***Haloanaerobium kushneri* sp. nov., an obligately halophilic, anaerobic bacterium from an oil brine**

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Three strains, designated VS-751^T, VS-511 and VS-732, of a strictly anaerobic, moderately halophilic, Gram-negative, rod-shaped bacterium were isolated from a highly saline (15–20%) brine from an oil reservoir in central Oklahoma, USA. The optimal concentration of NaCl for growth of these three strains was 2 M (12%), and the strains also grew in the presence of an additional 1 M MgCl₂. The strains were mesophilic and grew at a pH range of 6–8. Carbohydrates used by all three strains included glucose, fructose, arabinose, galactose, maltose, mannose, cellobiose, sucrose and inulin. Glucose fermentation products included ethanol, acetate, H₂ and CO₂, with formate produced by two of the three strains. Differences were noted among strains in the optimal temperature and pH for growth, the maximum and minimum NaCl concentration that supported growth, substrate utilization and cellular fatty acid composition. Despite the phenotypic differences among the three strains, analysis of the 16S rRNA gene sequences and DNA–DNA hybridizations showed that these three strains were members of the same genospecies which belonged to the genus *Haloanaerobium*. The phenotypic and genotypic characteristics of strains VS-751^T, VS-511 and VS-732 are different from those of previously described species of *Haloanaerobium*. It is proposed that strain VS-751^T (ATCC 700103^T) be established as the type strain of a new species, *Haloanaerobium kushneri*.

Keywords: halophile, anaerobe, *Haloanaerobiaceae*, *Haloanaerobium kushneri*

INTRODUCTION

Hypersaline environments are inhabited by a wide variety of aerobic and anaerobic micro-organisms. Anaerobic bacteria from these environments include phototrophs, acetogens, methanogens, sulfate-reducers, and members of the *Haloanaerobiaceae* and *Halobacteroidaceae* (Ollivier *et al.*, 1994; Rainey *et al.*, 1995). In the family *Haloanaerobiaceae*, the genus with the largest number of described species is *Haloanaerobium*, which includes: *Haloanaerobium praevalens* (Rainey *et al.*, 1995; Zeikus *et al.*, 1983), *Haloanaerobium acetoethylicum* (formerly *Halobacteroides*

acetoethylicus) (Patel *et al.*, 1995; Rengpipat *et al.*, 1988), *Haloanaerobium alcaliphilum* (Rainey *et al.*, 1995; Tsai *et al.*, 1995), *Haloanaerobium lacusroseus* (Cayol *et al.*, 1995), *Haloanaerobium saccharolyticum* subsp. *saccharolyticum* (formerly *Haloicola saccharolyticum* subsp. *saccharolyticum*) (Rainey *et al.*, 1995; Tourova *et al.*, 1995; Zhilina *et al.*, 1992), *Haloanaerobium saccharolyticum* subsp. *senegalensis* (formerly *Haloicola saccharolyticum* subsp. *senegalensis*) (Cayol *et al.*, 1994; Rainey *et al.*, 1995), *Haloanaerobium congolense* (Ravot *et al.*, 1997) and *Haloanaerobium salsugo* (Bhupathiraju *et al.*, 1994a). These are all halophilic, obligately anaerobic, Gram-negative, fermentative bacteria.

H. salsugo (Bhupathiraju *et al.*, 1994a) and a new genus of halophilic aerobic bacteria, *Arhodomonas* (Adkins *et al.*, 1993), were isolated from highly saline brine produced from a sandstone oil reservoir in Payne

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The GenBank accession numbers for the (1500 nucleotide) 16S rRNA sequences for strain VS-751^T and *H. acetoethylicum* ATCC 43120^T are U86446 and U86448.

County, OK, USA, during a pretest study for a microbially-enhanced oil recovery pilot field project (Bhupathiraju *et al.*, 1991, 1993). A diverse array of aerobic heterotrophs, anaerobic heterotrophs, methanogens, sulfate reducers, and bacteria with metabolisms useful for enhanced oil recovery have also been recovered from produced brine from this oil reservoir (Bhupathiraju *et al.*, 1991, 1993). These studies support the conclusion that active and diverse microbial communities are present in hypersaline oil reservoirs.

