Haloquadratum walsbyi gen. nov., sp. nov., the square haloarchaeon of Walsby, isolated from saltern crystallizers in Australia and Spain

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Strains C23^T and HBSQ001 were isolated from solar salterns and are novel square-shaped, aerobic, extremely halophilic members of the domain *Archaea* and family *Halobacteriaceae*. Cells stained Gram-negative and grew optimally in media containing 18 % salts at around neutral pH. Mg^{2+} is not required. The DNA G+C content of both isolates was 46.9 mol% and DNA–DNA cross-hybridization showed a relatedness of 80 %. Their 16S rRNA gene sequences showed only 2 nucleotide differences (99.9 % identity) and phylogenetic tree reconstructions with other recognized members of the *Halobacteriaceae* indicated that they formed a distinct clade, with the closest relative being *Halogeometricum borinquense* PR 3^T (91.2 % sequence identity). The major polar glycolipid of both isolates was the sulfated diglycosyl diether lipid S-DGD-1. Electron cryomicrosopy of whole cells revealed similar internal structures, such as gas vesicles and polyhydroxyalkanoate granules, but the cell wall of isolate HBSQ001 displayed a more complex S-layer compared with that of isolate C23^T. The phenotypic characterization and phylogenetic data support the placement of isolates C23^T and HBSQ001 in a novel species in a new genus within the *Halobacteriaceae*, for which we propose the name *Haloquadratum walsbyi* gen. nov., sp. nov. The type strain of *Haloquadratum walsbyi* is C23^T (=JCM 12705^T=DSM 16854^T).

INTRODUCTION

Salt lakes occur worldwide and present a diverse range of environmental conditions to the organisms dwelling within them, including substantial variation in pH, salinity (including variations in ionic composition) and temperature (reviewed by Oren, 2002). At salinities approaching saturation, the majority of micro-organisms are extremely halophilic archaea belonging to the family *Halobacteriaceae* (Oren, 2002) and, at high cell densities, the carotenoid pigments of these haloarchaea often give such waters a distinctive pink colour. Certain bacteria, such as strains of *Salinibacter* and *Salicola*, may also be present, but are usually a minor component (Antón *et al.*, 2000, 2002; Maturrano *et al.*, 2006).

In 1980, the extraordinary square haloarchaea of Walsby (SHOW) were first described in water samples taken from hypersaline pools (*sabkha*) near the Red Sea (Walsby, 1980). The cells were regular, extremely thin squares that contained gas vesicles and often formed extended sheets (Stoeckenius, 1981; Parkes & Walsby, 1981). Such cells were commonly found as the dominant cell type in these and other hypersaline waters, including natural salt lakes and saltern crystallizer ponds, and were able to be characterized by 16S rRNA gene PCR amplification and sequencing (Benlloch *et al.*, 1995, 2001). The sequence information enabled the development of specific fluorescent *in situ* hybridization (FISH) probes (Antón *et al.*, 1999) and, later, sequence tags to screen cultures by PCR (Burns *et al.*, 2004a). However, it

Abbreviations: PHA, polyhydroxyalkanoate; SHOW, square haloarchaea of Walsby.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of isolates C23^T and HBSQ001 are respectively AY676200 and AM180088.

was not until 2004 that the first isolates were obtained in pure culture, by two independent groups (Bolhuis *et al.*, 2004; Burns *et al.*, 2004b). Recently, the genome sequence of strain HBSQ001 has been published (Bolhuis *et al.*, 2006).

In this study, the phenotypic and phylogenetic characteristics of two different SHOW isolates were determined. These indicated that they belong to a novel species and a new genus within the *Halobacteriaceae*.

METHODS

Strains. Isolation and preliminary descriptions of the two SHOW isolates C23^T (=JCM 12705^T=DSM 16854^T) and HBSQ001 (=JCM 12895=DSM 16790) have been reported previously (Burns *et al.*, 2004b; Bolhuis *et al.*, 2004). Other reference strains included in this study were *Haloferax mediterranei* ATCC 33500^T, *Haloferax volcanii* NCIMB 2012^T, *Halogeometricum borinquense* JCM 10706^T, *Halobacterium salinarum* NRC-1 (=JCM 11081) and *Haloterrigena turkmenica* JCM 9101^T.

