Haloanaerobacter salinarius* sp. nov., a novel halophilic fermentative bacterium that reduces glycine-betaine to trimethylamine with hydrogen or serine as electron donors; emendation of the genus *Haloanaerobacter

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A novel halophilic fermentative bacterium has been isolated from the black sediment below a gypsum crust and a microbial mat in hypersaline ponds of Mediterranean salterns. Morphologically, physiologically and genetically this organism belongs to the genus *Haloanaerobacter*. *Haloanaerobacter* strain SG 3903^T (T = type strain) is composed of non-sporulating long flexible rods with peritrichous flagella, able to grow in the salinity range of 5–30% NaCl, with an optimum at 14–15%. The strain grows by fermenting carbohydrates or by using the Stickland reaction with either serine or H₂ as electron donors and glycine-betaine as acceptor, which is reduced to trimethylamine. The two species described so far in the genus *Haloanaerobacter* are not capable of Stickland reaction with glycine-betaine + serine; however, *Haloanaerobacter chitinovorans* can use glycine-betaine with H₂ as electron donor. Strain SG 3903^T thus represents the first described strain in the genus *Haloanaerobacter* capable of the Stickland reaction with two amino acids. Although strain SG 3903^T showed 67% DNA–DNA relatedness to *H. chitinovorans*, it is physiologically sufficiently different from the two described species to be considered as a new species which has been named *Haloanaerobacter salinarius* sp. nov.

Keywords: marine salterns, halophilic fermentative bacteria, *Haloanaerobacter*, glycine-betaine, Stickland reaction

INTRODUCTION

The anoxic sediments of hypersaline environments are often characterized by a large number of halophilic anaerobic bacteria belonging to the domain Bacteria. Among them the most frequently isolated are fermentative bacteria (Rainey *et al.*, 1995) and anoxygenic phototrophs (Ollivier *et al.*, 1994; Imhoff & Süling, 1996). Many types of halophilic bacteria have been isolated from the Dead Sea, inland salt lakes or

subterranean waters in oilfields (Oren, 1992; Ollivier *et al.*, 1994; Rainey *et al.*, 1995; Imhoff & Süling, 1996; Ravot *et al.*, 1997). Some of them were isolated from thalassohaline environments such as solar salterns; they include phototrophic bacteria of the family *Chromatiaceae* (Caumette *et al.*, 1988, 1991a, 1997), sulfate-reducing bacteria (Caumette *et al.*, 1991b) and fermentative bacteria (Zhilina & Zavarzin, 1990; Zhilina *et al.*, 1991a, b, 1992; Liaw & Mah, 1992; Simankova *et al.*, 1993).

During ecological investigations in solar salterns of the French Mediterranean coast (Salin-de-Giraud, Camargue, Rhône Delta), we isolated several new species of phototrophs and sulfate reducers that developed at the sediment surface of certain hypersaline ponds characterized by gypsum deposits and

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The EMBL accession number for the 16S rRNA sequence of SG 3903^T is Y14212.

salinities ranging from 15 to 30% (Caumette, 1993; Caumette *et al.*, 1994). Recently a number of fermentative halophilic bacterial strains belonging to the order *Haloanaerobiales* were isolated from the black anoxic sediment of these ponds, where they co-exist with the sulfate reducers. So far, the order *Haloanaerobiales* contains 17 species, grouped into nine genera and two families, *Haloanaerobiaceae* and *Halobacteroidaceae* (Bhupathiraju *et al.*, 1994; Cayol *et al.*, 1995; Rainey *et al.*, 1995; Tsai *et al.*, 1995; Zhilina *et al.*, 1996; Ravot *et al.*, 1997). These species grow mainly by fermenting carbohydrates; one of them (*Acetohalobium arabaticum*) was found to be able to use amino acids such as glycine-betaine (Zhilina & Zavarzin, 1990) which is a common compound in hypersaline environments and is used as compatible solute by a large variety of halophilic organisms and micro-organisms (Yancey *et al.*, 1982; Galinski & Trüper, 1994). Among our strains isolated from the Salin-de-Giraud salterns, several were able to use glycine-betaine as substrate; morphologically and physiologically, they were similar to *Acetohalobium arabaticum*. Others were found to be able to use glycine-betaine only in the presence of hydrogen or serine. One of these strains, strain SG 3903^T, was selected for further characterization. According to morphological, physiological and genetical studies, this strain was closely related to the species *Haloanaerobacter chitinovorans*, but sufficiently different to be proposed as a new species. This paper describes its characteristics in comparison with *Haloanaerobacter chitinovorans*, in particular its ability to carry out the Stickland reaction with glycine-betaine as electron acceptor and hydrogen or serine as electron donors. This strain SG 3903^T is consequently proposed as a new species with the name *Haloanaerobacter salinarius* sp. nov.

