Isolation and characterization of *Desulfocurvus thunnarius* sp. nov., a sulfate-reducing bacterium isolated from an anaerobic sequencing batch reactor treating cooking wastewater

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A novel anaerobic, chemo-organotrophic, sulfate-reducing bacterium, designated strain Olac 40\(^T\), was isolated from a Tunisian wastewater digester. Cells were curved, motile rods or vibrios (5.0–7.0×0.5 μm). Strain Olac 40\(^T\) grew at temperatures between 15 and 50 °C (optimum 40 °C), and between pH 5.0 and 9.0 (optimum pH 7.1). It did not require NaCl for growth but tolerated it up to 50 g l\(^{-1}\) (optimum 2 g l\(^{-1}\)). In the presence of sulfate or thiosulfate, strain Olac 40\(^T\) used lactate, pyruvate and formate as energy sources. Growth was observed on H\(_2\) only in the presence of acetate as carbon source. In the presence of sulfate or thiosulfate, the end products of lactate oxidation were acetate, sulfide and CO\(_2\). Sulfate, thiosulfate and sulfite were used as terminal electron acceptors, but not elemental sulfur, nitrate or nitrite. The genomic DNA G+C content of strain Olac 40\(^T\) was 70 mol%. The profile of polar lipids consisted of phosphatidylglycerol, phosphatidylethanolamine, aminophospholipid and four phospholipids. The main fatty acids were C\(_{16}\):0, anteiso-C\(_{15}\):0 and iso-C\(_{15}\):0. Phylogenetic analysis of the 16S rRNA gene sequence indicated that strain Olac 40\(^T\) was affiliated with the family *Desulfovibrionaceae* within the class *Deltaproteobacteria*. On the basis of 16S rRNA gene sequence comparisons and physiological characteristics, strain Olac 40\(^T\) is proposed to be assigned to a novel species of the genus *Desulfocurvus*, for which the name *Desulfocurvus thunnarius* is proposed. The type strain is Olac 40\(^T\) (=DSM 26129\(^T\)=JCM 18546\(^T\)).

Sulfate-reducing bacteria (SRB) are versatile in their use of various electron acceptors and electron donors and they can also thrive in a range of different environmental conditions (Fauque & Ollivier, 2004; Muyzer & Stams, 2008; Barton & Fauque, 2009). They are ubiquitous, and can be found in many natural terrestrial, marine and subterranean ecosystems together with engineered ones, such as anaerobic wastewater treatment plants, when sulfate is present (Birkeland, 2005; Ben Dov et al., 2007; Ollivier et al., 2007; Muyzer & Stams, 2008; Ollivier & Guyot, 2009). Sulfate reduction may account, particularly in marine anoxic sediments, for more than 50% of the mineralization of organic matter (Jørgensen, 1977) thus being indicative of the significant ecological role to be played by SRB regarding the C an S cycles on Earth. It is only recently that a novel species of a novel genus of SRB, *Desulfocurvus vexinensis*, has been reported (Klouche et al., 2009). This bacterium, isolated from a well that collected water from a deep saline aquifer used for underground gas storage at a depth of 830 m in the Paris Basin, France (Basso et al., 2009; Klouche et al., 2009), belongs to the phylum Proteobacteria, class *Deltaproteobacteria*, order *Desulfovirionales*, family *Desulfovibrionaceae*. It is an anaerobic, chemo-organotrophic, incomplete-oxidizing, SRB.

