Clostridium methoxybenzovorans sp. nov., a new aromatic o-demethyllating homoacetogen from an olive mill wastewater treatment digester

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A strictly anaerobic, spore-forming bacterium (3.0-5.0 × 0.4-0.8 μm), designated strain SR3T (T = type strain), which stained Gram-positive and possessed a Gram-positive type cell wall was isolated from a methanogenic pilot-scale digester fed with olive mill wastewater (Sfax, Tunisia). It utilized a number of carbohydrates (glucose, fructose, sorbose, galactose, myo-inositol, sucrose, lactose, cellobiose), organic compounds (lactate, betaine, sarcosine, dimethylglycine, methanethiol, dimethylsulfide), alcohol (methanol) and all methoxylated aromatic compounds only in the presence of yeast extract (0.1%). The end products from carbohydrate fermentation were H₂, CO₂, formate, acetate and ethanol, that from lactate was methanol, those from methoxylated aromatics were acetate and butyrate, and that from betaine, sarcosine, dimethylglycine, methanethiol and dimethylsulfide was only acetate. Strain SR3T was non-motile, had a G+C content of 44 mol% and grew optimally at 37 °C and pH 7.4 on a glucose-containing medium.

Phylogenetically, the closest relatives of strain SR3T were the non-methoxylated aromatic-degrading Clostridium xylanolyticum, Clostridium aerotolerans, Clostridium sphenoides and Clostridium celerecrescens (mean similarity of 98%). On the basis of the phenotypic, genotypic and phylogenetic characteristics of the isolate, it is proposed to designate strain SR3T as Clostridium methoxybenzovorans sp. nov. The type strain is SR3T (= DSM 12182T).

Keywords: Clostridium methoxybenzovorans, o-demethylation, aromatic compounds, syringic acid, methoxyl group

INTRODUCTION

A heterogeneous collection of methoxylated aromatic compounds forms the major components of plant cell walls and these compounds are widespread in nature. A number of these compounds are degraded by some anaerobic, acetogenic and homoacetogenic bacteria. Sporobacter termitidis (Grech-Mora et al., 1997) and Holophaga foetida (Liesack et al., 1994) are two such homoacetogens that cleave the rings of the methoxylated aromatic compounds syringate, sinapate, 3,4,5-trimethoxycinnamate and 3,4,5-trimethoxybenzoate to produce acetate, methanethiol and dimethylsulfide. In these cases, o-demethylation is the key reaction preceding aromatic ring cleavage. However, other acetogenic bacteria have been described that demethylate aromatic compounds to their corresponding hydroxylated derivatives and gain energy by the conversion of the o-methyl group to acetic acid, but are unable to cleave the aromatic ring. Examples of such cases include Sporomusa termitida (Breznak et al., 1988), Sporomusa malonica (Dehning et al., 1989), Sporomusa silvacetica (Kuhner et al., 1997), Moorella thermoaerotrophica (formerly Clostridium thermoaerotrophicum) (Collins et al., 1994; Wiegel et al., 1981), Moorella thermacetica (formerly Clostridium thermo-
methoxybenzovorans. Demethylation also occurs in natural ecosystems and subsurface sediments (Liu et al., 1993), aggregated forest soils (Kühner et al., 1997) and human faeces (Wolin & Miller, 1993). We report in this paper the isolation and taxonomic description of Clostridium methoxybenzovorans sp. nov., an isolate from an anaerobic methanogenic pilot-scale digester fed with olive mill wastewater, capable of o-demethylating a wide range of methoxylated aromatic compounds.

**METHODS**

**Sources of samples and organisms.** Liquid samples from a 300 l pilot-scale anaerobic methanogenic digester, which was fed with olive mill wastewater (Sfax, Tunisia), were collected anaerobically using N₂-flushed syringes and inoculated immediately into basal medium containing 5 mM syringate as described below. Strain SR3T was isolated from this enrichment culture and is the subject of this paper. Clostridium aerotolerans (DSM 5434), Clostridium celercretescens (DSM 5628³) and Clostridium xylanolyticum (DSM 6555) were used as reference cultures and were purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The reference cultures were routinely grown using glucose-containing basal medium.