Media and growth conditions. Two types of characterization media were used. Medium CM1 contained (per litre): 995 ml 25 % (w/v) salt water (SW) and 5 ml 23 % (w/v) MGM. The recipes for SW and MGM have been published previously (Nuttall & Dyall-Smith, 1993; Porter *et al.*, 2005) and are available online in *The Halohandbook* (Dyall-Smith, 2006). The solution was autoclaved (121 °C, 101 kPa, 15 min) and the following were added from filter-sterilized stock solutions: 5 ml 1 M NH_4Cl, 2 ml 0.5 M K_2HPO_4, 1 ml SL10 trace metal solution (Widdel *et al.*, 1983), 3 ml Vit10 vitamin solution (Vit10 is a combination of 0.25 ml vitamin solution 1 and 0.75 ml vitamin solution 2 ml^{-1} as described in Janssen *et al.*, 1997) and 4.4 ml 25 % (w/v) sodium pyruvate (10 mM final concentration). Medium CM2 was identical to CM1 except that the base medium comprised (per litre) 950 ml 25 % (w/v) SW and 50 ml 23 % (w/v) MGM. Medium MGM was prepared as described in *The Halohandbook* always using 23 % salts. Solid MGM media used unwashed 1.5 % (w/v) agar (Difco-Bacto) as the gelling agent. All cultures were incubated unshaken at 37 °C, unless stated otherwise. Growth of liquid cultures was followed spectrophotometrically (Bausch & Lomb Spectronic 20) at 600 nm.

Light microscopy and electron cryomicroscopy. Light microscopy was performed with a Leitz Diaplan microscope fitted with phase-contrast optics and was used to check cell morphology, Gram stain and motility. For electron cryomicroscopy, a 4 µl drop of cell culture was applied to glow-discharged Quantifoil grids (Quantifoil Micro Tools) and then plunge-frozen in liquid ethane using a Vitrobot (FEI). Images were recorded in an FEG G2 Polara FEI transmission electron microscope operating at 300 keV. Liquid nitrogen was used as the cryogen. For the projection images shown in Fig. 1, the defocus was ~30 µm.

Electron cryotomography. For the tomographic slices (in Fig. 1e, g), tilt series were acquired from −58° to +58° in steps of 2° with a total dose of ~50 electrons Å^{-2} for HBSQ001 and ~80 electrons Å^{-2} for C23^T, an image pixel size of 1.2 nm, an energy slit-width of 20 eV and a defocus of ~30 µm. Three-dimensional reconstructions were calculated using IMOD using images binned twofold (McIntosh *et al.*, 2005). For the measurement of distances between membrane layers of C23^T and HBSQ001, tomograms of cells of strains C23^T and HBSQ001 were first segmented manually to generate a shell following the outer layer of cytoplasmic membrane (position of zero). The shell surface consisted of thousands of small triangles. The density values along the normal line of each triangle plane were then measured and averaged.

Phenotypic tests. The minimal standards for the description of new taxa within the order *Halobacteriales* were followed (Oren *et al.*, 1997). The methods have been described previously (Torreblanca *et al.*, 1986; Gutierrez *et al.*, 2002) and are based on standard microbiological protocols (Gerhardt *et al.*, 1994) but adapted for use with extremely halophilic micro-organisms. Variations are described below. The tests performed are included in the species description. *Hfx. volcanii* NCIMB 2012^T was used as a control in most tests.

For the hydrolysis of casein, cells were cultured for 1 week in 10 ml medium CM1 with a low level (5 mM) of pyruvate and with 0.5 % (w/v) casein from a filter-sterilized stock solution. Azocasein (0.1 % w/v) was then added and the cultures were incubated for a further 9 days. Cells and salt-precipitated casein (and azocasein) were removed by centrifugation and azocasein hydrolysis was measured spectrophotometrically (LKB Biochrom Ultrospec II) at 405 nm.

For substrate utilization tests, cells were inoculated into medium CM1 containing 10 mM substrate (see species description) except for cellulose, chitin and starch, which were added at 0.1 % (w/v). In cultures that grew, the final pH was measured using a calibrated pH meter and compared with the uninoculated control of the same substrate.