In this study, a novel mesophilic, halotolerant, SRB pertaining to the genus *Desulfocurvus* and isolated from sludge samples of a Tunisian anaerobic bioreactor is described. The samples were collected under anaerobic conditions from the sludge of a 2 l anaerobic sequencing batch reactor.
(temperature: 37 °C, pH: 7.6, flow rate: 100 ml day⁻¹) fed continuously with cooking tuna wastewater at a flow rate of 100 ml per day (30 g NaCl l⁻¹) and transported to the laboratory at ambient temperature. Micro-organisms were isolated and cultivated under strict anaerobiosis, according to the Hungate technique (Hungate, 1969). The basal medium (BM) for isolation containing (g l⁻¹): NH₄Cl (1.0), K₂HPO₄ (0.3), KH₂PO₄ (0.3), KCl (0.1), CaCl₂, H₂O (0.1), NaCl (40), yeast extract (Difco) (0.1), cysteine-hydrochloride (0.5), thiosulfate (3.16); 1 ml trace mineral element solution (Widdel & Pfennig, 1982) and 1 ml 0.1 % resazurin. The pH was adjusted to 7.6 with 10 M KOH solution. The BM was boiled under a stream of O₂-free N₂ gas, cooled to room temperature. Aliquots of 5 ml were dispensed into Hungate tubes, degassed under N₂/CO₂ gas, cooled to room temperature. Aliquots of 5 ml were added to the tubes. Desulfococcus vexinensis As36T (=DSM 17965T) was cultivated in the same medium.

Enrichments were performed in Hungate tubes containing BM inoculated with 10 % of sample and incubated at 37 °C. The culture was purified by repeated use of the Hungate Roll tubes method, using 1.6 % agar solid medium, and then transferred into liquid medium (Hungate, 1969). The pH, temperature and NaCl concentration ranges for growth were determined using BM supplemented with 20 mM lactate. For studies of NaCl requirements, NaCl was weighed directly in the tubes for concentrations ranging from 0 to 5 % NaCl before dispensing BM exempt of NaCl. To determine the optimum pH for growth, the pH of BM in Hungate tubes (5 ml) was adjusted by injecting appropriate aliquots of 0.1 M HCl (acidic pH), 10 % NaHCO₃ or 8 % Na₂CO₃ (basic pH) from sterile stock solutions into the tubes. Desulfococcus vexinensis As36T (=DSM 17965T) was cultivated in the same medium.

For electron microscopy, exponentially grown cells were negatively stained with 1 % sodium phosphotungstic acid (pH 7.0) as described by Fardeau et al. (1997). Whole cells were observed with a Hitachi model H 600 electron microscope at an accelerating voltage of 75 kV.

Methanol, ethanol, propanol, butanol, glycerol, glucose, fructose, acetate, propionate, butyrate, succinate, fumarate, malate, citrate, formate, lactate, pyruvate and Casamino acids were tested as substrates in BM at a final concentration of 20 mM and H₂CO₃ (80:20, v/v) and H₂/CO₂ + 2 mM acetate at 2 bar. To test for electron acceptors, sodium thiosulfate (20 mM), sodium sulfate (20 mM), sodium sulfite (2 mM), elemental sulfur (10 g l⁻¹), nitrate (20 mM) and nitrite (2 mM) were added to the medium. H₂S production was determined photometrically as colloidal CuS according to Cord-Ruwisch (1985). End products of metabolism were measured by HPLC after 2 days of incubation at 37 °C (Fardeau et al., 2000). Growth of strain Olac 40T strain As36 was stopped at the end of exponential phase and cultures were sent to DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) for fatty acid analysis. Fatty acids were extracted using the method of Miller (1982), with the modifications of Kuykendall et al. (1988), and the profile of cellular fatty acids was analysed by GC using the Microbial Identification System (MIDI, Sherlock version 6.1; database, TSBA40; gas chromatograph, model 6890N, Agilent). Analysis of the polar lipids was carried out by the Identification Service of the DSMZ for the two strains, Olac 40T and As36. Polar lipids were separated by two-dimensional TLC and detected using molybdatophosphoric acid and heating at 200 °C for 10 min. The presence of c-type cytochromes and desulfoviridin (the dissimilatory high-spin bisulfite reductase) were determined on the crude bacterial extract according to the method described by Postgate (1956).