METHODS

Sources of strains. Strain SG 3903^T was isolated from the microbial mat at the sediment surface of hypersaline ponds where total salinity ranges from 13 to 20% depending of the seasons, in the saltern of Salin-de-Giraud (Camargue, France).

Haloanaerobacter lacunaris strain DSM 6640^T and *Haloanaerobacter chitinovorans* OGC 229^T (DSM 9569^T) were obtained from the DSM. *Haloanaerobacter lacunaris* (Zhilina *et al.*, 1991b) originated from the silt of Lake Chokrak, Crimea (25% total salinity) and *Haloanaerobacter chitinovorans* (Liaw & Mah, 1992) was isolated from the sediment of salterns in Chula Vista, CA, USA (20–30% total salinity).

Media, isolation and culture conditions. The basal synthetic medium used for the growth of strain SG 3903^T contained, per litre of distilled water: NaCl, 150 g; MgCl₂·6H₂O, 15 g; KCl, 3 g; NH₄Cl, 0.5 g; KH₂PO₄, 0.35 g; CaCl₂·2H₂O, 0.05 g; yeast extract, 0.1 g; 0.1% resazurin solution, 1 ml; trace-element solution SL12 (Overmann *et al.*, 1992), 1 ml; selenite-tungstate solution (Na₂SeO₃·5H₂O, 6 mg l⁻¹; Na₂WO₄·2H₂O, 8 mg l⁻¹; NaOH, 0.4 g l⁻¹), 1 ml; NaHCO₃, 2 g; Na₂S·9H₂O, 0.5 g; vitamin V7 solution (Pfennig *et al.*, 1981), 1 ml; pH 7.2–7.4.

The medium was prepared under a mixture of gas (N₂/CO₂; 90:10, v/v) according to the method of Pfennig *et al.* (1981). Prior to utilization, the medium was supplemented with organic substrates as energy and carbon sources (see Table 1 for substrate utilization).

Pure cultures of strain SG 3903^T were obtained from repeatedly prepared deep agar dilution series (Pfennig & Trüper, 1981) in Hungate tubes with H₂/CO₂ (90:10, v/v) in the gas phase, and glycine-betaine as carbon source and electron acceptor. The tubes were incubated at 30 °C in the dark.

Purity of the cultures was checked microscopically and by inoculation into different media specific for aerobic bacteria and sulfate reducing bacteria.

Pure strains were grown in liquid cultures under a gas phase (H₂/CO₂; 90:10, v/v) in 60 ml serum bottles stoppered with butyl rubber stoppers, using the Hungate anaerobic technique. The basal medium was supplemented with glycine-betaine + H₂ or with glycine-betaine + serine. After growth at 30 °C, the strains were then stored at 4 °C in the dark, for periods of 2–4 months.

Microscopy. Microscopic observations and photomicrographs were made with an Olympus photomicroscope (OM2) according to the method of Pfennig & Wagener (1986). Flagella were observed by transmission electron microscopy with a JEOL 1200 ES electron microscope after negative staining with 1% (w/v) tungstic acid neutralized to pH 7.2. The fine structure of the cells was studied by transmission electron microscopy after fixation of a cell pellet with osmic acid and ultrathin sectioning of the cells according to Glazer *et al.* (1971).

Physiological tests. Utilization of carbon sources and electron donors was tested in triplicate in basal liquid medium with substrate concentrations as given in Table 1. Growth tests for utilizable substrates, the utilization of optimum concentrations of NaCl and MgCl₂·6H₂O, optimum pH, optimum temperature and sulfide tolerance were performed in completely filled 25 ml screw-cap tubes as described by Caumette *et al.* (1988).

For aerobic growth tests, the basal medium without hydrogen carbonate and sulfide was buffered with 0.4 M Tris/HCl and supplemented with glucose as substrate. The growth was checked in test tubes open to air through a cotton stopper. Antibiotic and antibacterial susceptibility was tested in completely filled screw-cap tubes with the growth medium for maintenance of strains supplemented with the following substances: anisomycin (40 µg ml⁻¹), chloramphenicol (20 µg ml⁻¹), tetracycline (40 µg ml⁻¹), erythromycin (40 µg ml⁻¹) kanamycin (40 µg ml⁻¹) and sodium taurocholate (50 µg ml⁻¹) according to the method described by Oren (1990).