Several fermentative, anaerobic, halophilic bacteria recovered from this brine were studied further. Three strains, VS-751^T, VS-732 and VS-511, are described in the present report. These are shown to be strains of a single species which is genotypically and phenotypically distinct from the described species in the genus *Haloanaerobium*. We propose the name *Haloanaerobium kushneri* for these strains with strain VS-751^T as the type strain of *H. kushneri*. A portion of this work has appeared previously (Bhupathiraju *et al.*, 1994b).

METHODS

Strains. *Haloanaerobium acetoethylicum* [formerly *Halobacteroides acetoethylicus* (Rainey *et al.*, 1995; Rengpipat *et al.*, 1988)] ATCC 43120^T and *Haloanaerobium praevalens* ATCC 33744^T were obtained from American Type Culture Collection, Manassas, VA, USA. *Haloanaerobium saccharolyticum* [formerly *Haloicola saccharolytica* (Rainey *et al.*, 1995; Zhilina *et al.*, 1992)] subsp. *saccharolyticum* (= DSM 6643^T) was kindly provided by Dr T. N. Zhilina. *Haloanaerobium salsugo* (= ATCC 51327^T) was from our culture collection. Strains VS-751^T, VS-511 and VS-732 were isolated from a hypersaline oil brine in Payne County, Oklahoma, as previously described (Bhupathiraju *et al.*, 1991, 1993, 1994a, b). *Haloanaerobium kushneri* strain VS-751^T was deposited in the American Type Culture Collection as ATCC 700103^T.

Media and culture methods. The basal medium contained (g l⁻¹): NaCl, 120; NH₄Cl, 1.0; KCl, 0.1; KH₂PO₄, 0.1; MgSO₄ · 7H₂O, 0.2; CaCl₂ · 2H₂O, 0.04; PIPES dipotassium salt, 1.5; yeast extract, 0.25; resazurin, 0.0005; cysteine hydrochloride, 0.5; and Na₂S · 9H₂O, 0.5, as well as 5 ml trace metal solution (Tanner, 1997), 10 ml vitamin solution (Tanner, 1997) and a N₂ gas phase. The pH was 7.1–7.3. A complex medium contained the basal medium described above supplemented with (g l⁻¹): Casamino acids, 1.0; yeast extract, 1.0; and glucose, 5.0. Solid media were prepared by the addition of 20 g purified agar l⁻¹ (BBL). Methods for the preparation and use of anaerobic media were those of Balch & Wolfe (1976). Cultures were routinely maintained in the complex medium by transfer once every week.

Metabolic and growth characteristics. All experiments were performed in duplicate or triplicate at 37 °C, unless otherwise indicated. The temperature and NaCl ranges for growth were determined with complex medium. The effect of magnesium ion on growth was examined with complex medium containing 2 M NaCl. The effect of pH on growth was tested in complex medium with 100 mM MOPS buffer and a N₂ gas phase. The pH was adjusted with 10 M NaOH or HCl. The ability of strains VS-751^T, VS-511, VS-732, *H. acetoethylicum* ATCC 43120^T and *H. praevalens* ATCC 33744^T to utilize different substrates was tested in basal

medium with the test compounds at 10 mM. Complex substrates were added at a concentration of 5 g l⁻¹. The use of a compound or combination of compounds was scored as positive when growth occurred in at least three serial transfers. The effect of external electron acceptors on growth was tested in the basal medium with glucose (10 mM) as an electron donor. Compounds tested as electron acceptors included elemental sulfur, sodium sulfate, sodium thio-sulfate, sodium sulfite, sodium nitrate, and sodium nitrite at a concentration of 3 g l⁻¹. Amino acid combinations tested included alanine-glycine, leucine-proline, isoleucine-tryptophan, valine-ornithine, phenylalanine-leucine and histidine-sarcosine.

