Dethiosulfovibrio salsuginis sp. nov., an anaerobic, slightly halophilic bacterium isolated from a saline spring

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A mesophilic, strictly anaerobic, slightly halophilic bacterium, designated strain USBA 82^T, was isolated from a terrestrial saline spring in the Colombian Andes. The non-spore-forming curved rods (5–7×1.3 µm) with pointed or rounded ends, stained Gram-negative and were motile by means of laterally inserted flagella. The strain grew optimally at 30 °C (growth range 20–40 °C), pH 7.3 (growth range pH 5.5–8.5) and 2 % (w/v) NaCl (growth range 0.1–7 % NaCl). The strain fermented peptides, amino acids and a few organic acids, but growth was not observed on carbohydrates, alcohols or fatty acids. The strain reduced thiosulfate and sulfur to sulfide. Sulfate, sulfite, nitrate and nitrite were not used as electron acceptors. On peptone alone, acetate, succinate, propionate and traces of ethanol were formed, but in the presence of thiosulfate, acetate and succinate were formed. The G+C content of the chromosomal DNA was 52 mol% (*T_m*). 16S rRNA gene sequence analysis indicated that strain USBA 82^T was affiliated to *Dethiosulfovibrio peptidovorans* within the phylum *Synergistetes* with a similarity value of approximately 93 %. Based on the differences between the new strain and the type species of the genus *Dethiosulfovibrio*, we suggest that strain USBA 82^T represents a novel species of the genus for which the name *Dethiosulfovibrio salsuginis* sp. nov. is proposed. The type strain is USBA 82^T (=DSM 21565^T=KCTC 5659^T).

The phylum *Synergistetes* (Jumas-Bilak *et al.*, 2009) currently includes members of the genera *Dethiosulfovibrio*, *Synergistes*, *Anaerobaculum*, *Aminobacterium*, *Thermanaerovibrio*, *Aminomonas*, *Thermovirga*, *Aminiphilus*, *Jonquetella* and *Cloacibacillus* (Rees *et al.*, 1997; Baena *et al.*, 1998, 1999a, b; Dahle & Birkeland, 2006; Díaz *et al.*, 2007; Jumas-Bilak *et al.*, 2007; Ganesan *et al.*, 2008). A distinguishing feature that is common to all members of this phylum is the capacity to degrade amino acids (Vartoukian *et al.*, 2007). *Dethiosulfovibrio peptidovorans* is the type species of the genus *Dethiosulfovibrio*. It was obtained from a corroding offshore oil well in Congo and is a non-saccharolytic peptide- and amino acid-degrading species which grows on several different amino acids in the presence of elemental sulfur or thiosulfate. During such growth, an increase in peptide utilization, cell yields and growth rates has been noted (Magot *et al.*, 1997). In this report, we describe the characterization of a new *Dethiosulfovibrio* strain, isolated from the 'Salpa' saline spring.

As part of our ongoing research into the microbial diversity of the Salpa terrestrial saline spring, samples were collected

in sterile containers, the containers were capped, stored over ice, transported to the laboratory and maintained at 4 °C until used. The Salpa saline spring is located in the Andean region of Colombia at 2500 m above sea level. The spring has a constant temperature of 21 °C and a pH of 6.5 throughout the year. The predominant dissolved ion is sulfate (20 g l⁻¹) and the conductivity is approximately 50 mS cm⁻¹ (Alfaro, 2002).

Enrichments were initiated in a medium prepared by filtering saline spring water through polycarbonate membranes (Durapore) with a pore size of 0.22 µm. The medium was supplemented with peptone (0.2 %, w/v), yeast extract (0.02 %, w/v) and the trace element solution (1 ml l⁻¹) as described by Imhoff-Stuckle & Pfenning (1983). The medium was boiled and then cooled to room temperature under a stream of oxygen-free nitrogen. An 8 ml aliquot was dispensed into Hungate tubes under oxygen-free nitrogen gas and sterilized by autoclaving at 121 °C for 20 min at a pressure of 1–1.5 kg cm⁻². The enrichment medium was inoculated with 2 ml water samples, incubated at 36 °C for up to two weeks and examined by phase-contrast light microscopy (Eclipse 50i; Nikon).

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain USBA 82^T is EU719657.

To isolate pure cultures, serial dilutions of the enrichment cultures were made in an artificial basal medium (BM) fortified with 2% (w/v) Noble agar using the roll-tube technique. BM contained (l^{-1} deionized water): 1 g NH_4Cl , 0.3 g K_2HPO_4 , 0.3 g KH_2PO_4 , 3 g $MgCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g KCl , 23 g $NaCl$, 1 ml trace element solution (Imhoff-Stuckle & Pfenning, 1983), 0.5 g cysteine-HCl, 1.0 g yeast extract and 0.5% (w/v) peptone. The pH of the medium was adjusted to 7.1 with 1 M NaOH solution and then boiled, cooled and dispensed as described previously (Baena *et al.*, 1998). Three morphologically similar cultures were isolated and 16S rRNA gene sequences of all of the isolates showed an identical sequence (100% similarity). One of the three isolates, designated strain USBA 82^T, was selected and studied further. The pure cultures were stored at $-80\text{ }^{\circ}C$ in glycerol-BM (20:80).

