Halomonas rifensis sp. nov., an exopolysaccharide-producing, halophilic bacterium isolated from a solar saltern

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A polyphasic taxonomic study was conducted on strain HK31^T, a moderately halophilic bacterium isolated from a solar saltern in Chefchaouen, Morocco. The strain was a Gram-reaction-negative, oxidase-positive rod, which was motile by means of peritrichous flagella. The strain required NaCl for growth and grew in salt concentrations (mixture of sea salts) of 0.5–20% (w/v) (optimum 5–7.5%, w/v), at 25–45 °C (optimum 32 °C) and at pH 5–10 (optimum pH 6–9). Strain HK31^T did not produce acids from sugars and its metabolism was respiratory, using oxygen as terminal electron acceptor. The strain was positive for the accumulation of poly- β -hydroxyalkanoate granules and formed mucoid colonies due to the excretion of an exopolysaccharide. The DNA G + C content was 61.5 mol%. 16S rRNA gene sequence analysis indicated that it belonged to the genus *Halomonas* in the class *Gammaproteobacteria*. The most phylogenetically related species was *Halomonas anticariensis*, with which strain HK31^T showed a 16S rRNA gene sequence similarity of 96.48%. Its major fatty acids were C_{18:1} ω 7c, C_{16:0}, C_{19:0} cyclo ω 8c, C_{16:1} ω 7c/iso-C_{15:0} 2-OH and C_{12:0} 3-OH and the predominant respiratory lipoquinone was ubiquinone with nine isoprene units (Q-9). Based on the evidence provided in this study, strain HK31^T (=CECT 7698^T =LMG 25695^T) represents a novel species of the genus *Halomonas*, for which the name *Halomonas rifensis* is proposed.

The family *Halomonadaceae* of the class *Gammaproteobacteria*, to date, contains 10 genera (Euzéby, 2010; Franzmann *et al.*, 1988): *Aidingimonas* (Wang *et al.*, 2009), *Carnimonas* (Garriga *et al.*, 1998), *Chromohalobacter* (Ventosa *et al.*, 1989), *Cobetia* (Arahal *et al.*, 2002), *Halomonas* (Dobson & Franzmann, 1996), *Kushneria* (Sánchez-Porro *et al.*, 2009), *Modicisalibacter* (Ben Ali Gam *et al.*, 2007) and *Salinicola* (Anan'ina *et al.*, 2007), which are composed of halophilic and halotolerant bacteria, and *Halotalea* (Ntougias *et al.*, 2007) and *Zymobacter* (Okamoto *et al.*, 1993), which are composed of non-halophilic bacteria. The genus *Halomonas* currently contains more than 60 species of halophilic bacteria, most of which have been isolated from saline environments such as salterns, saline soils, seawater and marshes. Its members are Gram-negative, rod-shaped, non-sporulating chemo-organotrophs that have a predominantly respiratory metabolism, using oxygen or

nitrate as terminal electron acceptors. Some species of this genus are denitrifiers and a few of them have a fermentative metabolism. Members of the genus require 3–15% (w/v) NaCl for optimum growth and can grow using a variety of compounds as sole carbon and energy sources (Mata *et al.*, 2002; Vreeland, 2005; Arahal *et al.*, 2007). Over the last two decades, some species of the genus *Halomonas* have provoked interest amongst biotechnologists because of their ability to produce exo-enzymes (Margesin & Schinner, 2001; Ventosa & Nieto, 1995), exopolysaccharides (Arias *et al.*, 2003; Mata *et al.*, 2006; Quesada *et al.*, 2004) and other commercially valuable products.

During the course of ecological and taxonomic studies sampling hypersaline environments, a wide search was aimed at isolating and characterizing EPS-producing halophilic bacteria, which consequently led to the description of seven novel EPS-producing species of the genus *Halomonas* (Quesada *et al.*, 1990; Bouchotroch *et al.*, 2001; Martínez-Cánovas *et al.*, 2004a, b; Martínez-Checa *et al.*, 2005; González-Domenech *et al.*, 2008a, b). As part of this study, a hypersaline habitat in Morocco was analysed, from which

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HK-31^T is HM026177

Three supplementary figures are available with the online version of this paper.