Glucose fermentation products were determined in basal medium containing 10 g glucose l⁻¹ and a N₂ gas phase. Volatile fatty acids and alcohols in the liquid phase and CO₂ and H₂ in the gas phase were analysed at the beginning of the experiment and after 4 d incubation.

Gram stains were performed using both heat-fixed smears and smears fixed with acetic acid as described previously (Dussault, 1955). Antibiotic susceptibility tests were performed by adding filter-sterilized antibiotics to growth medium at a final concentration of 10 µg ml⁻¹. Sodium azide was tested at 50 µg ml⁻¹. Tests for catalase activity, oxidase activity, motility and spore formation were performed as described previously (Smibert & Krieg, 1994).

Analytical techniques. Growth was monitored by measuring the increase in OD₆₆₀ (Balch & Wolfe, 1976). Glucose was measured by the glucose oxidase method (Sigma). Volatile fatty acids, alcohols and gases were analysed as described previously (Bhupathiraju *et al.*, 1991, 1994a).

Microscopy. Transmission electron microscopy was performed using a JEOL JEM 2000 FX transmission electron microscope. Micrographs were prepared from exponential-phase cells grown in complex medium. The cells were fixed with 1% glutaraldehyde in 12% NaCl, spread onto carbon-coated Formvar grids and stained with 1% phosphotungstate (pH 7.0).

Cellular fatty acid analysis. The cellular fatty acid composition of cells of strains VS-751^T, VS-511, VS-732, *H. acetoethylicum* ATCC 43120^T and *H. praevalens* ATCC 33744^T grown in the complex medium containing 10% NaCl was determined. Cellular fatty acid analysis was performed by Microcheck. Whole-cell fatty acids were analysed as fatty acid methyl esters with a MIDI microbial identification system. Fatty acid methyl esters were synthesized and analysed as described previously (Miller, 1982; Moss *et al.*, 1998; Sassar, 1990).

DNA base composition. DNAs were isolated and purified as described previously (Marmur, 1961). The G+C content was determined by the HPLC method of Mesbah *et al.* (1989). Non-methylated λ phage DNA was used for calibration.

DNA–DNA hybridization. Isolated DNA from strains VS-751^T, VS-511 and VS-732 and other *Haloanaerobium* species were nick translated with [³²P]dCTP using a nick translation kit (Boehringer Mannheim Biochemica). The labelled DNA was purified with Quick Spin Columns (Boehringer Mannheim Biochemica) and ethanol precipitation. DNA–DNA reassociation was performed by using the free-solution method and analysed by the S1 nuclease procedure (Johnson, 1994). Reassociation mixtures contained 12.5% formamide and reassociation was performed at T_m – 25 °C of the radiolabelled DNA. The T_m was calculated from the mol% G+C content using the equation T_m =

64.9 °C + (0.5025 × [mol % G + C]) and was further reduced by 0.62 °C for each percentage formamide in solution.

16S rRNA sequence analysis. The 16S rRNAs were sequenced by methods described previously (Lane *et al.*, 1985; Oyaizu *et al.*, 1987). Sequences were analysed by distance matrix analysis with a program for fitting trees to distance data (De Soete, 1983; Jukes & Cantor, 1969; Oyaizu *et al.*, 1987). A minimum of 1500 nucleotide positions were used in tree construction.

RESULTS AND DISCUSSION

Genetic analysis

Strains VS-751^T, VS-511 and VS-732 had the same 16S rRNA gene sequence. Distance matrix analysis showed that these three strains were closely related to members of the genus *Haloanaerobium* (Fig. 1). The 16S rRNA gene sequence of strain VS-751^T was 1.9% different from that of *Haloanaerobium acetoethylicum* and 2.1% different from that of *Haloanaerobium praevalens*.