Growth was measured by following the optical density of cultures at 450 nm (Bausch & Lomb; Spectronic 20) over a period of 10 d.

Vitamin requirement was tested in 60 ml serum bottles by growth tests through five consecutive transfers in synthetic medium free of vitamins and yeast extract. Catalase was tested by adding a few drops of 3% (v/v) H₂O₂ to a cell pellet on a microscope slide. Utilization of sulfate, sulfite, thio-sulfate or nitrate was tested in Postgate medium B (Postgate, 1984), previously made free of sulfate, in 60 ml serum bottles, with glucose as electron donor. After growth, sulfur

compound utilization was determined by sulfide production, as revealed by the formation of a black precipitate of FeS in the liquid culture; nitrate reduction was determined by nitrite production revealed by the formation of a red colour upon addition of Griess reagent.

Analytical procedures. The presence of H₂ and CO₂ among fermentation products was detected with a Chrompack CP 9001 gas chromatograph equipped with a thermal conductivity detector (135 °C) and a semi-capillary column Poraplot Q (25 m long, 0.53 mm i.d.) operated at 35 °C with N₂ (12 ml min⁻¹) or helium (12 ml min⁻¹) as the carrier gases for H₂ or CO₂ detections, respectively.

A flame-ionization detector (220 °C) was used for alcohol determination with the same column operated at 150 °C with helium (12 ml min⁻¹) as the carrier gas.

Methylamines were determined by gas chromatography using the same gas chromatograph equipped with a 3 m × 3.1 mm column (Chromosorb 103, 80/100 mesh) and with a flame-ionization detector (200 °C). The column was operated at 110 °C with helium (25 ml min⁻¹) as the carrier gas. Before injection, samples of cell-free culture medium were prepared according to the method of Hippe *et al.* (1979).

Organic acids were determined with a HPLC apparatus, equipped with a Shimadzu LC 6A pump, a PYE Unicam UV detector (at 210 nm) and a Rezex-type organic acid (Phenomenex) column (300 × 7.8 mm); 0.005 M H₂SO₄ was used as the solvent at a flow rate of 0.5 ml min⁻¹. The volume of the injection loop was 20 µl.

Glycine-betaine and serine were analysed with the same HPLC fitted with a Hypersil-NH₂ column (Touzart and Matignon); acetonitrile/10 mM phosphate buffer pH 7 (75/25) solution was used as the solvent at a flow rate of 1 ml min⁻¹.

DNA base composition, sequence of the 16S rDNA gene and DNA–DNA hybridization

Isolation of genomic DNA. Strain SG 3903^T was grown on H₂ + glycine-betaine, lyophilized, and stored under 2-propanol. Before use, the cells were centrifuged to remove 2-propanol, and resuspended and washed in 0.1% SDS/10 mM Tris/HCl/1 mM EDTA, pH 8.0 (Vargas *et al.*, 1995). Genomic DNA was then prepared as described by Ausubel *et al.* (1989).

Determination of G+C content. The G+C content of genomic DNA from SG 3903^T was determined by high-performance liquid chromatography as described by Mesbah *et al.* (1989), using bacteriophage lambda DNA as standard.

Amplification of 16S rDNA by PCR. A 1.4 kb fragment of the gene encoding 16S rRNA was amplified by PCR using the following primers specified by Amann *et al.* (1995): sense primer, 5'-AGAGTTTGATCCTGGCTCA3'. Bacteria, positions 8–26 (*Escherichia coli* numbering); antisense primer, 5'-ACGGGCGGTGTGTA(G)C3', Universal, positions 1406–1392 (*E. coli* numbering). The PCR reaction mixture contained (in 100 µl): 0.5 µg genomic DNA; 200 pmol each primer; 50 mM KCl; 10 mM Tris/HCl, pH 9.0; 0.1% Triton X-100; 1.25 mM MgCl₂; 0.2 mM each dNTP; 5% (v/v) DMSO; 2.5 U *Taq* DNA polymerase (Promega). An initial cycle composed of 3 min denaturation at 94 °C, 2 min annealing at 50 °C, and 3 min extension at

72 °C was followed by 34 cycles of 1 min at 94 °C, 2 min at 50 °C and 3 min at 72 °C.

Cloning and sequencing of the PCR product. The PCR reaction mixture was extracted with 100 µl chloroform/isoamyl alcohol (24:1) and purified by centrifugation through a MicroSpin S-400 HR column (Pharmacia Biotech). After treatment with T4 DNA polymerase to remove protruding dA residues (Sambrook *et al.*, 1989), the 1.4 kb PCR product was cloned into the *Sma*I site of pUC19. The cloned insert was then sequenced entirely on both strands, using Sequenase enzyme (USB Biochemicals). The resulting sequence (1383 nucleotides) is EMBL accession number Y14212.