Table 1. Characteristics that distinguish strain HK-31^T from related type strains of the genus *Halomonas*

Strains: 1, HK31^T; 2, *H. anticariensis* CECT 5854^T; 3, *H. pantelleriensis* DSM 9661^T; 4, *H. lutea* KCTC 12847^T; 5, *H. kribbensis* KCTC 12584^T; 6, *H. elongata* ATCC 33173^T. All strains included in this table are negative for amylase activity, Tween 80 hydrolysis, haemolysis, respiration on nitrite, gas production from nitrate and growth on caprylate, DL-lactate, L-methionine and L-phenylalanine; positive for motility, growth on L-serine and cetrimide agar; and susceptible to rifampicin (5 µg), nalidixic acid (30 µg) and tobramycin (10 µg). +, Positive; –, negative.

Characteristic	1	2	3	4	5	6
Morphology	Rod	Rod	Rod	Short rod	Coccus or short rod	Rod
Cell size (µm)	0.8–1.0 × 3.0–4.4	0.75–1 × 3.0–3.5	0.4–0.7 × 1.4–2.6*	0.7–0.8 × 2.5–3	1.4–1.8 × 1.8–2.6‡	0.8 × 1.1
Pigmentation	Cream	Cream	Cream	Orange	Yellow–cream	White
Exopolysaccharide production	+	+	–	–	–	–
Flagellation	Peritrichous	Peritrichous	Peritrichous*	Single flagellum†	Single flagellum‡	Polar/Peritrichous§
Sea-salt concentration for growth (% w/v)						
Range	0.5–20	0.5–15	1–15	1–20	1–14	0–20
Optimum	5–7.5	7.5	5	5–10	5	3–8
pH for growth						
Range	5–10	6–9	6–11	5–9	5.5–9.5	5–10
Optimum	6–9	7–8	9	7.5	8–8.5	6–8
Temperature for growth (°C)						
Range	25–45	20–45	10–45	4–45	10–40	4–45
Optimum	32	32–37	33–35	37	32–35	32–37
Hydrolysis of:						
Gelatin	–	–	–	–	+	–
DNA	–	+	–	–	–	+
Casein	–	–	–	+	+	–
Tween 20	+	+	+	–	–	+
Tyrosine	–	–	+	+	–	–
Tyrosine pigment production	+	–	–	–	–	–
Hydrogen sulfide production	–	–	–	–	–	–
Lecithinase	+	–	+	–	–	+
Phosphatase	+	+	–	–	–	+
Urease	–	+	+	–	–	+
Respiration on nitrate	+	–	+	+	+	–
Aerobic nitrate reduction	–	–	–	–	–	+
Ornithine decarboxylase	–	–	–	–	–	+
Growth on MacConkey agar	+	+	+	–	+	+
Utilization of:						
Acetate, succinate	+	+	+	–	+	+
Adonitol	+	–	+	–	+	+
Aesculin	–	–	–	–	+	+
L-Arabinose, D-fructose, D-galactose	+	+	–	+	–	+
Cellobiose	+	–	+	+	+	+
Citrate	–	+	+	–	+	+
Ethanol	+	+	+	+	+	–
L-Alanine, D-gluconate, D-glucose, L-histidine, D-mannitol, trehalose	+	+	+	+	–	+
Glycerol	+	+	–	+	+	–
Inositol, D-mannose	+	+	–	–	–	+
Lactose	–	–	–	+	–	+
Propionate	+	–	–	–	+	+
Raffinose	+	–	–	–	–	+
L-Rhamnose, sorbitol	+	–	+	+	–	+
D-Salicin	+	–	+	–	–	–
Starch	+	–	–	+	–	+

Table 1. cont.

Characteristic	1	2	3	4	5	6
L-Valine	—	—	—	—	—	+
Susceptibility to:						
Amoxicillin (25 µg)	+	+	+	+	+	—
Ampicillin (10 µg)	+	+	+	+	+	—
Erythromycin (15 µg)	—	—	+	+	+	—
Penicillin G (10 µg)	+	—	—	+	+	—
Polymyxin B (300 IU)	+	+	+	+	+	—
Tetracycline (30 µg)	+	+	—	+	+	—
DNA G + C content (mol%)	61.5	61.4	65.0*	60.8†	66‡	64.2§

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

†Data from Wang *et al.* (2008).

‡Data from Jeon *et al.* (2007).

§Data from Vreeland *et al.* (1980).

strain HK31^T was isolated. The strain was subjected to a polyphasic taxonomic investigation, the results of which identified it as a novel species of the genus *Halomonas*.