For testing antibiotic sensitivity, cells were inoculated into CM2 media with the addition of 10 mM pyruvate and 50 µg antibiotic ml^{-1}. Cells were incubated for 3 weeks at 37 °C.

Anaerobic respiration was tested using medium CM1, but without MGM, and with only one-tenth the level of NH_4Cl. DMSO or $NaNO_3$ or neither (to test for fermentation) were added to a final concentration of 10 mM. Media were dispensed aseptically into 30 ml plastic tubes, each containing an inverted Durham tube. Cultures were incubated under either anaerobic conditions using Anaerogen sachets (Oxoid) or aerobically, for 3 weeks at 37 °C without shaking, after which the presence of nitrogen gas bubbles and nitrite were determined.

For the indole test, cells were grown in medium CM1 with the addition of 1 % (w/v) peptone for 1 week at 37 °C before testing for indole using Kovacs' reagent.

Growth at different temperatures was tested in CM2 medium. Cultures were made in duplicate and incubated at 4, 25, 30, 37, 40, 42, 45 and 55 °C for 2 weeks. Growth was followed spectrophotometrically at 600 nm.

For growth at different salinities, SW solutions of increasing salinity (2 % increments) were prepared, keeping the same ratio of salts as in 30 % SW (above). The supplements normally added to CM2 medium were added to each salinity increment. After inoculation, cultures were incubated for up to 3 weeks at 37 °C.

The magnesium requirement was assessed by increasing Mg^{2+} against a constant NaCl concentration. Solutions contained 2.37 M NaCl and 0.08 M KCl and either $MgCl_2$ or $MgSO_4$ ranging from 0 to 1.5 M in steps of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.25, 1.5 M. MGM was added to one-tenth the usual concentration (0.5 g peptone, 0.1 g yeast extract l^{-1}) before autoclaving. Cultures were incubated, unshaken, at 37 °C for 3 weeks.

Lipids were extracted with chloroform/methanol and separated by TLC as described previously (Kamekura, 1993; Gutierrez *et al.*, 2002).

G+C content and DNA–DNA hybridization. The G+C content of whole-cell DNA was determined by HPLC (Tamaoka, 1994). Relatedness by DNA–DNA hybridization was performed by a fluorometric method (Ezaki *et al.*, 1989).

16S rRNA gene sequencing and phylogenetic tree reconstructions. For isolate C23^T, 16S rRNA genes were amplified by

