pH 7.0–9.0; and 10 mM CAPS for pH 10.0 and 11.0. The Mg^{2+} requirement for growth was determined using modified M372 as a basal medium without $MgSO_4 \cdot 7H_2O$, which was supplemented with 10 mM bis-Tris propane. The different concentrations of $MgSO_4 \cdot 7H_2O$ were 0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 M.

Cell morphology and size of the isolate were determined using a light microscope (Eclipse 80*i*; Nik on). Gram staining was performed using an industrial Gram-staining kit according to the method of Dussault

(1955). Cell lysis in distilled water was tested by microscopic examination immediately after cells had been suspended in distilled water. The tests for catalase and oxidase activities, nitrate reduction, indole production, urease activity and hydrolysis of casein and starch were conducted as described by Benson (2002) using M372 as the basal medium. The ability to hydrolyse Tween 40 and 80 was tested according to the method described by González et al. (1978). Gelatine hydrolysis was performed according to the method described by Smibert and Krieg (1994) using M372 as the basal medium. Anaerobic growth tests were performed using M372 medium in the presence of nitrate (30 mM) and L-arginine (5 g l^{-1}), trimethylamine *N*-oxide (TMAO) (5 g l^{-1}), and dimethyl sulfoxide (DMSO) (5 g l^{-1}) at 37 °C in an anaerobic chamber (Coy), with N_2:CO_2:H_2 of 90:5:5 (v/v/v). The ability to utilize various substrates as sole carbon and energy sources was tested using modified M372 (0.1 g yeast extract, 2 g KCl, 20 g $MgSO_4 \cdot 7H_2O$, 36 mg $FeCl_2 \cdot 4H_2O$, 0.36 mg $MnCl_2 \cdot 4H_2O$, 200 g NaCl and 20 g agar per liter) supplemented with 10 mM bis-Tris propane and the following substrates (final concentration, 1 %): acetate, L-alanine, L-arginine, L-aspartate, citrate, D-fructose, fumarate, D-galactose, D-glucose, L-glutamate, glycerol, glycine, DL-lactate, lactose, L-lysine, L-malate, maltose, mannitol, D-mannose, L-ornithine, pyruvate, D-ribose, sorbitol, L-sorbose, starch, succinate, sucrose or D-xylose.

To test antibiotic sensitivity, the strain was inoculated on agar medium plates using antibiotic discs (μg per disc, unless indicated): ampicillin (20), bacitracin (0.1 IU), chloramphenicol (50), ciprofloxacin (10), erythromycin (25), gentamicin (20), kanamycin (50), nalidixic acid (50), neomycin (50), nitrofurantoin (100), norfloxacin (20), novobiocin (50), nystatin (100), penicillin G (20 IU), rifampicin (10), streptomycin (10), tetracycline (50), trimethoprim (20) and vancomycin (50). The strain was incubated for 2 weeks at 37 °C.

Determination of the 16S rRNA and RNA polymerase subunit B (*rpoB′*) and RNA polyphylogenetic analysis