Strain HK31^T was isolated from a soil sample taken from a solar saltern in the Brikcha Rural Commune village in Chefchaouen in the Rif Mountains, northern Morocco.

Recommendations of the proposed Minimal Standards for describing new taxa of the family *Halomonadaceae* (Arahal *et al.*, 2007) were taken into account when performing phenotypic characterization on the novel strain. The miniaturized systems API 20 E and API 20 NE were used to study biochemical and nutritional traits; other tests were performed using traditional methods described previously (Ventosa *et al.*, 1982; Quesada *et al.*, 1983; Mata *et al.*, 2002), the results of which are provided in Table 1 and in the species description.

The G + C content of the genomic DNA was estimated from the midpoint melting temperature (T_m) value of the DNA (Marmur & Doty, 1962), determined by the graphic method (Ferragut & Leclerc, 1976), and calculated by using the equation of Owen & Hill (1979). The DNA G + C content of strain HK31^T was 61.5 mol%, based on the G + C content of reference DNA from *Escherichia coli* NCTC 9001^T (50.9 mol%) (Owen & Pitcher, 1985).

Phylogenetic analysis based on 16S rRNA gene sequences was performed following standard procedures. The 16S rRNA gene of strain HK31^T was amplified by PCR and the product was cloned into pGEM-T cloning vector (Promega) according to the manufacturer's instructions and transformed into *E. coli* DH5- α . The 16S rRNA gene sequence (1471 bp) was then determined by primer walking using a BigDye Terminator Cycle Sequencing kit in an ABI 3100 DNA sequencer (Applied Biosystems). The sequence was then compared with reference 16S rRNA gene sequences available in the GenBank database using BLAST. Pairwise 16S rRNA gene sequence identities were calculated using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007).

Phylogenetic analyses were performed using MEGA version 4 software (Tamura *et al.*, 2007) after multiple alignments of the data in the CLUSTAL W (Thompson *et al.*, 1997) and PHYLIP version 3.69 (Felsenstein, 1981) programs. Phylogenetic analysis was carried out as recommended by Arahal *et al.*, 2007. Distances were calculated and clustering was determined, using the neighbour-joining, maximum-parsimony and maximum-likelihood methods to reconstruct phylogenetic trees supported by bootstrap values based on 1000 replications. The gene fragment analysed contained the 18 signature nucleotides defined for members of the family *Halomonadaceae* and the four signature nucleotides defined for the genus *Halomonas* (Dobson & Franzmann, 1996). Phylogenetic trees based on the neighbour-joining method (Fig. 1) and the maximum-likelihood and maximum-parsimony methods (Supplementary Figs S1 and S2, available in IJSEM Online) all showed a highly similar tree topology, indicating that strain HK31^T belongs to the genus *Halomonas*. Strain HK31^T showed the highest 16S rRNA gene sequence similarity to *Halomonas anticariensis* CECT 5854^T (96.48 %). Other species in the same clade included *H. pantellerensis*, *H. kribbensis* and *H. lutea*, with which strain HK31^T shared 96.1, 95.7 and 95.4 % 16S rRNA gene sequence similarity, respectively. Evolutionary distances, including a correction factor for reverse mutations (Jukes & Cantor, 1969), were calculated for sequence pairs by using a 'mask' (Lane, 1991) for dissimilar or uncertain nucleotide positions.

Transmission electron microscopy was used, as described by Bouchotroch *et al.* (2001), to determine cell size, cell morphology and the presence of peritrichous flagella (Supplementary Fig. S3). For fatty acid and quinones analyses, cells were grown at 32 °C in MY medium (Moraine & Rogovin, 1966) with 7.5 % (w/v) sea-salt solution (Rodríguez-Valera *et al.*, 1981). Fatty acids and quinones were analysed at the DSMZ by using high-resolution GLC and HPLC, respectively. Strain HK31^T contained a combination of fatty acids characteristic of species of the genus *Halomonas* (Dobson & Franzmann,

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain HK31^T compared with other members of the family Halomonadaceae. The tree was obtained using the neighbour-joining algorithm. Bar, 1% sequence divergence. Bootstrap values >70% (based on 1000 replications) are shown at branch points.