Genetic analysis. The 16S rRNA/rDNA sequences of 14 species of fermentative halophilic anaerobic bacteria were obtained from the EMBL Database, using the accession numbers quoted by Rainey *et al.* (1995). The following strains were used (sequence accession numbers in parentheses): *Haloanaerobium praevalens* DSM 2228^T (M59123), *Haloanaerobium alcaliphilum* DSM 8275^T (X81850), *Haloanaerobium saccharolyticum* subsp. *saccharolyticum* DSM 6643^T (X89069), *Haloanaerobium saccharolyticum* subsp. *senegalense* DSM 7379^T (X89070), *Haloanaerobium acetoethylicum* DSM 3532^T (X89071), *Haloanaerobium salsuginis* ATCC 51327^T (L22890), *Halocella cellulolytica* DSM 7362^T (X89072), *Halothermothrix orenii* OCM 544^T (L22016), *Haloanaerobacter chitinovorans* OGC 229^T (X89076), *Haloanaerobacter lacunaris* DSM 6640^T (X89075), *Halobacteroides halobius* DSM 5150^T (X89074), *Orenia marismortui* DSM 5156^T (X89073), *Sporohalobacter lortetii* DSM 3070^T (M59122), *Acetohalobium arabaticum* DSM 5501^T (X89077) and *Megasphaera elsdenii* (M26493). The database entry for *Haloanaerobium acetoethylicum* (X89071) was found to be identical to that for *Haloanaerobium saccharolyticum* subsp. *senegalense* (X89070), but this has since been corrected (F. Rainey, personal communication). These sequences were aligned with the strain SG 3903^T sequence by the CLUSTAL W method, using the MEGALIGN program of the DNASTAR software package. The alignment was then modified to remove regions containing unidentified bases or gaps of more than three nucleotides. The subsequent analysis was based on a comparison of approximately 1240 nucleotides.

DNA–DNA hybridization. The DNA–DNA hybridization was performed between strain SG 3903^T and *Haloanaerobacter chitinovorans* OGC 229^T. The test was carried out at the Identification Service of DSMZ (Braunschweig, Germany). DNA was isolated by chromatography on hydroxyapatite by the procedure of Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), with the modification described by Huss *et al.* (1983) and Escara & Hutton (1980) using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER .BAS program by Jahnke (1992).

RESULTS

Enrichment and isolation

Different kinds of fermentative bacteria were enriched from black anoxic sediment below a thin laminated photosynthetic microbial mat and a gypsum crust in hypersaline ponds of Salin-de-Giraud (Camargue,

Fig. 1. Phase-contrast photomicrograph of *Haloanaerobacter salinarium* strain SG 3903^T in liquid culture with (a) glycine-betaine + H₂ or (b) glucose as substrates. Bar, 10 µm.

France) where the salinity ranged between 13 and 20%. The laminated microbial mat was composed of halophilic phototrophic purple bacteria, as described previously (Caumette *et al.*, 1988, 1991a). From the anoxic sediment, rich in acid-volatile sulfide due to a high sulfate reduction activity (Caumette *et al.*, 1994), the enrichment culture of fermentative bacteria showed a large variety of cell types.

Several strains were purified and isolated by using different fermentable substrates such as glucose, glycine-betaine or glycine-betaine + hydrogen. Among the pure strains obtained, some were facultative anaerobes resembling vibrios, others were strict anaerobes able to ferment mainly glucose or, in a few cases, glycine-betaine. Some strains needed H₂ to degrade glycine-betaine to trimethylamine; one of these, strain SG 3903^T, was selected for further characterization.

Morphology and fine structure

The individual cells of strain SG 3903^T were long, thin, flexible rods, 0.3–0.4 µm in width and 5–8 µm in length, in young cultures grown with glycine-betaine + H₂ (Fig. 1a). When cultured with glucose as substrate, single cells were larger and less flexible (Fig. 1b). After the end of exponential growth, they rapidly formed sphaeroplasts.

The cells were motile. Negatively stained cells showed peritrichous flagella (data not shown). Electron microscopic examination of thin sections of strain SG 3903^T revealed a typical Gram-negative layered cell envelope

Fig. 2. Electron micrographs of *Haloanaerobacter salinarium* strain SG 3903^T. Ultrathin sections showing (a) the Gram-negative structure and the formation of sphaeroplasts and (b) cell division. Bars, 1 µm.