**Culture media.** The anaerobic techniques of Hungate (Hungate, 1969; Macy et al., 1972; Miller & Wolin, 1974) were used throughout this work. The basal medium contained (1 l): 1 g NH₄Cl; 0.3 g K₂HPO₄; 0.3 g KH₂PO₄; 0.6 g NaCl; 0.1 g CaCl₂, 2H₂O; 0.2 g MgCl₂, 6H₂O; 0.1 g KCl; 0.5 g cysteine·HCl; 1 g yeast extract (Difco); 1.5 ml trace element mineral solution of Widdel & Pfennig (1981); and 1 mg resazurin. The pH was adjusted to 7 with 10 M KOH solution, the medium was boiled under a stream of O₂-free N₂ gas and cooled to room temperature. Aliquots of 5 ml were dispensed into Hungate tubes and subsequently sterilized by autoclaving at 110 °C for 45 min. Prior to culture inoculation, 0.2 ml 5% (v/v) NaHCO₃ and 0.05 ml 2.5% (v/v) Na₃·9H₂O were injected into the 5 ml pre-sterilized medium from sterile stock solutions. Substrates were injected from concentrated anaerobic sterile stock solutions to give the desired final concentration when necessary.

**Enrichment, isolation and routine culturing.** Samples (0.5 ml) were inoculated into 5 ml basal medium containing 5 mM syringic acid and then incubated at 37 °C. The enrichment was subcultured several times under the same conditions prior to isolation. For isolation, the culture was serially diluted tenfold and single well-isolated colonies that developed in roll tubes (basal medium containing syringic acid and 1.5% agar) were picked. This procedure was repeated several times until only one type of colony (white) was observed. Routine culturing of strain SR3T was achieved in basal medium containing 5 mM syringic acid.

**Cellular studies.** Light and electron microscopy were performed as previously described (Faradeau et al., 1997). For heat resistance, cells grown in basal medium containing glucose were exposed to temperatures of 80, 90 and 100 °C for 10 min. The cells were cooled quickly to ambient temperature, inoculated into fresh glucose-containing medium and growth was recorded after 24 h incubation at 37 °C. Conditions for sporulation that were tested included growth in the presence of glucose or syringate, or with no added carbon source.

**Growth parameters.** For all experiments, basal medium containing 20 mM glucose was used. The pH of the pre-reduced anaerobic medium was adjusted by injecting appropriate amounts of 5% NaHCO₃, 5% Na₂CO₃ or 0.1 M HCl to give an initial pH range between 5.0 and 9.5. Different amounts of NaCl were weighed directly in Hungate tubes prior to dispensing 5 ml medium to give the desired NaCl concentration (range 0–40 g l⁻¹). The temperature range for growth was determined between 10 and 50 °C.

**Substrate utilization.** All experiments were performed with inoculum which had been subcultured at least once under the same test conditions. All experiments were performed in duplicate unless indicated. For substrate utilization tests, 20 mM carbohydrates (glucose, fructose, xylose, sorbose, galactose, myo-inositol, sucrose, lactose, cellulose), 10 g l⁻¹ each of xylan and cellulose, 20 mM organic acids (formate, fumarate, pyruvate, crotonate, malonate, succinate), 5 mM aromatic compounds (monomethoxylated compounds (2-, 3-, 4-methoxymethoxybenzoates, 2-, 3-, 4-methoxymethylbenzaldehydes, 2-methoxyphenol), dimethoxylated compounds (2,3-, 2,4-, 2,5-, 2,6-, 3,4-, 3,5-dimethoxymethoxybenzoates, 3,4-dimethoxybenzaldehyde), trimethoxylated aromatics (3,4,5-trimethoxy cinnamate, 3,4,5-trimethoxybenzoate, 3,4,5-trimethoxyphenylacetate, 3,4,5-trimethoxyphenylpropionate, 3,4,5-trimethoxyphenyl, 1,3,5-trimethoxybenzene, 3,4,5-trimethoxyphenoxyphenone) and mixed methoxylated/hydroxylated compounds (4-hydroxy 3,5-dimethoxymethoxybenzoate, 4-hydroxy 3,5-dimethoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxymethoxybenzoate, 4-hydroxy 3-methoxycinnamat...
Clostridium methoxybenzovorans sp. nov.

160A spectrophotometer and growth was measured at 580 nm. Aromatic compounds were measured by HPLC by using a chromatograph (ConstaMetric 200; LDC-Analytical) equipped with a C18 Symmetry 5 μm-particle-size column 250 mm long, 4.6 mm i.d. (Waters Chromatography). The column temperature was maintained at 35 °C. An isocratic mobile phase of 30:69:5:0:5 (by vol.) acetonitrile/distilled water/acetic acid was used at a flow rate of 0.6 ml min⁻¹. The volume of the injection loop was 20 μL. Aromatic compounds were quantified at 240 nm with a Shimadzu SPD-6A UV detector connected to a CR-6A Shimadzu integrator. Volatile fatty acids, ethanol, glycerol, and carbohydrates were measured by HPLC (Spectra Series 100 model; Thermo Separation Products) equipped with an Aminex HPX-87X 300 mm long, 7.8 mm i.d. column (Bio-Rad) connected to a differential refractometer (RID-6A; Shimadzu). Analysis was performed using a CR-6A Shimadzu integrator. The mobile phase was 0.0252 M H₂SO₄ at a flow rate of 0.5 ml min⁻¹, and the column temperature was 35 °C. The volume of the injection loop was 20 μL. H₂ and CO₂ were measured as described previously (Fardeau et al., 1993).

