**Halovulum dunhuangense** gen. nov., sp. nov., isolated from a saline terrestrial spring

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A bacterial strain, YYQ-30^T_1, isolated from a mixed water–sand–sediment sample collected from a terrestrial spring located in Dunhuang, China, was characterized with respect to its morphology, physiology and taxonomy. Cells of the strain were Gram-stain-negative, aerobic, oxidase- and catalase-positive, non-flagellated, oval to rod-shaped (0.5–1.0 μm wide and 1.1–6.6 μm long) and divided by binary fission. Growth was observed in the presence of 0–10.0 % (w/v) NaCl with optimal growth at 0–3.0 %, at pH 6.0–9.0 (optimum pH 7.0–8.5) and at 10–45 °C (optimum 30–37 °C). The isolate could reduce nitrate to nitrite and hydrolyse aesculin and gelatin (weakly), but was unable to degrade Tween 80 or starch. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain YYQ-30^T_1 belongs to the family *Rhodobacteraceae* and forms a distinct lineage with the type strain of *Albimonas donghaensis* and forms a branch within a cluster constituted by the type strains of species of the genera *Albimonas, Rhodovulum, Albidovulum, Haematobacter* and *Tropicimonas*; levels of 16S rRNA gene sequence similarity between strain YYQ-30^T_1 and members of related genera ranged from 94.1 to 89.7 %. Strain YYQ-30^T_1 contained Q-10 as the predominant ubiquinone and summed feature 8 (C_{18:1ω7c} and/or C_{18:1ω6c}; 70.0 %), C_{18:0} (9.5 %), summed feature 2 (one or more of C_{14:0 3-OH}, iso-C_{15:0 1} and C_{12:0 aldehyde}; 6.9 %) and 11-methyl C_{18:1ω7c} (6.0 %) as the principal fatty acids. The polar lipids comprised diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, three unidentified phospholipids, two unidentified aminolipids and five unknown lipids. The *pufLM* gene was detected. The G+C content of the genomic DNA was 71.7 mol%. Based on the phylogenetic, chemotaxonomic and phenotypic data obtained in this study, strain YYQ-30^T_1 is considered to represent a novel species in a new genus within the family *Rhodobacteraceae*, for which the name *Halovulum dunhuangense* gen. nov., sp. nov. is proposed. The type strain of *Halovulum dunhuangense* is YYQ-30^T_1 (=LMG 27418^T_1=MCCC 1A06483^T_1).

During an investigation of bacterial diversity of a terrestrial spring, strain YYQ-30^T_1 was isolated and characterized taxonomically along with several other bacterial isolates. Comparative 16S rRNA gene sequence analysis indicated that strain YYQ-30^T_1 belongs to the family *Rhodobacteraceae*. However, it could not be assigned to any genus because of its low sequence similarity to any type strain (<94.1 %). The type genus of the family *Rhodobacteraceae*, *Rhodobacter*, was proposed by Imhoff et al. (1984) and, at the time of writing, the family comprised 105 genera (http://www.bacterio.net/rhodobacteraceae.html). Consequently, the aim of the present work was to determine the exact taxonomic position of strain YYQ-30^T_1 by using a polyphasic approach.

A mixed water–sand–sediment sample was collected from a terrestrial spring named Crescent Moon Spring located at the foot of Echoing-Sand Mountain in the desert of Dunhuang County, Gansu Province, China (40.5°N 94.40°E) and used for bacterial isolation, with the sample spread on M2 agar plates and incubated at 25 °C for 2 weeks. Medium M2 contained (per litre seawater) 5.0 g sodium acetate, 0.5 g tryptone, 0.5 g yeast extract, 0.5 g glucose, 0.5 g sucrose, 0.05 g sodium citrate, 0.05 g DL-malic acid, 1.0 g

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†These authors contributed equally to this work.

The GenBank/EMBL/DDJB accession numbers for the 16S rRNA and *pufLM* gene sequences of strain YYQ-30^T_1 are KJ191196 and KP698407.