The discovery that strains VS-751^T, VS-511 and VS-732 had identical 16S rRNA gene sequences was not expected given the phenotypic differences among the three strains in motility, substrate utilization, cellular fatty acid composition, and other phenotypic proper-

ties (below; Bhupathiraju *et al.*, 1991). However, the results of DNA–DNA hybridization showed that strains VS-751^T, VS-511 and VS-732 were members of a single genospecies, with percentage DNA–DNA hybridization values of 89% or higher (Table 1). Also, these three strains were not related at the species level to the two *Haloanaerobium* species with the most similar 16S rRNA gene sequences, *H. acetoethylicum* and *H. praevalens*.

The mol% G + C content of the DNA from strains VS-751^T, VS-511 and VS-732 was (± standard deviations of four replicates) 34.1 ± 0.2, 36.9 ± 0.1 and 32.4 ± 0.2, respectively.

Cellular characteristics

Strains VS-751^T, VS-511 and VS-732 were all non-spore-forming, Gram-negative, rod-shaped bacteria. Colonies on complex medium are circular, entire, opaque, white in colour and 2 mm in diameter. Cells of strains VS-751^T and VS-732 were actively motile and measured 0.7 × 2.0–3.3 µm and 0.5 × 0.7–1.9 µm, respectively. Strain VS-511 was not motile and the size of the cells was 0.8 × 1.3–2.6 µm. Cells of all three strains appeared singly or in pairs. Strain VS-751^T had peritrichously arranged flagella (Fig. 2).

The variability in cell size among strains VS-751^T, VS-511 and VS-732, while seemingly unusual, is similar to the variability in cell size observed in other halophilic eubacteria, which are sometimes described as pleomorphic, especially in older cultures. For example, the cell size of the two subspecies of *Haloanaerobium saccharolyticum* varied from 0.5–0.7 × 1.0–1.5 µm for one subspecies to 0.4–0.6 × 2.0–5.0 µm for the other subspecies (Cayol *et al.*, 1994; Zhilina *et al.*, 1992).

Growth and metabolic characteristics

Strains VS-751^T, VS-511 and VS-732 required anaerobic conditions for growth. The strains were obligately halophilic, with an optimal NaCl requirement of 12% (2 M) (Fig. 3). The strains also grew in medium containing 1 M MgCl₂·6H₂O in addition to 2 M NaCl. Strains VS-751^T (Fig. 3) and VS-511 grew at NaCl concentrations between 9 and 18%, and strain VS-732 grew at NaCl concentrations between 6 and 25%. The growth rates under optimal NaCl conditions at 37 °C and an initial pH of 7.2 for strains VS-751^T, VS-511 and VS-732 were 0.095, 0.086 and 0.063 h⁻¹, respectively.

The temperature ranges (with the optimum temperature given in parentheses) for strains VS-751^T, VS-511 and VS-732 were 20–45 °C (40 °C), 20–37 °C (35 °C) and 20–37 °C (35 °C), respectively. The pH range for growth of all three strains was 6.0–8.0. The pH optima for strains VS-751^T, VS-511 and VS-732 were 6.5–7.5, 7.0 and 6.5, respectively.

The substrates that supported the growth of these strains were predominantly carbohydrates, in common with other species of *Haloanaerobium* (Rainey *et al.*,

Fig. 1. Phylogenetic relationships of *H. kushneri* VS-751^T to other bacteria. The total horizontal distance between two species indicates the level of difference in their sequences. Bar indicates an evolutionary distance of 0.1. Type species used for this analysis (with gene accession numbers in parentheses) are as follows: *Haloanaerobium lacusroseus* strain DSM 10165^T (L39767) (Cayol *et al.*, 1995); *Haloanaerobium salsugo* ATCC 43120^T (L22890); *Haloanaerobium praevalens* ATCC 33744^T (M59123) and *Sporohalobacter lortetii* ATCC 35059^T (M59122) (Bhupathiraju *et al.*, 1994a); *Haloanaerobium congolense* DMS 11287^T (Ravot *et al.*, 1997); *Haloanaerobium alcaliphilum* DMS 8275^T (X81850) (Tsai *et al.*, 1995); *Halothermothrix orenii* OMC 544^T (L22016) (Cayol *et al.*, 1994); *Haloanaerobium saccharolyticum* DSM 6643^T (X89069); *Halocella cellulolytica* DSM 7362^T (X89072); *Halobacteroides halobius* DSM 5150^T (X89074) (Rainey *et al.*, 1995); and *Megasphaera elsdenii* (M26493) (Zhao *et al.*, 1989).