The determination of the G+C content of the DNA and DNA–DNA hybridization were performed at the DSMZ. Genomic DNA for analysis of the base composition and DNA–DNA hybridization was isolated after disruption of bacterial cells by using a French press (Thermo Spectronic) and purified by chromatography on hydroxypatite using the procedure of Cashion et al. (1977). The DNA G+C content was determined by using HPLC as described by Mesbah et al. (1989). DNA–DNA hybridization was carried out as described by De Ley et al. (1970) under consideration of the modifications described by Huf et al. (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostated 6 x 6 multichannel and a temperature controller with in situ temperature probe (Varian).

The extraction and purification of total DNA followed by the amplification and sequencing of the 16S rRNA gene were previously described (Khelifi et al., 2010). The 16S rRNA gene sequence was then compared with available sequences in the GenBank database using the BLASTN search (Altschul et al., 1997). A multiple alignment was built using the MUSCLE program (Edgar, 2004) implemented in MEGA5 (Tamura et al., 2011). Positions of sequences with alignment uncertainty and gaps were omitted from the analysis. Evolutionary analyses were conducted in MEGA5 using the maximum-likelihood method based on the Kimura two-parameter model (Kimura, 1980). A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter=0.3965)]. The analysis involved 14 nucleotide sequences. There were a total of
1403 positions in the final dataset. Branch robustness of the resulting maximum-likelihood tree was estimated by the non-parametric bootstrap procedure implemented in MEGA5 (500 replicates of the original dataset). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

Several colonies developed after incubation at 37 °C and were picked separately. Colonies were black and circular with diameters ranging from 1.0 to 2.0 mm after 3–5 days of incubation at 37 °C. The process of serial dilution was repeated several times until the isolates were deemed to be axenic. Several strains were isolated; their morphology and metabolic profiles were similar and the same phylogenetic inference was obtained for all of them. One strain, designated Olac40\textsuperscript{T}, was selected and used for further metabolic and physiological characterization.

Cells of strain Olac 40\textsuperscript{T} were curved rods or vibrios staining Gram-negative (5–7 µm by 0.5 µm) when grown on a medium containing lactate as electron donor and thiosulfate as terminal electron acceptor (Fig. 1a). Ultrathin sections showed a typical Gram-negative cell wall with a thin peptidoglycan layer and an outer membrane (Fig. 1b). Cells were motile by a single polar flagellum (Fig. 1c).

Strain Olac 40\textsuperscript{T} was anaerobic but tolerated up to 1 % O\textsubscript{2}. The physiological optimal growth conditions were determined in duplicate experiments conducted in BM containing lactate (20 mM) and thiosulfate (20 mM) as previously described (Fardeau \textit{et al.}, 2000). The optimal temperature for growth was 40 °C (range 15–50 °C). Optimum pH was 7.1 (range pH 5.0–9.0). The strain could grow without NaCl, but tolerated it up to 50 g l\textsuperscript{−1} with an optimum at 2 g l\textsuperscript{−1}. Substrate utilization was tested with 1 g yeast extract l\textsuperscript{−1} added to BM. Very few of the tested compounds for growth were oxidized. They include only lactate, pyruvate, formate and H\textsubscript{2}, which was used only in the presence of acetate (2 mM) as carbon source. Thiosulfate, sulfate and sulfite, but not elemental sulfur, nitrate or nitrite served as terminal electron acceptors. They were reduced to sulfide. Methanol, ethanol, propanol, butanol, glycerol, glucose, fructose, acetate, propionate, butyrate, succinate, fumarate, malate, citrate and Casamino acids did not support growth. Under optimal conditions with lactate as electron donor and thiosulfate as electron acceptor, the maximum growth rate of strain Olac 40\textsuperscript{T} was 0.26 h\textsuperscript{−1}.

Although growth occurred in minimal medium with lactate as the only energy and carbon sources, yeast extract, biotryptcase and also vitamins (Balch \textit{et al.}, 1979) improved growth on this substrate.