Cell morphology was determined by phase-contrast microscopy. Samples for electron microscopy were prepared as described by Patel *et al.* (1985). Cells of strain USBA 82^T were slightly curved rods with pointed or rounded ends ($5\text{--}7 \times 1.5\text{ }\mu m$) and occurred singly or in pairs. Cells stained Gram-negative and were motile by laterally inserted flagella. Spores were not observed and exponential phase cultures were killed when heated to $90\text{ }^{\circ}C$ for 5 min.

All growth studies were performed in duplicate in BM as described above. The novel strain was subcultured at least once under the same experimental conditions before use. To investigate the pH range for growth, studies were performed in BM adjusted to pH values between 4.0 and 9.0 with anoxic stock solutions of $NaHCO_3$ (10%, w/v), Na_2CO_3 (10% w/v) or citrate buffer (1 M). Strain USBA 82^T grew between pH 5.5 and 8.5, with optimum growth at pH 7.3. The temperature range for growth was between 20 and $40\text{ }^{\circ}C$, with optimum growth occurring at $30\text{ }^{\circ}C$. To determine the NaCl tolerance of the strain, NaCl was added directly into the tubes to obtain the desired concentration and the BM was then added. Strain USBA 82^T grew in concentrations of 0.1–7% NaCl, but not in NaCl concentrations below 0.1% or above 8%. Cells grew optimally at 2% NaCl (w/v).

Thiosulfate (10 mM), sulfate (10 mM), sulfite (5 mM), elemental sulfur (3%, w/v), sodium nitrate (20 mM) and sodium nitrite (5 mM) were tested as electron acceptors in BM lacking yeast extract and supplemented with 0.2% (w/v) peptone rather than the usual 0.5% (w/v). H_2S production from the reduction of sulfate, sulfite, elemental sulfur and thiosulfate was determined using the method of Cord-Ruwisch (1985). The reduction of nitrate, nitrite and ammonia was determined as described by Ogg & Patel (2009). Strain USBA 82^T was able to weakly reduce elemental sulfur and thiosulfate, but not sulfate, sulfite, nitrate or nitrite. Strain USBA 82^T was unable to grow under aerobic conditions.

Substrate utilization tests were performed in the presence or absence of thiosulfate and sulfur in BM lacking peptone

and amended with 0.025% (w/v) yeast extract rather than 1.0%. Substrates from autoclaved or filter-sterilized anaerobic stock solutions of monosaccharides, disaccharides, polysaccharides, sugar alcohols and peptides were added to a final concentration of 0.2% (w/v) and organic acids and amino acids were added to a concentration of 10 mM. Growth was measured by inserting Hungate tubes into a Novaspec LKB spectrophotometer (Pharmacia-Biotech Pty. Ltd) and monitoring the optical density at 580 nm. Cultures were recorded as positive for growth if they exhibited an increase in optical density relative to controls (which lacked any added carbon sources). Strain USBA 82^T fermented Casamino acids, peptone, tryptone, serine, histidine, threonine, arginine, glutamate, pyruvate and citrate. Growth did not occur on lysine, glycine, proline, valine, alanine, cysteine, leucine, isoleucine, lactate, succinate, formate, acetate, propionate, butyrate, fructose, cellobiose, xylose, glucose, mannose, mannitol, maltose, ribose, sucrose, lactose, galactose, arabinose, raffinose, glycerol, methanol, ethanol or inositol either in the presence or the absence of thiosulfate or sulfur.

End products from peptone fermentation and respiration were measured by HPLC (LC-20AT; Shimadzu Prominence) equipped with a reverse-phase C18 ultra aqueous column ($150\text{ mm} \times 4.6\text{ mm ID}$) and a diode array detector (SPD-M20A) at 210 nm. The end products detected from peptone fermentation were acetate, succinate, propionate and traces of ethanol. In the presence of thiosulfate, the only end products detected were acetate and succinate.

Using the whole genome amplification method (Ogg & Patel, 2009), the G+C content of the DNA of strain USBA 82^T was calculated to be 52 mol% (T_m).

DNA purification, PCR amplification and sequencing of the 16S rRNA gene were performed as described by Andrews & Patel (1996). Raw sequence data were imported into BioEdit, version 5.0.9 (Hall, 1999), the base calling was carefully examined and a contiguous consensus sequence of 1388 bp was generated. Using this consensus sequence, the closest relatives identified against the GenBank database (Altschul *et al.*, 1997; Benson *et al.*, 1999) using BLASTN and against the Ribosomal Database Project using the Sequence Aligner Program (Maidak *et al.*, 2001) were downloaded. The sequences were aligned and positions of sequence and alignment ambiguity were omitted. A pairwise evolutionary distance matrix was generated using the method of Jukes & Cantor (1969) and a dendrogram was constructed using the neighbour-joining method (Saitou & Nei, 1987) as implemented in TREECON (Van de Peer & De Wachter, 1994). Confidence in the tree topology was determined by using 1000 bootstrapped trees (Felsenstein, 1985). Phylogenetic analysis placed strain USBA 82^T in the phylum *Synergistetes*, closest to *Dethiosulfovibrio peptidovorans* DSM 11002^T (93% gene sequence similarity) (Magot *et al.*, 1997) (Fig. 1).