Total cellular DNA was extracted from strain CBA1103^T and three related reference strains, *Hbs. rarus* JCM 18362^T, *Hbs. limi* JCM 16811^T and *Hbs. litoreus* JCM 17118^T, and purified as described by Sambrook et al. (1989). The 16S rRNA gene of strain CBA1103^T was amplified by PCR using an archaea-specific primer set, 0018F and 1518R, as described previously (Cui et al. 2009) and the PCR product was sequenced as described previously (Roh et al. 2008). The amplification of *rpoB′* gene was performed with the HrpoB2 1420F (5′-TGTGGGCTNGTGAAGAA CTT-3′) and HrpoA 153R (5′-GGGTCCATCAGCCC-CATGTC-3′) primers, and the PCR product was sequenced using the following primers: HrpoB-458F (5′-TTACSATGGGNKCRGGGATG-3′), HrpoB-671R (5′-GCGTCCTCGATGTTGAANCCC-3′), HrpoB-721F (5′-TTCTTCCGNCANTACGAGGG-3′), HrpoB-1148F (5′-AGGAGGACATGCAGGAGGACATGCCNTTY ACC-3′), HrpoB-1166R (5′-GTRAASGGCATGTCC TCCTG-3′) and HrpoB-1457R (5′-ACCATGTGRTA SAGYTT STG-3′) (Minegishi et al. 2010). The assembly of the amplified 16S rRNA and *rpoB′* gene sequences of strain CBA1103^T was performed as described previously (Roh et al. 2008). The sequence alignments were performed using SILVA aligner (http://www.arb-silva.de/aligner), which considers both sequence similarity and rRNA secondary structure (Pruesse et al. 2012). The phylogenetic neighbours of the 16S rRNA gene were identified and the pairwise sequence similarities were calculated using EzTaxon-e (Kim et al. 2012). The *rpoB′* gene sequences of strain CBA1103^T and related taxa were obtained from GenBank for analysing the phylogenetic tree. The 16S rRNA and *rpoB′* gene sequences of strain CBA1103^T were aligned and edited using the multiple sequence alignment program Clustal_W. The phylogenetic neighbours were determined using MEGA 5 software (Thompson et al. 1997). A distance matrix was determined using the two-parameter model of Kimura (1980). Phylogenetic tree reconstructions were generated using the neighbour-joining (NJ) (Saitou and Nei 1987), minimum-evolution (ME) (Nei et al. 1998), and maximum likelihood (ML) (Felsenstein 1981) methods. To evaluate the stability of the phylogenetic trees, bootstrap analysis was conducted using a consensus tree developed using the NJ, ME and ML methods (1,000 replicates each).

Determination of the DNA G+C content, DNA–DNA hybridisation (DDH)

The DNA G+C content was determined as described by González and Saiz-Jimenez (2002). DDH was used to

determine the genetic relatedness of strain CBA1103^T, *Hbs. rarus* JCM 18362^T, *Hbs. limi* JCM 16811^T and *Hbs. litoreus* JCM 17118^T. DDH was performed using a fluorometric method with photobiotin-labelled DNA probes and a microwell plate (MaxiSorp, FluoroNunc), as described by Ezaki et al. (1989). The values were determined from five replicates.

Table 1 Differential characteristics of strain CBA1103^T and closely related species in the genus *Halobellus*

Characteristic	1	2	3	4
NaCl range for growth (%)	10–30	10–30	8–30	5–30
Optimum NaCl	15	15	15–25	18
Mg^{2+} range for growth (M)	0–0.5	0–0.3	0–1.0	0–1.0
Optimum Mg^{2+}	0.2	0.05	0–0.1	0.05
Temperature range for growth (°C)	20–55	20–50	25–55	25–50
Optimum temperature (°C)	37	37	45	37
pH range for growth	6–9	6–9	5.5–9.5	6–8.5
Optimum pH	7–8	7–7.5	7	7
Hydrolysis of				
Starch	−	−	−	+
Tween 80	−	+	−	−
Reduction of nitrate to nitrite	−	+	+	−
Anaerobic growth with nitrate	−	+	−	+
Utilization of[a]				
L-Alanine	−	−	+	−
Fumarate	−	−	+	−
D-Galactose	+	−	+	−
D-Glucose	−	−	+	−
L-Glutamate	+	−	+	−
Glycine	+	−	+	−
DL-Lactate	−	−	+	+
L-Lysine	+	−	−	−
L-Malate	+	+	−	−
Maltose	−	−	−	+
Mannitol	−	−	+	+
D-Mannose	+	−	+	−
Sorbitol	−	−	+	+
Starch	−	−	−	+
L-Ornithine	−	+	−	−
DNA G+C content (mol%)	67.0	66.1	69.2	66.8

Taxa: 1, *Halobellus rufus* CBA1103^T sp. nov.; 2, *Hbs. rarus* JCM 18362^T; 3, *Hbs. limi* JCM 16811^T; 4, *Hbs. litoreus* JCM 17118^T. Data were obtained from this study, Zhang et al. (2013), Cui et al. (2012) and Zhao et al. (2013), unless otherwise indicated. All of the strains lysed in distilled water; positive for catalase, oxidase and utilization of pyruvate; negative for hydrolysis of gelatine and casein, indole formation, anaerobic growth with arginine and DMSO and utilization of acetate, L-arginine, L-aspartate, citrate, D-fructose, glycerol, lactose, D-ribose, L-sorbose, succinate, sucrose and D-xylose. +, positive; −, negative