**Determination of G+ C content.** The G+C content of DNA was determined by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The DNA was isolated and purified by chromatography on hydroxyapatite and the G+C content was determined by using HPLC as described by Mesbah et al. (1996). Non-methylated lambda DNA (Sigma) was used as the standard.

**DNA extraction and amplification of 165 rRNA gene.** DNA was extracted from the isolate as described previously (Andrews & Patel, 1996; Redburn & Patel, 1993). The universal primers Fd1 and Rd1 were used to obtain a PCR product of approximately 1.5 kb corresponding to base positions 8–1542 based on Escherichia coli numbering of the 16S rDNA (Winker & Woese, 1991). A 50 μl reaction mixture contained 1–20 ng genomic DNA, 1 μM of each primer, 5 μl x 10 buffer, 200 μM dNTP, 3.5 mM MgCl₂ and 2.5 U Taq polymerase (Promega). PCR was carried out by an initial denaturation at 94 °C for 7 min, then 29 cycles of annealing at 55 °C for 2 min, extension at 72 °C for 4 min, denaturation at 94 °C for 1 min, and finally an extension cycle of 55 °C for 2 min and 72 °C for 20 min.

**Direct sequencing of PCR products.** PCR products were purified using a QIAquick kit (Qiagen). The DNA concentration of the purified PCR product was estimated by comparison with the Low Mass Ladder (Gibco-BRL) on an ethidium bromide-containing agarose gel. Sequencing was carried out on an ABI 373A sequencer using the ABI PRISM Dye Terminator Cycle Sequencing kit containing AmpliTaq FS DNA polymerase under the following conditions. A 10 μl reaction mixture contained 35 ng PCR product, 4 μl cycle sequencing reaction mix, 3.2 pmol primer (Andrews & Patel, 1996) and 2.5 μg BSA. Thermal cycling was carried out using a Rapid Cycler (Idaho Technology) at a temperature transition slope of 2, an initial denaturation of 94 °C for 15 s, followed by 25 cycles of denaturation at 94 °C for 0 s, annealing at 50 °C for 10 s and extension at 60 °C for 3 min.

**Sequence alignments and phylogenetic inferences.** The new sequence data that were generated were assembled, aligned to an almost full-length consensus 16S rRNA gene sequence and checked for accuracy manually using the alignment editor ae2 (Maidak et al., 1996). These were compared with other sequences in the GenBank database (Benson et al., 1993) using BLAST (Altschul, 1997), and in the Ribosomal Database Project version 5.0 using SIMILARITY-RANK and SUGGEST_TREE (Maidak et al., 1996). Reference sequences most related to our newly generated sequences were extracted from these databases and aligned. Positions of sequence and alignment uncertainty were omitted from the analysis. Pairwise evolutionary distances based on 1037 unambiguous nucleotides were computed using DNADIST (Jukes & Cantor option) and neighbour-joining programs that form part of the PHYLIP suite of programs (Felsenstein, 1993).

**RESULTS**

**Enrichment and isolation.** Enrichment cultures developed in medium containing 5 mM syringic acid within 2 weeks of incubation at 37 °C as shown by growth and acetate production. After several transfers in the liquid medium, the enrichment developed a stable microbial population capable of degrading syringic acid. Several isolates were obtained using the roll-tube method (Hungate, 1969) and one of these cultures, designated strain SR3T, was studied further.

**Morphology.** Cells of strain SR3T were rod-shaped and 3.0–5.0 x 0.4–0.8 μm. Terminally located spherical spores which distended the cells were present (Fig. 1a). Spores were numerous in the medium that contained only yeast extract (0.1 %) but very few were observed in the medium which contained yeast extract and syringate or yeast extract and glucose. Positive growth was obtained from inoculum prepared from cultures that had been heated to temperatures of 100 °C for 10 min, indicating resistance to heat. The cells stained Gram-positive. This was verified by the presence of a thick bilayered cell wall structure in electron micrographs of thin sections of the cells (Fig. 1b). Cells were non-motile and flagella were not observed in negatively stained cells under an electron microscope.