Strain USBA 82^T and *D. peptidovorans* DSM 11002^T shared the ability to ferment yeast extract, peptone, tryptone,

Fig. 1. Neighbour-joining dendrogram showing the phylogenetic position of strain USBA 82^T and its closest relatives. GenBank accession numbers are given in parentheses. Bootstrap values >90% are shown. Bar, 10 nucleotide changes per 100 nucleotides.

Casamino acids, serine and histidine and the inability to use carbohydrates, fatty acids and organic acids as sole carbon and energy sources. Both strains reduced the electron acceptors sulfur and thiosulfate to sulfide. Sulfur or thiosulfate was obligately required by *D. peptidovorans* for growth on the amino acids arginine, glutamate and valine, but not by strain USBA 82^T which grew on these amino acids fermentatively (Table 1). Strain USBA 82^T could also be differentiated from *D. peptidovorans* by its ability to ferment threonine and citrate and its inability to use alanine, isoleucine and leucine in the presence or absence of sulfur or thiosulfate (Table 1). On the basis of its phenotypic characteristics, the phylogeny based on 16S rRNA gene sequence analysis and its G+C content, strain USBA 82^T may be categorized as a novel member

of the genus *Dethiosulfovibrio*, for which the name *Dethiosulfovibrio salsuginis* sp. nov. is proposed.

Description of *Dethiosulfovibrio salsuginis* sp. nov.

Dethiosulfovibrio salsuginis (sal.su'gi.nis. L. gen. n. *salsuginis* of salt water).

Cells stain Gram-negative and are slightly curved or vibrioid-like rods with rounded or pointed ends, 5–7 × 1.5 µm. Cells are highly motile by means of lateral flagella. Endospores are not formed. Obligately anaerobic. Growth occurs in media containing 0.1–6.0% (w/v) NaCl; 2% (w/v) is optimal for growth. NaCl is tolerated up to 7%, but no growth occurs at 8% or below 0.01%. The pH range

Table 1. Physiological characteristics of strain USBA 82^T and the type species of the genus *Dethiosulfovibrio*

Strains: 1, USBA 82^T; 2, *D. peptidovorans* DSM 11002^T (data from Magot *et al.*, 1997). +, Positive; –, negative; +¹, used only in the presence of thiosulfate or sulfur; ND, not determined. Neither of the strains use lactate, formate, acetate, propionate, butyrate, succinate, ethanol, methanol, glycerol or mannitol. Both taxa reduce sulfur and thiosulfate to sulfide, but do not reduce sulfate to sulfide.

Characteristic	1	2
Sampling site	Saline spring	Corroding offshore oil well
Cell morphology	Curved rods or spirals	Vibrio
Cell size (µm)	1.5 × 5–7	1 × 3–5
DNA G + C content (mol%)	52	56
Temp. range (optimum) (°C)	20–40 (30)	20–45 (42)
NaCl range (% w/v) (optimum)	0.1–7 (2)	(3)
pH range (optimum)	5.5–8.5 (7.3)	5.5–8.8 (7.0)
Utilization of:		
Alanine	–	+ ¹
Arginine	+	+ ¹
Citrate	+	–
Cysteine	–	ND
Glutamate	+	+ ¹
Isoleucine	–	+ ¹
Leucine	–	+ ¹
Lysine	–	–
Pyruvate	+	ND
Threonine	+	–
Valine	–	+ ¹

for growth is 5.5–8.5 with an optimum at pH 7.3. The temperature range for growth is 20–40 °C with an optimum at 30 °C. The type strain is able to reduce elemental sulfur (weakly) and thiosulfate when growing on peptone, but is not able to reduce sulfate, sulfite, nitrate or nitrite. Ferments pyruvate, citrate, Casamino acids, peptone, tryptone, serine, histidine, threonine, arginine and glutamate. Growth does not occur on lysine, glycine, proline, valine, alanine, cysteine, leucine, isoleucine, lactate, succinate, formate, acetate, propionate, butyrate, fructose, cellobiose, xylose, glucose, mannose, mannitol, maltose, ribose, sucrose, lactose, galactose, arabinose, raffinose, glycerol, methanol, ethanol or inositol either the presence or the absence of thiosulfate or sulfur. The main end products of fermentation are acetate and succinate.

The type strain, USBA 82^T (=DSM 21565^T=KCTC 5659^T), was isolated from the Salpa saline spring. The DNA G + C content of the type strain is 52 mol%.

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