[a] Data from this study

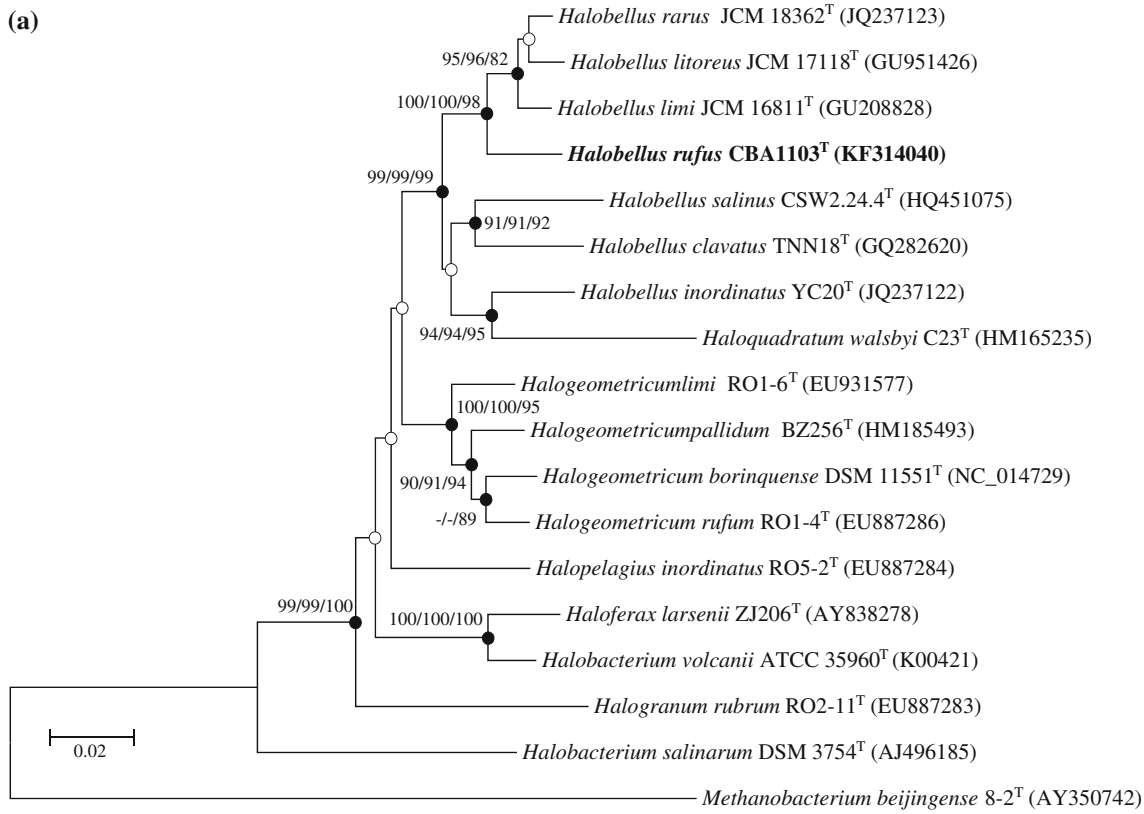
Fig. 1 Phylogenetic tree based on the neighbour-joining (NJ) algorithm for the 16S rRNA (**a**) and *rpoB′* (**b**) gene sequences of strain CBA1103^T and closely related taxa. The *numbers* on the nodes indicate the bootstrap values (>70 %) calculated using the NJ/minimum-evolution (ME)/maximum-likelihood (ML) probabilities. The *closed circles* represent nodes recovered by both the ME and ML methods, while the *open circles* indicate nodes recovered using either the ME or ML methods. *Methanobacterium beijingense* 8-2^T and *Haloquadratum walsbyi* DSM 16790^T served as the outgroups in the phylogenetic trees of the 16S rRNA and *rpoB′* genes, respectively. *Bar* 0.02 and 0.05 accumulated changes per nucleotide, respectively