Table 1. Levels of DNA relatedness between strains VS-751^T and VS-732 and other species in the genus *Haloanaerobium*

Data are means $\pm$ standard deviation of three replicates. A value of 100% indicates homologous hybridization.

Source of unlabelled DNA	DNA hybridization (%) with ³² P-labelled DNA from:	
	VS-751 ^T	VS-732
Strain VS-751 ^T	100	91 $\pm$ 1
Strain VS-732	98 $\pm$ 1	100
Strain VS-511	98 $\pm$ 1	89 $\pm$ 1
<i>H. acetoethylicum</i> ATCC 43120 ^T	37 $\pm$ 3	40 $\pm$ 1
<i>H. saccharolyticum</i> subsp. <i>saccharolyticum</i> DSM 6643 ^T	28 $\pm$ 1	25 $\pm$ 2
<i>H. salsugo</i> ATCC 51327 ^T	25 $\pm$ 3	22 $\pm$ 2
<i>H. praevalens</i> ATCC 33744 ^T	18 $\pm$ 2	16 $\pm$ 1

Fig. 2. Transmission electron micrograph of a negatively stained cell of *H. kushneri* VS-751^T. Bar, 1 μ m. **Fig. 3.** Effect of NaCl concentration on maximum absorbance (●) and growth rate (○) of strain VS-751^T.

1995). Strain VS-751^T used arabinose, cellobiose, fructose, galactose, glucose, inulin, lactose, maltose, mannose, peptone, pyruvate, sucrose and trypticase.

There was variability observed among the strains in types of substrates used. In addition to using the substrates used by strain VS-751^T, strain VS-511 also used fucose, glucosamine, glycerol, mannitol, sorbose and xylose. The substrate utilization profile of strain VS-732 was similar to that of strain VS-751^T, except strain VS-732 also used fucose and xylose, but not lactose and pyruvate. Substrates that did not support growth of any of the three strains included L-alanine, L-ascorbate, adonitol, betaine, butyrate, cellulose, chitin, crotonate, dulcitol, formate, fumarate, glutamate, glycine, glycogen, inositol, lactate, L-lysine, malate, methanol, methionine, pectin, proline, propionate, starch, succinate, sarcosine and sorbitol. The amino acid mixtures of alanine-glycine, leucine-proline, isoleucine-tryptophan, valine-ornithine, phenyl-alanine-leucine and histidine-sarcosine did not support growth of any of the strains.

Strain VS-751^T differed from *H. praevalens* ATCC 43120^T in its ability to use arabinose, cellobiose, galactose, lactose, pyruvate and sucrose, and in its inability to use glucosamine. Strain VS-751^T differed from *H. acetoethylicum* ATCC 33744^T in its ability to use galactose and its inability to use glucosamine and xylose.

The major fermentation products from glucose were ethanol, acetate, CO₂ and H₂ (Table 2). Strains VS-751^T and VS-732 also produced formate. Growth of all three strains was inhibited by chloramphenicol, tetracycline or penicillin, but not by cycloheximide or azide. Nitrate, nitrite, sulfate, sulfite, thiosulfate and elemental sulfur did not stimulate growth of any of the strains. All three strains were oxidase and catalase-negative.