1996). The major fatty acids were C_{18:1}ω7c (38.25%), C_{16:0} (26.73%), C_{19:0} cyclo ω8c (14.85%), C_{16:1}ω7c/iso-C_{15:0} 2-OH (6.63%) and C_{12:0} 3-OH (5.85%). Other fatty acids included C_{14:0} (0.23%), C_{17:0} cyclo (0.35%), C_{18:0} (0.88%), C_{18:1}ω5c (0.21%) and C_{20:2}ω6,9c (0.25%). Analysis of the quinones showed that the novel strain contained ubiquinone 9 (Q-9) as the predominant respiratory lipoquinone (100%).

Based on the evidence provided herein, strain HK31^T represents a novel species of the genus *Halomonas*, for which the name *Halomonas rifensis* is proposed.

Description of *Halomonas rifensis* sp. nov.

Halomonas rifensis (ri.fen'sis. N.L. adj. *rifensis* pertaining to the Rif mountains in northern Morocco, where the strain was isolated).

Cells are Gram-reaction-negative rods, 0.8–1.0 × 3.0–4.4 μm, that occur either singly or in pairs, are motile by peritrichous flagella, produce exopolysaccharide, accumulate poly-β-hydroxyalkanoate and do not form endospores. Forms circular, convex, creamy-white colonies. Growth pattern in liquid medium is uniform. Moderately halophilic; capable of growth in salt concentrations (mixture

of sea salts) of 0.5–20% (w/v) (optimum 5–7.5%, w/v); incapable of growing without NaCl. Grows at 25–45 °C (optimum 32 °C) and at pH 5–10 (optimum pH 6–9). Does not produce acid from any of the sugars or alcohols tested, including L-arabinose, amygdalin, D-glucose, inositol, D-mannitol, melibiose, L-rhamnose, sucrose and D-sorbitol. Chemoorganotrophic and produces catalase and oxidase. Metabolism is respiratory with oxygen and nitrate but not with nitrite and fumarate as terminal electron acceptors. Indole, methyl red, Voges–Proskauer and ONPG tests are negative. Does not produce hydrogen sulfide from L-cysteine. Aerobic nitrate reduction is negative. Hydrolyses Tween 20 but not casein, DNA, gelatin, Tween 80 or tyrosine. Produces pigment from tyrosine. Produces lecithinase, phosphatase and tryptophan deaminase but not amylase, arginine dihydrolase, haemolysin, lysine decarboxylase, ornithine decarboxylase or urease. Grows on MacConkey agar and cetrimide agar. The following compounds are utilized as sole carbon and energy sources: acetate, adonitol, L-arabinose, L-aspartate, benzoate, cellobiose, ethanol, formate, fumarate, D-fructose, D-galactose, D-gluconate, D-glucose, D-glutamate, glycerol, hippurate, DL- β -hydroxybutyrate, inositol, malate, malonate, maltose, D-mannitol, D-mannose, melezitose, melibiose, propionate, pyruvate, raffinose, L-rhamnose, D-salicin, sorbitol, starch, succinate, trehalose and D-xylose. The following compounds are not used as sole carbon and energy sources: adipic acid, aesculin, caprylate, citrate, DL-lactate, lactose, phenylacetic acid, 2-propanol, ribose, D-salicin and tartrate. N-acetylglucosamine, L-alanine, creatine, L-histidine, L-isoleucine, L-leucine, L-proline, L-serine and L-threonine are used as sole sources of carbon, nitrogen and energy. L-arginine, L-cysteine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-tryptophan and L-valine are not used as sole sources of carbon, nitrogen and energy. Susceptible to (μ g unless stated otherwise) amoxicillin (25), ampicillin (10), cefalothin (30), gentamicin (10), nalidixic acid (30), penicillin G (10), polymyxin B (300 IU), tetracycline (30), tobramycin (10), rifampicin (5) and sulphamide (300) and resistant to erythromycin (15). The principal fatty acids are C_{18:1} ω 7c, C_{16:0}, C_{19:0} cyclo ω 8c, C_{16:1} ω 7c/iso-C_{15:0} 2-OH and C_{12:0} 3-OH. The predominant respiratory quinone is Q-9.

The type strain, HK31^T (=CECT 7698^T =LMG 25695^T), was isolated from a solar saltern in Chefchaouen in the Rif Mountains, Morocco. The DNA G + C content of the type strain is 61.5 mol% (T_m method).

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