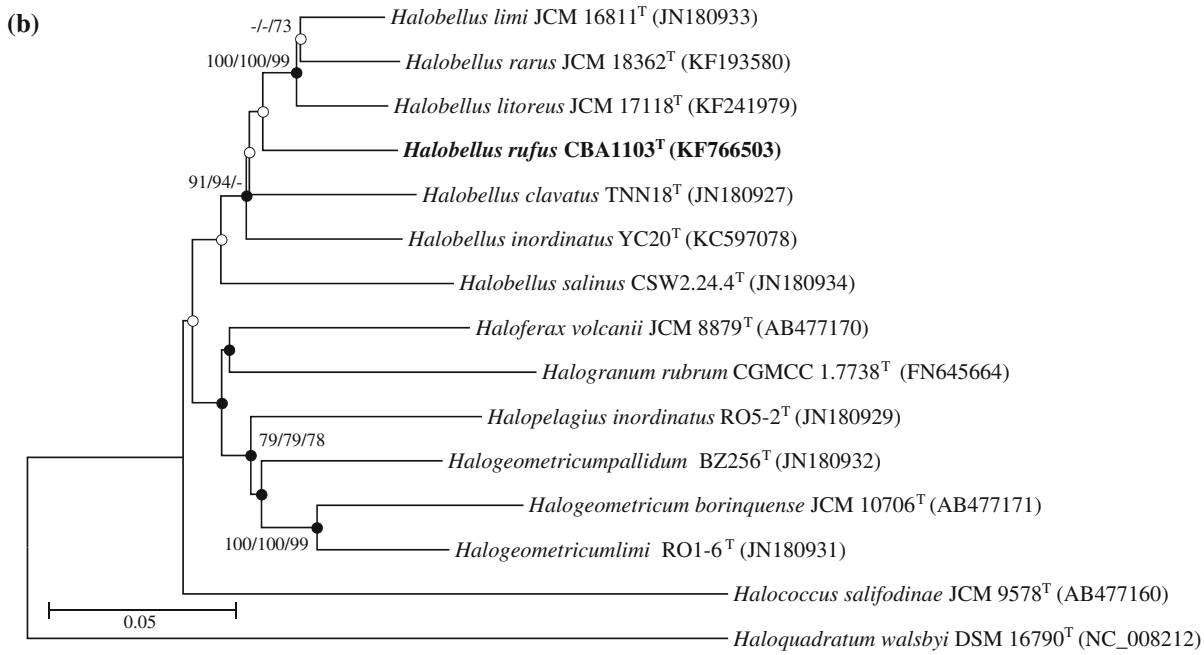
Polar lipid analysis

The polar lipids were extracted and detected using specific reagents (Dittmer and Lester 1964; Xin et al. 2000) sprayed on a Merck silica gel 60 F_{254} plate, as described by Oren et al. (1996). The designations of all of the lipid spots were provided according to Cui et al. (2011). The designations of the polar lipid spots were compared with those of the reference strains.

Results and discussion

The colonies of strain CBA1103^T appeared red and the cells were observed to be Gram-stain negative and pleomorphic (Supplementary Fig. S1). Strain CBA1103^T was found to grow at the following conditions: 20–55 °C (optimum 37 °C), pH 6.0–9.0 (optimum pH 7.0), 10–30 % (w/v) NaCl concentration (optimum 15 %) and 0–0.5 M $MgSO_4 \cdot 7H_2O$ (optimum 0.1 M). The strain does not require Mg^{2+} for growth. Strain CBA1103^T was determined not to reduce nitrate to nitrite under aerobic conditions and to exhibit catalase and oxidase activities. Strain CBA1103^T did not grow in M372 medium supplemented with any of the following compounds: DMSO, TMAO, L-arginine and nitrate under anaerobic conditions. Strain CBA1103^T did not hydrolyse Tween 40 and 80, casein, starch or gelatine. Additionally, indole production and urease activity were negative. Strain CBA1103^T was found to utilize D-galactose, L-glutamate, glycine, L-lysine, L-malate, D-mannose and pyruvate as sole carbon source. Cells of strain CBA1103^T lysed immediately upon dilution in distilled water. The detailed characteristics of strain CBA1103^T are presented in the species description and are compared with related *Halobellus* species in Table 1. Strain CBA1103^T was observed to be sensitive to bacitracin, chloramphenicol, ciprofloxacin,