The differences among strains VS-751^T, VS-511 and VS-732 in certain growth and metabolic characteristics is not inconsistent with these strains being members of the same species. There are clear differences in substrate utilization, NaCl concentration tolerance and doubling times under the same growth conditions

Table 2. Glucose fermentation products

Each value reported is mol product formed per mol glucose fermented. Carbon recoveries were 103, 98 and 91 % for strains VS-751^T, VS-511 and VS-732, respectively. Hydrogen recoveries were 101, 102 and 88 % for strains VS-751^T, VS-511 and VS-732, respectively.

Product	VS-751 ^T	VS-511	VS-732
Ethanol	1.4	1.4	1.0
Acetate	0.58	0.67	0.70
Formate	0.32	ND	0.32
CO ₂	1.9	1.7	1.7
H ₂	0.52	0.53	0.48

ND, Not detected.

Table 3. Cellular fatty acid compositions of halophilic anaerobes

Strains: 1, VS-751^T; 2, VS-511; 3, VS-732; 4, *H. praevalens* ATCC 33744^T†; 5, *H. acetoeithylicum* ATCC 43120^T†

Fatty acid*	Cellular fatty acid composition (%) of strain:				
	1	2	3	4†	5†
C _{10:0} 3-OH	2.7	2.4	2.2	0.9	3.2
C _{12:0} 3-OH or C _{13:0}	4.1	3.3	2.7	2.7	3.5
C _{14:0}	9.3	6.6	9.1	8.4	11.2
C _{16:0}	34.9	35	22.1	25.8	20.4
C _{16:1} <i>cis</i> 7	8.1	7.4	12.0	11.4	12.0
C _{16:1} <i>cis</i> 9	22.5	21.6	38.5	36.8	37.6
C _{16:1} <i>cis</i> 11	4.0	4.3	6.7	6.8	5.3
C _{17:0} cyc	7.8	9.4	1.0		1.1

* 3-OH, 3-hydroxy; cyc, cyclic.

† Cultures grown in the same medium at the same time as strains VS-751^T, VS-511 and VS-732, and data published in Bhupathiraju *et al.* (1994a).

between the subspecies of *H. saccharolyticum* (Cayol *et al.*, 1994).

Cellular fatty acid composition

The cellular fatty acid compositions of strains VS-751^T, VS-511, VS-732, *H. acetoeithylicum* ATCC 43120^T and *H. praevalens* ATCC 33744^T, cultured under the same conditions and harvested while in exponential growth, are given in Table 3. Fatty acids, hydroxy fatty acids and aldehydes [from plasmalogens (Jantzen & Hofstad, 1981)] were found, with variants of C₁₆ compounds prevalent, as was observed in the cellular fatty acid compositions of *H. praevalens* ATCC 33744^T and *H. acetoeithylicum* ATCC 43120^T (Table 3) and *H. salsugo* (Bhupathiraju *et al.*, 1994a).

Again, while variations in the cellular fatty acid compositions of strains VS-751^T, VS-511 and VS-732 are clearly evident, a similar variability in fatty acid composition was found for strains of *H. saccharolyticum* (Cayol *et al.*, 1994). While these results are not inconsistent with these three strains being members of the same species, they do suggest that cellular fatty acid composition should not be used to determine relatedness of strains of these or other species of *Haloanaerobium*.

Characteristics that differentiate strains VS-751^T, VS-511 and VS-732 from the described fermentative halophilic anaerobic bacteria

Strains VS-751^T, VS-511 and VS-732 were assigned as members of the genus *Haloanaerobium* by both 16S rRNA gene sequence analysis and phenotypic characterization (Rainey *et al.*, 1995). Some of the differences between the VS-751^T, VS-511 and VS-732 strains and the other species of *Haloanaerobium* are summarized in Table 4. The products of glucose metabolism of strains VS-751^T, VS-511 and VS-732 differ from those of *H. alcaliphilum*, *H. praevalens*, *H. saccharolyticum* and *H. congolense*. In addition, the maximum pH for growth for strains VS-751^T, VS-511 and VS-732 is 8.0 while *H. alcaliphilum*, *H. praevalens* and *H. congolense* can grow at pH values greater than 8.0 (Ravot *et al.*, 1997; Zeikus *et al.*, 1983; Tsai *et al.*, 1995). The growth of *H. lacusroseus* was inhibited by magnesium chloride (Cayol *et al.*, 1995) while the three strains examined here tolerated at least 1 M magnesium chloride. *H. congolense* uses thiosulfate and elemental sulfur as electron acceptors (Ravot *et al.*, 1997) while strains VS-751^T, VS-511 and VS-732 do not use these compounds.