Two supplementary figures and a supplementary table are available with the online Supplementary Material.
NH₄NO₃, 0.2 g NH₄Cl and 0.5 g KH₂PO₄, adjusted to pH 7.5–7.6 (Lai et al., 2009b). One colony was picked and the purity was confirmed by the uniformity of cell morphology after repeated streaking. Cell morphology was examined by light microscopy (Nikon; model 50i). Strain YYQ-30ᵀ was maintained as a 20 % (v/v) glycerol suspension at (80 °C. For morphological and biochemical characterization, strain YYQ-30ᵀ was cultivated on marine agar 2216 (MA; BD Difco) unless otherwise indicated. Albinomonas donghaensis DSM 17890ᵀ, obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, was used as a reference strain.

Extraction of genomic DNA for 16S rRNA gene sequence determination, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out as described previously (Ausubel et al., 1995; Liu & Shao, 2005). Searches for similar 16S rRNA gene sequences were conducted by using the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and EzTaxon-e server (Kim et al., 2012a). Phylogenetic analysis was performed using software MEGA version 5.0 (Tamura et al., 2011) after multiple alignments of the data by CLUSTAL W with distance options according to Kimura’s two-parameter model and clustering with the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and minimum-evolution (Rzhetsky & Nei, 1992) methods. In each case, bootstrap values were calculated based on 1000 replications.

A nearly full-length 16S rRNA gene sequence (1381 nt) from strain YYQ-30ᵀ was determined and compared with the database of sequences from type strains of prokaryotic species with validly published names (http://eztaxon-e.ezbiocloud.net/). The result revealed that strain YYQ-30ᵀ is a member of the family Rhodobacteraceae and showed the highest 16S rRNA gene sequence similarity to Rhodovulum euryhalinum DSM 48685ᵀ (94.1 %). As shown in Fig. 1, a phylogenetic tree was reconstructed based on the 16S rRNA gene sequences of representatives of 29 genera (31 type strains) of the family Rhodobacteraceae, with Oceanibaculum indicum P24ᵀ as an outgroup. It is clear that strain YYQ-30ᵀ belongs to the family Rhodobacteraceae and forms a distinct lineage with the species of the genus Albinomonas (92.1 % similarity to the type strain of the type species of genus Albinomonas) that branched from a cluster of 26 closely related genera with moderate bootstrap support (60.0 %). Topologies of phylogenetic trees reconstructed using the maximum-likelihood and minimum evolution algorithms were similar to that of the neighbour-joining tree. Comparative analysis of 16S rRNA gene sequences showed that the levels of sequence similarity between strain YYQ-30ᵀ and the type strains of species of related genera are 89.7–94.1 %, which is below the threshold (94.5 %) determined recently for genus delineation (Yarza et al., 2014). On the basis of the phylogenetic evidence and relatively low levels of 16S rRNA gene sequence similarity to its phylogenetic neighbours, strain YYQ-30ᵀ should be assigned to a novel genus within the family Rhodobacteraceae.

Cell morphology was observed by using phase-contrast light microscopy (model 50i; Nikon) and transmission electron microscopy (model JEM-1230; JEOL) with cells from the early exponential growth phase on MA. Flagella were examined after negative staining with phosphotungstic acid. Gram staining was performed by using a Gram staining kit (Hangzhou Tianhe Microorganism Reagent Co.) according to the manufacturer’s instructions. Catalase and oxidase activities were assessed by means of bubble production in 10 % (v/v) aqueous hydrogen peroxide solution and oxidation of 1 % (w/v) Na₂N₉N₉N₉-tetramethyl 1,4-phenylenediamine, respectively. Hydrolysis of casein, starch and Tween 20, 40, 60 and 80 was investigated on modified MA respectively supplemented with 1 % casein, 1 % soluble starch or 1 % Tween 20, 40, 60 or 80. Growth was tested at 4, 16, 20, 25, 30, 37, 41 and 45 °C in medium 216L; medium 216L contained (per litre seawater) 1 g sodium acetate, 10 g tryptone, 2 g yeast extract, 0.5 g sodium citrate, 0.2 g NH₄NO₃, pH 7.5 (Lai et al., 2009a). The pH range for growth was determined at pH 3.0–11.0 (in increments of 0.5 pH units) in marine broth 2216 (MB; Difco) adjusted with citrate/phosphate (pH 3.0–7.0), Tris/HCl (pH 7.5–8.5) or glycine/NaOH (pH 9.0–11.0) buffers. Tolerance of NaCl was studied in HLB medium devoid of NaCl, obtained by replacing seawater with distilled water, supplemented with 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15 and 18 % (w/v) NaCl. HLB was modified from Luria–Bertani medium (Sambrook et al., 1989), with the NaCl concentration increased to 30 g l⁻¹. Antibiotic susceptibility tests were performed on MA at 30 °C for up to 7 days by using the disc-diffusion method with discs impregnated with the following antibiotics (all from Oxoid; μg per disc unless indicated): ceftriaxone (30), ceftazidime (30), chloramphenicol (30), gentamicin (10), erythromycin (15), ceftoperazone (75), ciprofloxacin (5), clindamycin (2), doxycycline hydrochloride (30), tetracycline (30), cefalexin (30), ampicillin (10), metronidazole (5), cefazolin (30), lincomycin (2), minocycline (30), norfloxacin (10), kanamycin (30), vancomycin (30), trimethoprim (25), piperacillin (100), ofloxacin (5), rifampicin (5), carbenicillin (100), polymyxin B (30 IU), streptomycin (10), oxacillin (1) and penicillin G (10). Additional enzyme activities and biochemical properties were examined using API ZYM, API 20NE and API 20E strips following the manufacturer’s instructions (bioMérieux), except that cells to inoculate the API strips were suspended in sterile seawater. Microaerobic (6 % O₂) and anaerobic growth was observed on MA supplemented with nitrate (1 g l⁻¹) incubated in a jar with the Anoxomat Mark II Anaerobic System (Mart Microbiology).