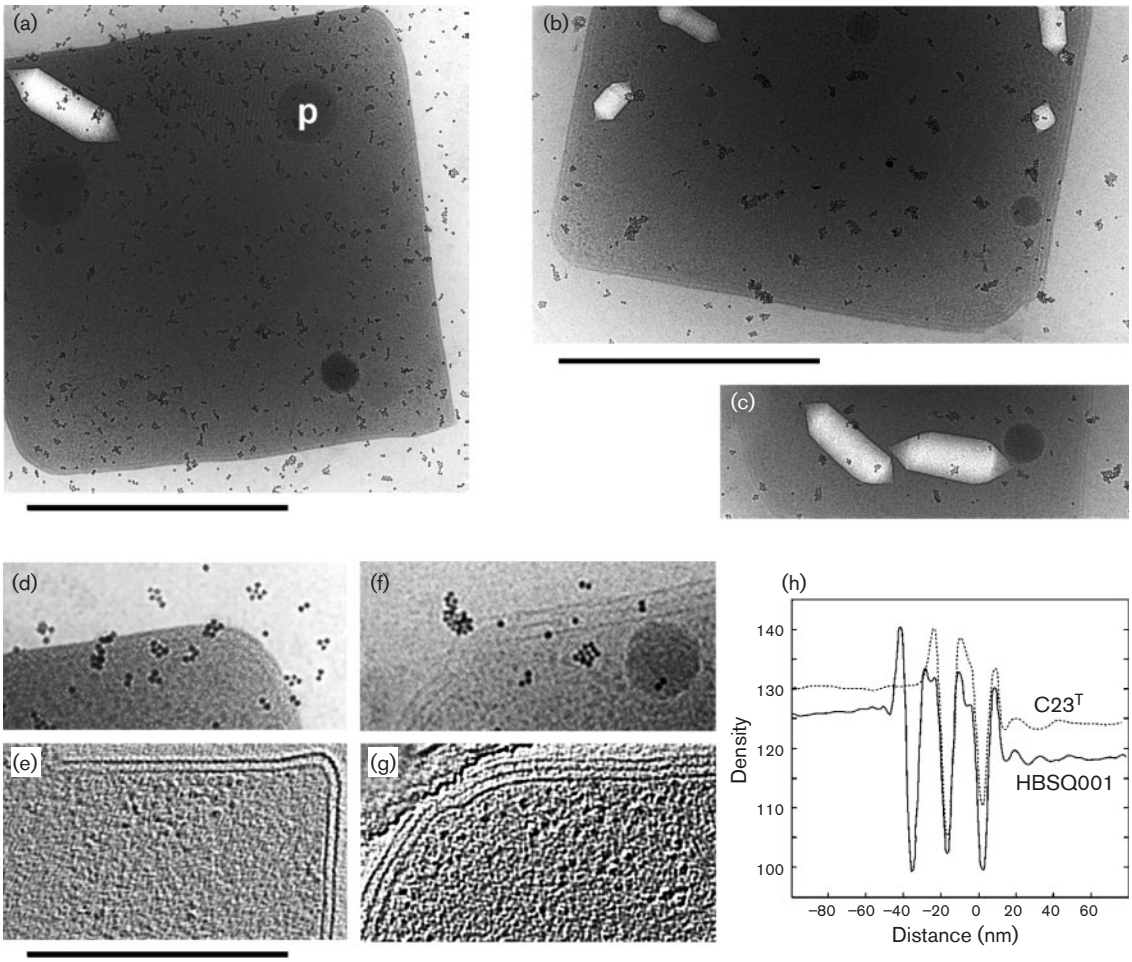

Fig. 1. Electron cryomicroscopy of C23^T and HBSQ001. Cells were embedded in vitreous ice and images recorded in an FEG G2 Polara FEI transmission electron microscope. (a) Cell of strain C23^T showing a gas vesicle and PHA granules (one is labelled p); (b) cell of strain HBSQ001 with PHA granules and immature or damaged gas vesicles; (c) mature gas vesicles of strain HBSQ001; (d) enlargement of cell wall of isolate C23^T showing the membrane and S-layer structure of the cell wall; (e) 4.8 nm thick tomographic slice through the middle of a three-dimensional reconstruction of a cell of strain C23^T, highlighting the bilaminar structure of the cell wall; (f) enlargement of cells of strain HBSQ001 showing the trilaminar structure of the cell wall; (g) 4.8 nm thick tomographic slice through the middle of a cell of strain HBSQ001 showing the trilaminar cell wall; (h) measurement of distances between cell wall layers of C23^T (dotted line) and HBSQ001 (solid line) using densitometry across cell images taken by cryotomography (see Methods). The horizontal axis is the distance (in nm) away from the cytoplasmic membrane shell. The vertical axis is the mean density value. The small black dots scattered across the images in (a–d) and (f) are 10 nm diameter colloidal gold particles. The scale bars shown under (a) and (b) represent 1000 nm, and (b) and (c) share the same bar. The scale bar beneath (d) and (e) (500 nm) is the same for (f) and (g).

PCR and sequenced as described in Burns *et al.* (2004a). The 16S rRNA gene sequence of isolate HBSQ001 was derived from the genome sequence (GenBank accession no. AM180088). Sequences were aligned and phylogenetic trees were constructed using programs within the ARB phylogeny package (Ludwig *et al.*, 2004).

RESULTS AND DISCUSSION

Two SHOW strains were isolated from solar salterns, one in Australia and the other in Spain, and their general characteristics, light and electron microscopy, have been described previously (Burns *et al.*, 2004a; Bolhuis *et al.*, 2004). Different isolation methods were used: isolate C23^T (Australia) was recovered using an extinction–dilution culturing technique and isolate HBSQ001 (Spain) was obtained by serial enrichment over a 2-year period. In both cases, low-nutrient media containing pyruvate were used.