The species with the most similar 16S rRNA sequence to strains VS-751^T, VS-511 and VS-732 was *H. acetoeithylicum*. The growth of *H. acetoeithylicum* was inhibited by magnesium ion concentrations higher than 0.2 M (Rengpipat *et al.*, 1988) while strains VS-751^T, VS-511 and VS-732 grew in the presence of 1 M magnesium ions. *H. acetoeithylicum* produced more acetate than ethanol from glucose fermentations (Rengpipat *et al.*, 1988) while strains VS-751^T, VS-511 and VS-732 produced more ethanol than acetate from glucose. The results of the DNA–DNA hybridization analysis showed that the VS strains differed at the species level from *H. acetoeithylicum* (Table 1). *H. praevalens*, which had a 16S rRNA gene sequence of 2.1 % different from that of strains VS-751^T, VS-511 and VS-732, was also differentiated from these strains by DNA–DNA hybridization analysis.

H. salsugo (Bhupathiraju *et al.*, 1994a) was phenotypically the most similar to strains VS-751^T, VS-511 and VS-732. *H. salsugo* produced more acetate than ethanol from glucose (Bhupathiraju *et al.*, 1994a), while the opposite was observed for strains VS-751^T, VS-511 and VS-732. The salt optimum for *H. salsugo* was 9 % NaCl (Bhupathiraju *et al.*, 1994a) compared

Table 4. Differential characteristics of species of the genus *Haloanaerobium*

Species: 1, *H. kushneri* VS-751^T; 2, *H. acetoethylicum* ATCC 43120^T; 3, *H. alcaliphilum* DSM 8275^T; 4, *H. congolense* DSM 11287^T; 5, *H. lacusroseus* DSM 10165^T; 6, *H. praevalens* ATCC 33744^T; 7, *H. saccharolyticum* subsp. *saccharolyticum* DSM 6643^T; 8, *H. saccharolyticum* subsp. *senegalensis* DSM 7379^T; 9, *H. salsugo* ATCC 51327^T. Data from this study and Bhupathiraju *et al.* (1994a), Cayol *et al.* (1994, 1995), Ravot *et al.* (1997), Rainey *et al.* (1995), Rengpipat *et al.* (1988), Tourouva *et al.* (1995), Tsai *et al.* (1995), Zeikus *et al.* (1983) and Zhilina *et al.* (1992). +, Supported growth; –, did not support growth; ND, not determined; NR, not reported; NA, not applicable.

Character	1	2	3	4	5	6	7	8	9
Motility	Yes	Yes	Yes	No	Yes	No	Yes	Yes	No
Use of substrates									
L-Arabinose	+	+	–	–	–	–	+	–	+
Cellobiose	+	+	–	ND	+	–	+	+	–
Galactose	+	–	–	+	+	–	ND	–	+
Glycerol	–	–	–	ND	+	–	ND	+	–
Pectin	–	–	–	ND	ND	+	–	–	–
L-Sorbose	–	–	ND	ND	–	–	–	–	+
Starch	–	–	–	ND	+	–	–	ND	–
Sucrose	+	+	+	+	+	–	+	+	+
Products of glucose fermentation	Ethanol, acetate, (formate)*, H ₂ , CO ₂	Acetate, ethanol, H ₂ , CO ₂	Acetate, lactate, butyrate, H ₂ , CO ₂	Acetate, H ₂ , CO ₂	Ethanol, acetate, H ₂ , CO ₂	Acetate, butyrate, H ₂ , CO ₂	Acetate, H ₂ , CO ₂	Acetate, H ₂ , CO ₂	Acetate, ethanol, H ₂ , CO ₂
Ethanol/acetate ratio	2.4	0.87	NA	NA	NR	NA	NA	NA	0.71

* Formate was produced from glucose by two of the three strains of *H. kushneri*, including the type strain.

to 12% for these three strains. The DNA–DNA hybridization analysis (Table 1) showed that the three VS strains are differentiated at the species level from *H. salsugo*.