Cells of strain YYQ-30ᵀ were Gram stain-negative, non-spore-forming, oval to rod-shaped, 0.5–1.0 μm wide and 1.1–6.6 μm long (Fig. S1, available in the online Supplementary Material). On MA, strain YYQ-30ᵀ formed smooth, creamy colonies with regular edges, 0.3–0.6 mm
in diameter, after 4 days of incubation at 30 °C. The colonies were slightly raised in the centre. Tests for catalase and oxidase activity were positive. It hydrolysed Tweens 20 (weakly), 60 (weakly) and 80, but hydrolysis of casein or starch was not observed. Growth occurred at 10–45 °C (optimum 30–37 °C), at pH 6.0–9.0 (optimum pH 7.0–8.5) and in the presence of 0–10 % NaCl (optimum 0–3 %). Strain YYQ-30T was sensitive to ampicillin, chloromycetin, carbenicillin, cefradin, cefobid, ciprofloxacin, cefalexin, rocephin, vibramycin, erythromycin, kanamycin, cefazolin, neomycin, norfloxacin, ofloxacín, oxacillin, penicillin G, polymyxin B, piperacillin, rifampicin, streptomycin and tetracycline, weakly sensitive to minomycin and resistant to gentamicin, clindamycin, furazolidone, metronidazole, lincomycin, cotrimoxazole and vancomycin. Strain YYQ-30T grew under microaerobic conditions but could not tolerate anaerobic conditions. The results of API ZYM, API 20NE and API 20E tests are shown in Table 1 and the species description.

The genetic potential for anoxygenic phototrophy was determined by PCR amplification of the photosynthetic genes of strain YYQ-30T.

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain YYQ-30T and representatives of related taxa, based on 16S rRNA gene sequences. Filled circles indicate nodes that were also recovered in maximum-likelihood and minimum-evolution trees based on the same sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0.005 substitution rate (K_nuc) units. Oceanibaculum indicum P24T was used as an outgroup.
Table 1. Physiological characteristics of strain YYQ-30T and related genera of the family Rhodobacteraceae

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DNA G+C content (mol%) | 71.7 | 69–72 | 58–69 | 63.6–70.6 | 65–65.5 | 66.5–69.6 | 67.2 | 64.8 | 60.2–69.4 | 55.4–66.2 |

*The type strains of the following species display different results from those shown, as indicated: a, Gemmobacter aquaticus (+); b, Roseovarius aestuarii and Roseovarius halocynthiae (+); c, Rhodovulum mangrovei (+); d, Roseovarius rubinhibens (v); e, Roseovarius indicus (ND); f, Roseovarius pacificus (+), Roseovarius indicus (ND); g, Roseovarius marisflavi (−).