Visible absorption spectra of a cell-free extract of strain Olac 40\textsuperscript{T} showed the presence of low redox potential c-type cytochromes (very likely the tetraheme cytochrome c\textsubscript{3}) with absorption peaks at 522, 551 and 418 nm in the dithionite reduced form. The characteristic absorption band (at 628 nm) of desulfoviridin (the dissimilatory high-spin bisulfite reductase characteristic of the genus \textit{Desulfovibrio}) was not detected in the cell-free extract. The reddish colour of the crude extract may possibly indicate the presence of desulforubidin, the other cytoplasmic dissimilatory high-spin bisulfite reductase isolated in mesophilic non-sporulating species of SRB (Fauque \textit{et al.}, 1991; Fauque & Barton, 2012).

The major fatty acids of strain Olac 40\textsuperscript{T} were: C\textsubscript{16:0} (29.2 %), anteiso-C\textsubscript{15:0} (21.2 %) and iso-C\textsubscript{15:0} (19.3 %); for \textit{Desulfocurvus vexinensis} As36\textsuperscript{T}, major fatty acids were anteiso-C\textsubscript{15:0} (25.0 %), C\textsubscript{16:0} (22.3 %), iso-C\textsubscript{15:0} (21.1 %) (Table 1). The polar lipid profile of strain Olac 40\textsuperscript{T} consisted of phosphatidylglycerol, four phospholipids, phosphatidylethanolamine and aminophospholipid. The

![Fig. 1. (a) Phase-contrast photomicrograph showing cells of strain Olac 40\textsuperscript{T} (bar, 10 µm). (b) Thin section electron micrograph of strain Olac 40\textsuperscript{T} showing the Gram-negative type of cell wall (bar, 0.2 µm). (c) Electron micrograph of strain Olac 40\textsuperscript{T} showing a single polar flagellum (bar, 0.5 µm).](http://ijs.sgmjournals.org)
The polar lipid profile was the same for *Desulfocurvus vexinensis* As36 T but with only one phospholipid (Fig. S1, available in IJSEM Online). The DNA G+C content of strain Olac 40 T was 70 mol%.

The phylogenetic tree obtained by the maximum-likelihood method, as shown in Fig. 2, showed that strain Olac 40 T is a new member of the genus *Desulfocurvus*, sharing 99% sequence similarity with the single species of the genus, *Desulfocurvus vexinensis*. Its other two closest relatives were the proposed species ' *Desulfovibrio ferrophilus* ' IS5 (Dinh et al., 2004) with 93.9% similarity and *Desulfovibrio senezii* DSM 8436 T (Tsu et al., 1998) with 90.8% similarity. However, in contrast to species of the genus *Desulfovibrio*, neither strain Olac 40 T nor *Desulfocurvus vexinensis* possesses desulfoviridin (Kuever et al., 2005; Fauque & Barton, 2012). DNA–DNA hybridization experiments revealed that strain Olac 40 T showed only 41.2% reassociation with *Desulfocurvus vexinensis* thus indicating that strain Olac 40 T represents a distinct species within the genus *Desulfocurvus*. Strain Olac 40 T is therefore proposed as a representative of a novel species of the genus *Desulfocurvus*. This is supported by the DNA G+C content of strain Olac 40 T, which is slightly higher than that of *Desulfocurvus vexinensis* (Klouche et al., 2009) (Table 2). Similarly to *Desulfocurvus vexinensis*, strain Olac 40 T used a limited range of substrates including lactate, pyruvate and formate, but unlike *Desulfocurvus vexinensis* oxidized hydrogen. Strain Olac 40 T also differed from *Desulfocurvus vexinensis* by its tolerance to 1 % O2. The profiles of fatty acids and polar lipids were slightly different for the two strains. A comparison of the main characteristics of strain Olac 40 T and *Desulfocurvus vexinensis* is given in Tables 1 and 2. Based on phenotypic, and genetic characteristics of strain Olac 40 T, we propose it to be assigned to a novel species of the genus *Desulfocurvus*, *Desulfocurvus thunnarius* sp. nov.