(a)
Halobellus rarus JCM 18362T (JQ237123)
95/96/82
Halobellus litoreus JCM 17118T (GU951426)
100/100/98
Halobellus limi JCM 16811T (GU208828)
Halobellus rufus CBA1103T (KF314040)
99/99/99
Halobellus salinus CSW2.24.4T (HQ451075)
91/91/92
Halobellus clavatus TNN18T (GQ282620)
Halobellus inordinatus YC20T (JQ237122)
94/94/95
Haloquadratum walsbyi C23T (HM165235)
Halogeometricumlimi RO1-6T (EU931577)
100/100/95
Halogeometricumpallidum BZ256T (HM185493)
Halogeometricum borinquense DSM 11551T (NC_014729)
90/91/94
-/-/89
Halogeometricum rufum RO1-4T (EU887286)
Halopelagius inordinatus RO5-2T (EU887284)
99/99/100
100/100/100
Haloferax larsenii ZJ206T (AY838278)
Halobacterium volcanii ATCC 35960T (K00421)
Halogranum rubrum RO2-11T (EU887283)
0.02
Halobacterium salinarum DSM 3754T (AJ496185)
Methanobacterium beijingense 8-2T (AY350742)


(b)
-/-/73
Halobellus limi JCM 16811T (JN180933)
100/100/99
Halobellus rarus JCM 18362T (KF193580)
Halobellus litoreus JCM 17118T (KF241979)
Halobellus rufus CBA1103T (KF766503)
91/94/-
Halobellus clavatus TNN18T (JN180927)
Halobellus inordinatus YC20T (KC597078)
Halobellus salinus CSW2.24.4T (JN180934)
Haloferax volcanii JCM 8879T (AB477170)
Halogranum rubrum CGMCC 1.7738T (FN645664)
Halopelagius inordinatus RO5-2T (JN180929)
79/79/78
Halogeometricumpallidum BZ256T (JN180932)
Halogeometricum borinquense JCM 10706T (AB477171)
100/100/99
Halogeometricumlimi RO1-6T (JN180931)
Halococcus salifodinae JCM 9578T (AB477160)
0.05
Haloquadratum walsbyi DSM 16790T (NC_008212)

erythromycin, neomycin, nitrofurantoin, novobiocin and rifampicin, but resistant to ampicillin, gentamicin, kanamycin, nalidixic acid, norfloxacin, nystatin, penicillin G, streptomycin, tetracycline, trimethoprim and vancomycin.

The nearly complete 16S rRNA and *rpoB′* gene sequences of strain CBA1103^T were obtained (1473, 1830 bp, respectively). Analysis of the 16S rRNA gene sequence revealed that strain CBA1103^T can be considered to belong to the genus *Halobellus* as the following similarities were found: *Hbs. rarus* JCM 18362^T (97.3 % 16S rRNA gene sequence similarity), *Hbs. limi* JCM 16811^T (96.5 %), *Hbs. litoreus* JCM 17118^T (96.5 %), *Hbs. inordinatus* YC20^T (94.5 %), *Hbs. clavatus* TNN18^T (94.5 %) and *Hbs. salinus* CSW2.24.4^T (93.7 %). In the 16S rRNA gene phylogenetic tree, strain CBA1103^T clusters with *Hbs. rarus* JCM 18362^T, *Hbs. limi* JCM 16811^T and *Hbs. litoreus* JCM 17118^T, with high bootstrap values (100, 100 and 98 % in the NJ, ME and ML trees, respectively) (Fig. 1a). The similarities between the *rpoB′* gene sequence of strain CBA1103^T and the related taxa (*Hbs. litoreus* JCM 17118^T, *Hbs. limi* JCM 16811^T, *Hbs. rarus* JCM 18362^T, *Hbs. clavatus* TNN18^T, *Hbs. inordinatus* YC20^T, *Hbs. salinus* CSW2.24.4^T and *Halogeometricum pallidum* BZ256^T) are 93.7, 93.3, 92.7, 92.0, 92.0, 90.2 and 89.6 %, respectively (Fig. 1b). An *rpoB′* gene sequence similarity value of 90.0 % can be related to the threshold value that is currently accepted as indicating inclusion of a haloarchaeal member in the genus *Halobellus*.