Based on the results presented above, we propose that strains VS-751^T, VS-511 and VS-732 be established as a new species, *H. kushneri*, in the genus *Haloanaerobium*, with strain VS-751^T designated the type strain of this species.

The phenotypic variability among strains VS-751^T, VS-511 and VS-732 seems unusual since all three strains were isolated from the same hypersaline oil reservoir, which also contained another phenotypically similar species of *Haloanaerobium* (Bhupathiraju *et al.*, 1991, 1993, 1994a, b). It is easier to understand such variability when strains of the same species are isolated from geographically distinct environments, as with the subspecies of *H. saccharolyticum* (Cayol *et al.*, 1994; Zhilina *et al.*, 1992). It may be that the phenotypic variation observed for the three strains of *H. kushneri* reflects local heterogeneities in the reservoir. Spatially separated habitats are likely to form due to the diffusion-limited movement of cells and nutrients in the reservoir. Such habitats may select for strains with different phenotypic variation properties.

Description of *Haloanaerobium kushneri* sp. nov.

Haloanaerobium kushneri (kush'ner.i. M.L. gen. n. *kushneri* named after Donn J. Kushner, in recognition of his contributions to the microbiology of halophilic bacteria).

Straight, rod-shaped bacterium with cell dimensions of 0.5–0.8 × 0.7–3.3 µm. Growing cells appear singly and in pairs and can be motile. The cells stain Gram-

negative and are non-spore-forming. Colonies on complex medium are circular, entire, opaque, white in colour and 2 mm in diameter. Growth in liquid medium is uniform. Growth is inhibited by chloramphenicol, tetracycline and penicillin, but not by cycloheximide or sodium azide. Obligately halophilic. The optimal NaCl concentration for growth is 12%; growth occurs at NaCl concentrations of between 9 and 18%. Magnesium chloride concentrations up to 1 M do not inhibit growth. The temperature range is 20–45 °C, with optimum between 35–40 °C. The pH range is 6.0–8.0, with the optimum between 6.5 and 7.5. Strictly anaerobic and catalase- and oxidase-negative. Fermentative metabolism. The end products of glucose fermentation are ethanol, acetate, CO₂ and H₂ and, sometimes, formate. The following compounds support growth in a basal medium: L-arabinose, cellobiose, fructose, galactose, D-glucose, inulin, maltose, D-mannose, peptone, sucrose and trypticase. The utilization of fucose, glucosamine, glycerol, lactose, mannitol, pyruvate, sorbose, and xylose is variable among the strains of *H. kushneri*. The following compounds do not support growth in a basal medium: L-alanine, L-ascorbate, adonitol, betaine, butyrate, cellulose, chitin, crotonate, dulcitol, formate, fumarate, glutamate, glycine, glycogen, inositol, lactate, L-lysine, malate, methanol, methionine, pectin, proline, propionate, starch, succinate, sarcosine and sorbitol. Amino acid mixtures such as alanine-glycine, leucine-proline, isoleucine-tryptophan, valine-ornithine, phenylalanine-leucine and histidine-sarcosine are not utilized. The specific content of major cellular fatty acids is strain-dependent and primarily C_{16:0}, *cis*-9 C_{16:1}, *cis*-7 C_{16:1} and C_{14:0} fatty acids. The G+C content of the DNA is 32–37 mol% and is strain-dependent (as determined by HPLC). The habitat is

hypersaline petroleum reservoir production fluid from Payne County, OK, USA. The type strain is VS-751^T (= ATCC 700103^T).

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