Colonies took 4–8 weeks to grow on solid media. After 8 weeks, colonies were small (0.5–1.0 mm diameter), convex, round, with an entire edge, and intense red to pink in colour. Liquid cultures were also pink in colour, depending upon the cell density. Under optimal growth conditions, cells exhibit a perfect flat square or rectangular

shape, around 2 μm per edge and 0.2 μm thick. Much larger cell aggregates were also observed; HBSQ001 displays cell sheets of up to 40 × 40 μm (Bolhuis *et al.*, 2004) and C23^T can form sheets of at least 12 μm a side (data not shown). At salinities below ~23 %, the cell morphology deteriorates to a ragged square or other flat pleomorphs. Both strains are non-motile and stain Gram-negative. A large number of potential growth substrates were tested (see species description), but pyruvate was the only carbon source to permit reproducibly measurable growth of both isolates. Cells are oxidase- and catalase-negative using conventional testing. They do not hydrolyse starch or casein and do not show *β*-galactosidase activity. Indole is not produced in tryptophan-containing media. Neither strain was able to use nitrate or DMSO as an alternative electron acceptor under anaerobic conditions.

Electron cryomicroscopy resolved internal cell structures very clearly (Fig. 1). The two isolates showed similar types of gas vesicles and polyhydroxyalkanoate (PHA) granules but differed significantly in cell-wall structure. Isolate C23^T possessed a typical two-layer cell wall consisting of a simple S-layer above the cell membrane (Fig. 1a, d, e). The cell wall of isolate HBSQ001 displayed a more complex, apparently three-layered structure, unlike other haloarchaea (Fig. 1b, f, g, h). Fig. 1h shows the density profiles across both cell walls. In HBSQ001, two peaks were resolved outside the cytoplasmic membrane, corresponding to two layers of surface structure. Each layer appeared ~14 nm away from the next (peak-to-peak). In C23^T, there was only one surface layer covering the cytoplasmic membrane, also ~14 nm above the membrane.

The optimum growth temperature for both strains was 45 °C. The minimum growth temperature was 25 °C for C23^T and 30 °C for HBSQ001. No growth was observed at 55 °C. Growth occurred over a wide range of salinity, from a minimum of 12 % (w/v) for C23^T or 14 % (w/v) for HBSQ001, up to saturation. From 18–36 % salinity, the growth profiles of both isolates were relatively flat and did not show a steep rise and fall around an optimum concentration. The optimum salinity was 18 % (w/v) for HBSQ001 and C23^T. Neither strain required magnesium ions, but growth was poor in their absence. Optimum growth varied both by strain and Mg^{2+}-associated anion. C23^T had no specific optimum for $MgCl_2$, but concentrations above 1 M $MgCl_2$ yielded higher cell densities than lower Mg^{2+} concentrations, while growth peaked at 0.4–0.6 M $MgSO_4$. HBSQ001 grew optimally at 0.2 M $MgCl_2$, but required 0.6 M $MgSO_4$ to reach the same density. In both strains, growth declined markedly at high $MgCl_2$ concentrations compared with $MgSO_4$, suggesting that the high chloride ion concentration (>5 M) may have been inhibitory.

The two isolates had similar responses to pH, exhibiting growth over the range 5.5–8.5. Optimum growth was at pH 6.5 for C23^T and pH 7.0 for HBSQ001. Both SHOW strains were sensitive to the antibiotics anisomycin, chloramphenicol, erythromycin, novobiocin, rifampicin, simvastatin and tetracycline. They were resistant to ampicillin, bacitracin, cycloheximide, kanamycin, mycostatin, neomycin and streptomycin.

As shown in Fig. 2, the mobilities of the polar lipids detected by TLC were identical in the two isolates, although the proportion of phosphatidylglycerol (PG) observed in the HBSQ001 extract was lower than that in the C23^T sample. While visible in the original TLC, the PG spot for HBSQ001 did not reproduce in the photograph (Fig. 2, lane 4). In addition to PG and phosphatidylglycerophosphate methyl ester (PGP-Me), the isolates possessed a glycolipid that co-chromatographed with S-DGD-1 (Fig. 2, lanes 4 and 5), a lipid that occurs in *Haloferax* species (lanes 1 and 2). This is consistent with previous data derived from the extraction of lipids from natural samples containing almost pure populations of SHOW cells (Oren *et al.*, 1996).