(Fig. 2a, b). In aged cultures, distortion of the envelope and formation of degenerate cells and sphaeroplasts were detected (Fig. 2a). Endospores were not detected by either phase-contrast microscopy or electron microscopy, not even after pasteurization (80 °C, 20 min) of the samples. Colonies were translucent and glossy in surface agar and opaque in deep agar. They were white to slightly yellow with entire edges; their diameter ranged from 1 to 2 mm.

Growth and physiology

Strain SG 3903^T grew over a large range of salinity (Fig. 3a). Growth was observed at NaCl concentrations between 5 and 30% (w/v), with optimal growth at 14–15%. At the optimal NaCl concentration, the optimal temperature for growth of strain SG 3903^T was 45 °C (Fig. 3b) and growth occurred at temperatures between 10 and 50 °C. The optimum pH was 7.4–7.8 and growth was possible between pH 5.5 and pH 8.5. For optimal growth, strain SG 3903^T required a minimum of 1 mM Mg²⁺. No growth was obtained in the presence of oxygen.

The strain showed a high sulfide tolerance, up to 14 mM. Neither growth factors nor vitamins were required.

Strain SG 3903^T was not capable of dissimilatory reduction of inorganic nitrogen (NO₃⁻) or sulfur compounds (SO₄²⁻, SO₃²⁻, S₂O₃²⁻) thus showing a strict

Fig. 3. Growth of *Haloanaerobacter salinaris* strain SG 3903^T in liquid culture as a function of (a) salinity and (b) temperature. The growth rate has been calculated from exponential growth measured by OD₄₅₀.

fermentative metabolism. Catalase test was negative. The strain did not use sulfate or nitrate as sulfur or nitrogen sources but it could use cysteine as both nitrogen and sulfur source. Strain SG 3903^T was sensitive to chloramphenicol and erythromycin. Anisomycin, kanamycin, tetracycline, or Na-taurocholate did not inhibit growth. Strain SG 3903^T used the following fermentable substrates: glucose, fructose, galactose, mannose, trehalose, sucrose, maltose, raffinose, cellobiose, glucosamine, *N*-acetylglucosamine and mannitol (Table 1).

The strain was also able to use glycine-betaine in the presence of an electron donor (hydrogen or serine) via a Stickland reaction (Fig. 4). Other amino acids were tested but were not able to act as electron donors for the reduction of glycine-betaine. With other amino acid pairs, the Stickland reaction was not observed in the strain SG 3903^T. Neither glycine-betaine nor serine alone were used as fermentable substrates (Fig. 4).

Fermentation products are listed in Table 2. From glucose as substrate, the major products obtained were ethanol, acetate, formate, propionate, CO₂ and H₂. Lactate was detected in very low concentrations. When glycine-betaine + H₂ were used as substrates, strain SG 3903^T excreted acetate, 2-propanol, trimethylamine and CO₂. In the Stickland reaction with glycine-betaine and serine the strain produced acetate, trimethylamine, CO₂ and NH₃. Formate and acetate were also detected in very low concentrations. Monomethylamine, dimethylamine, dimethylglycine and sarcosine were never detected as fermentation products of glycine-betaine with either H₂ or serine as electron donors.

As strain SG 3903^T proved to be genetically closely

related to *Haloanaerobacter* (see below), *Haloanaerobacter chitinovorans* OGC 229^T and *Haloanaerobacter lacunaris* DSM 6640^T were tested for possible Stickland reactions. *Haloanaerobacter lacunaris* was not able to use glycine-betaine with either serine or hydrogen, whereas *Haloanaerobacter chitinovorans* used only glycine-betaine + hydrogen for growth.

Genomic characteristics and genetic relationships

The DNA base composition of strain SG 3903^T was 31.6 mol% G + C. A near complete sequence (1383 nucleotides) of the 16S rRNA gene of strain SG 3903^T was determined. The sequence (positions 8–1406, *E. coli* numbering according to Winker & Woese, 1991) was aligned and a genetic analysis was performed with 16S rRNA gene sequences of 14 representatives of the closest phyla in the domain *Bacteria*. A distance matrix based on the Jukes & Cantor (1969) method showing the percentage similarities and divergences between the different sequences has been constructed. The sequence from *Megasphaera elsdenii* was included as the outgroup for rooting the dendrogram. The dendrogram derived from the distance matrix is shown in Fig. 5. The tree clearly shows the branching of the fermentative halophilic anaerobic bacteria into two clusters (Patel *et al.*, 1995) or families (Rainey *et al.*, 1995). Strain SG 3903^T was most closely related to *Haloanaerobacter chitinovorans* (98.9% similarity) and *Haloanaerobacter lacunaris* (97.5% similarity). These last two species showed a similarity of 98.1% to each other. The closest species in the neighbouring genus was *Halobacteroides halobius*, which showed a similarity with strain SG 3903^T, *Haloanaerobacter chitinovorans* and *Haloanaerobacter lacunaris* of 85.6, 86.2 and 86.6%, respectively.