### Description of *Desulfocurvus thunnarius* sp. nov.

*Desulfocurvus thunnarius* [thun. na’ri.us. L. masc. adj. thunnarius of or belonging to a tunny (cooking tuna wastewater, Tunisia), where the species was first recovered].

Cells are anaerobic, motile rods or vibrios (5–7 × 0.5 μm), neutrophilic and slightly halotolerant (0–50 g NaCl l⁻¹, optimum at 2 g l⁻¹). The temperature range for growth is 20–50 °C, with an optimum of 40 °C. The optimum pH is

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**Table 1.** Comparison of the main fatty acids (%) of strain Olac 40 T and *Desulfocurvus vexinensis* As36 T

<table>
<thead>
<tr>
<th>Cellular fatty acid</th>
<th>Olac 40 T</th>
<th>As36 T</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>2.2</td>
<td>1.5</td>
</tr>
<tr>
<td>iso-C15:0</td>
<td>19.3</td>
<td>21.1</td>
</tr>
<tr>
<td>anteiso-C15:0</td>
<td>21.2</td>
<td>25.0</td>
</tr>
<tr>
<td>C16:0</td>
<td>29.2</td>
<td>22.3</td>
</tr>
<tr>
<td>iso-C16:0</td>
<td>1.6</td>
<td>0.8</td>
</tr>
<tr>
<td>anteiso-C17:0</td>
<td>5.0</td>
<td>4.5</td>
</tr>
<tr>
<td>iso-C17:0</td>
<td>11.7</td>
<td>13.6</td>
</tr>
<tr>
<td>Summed feature 10</td>
<td>3.2</td>
<td>3.0</td>
</tr>
<tr>
<td>C16:1ω7c</td>
<td>2.6</td>
<td>4.7</td>
</tr>
<tr>
<td>C18:0</td>
<td>2.3</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Summed feature 10 consisted of C18:1ω7c.

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**Fig. 2.** Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of strain Olac 40 T and related species of the genera *Desulfocurvus*, *Desulfovibrio*, *Desulfomicrobium* and *Desulfonatronum*. The tree was rooted by using *Desulfonatronum norvegicum* DSM 1741 T (AJ277897) as an outgroup. Numbers at nodes represent bootstrap values (>50 %) inferred by MEGA5 from 500 replicates. Bar, 5 nt changes per 100 nt.
Table 2. Comparison of the main characteristics of strain Olac 40^T and *Desulfocurvus vexinesis* As36^T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Olac 40^T</th>
<th>As36^T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (μm)</td>
<td>0.5 × 5–7</td>
<td>0.5 × 3–5</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>70</td>
<td>67.2</td>
</tr>
<tr>
<td>Temperature range for growth (optimum) (°C)</td>
<td>15–50 (40)</td>
<td>20–50 (37)</td>
</tr>
<tr>
<td>pH range for growth (optimum)</td>
<td>5–9 (7.1)</td>
<td>5–9 (6.9)</td>
</tr>
<tr>
<td>Salinity range for growth (optimum) (%)</td>
<td>0–5 (0.2)</td>
<td>0–2 (0.2)</td>
</tr>
<tr>
<td>Hydrogen as electron donor</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

7.1 (range pH 5–9). Lactate, pyruvate, formate and H₂, only in the presence of acetate as carbon source, are used as energy sources. The main end product of lactate catabolism is acetate. Methanol, ethanol, propanol, butanol, glycerol, glucose, fructose, acetate, propionate, butyrate, succinate, fumarate, malate, citrate and Casamino acids are not used as substrates. Sulfate, sulfite and thiosulfate serve as electron acceptors. The main fatty acids are C₁₆:₀ anteiso-C₁₅:₀ and iso-C₁₅:₀. c-Type cytochromes are present and desulfoviridin is absent in the crude extract.

The type strain is Olac 40^T (DSM 26129^T = JCM 18546^T), which was isolated from an anaerobic sequencing batch reactor treating tuna cooking wastewater. The genomic DNA G+C content of the type strain is 70 mol%.

References


