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

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Strains C23T 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 3T (91.2 % sequence identity). The major polar glycolipid of both isolates was the sulfated diglycosyl diether lipid S-DGD-1. Electron cryomicroscopy 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 C23T. The phenotypic characterization and phylogenetic data support the placement of isolates C23T 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 C23T (≡ JCM 12705T ≡ DSM 16854T).

**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 haloarchaeon of Walsby.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of isolates C23T 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 HB9Q001 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 HB9Q001 (\(=\) 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 volcani NCIMB 2012\(^T\), Halogeometricum borinquense ICM 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 & Dylam-Smith, 1993; Porter et al., 2005) and are available online in The Halohandbook (Dylam-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\(_4\)Cl, 2 ml 0.5 M K\(_2\)HPO\(_4\), 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 were unashed (1.5 % (w/v) agar (Difco-Bacto) as the gelling agent. All cultures were incubated unashed 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 ~25 μm.

**Electron tomography.** 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 HB9Q001 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 HB9Q001, tomograms of cells of strains C23\(^T\) and HB9Q001 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 (Torresblanca 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 (0.4 % 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\(_4\)Cl, 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’s 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, unashed, 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
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\textsuperscript{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

**Fig. 1.** Electron cryomicroscopy of C23\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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).
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 C23T 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 pleomorphic. 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 C23T 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 C23T, 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 C23T 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 C23T 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 C23T. Neither strain required magnesium ions, but growth was poor in their absence. Optimum growth varied both by strain and Mg2+-associated anion. C23T had no specific optimum for MgCl2, but concentrations above 1 M MgCl2 yielded higher cell densities than lower Mg2+ concentrations, while growth peaked at 0.4–0.6 M MgSO4. HBSQ001 grew optimally at 0.2 M MgCl2, but required 0.6 M MgSO4 to reach the same density. In both strains, growth declined markedly at high MgCl2 concentrations compared with MgSO4, 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 C23T 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 C23T 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 33500T (lane 1), Hfx. volcanii NCIMB 2012T (2), Hgm. borinquense JCM 10706T (3), strain HBSQ001 (4), strain C23T (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.](image_url)
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 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 3T (91.2 % sequence similarity). The 16S rRNA gene sequence similarity to *Hfx. volcanii* 2012T (90.3 %), *Hrr. sodomense* ATCC 33755T (85.6 %) and *Htg. turkmenica* ATCC 51198T (87 %), the control organism used in the DNA–DNA hybridization, was much lower.

The phenotypic characterization and phylogenetic data support the placement of isolates C23T 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.

### Table 1. DNA G+C content and DNA–DNA relatedness between strains C23T and HBSQ001

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<tr>
<th>Fixed strain</th>
<th>DNA G+C content (mol%)</th>
<th>DNA–DNA hybridization (%) with labelled strain:</th>
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<td></td>
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<td>C23T</td>
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<td>HBSQ001</td>
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<td>C23T</td>
<td>46.9</td>
<td>100</td>
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<tr>
<td>HBSQ001</td>
<td>46.9</td>
<td>80.3</td>
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Flattish, 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* 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, glucononurate, glucose, glyceral, glycine, glycolate, lactate, lactose, leucine, lysine, malate, malonate, mannitol, mannose, methanol, propanol, propionate, ribose, serine, succinate, succrose, 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 C₃₂₋ C₄₀ 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 C23T (= JCM 12705T = DSM 16854T). Strain HBSQ001 (= JCM 12895 = DSM 16790) is a reference strain.

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