As strain SG 3903^T and *Haloanaerobacter chitinovorans* were very closely related (98.9% similarity), a DNA–DNA hybridization was performed between strain SG 3903^T and *Haloanaerobacter chitinovorans* OGC 229^T. The DNA–DNA pairing value obtained was 67%.

DISCUSSION

Strain SG 3903^T is a Gram-negative, flexible, long, rod-shaped, carbohydrate fermenting, non-spore-forming, halophilic anaerobe which grows in the presence of 50–300 g NaCl l⁻¹. These characteristics and its G + C content (31.6 mol%) are consistent with assignment of strain SG 3903^T to the family *Halobacteroidaceae* (Rainey *et al.*, 1995). The genetic relatedness study based on comparison of 16S rDNA sequences showed that strain SG 3903^T is clearly a member of this family (Fig. 5). In the cluster of *Halobacteroidaceae*, strain SG 3903^T is included with the group forming the genus *Haloanaerobacter* which is, so far, composed of two species: *Haloanaerobacter chitinovorans* and *Haloanaerobacter lacunaris* (Liaw &

Table 1. Substrate fermentation by *Haloanaerobacter* strain SG 3903^T grown with 15% NaCl under optimal conditions

+, Substrate utilized; —, substrate not utilized. The following substrates were not used by strain SG 3903^T (mM except where stated): arabinose (6); inulin (6); dextran (6); xylan (6); salicin (6); gluconate (6); dextrin (6); lactose (6); melizitose (6); rhamnose (6); sorbose (6); xylose (6); glycogen (6); ascorbate (6); inositol (6); sorbitol (6); ethanol (6); methanol (6); dulcitol (6); adonitol (6); valine (6); sarcosine (10); threonine (6); ornithine (6); glycine (6); proline (6); leucine (6); isoleucine (6); tryptophan (6); phenylalanine (6); alanine (6); histidine (6); urea (6); gelatin (0.1%); pectin (0.1%); yeast extract (0.1%); crotonate (6). The following amino acid pairs were tested but were not utilized: glycine-betaine + glycine; glycine-betaine + alanine; glycine-betaine + leucine; glycine-betaine + isoleucine; glycine-betaine + valine; glycine-betaine + phenylalanine; glycine-betaine + histidine; glycine-betaine + threonine; glycine-betaine + proline; glycine-betaine + ornithine; glycine-betaine + tryptophan; alanine + glycine; leucine + proline; isoleucine + proline; valine + ornithine; phenylalanine + leucine; histidine + sarcosine.

Substrate	Utilization
Glucose (6 mM)	+
Fructose (6 mM)	+
Galactose (6 mM)	+
Mannose (6 mM)	+
Trehalose (6 mM)	+
Sucrose (6 mM)	+
Maltose (6 mM)	+
Raffinose (6 mM)	+
Cellobiose (6 mM)	+
Starch (0.1%)	—
Glucosamine (6 mM)	+
N-Acetylglucosamine (6 mM)	+
Chitin (0.1%)	—
Mannitol (6 mM)	+
Pyruvate (6 mM)	—
Malate (6 mM)	—
Lactate (6 mM)	—
Glutamate (6 mM)	—
Glycine-betaine (6 mM)	—
Glycine-betaine (6 mM) + H ₂ (10 ⁵ Pa)	+
Serine (6 mM)	—
Glycine-betaine (6 mM) + serine (6 mM)	+
Trimethylamine (6 mM)	—

Mah, 1992; Zhilina *et al.*, 1991b). Strain SG 3903^T, which may be considered as a new representative of the genus *Haloanaerobacter*, is most closely related to the species *Haloanaerobacter chitinovorans*. However, on the basis of its G + C content, *Haloanaerobacter* strain SG 3903^T (31.6 mol%) is closer to *Haloanaerobacter lacunaris* (32.4 mol%) than to *Haloanaerobacter chitinovorans* (34.8 mol%). In addition, the percentage of the DNA-DNA hybridization between *Haloanaerobacter chitinovorans* and our strain is 67%,

Fig. 4. Growth of *Haloanaerobacter salinaris* strain SG 3903^T in liquid cultures with different substrates (▲) with glycine-betaine alone; (△) with serine alone; (●) with glycine-betaine + serine (Stickland reaction); (○) with glycine-betaine + hydrogen.

indicating that strain SG 3903^T is close to the species *Haloanaerobacter chitinovorans*.