The genomic DNA G+C content value of strain CBA1103^T was determined to be 67.0 mol%, a value which is in the range of the genomic DNA G+C contents of members of the genus *Halobellus* (61.5–69.2 mol%). Compared with *Hbs. rarus* JCM 16832^T, *Hbs. limi* JCM 16811^T and *Hbs. rarus* JCM 18362^T, the DDH values of strain CBA1103^T were 36 ± 8, 29 ± 7 and 15 ± 3 %, respectively. DDH values of <70 % are considered to indicate species distinctness in current prokaryotic systematics (Wayne et al. 1987; Stackebrandt and Goebel 1994). Therefore, strain CBA1103^T can be considered a distinct species in the genus *Halobellus*.

The major lipids of strain CBA1103^T were identified as phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, and two glycolipids chromatographically identical to sulfated mannosyl glucosyl diether and mannosyl glucosyl diether, respectively. The minor lipids were three unidentified glycolipids and an unidentified phospholipid (Supplementary Fig. S2). The polar lipid profiles of this species was chromatographically identical to those obtained for *Hbs. rarus* JCM 18362^T and *Hbs. limi* JCM 16811^T (Supplementary Fig. S2a), which supports its inclusion in the genus *Halobellus*.

In conclusion, the results of the phenotypic, chemotaxonomic, phylogenetic and genomic analyses demonstrate that the extremely halophilic archaeal strain CBA1103^T represents a novel species of the genus *Halobellus*, and we propose the name *Hbs. rufus* sp. nov. for this species.

Description of *Hbs. rufus* sp. nov.

Halobellus rufus (ruf'us. L. masc. adj. *rufus* red)

Cells are Gram-stain negative and pleomorphic. Colonies on agar plates containing 15 % (w/v) NaCl are red and round. Growth occurs at 20–55 °C (optimum 37 °C) in the presence of 10–30 % (w/v) NaCl concentration (optimum 15 %) with 0–0.5 M Mg^{2+} (optimum 0.1 M) at a pH of 6.0–9.0 (optimum pH 7.0). Cells lyse immediately upon dilution in distilled water. Catalase and oxidase positive. Does not grow under anaerobic conditions with nitrate, L-arginine, DMSO or TMAO and nitrate is not reduced to nitrite under aerobic conditions. Does not synthesise indole or urease and hydrolyse starch, gelatine, Tween 40 and 80 or casein. The following substrates are utilized as single carbon sources for growth: D-galactose, L-glutamate, glycine, L-lysine, L-malate, D-mannose and pyruvate. No growth occurs when the following substrates: acetate, L-arginine, L-alanine, L-aspartate, citrate, D-fructose, fumarate, glycerol, D-glucose, DL-lactate, lactose, maltose, mannitol, L-ornithine, D-ribose, sorbitol, L-sorbose, starch, succinate, sucrose and D-xylose. The polar lipids are phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, sulfated mannosyl glucosyl diether, mannosyl glucosyl diether, three unidentified glycolipids and an unidentified phospholipid. The genomic DNA G+C content of the type is 67.0 mol%.

The type strain is CBA1103^T (=CECT 8423^T =JCM 19434^T), which was isolated from non-purified solar salt in the Republic of Korea. The GenBank/EMBL/DDBJ accession number for 16S rRNA and *rpoB′* gene

sequences of strain CBA1103^T are KF314040 and KF766503, respectively.

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