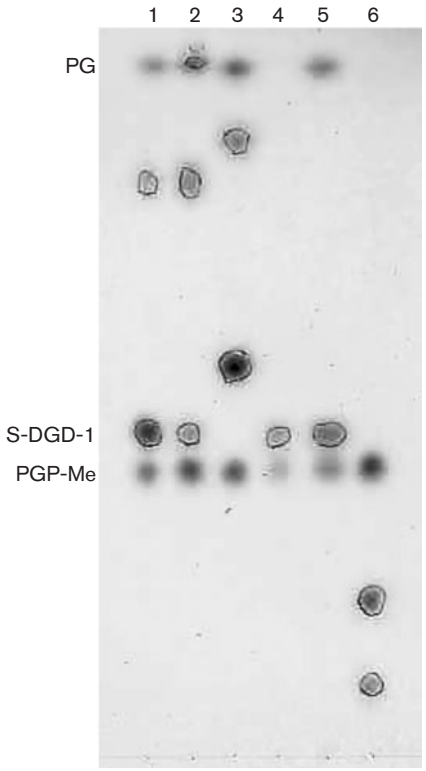

Fig. 2. TLC of polar lipids of *Hfx. mediterranei* ATCC 33500^T (lane 1), *Hfx. volcanii* NCIMB 2012^T (2), *Hgm. borinquense* JCM 10706^T (3), strain HBSQ001 (4), strain C23^T (5) and *Hbt. salinarum* NRC-1 (6). The origin is at the bottom. PG, Phosphatidylglycerol; PGP-Me, phosphatidylglycerophosphate methyl ester; S-DGD-1, sulfated diglycosyl diether lipid. Glycolipids were detected as purple spots and circled in pencil. Lane 4 showed a visible but faint PG spot that did not reproduce in the photograph. Cells were grown as described in Methods.

Table 1. DNA G+C content and DNA–DNA relatedness between strains C23^T and HBSQ001

Fixed strain	DNA G+C content (mol%)	DNA–DNA hybridization (%) with labelled strain:	
		C23^T	HBSQ001
C23^T	46.9	100	81.1
HBSQ001	46.9	80.3	100
Htg. turkmenica JCM 9101^T	62.9	0.2	0.2

The DNA G+C content of both isolates was 46.9 mol%, and whole-genome DNA–DNA hybridization gave a relatedness of ~80 % (Table 1). Their 16S rRNA gene sequences were almost identical, with only 2 base differences. These results indicate they are members of the same species. Phylogenetic tree reconstructions placed the two SHOW sequences in a separate clade within the *Halobacteriaceae* (Fig. 3). Although not shown in this figure, this clade also contained environmental clone sequences from SHOW organisms, including GenBank accession no. X84084 (Antón *et al.*, 1999). The closest cultivated isolate to this clade was T1.3 (93.2 %), isolated from a salt mine (McGenity *et al.*, 2000) (not shown). Among the recognized members of the *Halobacteriaceae*, the closest relative was *Hgm. borinquense* PR 3^T (91.2 % sequence similarity). The 16S rRNA gene sequence similarity to *Hfx. volcanii* NCIMB 2012^T (90.3 %), *Hrr. sodomense* ATCC 33755^T (85.6 %) and *Htg. turkmenica* ATCC 51198^T (87 %), the control organism used in the DNA–DNA hybridization, was much lower.

The phenotypic characterization and phylogenetic data support the placement of isolates C23^T and HBSQ001 in a novel species and a new genus within the *Halobacteriaceae*, for which we propose the name *Haloquadratum walsbyi* gen. nov., sp. nov.