**Table 1.** Physiological characteristics of strain YYQ-30T and related genera of the family Rhodobacteraceae

Taxa: 1, strain YYQ-30T; 2, Albinomas (n=2; data from Li et al., 2013a; Lim et al., 2008); 3, Rhodovulum (n=18; Chakravarthy et al., 2009; Hiraiishi & Ueda, 1994; Imhoff et al., 1984; Lakshmi et al., 2011; Nupur et al., 2014; Srinivas et al., 2012, 2014); 4, Albidovulum (n=2; Albuquerque et al., 2002; Yin et al., 2012); 5, Haemolobacter (n=2; Greub & Raoult, 2003; Helsel et al., 2007); 6, Tropicmonas (n=3; Harwati et al., 2009; Oh et al., 2012; Shin et al., 2012); 7, Paemirrhodobacter (n=1; Wang et al., 2014); 8, Dinorosobacter (n=1; Biebl et al., 2005); 9, Gemmobacter (n=10; Anil Kumar et al., 2007; Chen et al., 2013, Liu et al., 2010, 2014; Rothe et al., 1987, Tanaka et al., 2004); 10, Roseovarius (n=15; Boettcher et al., 2005; Choi et al., 2013; Jung et al., 2012; Kim et al., 2012b; Labrenz et al., 1999; Lai et al., 2011; Li et al., 2013b; Oh et al., 2009; Park & Yoon, 2013; Yoon et al., 2008). +, Positive; −, negative; v, variable; w, weakly positive; ND, no data. Results separated by a solidus (/) indicate that the first result is more widespread in the genus than the second; results separated by a comma indicate that the two results are found equally within the genus. Acylphosphatidylglycerol is absent from all taxa shown.

reaction centre genes (puFLM) using the primers puFLf (5'-CTKTCGACTCTCTGGTSGG-3') and puFLm (5'-CCCAT-SGTCAGCGCAGAA-3') (Béja et al., 2002). The specific 1.5 kb products were clearly amplified from strain YYQ-30T, but not from Albinomas donghaeensis DSM 27890T (data not shown).
The G+C content of the genomic DNA was determined by reversed-phase HPLC according to previously described methods (Mesbah & Whitman, 1989). The chromosomal DNA G+C content of isolate YYQ-30T was 71.7 mol%, which is within the range reported previously for phylogenetically related members of the family Rhodobacteraceae (58.6–72 mol%; Table 1).

For analysis of fatty acids, isolate YYQ-30T was cultivated on MA at 30 °C for 72 h. Fatty acids from the harvested cells were saponified, methylated and extracted using the standard MIDI protocol (Sherlock Microbial Identification System, version 6.0B). The prepared fatty acids were analysed by gas chromatography (Agilent Technologies 6850) and the individual components were identified by comparison with the retention times of authentic standards and quantified, using the TSBA6.0 database of the Microbial Identification System (Sasser, 1990). Polar lipids were extracted using a chloroform/methanol system followed by the second solvent, chloroform/methanol/acetic acid/water (85 : 12 : 15 : 4, by vol.) for TLC analysis. The plate, dotted with sample, was subjected to two-dimensional development, with the first solvent being chloroform/methanol/water (65 : 25 : 4, by vol.) followed by the second solvent, chloroform/methanol/acetic acid/water (85 : 12 : 15 : 4, by vol.).

The principal fatty acids of strain YYQ-30T were summed feature 8 (C18:1ω7c and/or C18:1ω6c; 70.0 %), C18:0 (9.5 %), summed feature 2 (one or more of C14:0 3-OH, iso-C16:1ω7c, I and C12:0 aldehyde; 6.7 %), 11-methyl C18:1ω7c (6.0 %) and C16:0 (3.1 %) (Table S1). The predominant respiratory quinone detected in strain YYQ-30T was ubiquinone 10 (Q-10), which is consistent with phylogenetic relatives in the family Rhodobacteraceae. The polar lipids of strain YYQ-30T comprised diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, three unidentified phospholipids, two unidentified aminolipids and five unknown lipids (Fig. S2).