Like *Haloanaerobacter chitinovorans* and *Haloanaerobacter lacunaris*, *Haloanaerobacter* strain SG 3903^T was isolated from a thalassohaline environment (hypersaline lagoon or saltern). These bacteria, with salinity optima between 12 and 18%, are well adapted to the salinity of their environments (Table 3). They grow by fermenting mainly carbohydrates. They are not able to use amino acids as sole substrates (Liaw & Mah, 1992). However, the data demonstrated that our strain *Haloanaerobacter* SG 3903^T was able to reduce glycine-betaine to trimethylamine with hydrogen or serine as electron donors (Fig. 4). In contrast, *Haloanaerobacter lacunaris* was not capable of glycine-betaine reduction whereas *Haloanaerobacter chitinovorans* could use glycine-betaine only with hydrogen. Thus, bacteria belonging to the genus *Haloanaerobacter* do not use exclusively carbohydrates as stated by Liaw & Mah (1992), but some strains or species can also use certain amino acids mainly glycine-betaine via a Stickland reaction with serine or H₂ as electron donors.

In our strain, *Haloanaerobacter* SG 3903^T, glucose and glycine-betaine were fermented according to the following equations (Table 2):

Table 2. Fermentation products and fermentation balance obtained after growth of *Haloanaerobacter* strain SG 3903^T with different substrates under optimal conditions

Substrates and products	Concn (mM)	Oxidation degree	Oxidation degree per equation	Sum
Substrate:				
Glucose	4.17	0	0	0
Products:				
Ethanol	3	-2	-6	
Acetate	2	0	0	
Formate	1	+1	+1	
Propionate	3	-1	-3	
Carbon dioxide	5	+2	+10	
Hydrogen	2	-1	-2	0
Substrates:				
Hydrogen	4.05	-1	-4.05	
Glycine-betaine	2.5	-4	-10	-14.05
Products:				
Acetate	0.95	0	0	
2-Propanol	1	-3	-3	
Trimethylamine	2.5	-4.5	-11.25	
Carbon dioxide	0.1	+2	+0.2	-14.05
Substrates:				
Serine	1.32	-0.5	-0.66	
Glycine-betaine	1	-4	-4	-4.66
Products:				
Acetate	2.32	0	0	
Trimethylamine	1	-4.5	-4.5	
Carbon dioxide	1.32	+2	+2.64	
NH ₃	1.32	-1.5	-1.98	-3.84

During the reduction of glycine-betaine with serine as electron donor, an additional product should be formed for a stoichiometric equilibrium in equation 3. This product was not detected during analysis.

The use of glycine-betaine is of ecological importance in hypersaline environments. Glycine-betaine is a compatible solute synthesized and stored in a large variety of organisms (Yancey *et al.*, 1982) and bacteria (Galinski & Trüper, 1994); it is one of the major osmolytes in halophilic bacteria and thus widely distributed in hypersaline ecosystems with microbial mats. It is thus not surprising to find bacteria able to use this compound as substrate in such environments.

So far, only one species of halophilic fermentative bacteria *Acetohalobium arabaticum* (Zhilina & Zavarzin, 1990) has been described as able to use glycine-betaine as sole substrate for growth.

However, two marine fermentative bacteria (*Clostridium halophilum* and *Clostridium litorale*) are able to use glycine-betaine via a Stickland reaction (Fendrich *et al.*, 1990), and many other non halophilic bacteria have been described as able to reductively cleave glycine-betaine into acetate and trimethylamine (Naumann *et al.*, 1983; Möller *et al.*, 1984, 1986; Heijthuisen & Hansen, 1989).

In hypersaline environments, two genera are thus currently considered to be able to degrade glycine-betaine: the genus *Acetohalobium* and the genus *Haloanaerobacter*. In the genus *Haloanaerobacter*, strain SG 3903^T is close to the species *Haloanaerobacter chitinovorans* but is sufficiently separated to be considered as a new species. Particularly, *Haloanaerobacter* strain SG 3903^T is able to use glycine-betaine via a real Stickland reaction with two amino acids (glycine-betaine and serine) whereas *Haloanaerobacter chitinovorans* strain OGC 229^T cannot; it can reduce glycine-betaine only with hydrogen. These unusual physiological traits are sufficient to consider strain SG 3903^T as a new representative of the genus *Haloanaerobacter* although the DNA-DNA homology between our strain and its closest relative in the genus (*Haloanaerobacter chitinovorans*) was only 67%.