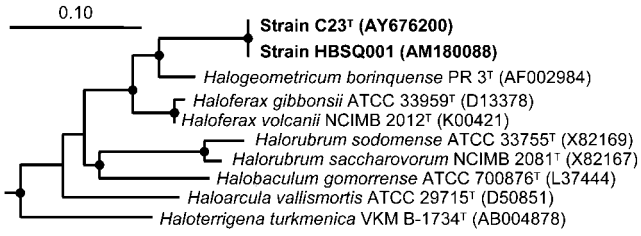

Fig. 3. Phylogenetic tree reconstruction based on complete or nearly complete 16S rRNA gene sequences (sequence accession numbers in parentheses). The tree shown was derived by maximum-likelihood (using the ARB package). Bar, 0.1 expected nucleotide substitutions per site. Bootstrap values (using distance matrix methods) were derived from 1000 replicates and significant nodes (>75 %) are indicated by filled circles at branch points. The outgroup sequences consisted of sequences representing most of the other genera of the *Halobacteriaceae* as well as that of *Methanosaeta concilii* ATCC 35969^T (GenBank accession no. X16932.1) (not shown).

Description of *Haloquadratum* gen. nov.

Haloquadratum (Ha.lo.quad.ra′tum. Gr. masc. n. *hals*, *haloes* salt; L. neut. n. *quadratum* square; N.L. neut. n. *Haloquadratum* salt square).

Flat, square cells that usually contain gas vesicles and PHA storage granules. Aerobic heterotrophs. Oxidase and catalase tests are negative. Cells stain Gram-negative. Phylogenetically belonging to the family *Halobacteriaceae*. Habitat: salt lakes and saltern crystallizer ponds. The DNA G+C content of the type species is 46.9 mol%. The type species is *Haloquadratum walsbyi*. Following the published guidelines (Oren & Ventosa, 2000), we propose the three-letter genus abbreviation *Hqr*.

Description of *Haloquadratum walsbyi* sp. nov.

Haloquadratum walsbyi (wals′by.i. N.L. gen. masc. n. *walsbyi* of Walsby, named after A. E. Walsby, who first published observations on this organism).

Displays the following properties in addition to those given in the genus description. Cells are square (~2 × 2 μm) and flat (0.2 μm thick) and can form large sheets. Under suboptimal conditions, such as reduced salinity, they show flat, pleomorphic forms. Colonies on agar medium are iridescent pink with an entire edge. Strictly aerobic; only oxygen is used as the final electron acceptor. Cannot utilize nitrate or DMSO as alternative electron acceptors. Does not grow anaerobically on L-arginine. Growth occurs at pH 6.0–8.5, 25–45 °C and 14–36 % (w/v) NaCl. Halophilic; cells lyse immediately in distilled water and a minimum of ~14 % (w/v) salts is required for growth. Optimal growth occurs under neutrophilic to alkaliphilic conditions, above 18 % salinity. Capable of growing in defined media, but is severely restricted in the substrates utilized. Grows best on pyruvate as sole carbon source. No growth enhancement occurs with acetate, alanine, arabinose, arginine, aspartate, benzoate, betaine, butanol, butyrate, cellobiose, citrate, ethanol, formate, fructose, fumarate, galactose, galacturonate, gluconuronate, glucose, glycerol, glycine, glycolate, lactate, lactose, leucine, lysine, malate, malonate, mannitol, mannose, methanol, propanol, propionate, ribose, serine, succinate, sucrose, tartrate, threonine, urea, valine or xylose at 10 mM or cellulose, chitin or starch at 0.1 % (w/v) as the sole carbon and energy source. Acid is not produced from carbohydrate utilization. Negative for β-galactosidase and indole production. Sensitive to anisomycin, chloramphenicol, erythromycin, novobiocin, rifampicin, simvastatin and tetracycline and resistant to ampicillin, bacitracin, cycloheximide, kanamycin, mycostatin, neomycin and streptomycin at 50 μg ml^{-1}. The polar lipids are $C_{20}C_{20}$ derivatives of PG, PGP-Me and S-DGD-1. The DNA G+C content of

the two known strains is 46.9 mol%. Both strains were isolated from solar saltern crystallizer ponds.

The type strain is C23^T (=JCM 12705^T=DSM 16854^T). Strain HBSQ001 (=JCM 12895=DSM 16790) is a reference strain.

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