The results of the phylogenetic analysis, phenotypic analysis and chemotaxonomic studies presented above support the view that strain YYQ-30T should be assigned to the family Rhodobacteraceae. Based on the phylogenetic analysis, a comparison was made between strain YYQ-30T and the members of related genera (Albinonas, Rhodovulum, Albidovulum, Haemotobacter, Tropicimonas, Paenirhodobacter, Dinoroseobacter, Gemmobacter and Roseovarius), and a number of differences were observed. For instance, strain YYQ-30T exhibits a unique polar lipid composition and differs from members of most closely related genera with respect to temperature growth range, NaCl growth range and higher DNA G+C content. In addition, compared with its close relatives Albinonas, Albidovulum and Tropicimonas, strain YYQ-30T lacks urease activity. The ability to reduce nitrate to nitrite and degrade ascelin and the inability to produce H2S and urease distinguish strain YYQ-30T from members of the genus Haemotobacter.

The inability to grow under anaerobic conditions, the presence of catalase activity, the reduction of nitrate to nitrite and the degradation of ascelin and the absence of H2S production and urease activity differentiate the novel strain from members of the genus Paenirhodobacter. Compared with members of Dinoroseobacter and Roseovarius, strain YYQ-30T gives a negative result for hydrolysis of Tween 80 and has a higher DNA G+C content, respectively. Phenotypic and genotypic characteristics that allow the differentiation of strain YYQ-30T from its phylogenetic relatives are listed in Table 1. Overall, considering the low 16S rRNA gene sequence similarity (<94.1 %) between strain YYQ-30T and members of closely related taxa, its distinct branching position in phylogenetic analyses and the cumulative differences that the isolate exhibits described above, our data unambiguously support the creation of a novel species of a new genus within the family Rhodobacteraceae, for which the name Halovulum dunhuangense gen. nov., sp. nov. is proposed.

Description of Halovulum gen. nov.

Halovulum (Hal{o}vulum. Gr. n. hals salt; L. dim. neut. n. ovulum a small egg; N.L. neut. n. Halovulum small saline egg).

Cells are Gram-stain-negative, non-spore-forming, oval to rod-shaped. Creamy-white colonies are formed on MA plates. The principal fatty acids are summed feature 8 (C18:1ω7c and/or C18:1ω6c), C18:0, summed feature 2 (one or more of C14:0 3-OH, iso-C16:1ω7c, I and C12:0 aldehyde), 11-methyl C18:1ω7c and C16:0. The predominant respiratory quinone is Q-10. The polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, three unidentified phospholipids, two unidentified aminolipids and five unknown lipids. The type species is Halovulum dunhuangense.

Description of Halovulum dunhuangense sp. nov.

Halovulum dunhuangense (dun.huang.en’se. N.L. neut. adj. dunhuangense of or belonging to Dunhuang City, where the type strain was isolated).

In addition to the characteristics reported for the genus, cells are 0.5–1.0 μm wide and 1.1–6.6 μm long, non-motile, non-flagellated and catalase- and oxidase-positive. On MA, forms smooth creamy colonies with regular edges, slightly raised in the centre, 0.7 mm in diameter, after 96 h of incubation at 35 °C, non-pigmented. Hydrolyses Twensi 00, 60 (weakly) and 80, but does not hydrolyse starch or casein. Grows at 10–45 °C (optimum 30–37 °C), at pH 6.0–9.0 (optimum pH 7.0–8.5) and in the presence of 0–10% NaCl (optimum 0–3%). Positive in the API ZYM test strip for alkaline phosphatase, esterase (C4), esterase lipase (C8), valine aminopeptidase and leucine aminopeptidase; negative for lipase (C14), cystine aminopeptidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphomuicidase, α-glucosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase,
z-mannosidase and z-fucosidase. Positive results are obtained in API 20NE tests for reduction of nitrate to nitrite, denitrification, β-glucosidase (aesculin hydrolysis), β-galactosidase and gelatinase (week reaction); negative results for production of H₂S and indole, D-glucose fermentation, arginine dihydrolase and urease activity and assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. In the API 20E test strip, results indicate positive reactions for acetoin production (Voges–Proskauer test), tryptophan deaminase (weak reaction) and gelatinase (weak reaction); negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization and fermentation/oxidation of glucose, mannitol, aspartokinase, andrati, gelatinase, rhamnose, sucrose, melibiose, amygdalin and arabinose. Growth is observed under microaerobic conditions but not under anaerobic conditions. The pufLM gene is detected.

The type strain, YYQ-30T (=LMG 27418T = MCCC 1A06483T), was isolated from a saline terrestrial spring. The G+C content of the chromosomal DNA of the type strain is 71.7 mol%.

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References


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