According to these unusual physiological features, we propose *Haloanaerobacter* strain SG 3903^T as the type strain of a new species with the name *Haloanaerobacter salinarius* sp. nov.

Emended description of the genus *Haloanaerobacter*

Haloanaerobacter gen. emend.: full description is by Liaw & Mah (1992), with additional features: some

Fig. 5. Dendrogram based on the distance matrix showing genetic relatedness between *Haloanaerobacter salinaris* strain SG 3903^T and related species or genera of the halophilic fermentative bacteria. The 16S rDNA sequence of *Megasphaera elsdenii* was used as an outgroup to root the tree. Bar, 5 nucleotide substitutions per 100 nucleotides.

Table 3. Comparison of the major properties of *Haloanaerobacter* species

+, Positive test result; –, negative test result; +/–, some strains with positive test; ND, not determined.

Character	<i>H. salinaris</i> SG 3903 ^T	<i>H. chitinovorans</i> OGC 229 ^T	<i>H. lacunaris</i> DSM 6640 ^T
Morphology	Long flexible rods	Long flexible rods	Short to long flexible rods
Cell size (µm)	0.3–0.4 × 5–8	0.5 × 1.4–8	0.7–1 × 0.5–6
Motility	+	+	+
Flagella	Peritrichous	Peritrichous	Peritrichous
G + C content (mol %)	31.6 (HPLC)	34.8 (Bd)	32.4 (<i>T_m</i>)
NaCl range (%)	5–30	3–30	10–30
Optimum [NaCl] (%)	14–15	12–18	15–18
Temperature range (°C)	10–50	23–50	5–52
Temperature optimum (°C)	45	30–45	35–40
pH range	6–8	ND	6–8
Optimum pH	7.4–7.8	7	6.5–7
Doubling time (h)	12	2.4	2.9–4.5
Carbohydrates utilized	+	+	+
Chitin degradation	–	+ / –	–
Amino-acid utilized	–	–	–
Stickland reaction:			
Glycine-betaine + serine	+	–	–
Glycine-betaine + H ₂	+	+	–
End products of glucose fermentation	H ₂ , CO ₂ , acetate, lactate, propionate, formate, ethanol	H ₂ , CO ₂ , acetate, isobutyrate	H ₂ , CO ₂ , acetate, ethanol

strains are able to use amino acids in Stickland reactions or with hydrogen as electron donor. Type species: *Haloanaerobacter chitinovorans*.

Description of *Haloanaerobacter salinarius* sp. nov.

Haloanaerobacter salinarius (sa.li.na'ri.us. L. adj. *salinarius* pertaining to *salinae* salterns, salt-works).

Cells are Gram-negative, colourless, non-sporulating rods, motile by peritrichous flagella; the cells are long, flexible, 0.3–0.4 µm width and 5–8 µm length, in young cultures; short degenerate cells and spheroplasts in old cultures. Surface colonies are circular, translucent, glossy, slightly yellow with entire edges. Colony diameters range from 1 to 2 mm. Agar-embedded colonies are opaque. Obligately halophilic. Growth occurs at NaCl concentrations of between 5 and 30%; optimal growth at 14–15%. The temperature range for growth is 10 to 50 °C with an optimal growth at 45 °C. The pH range is 5.5–8.5 with the optimum for growth at pH 7.4–7.8. Cells are susceptible to chloramphenicol and erythromycin, and are resistant to anisomycin, kanamycin, tetracycline and Na-taurocholate. Obligately anaerobic fermentative; catalase- and oxidase-negative. Glucose, fructose, galactose, mannose, trehalose, sucrose, maltose, raffinose, cellobiose, glucosamine, *N*-acetylglucosamine and mannitol are fermented. The bacteria use glycine-betaine as oxidant in the Stickland reaction which is reductively cleaved into trimethylamine and acetate with hydrogen or serine as electron donors. The major glucose fermentation products are ethanol, propionate, acetate, formate, CO₂ and H₂. The G + C content of the DNA is 31.6 mol% (HPLC). Habitat: anoxic organic sediment from solar salterns. Type strain: strain SG 3903^T, isolated from salt ponds in salterns of Salin-de-Giraud (Camargue, France) and deposited with the DSMZ, number DSM 12146^T and the ATCC, number ATCC 700559^T. 16S rRNA sequence has been deposited in the EMBL Database under the accession number Y14212.

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