**Characterization and physiology.** Strain SR3T was a mesophilic, strictly anaerobic, chemo-organotrophic bacterium. The optimal growth temperature was 37 °C and no growth was observed below 20 °C or above 45 °C. Strain SR3T had a pH range for growth of 5.5–9.0 with an optimum of 7.4. NaCl concentration in the medium affected the growth when it was more than 0.5% and it was inhibitory at 3.5%. Strain SR3T required yeast extract for growth.

Strain SR3T, but not C. celerecrescens, C. aerotolerans, C. xylanolyticicum or Clostridium sphenoides, was able to cleave the phenylether bond of a wide range of methoxylated monoaromatic compounds to give the corresponding hydroxylated aromatic derivatives. Strain SR3T was able to degrade all methoxylated monoaromatic compounds tested including mono-
**Fig. 1.** (a) Phase-contrast micrograph of strain SR3<sup>T</sup> showing terminal spores; bar, 10 μm. (b) Electron micrograph of an ultrathin section of strain SR3<sup>T</sup> showing the cytoplasmic membrane (cm), the inner layer (il) and the outer layer (ol); bar, 0.2 μm.

**Table 1.** Fermentation end products of strain SR3<sup>T</sup>

<table>
<thead>
<tr>
<th>Degraded substrate*</th>
<th>Product formed</th>
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<tbody>
<tr>
<td></td>
<td>Formate</td>
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<tr>
<td>Methoxy group†</td>
<td>–</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>–</td>
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<tr>
<td>Sorbose</td>
<td>+</td>
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<tr>
<td>Galactose</td>
<td>+</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>–</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
</tr>
<tr>
<td>Cellobose</td>
<td>+</td>
</tr>
<tr>
<td>Methanol</td>
<td>–</td>
</tr>
<tr>
<td>Lactate</td>
<td>–</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>+</td>
</tr>
<tr>
<td>Betaine</td>
<td>–</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>–</td>
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<tr>
<td>Dimethyl glycine</td>
<td>–</td>
</tr>
<tr>
<td>Dimethyl sulfide</td>
<td>–</td>
</tr>
<tr>
<td>Methanethiol</td>
<td>–</td>
</tr>
<tr>
<td>Casamino acids</td>
<td>–</td>
</tr>
<tr>
<td>Peptone</td>
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</table>

*All substrates were used at a final concentration of 20 mM except for peptone and Casamino acids (2 g l<sup>–1</sup>) and aromatic compounds (5 mM). The initial pH of the medium was pH 7.2 and all incubations were performed at 37 °C.

† Methoxy group: 2-, 3-, 4-methoxybenzoates, 2-, 3-, 4-methoxybenzaldehydes, 2-methoxyphenol, 2,3-, 2,4-, 2,5-, 2,6-, 3,4-, 3,5-dimethoxybenzoates, 3,4-dimethoxybenzaldehyde, 3,4,5-trimethoxycinnamate, 3,4,5-trimethoxybenzoate, 3,4,5-trimethoxyphenylacetate, 3,4,5-trimethoxyphenylpropionate, 3,4,5-trimethoxyphenol, 1,3,5-trimethoxybenzene, 3,4,5-trimethoxyphacetophenone, 4-hydroxy 3,5-dimethoxybenzoate, 4-hydroxy 3,5-dimethoxycinnamate, 3-hydroxy 4-methoxybenzoate, 4-hydroxy 3-methoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxycinnamate, 3-hydroxy 4-methoxybenzaldehyde, 4-hydroxy 3-methoxybenzaldehyde and 4-hydroxy 3-methoxycinnamylalcohol.
**Fig. 2.** Degradation of syringate by strain SR3T. The basal medium contained 5 mM syringate (○); the intermediate product formed was 5-hydroxyvanillate (●), which was further degraded to gallate (□).

**Fig. 3.** Unrooted phylogenetic dendrogram based on 16S rRNA sequence data indicating the position of *Clostridium methoxybenzovorans* strain SR3T within the radiation of representatives of the low-G+C-containing Gram-positive bacteria. All the sequences used in the analysis, with the exception of sequences of *Anaerobaculum thermoterrrenum* and *Dethiosulfovibrio peptidovorans* (GenBank accession nos US0711 and US2817, respectively), were obtained from the Ribosomal Database Project, version 5.0 (Maidak et al., 1996). The triangles indicate representative members of cluster V (*Thermoanaerobacter kivui* and *Thermoanaerobacter thermcopiae*), cluster III (*Acetobibrio cellulolyticus* and *Clostridium aldrichii*) and cluster IX (*Megasphaera elsdenii* and *Clostridium quercicolium*). Evolutionary distances (based on 1037 unambiguous nucleotides) and bootstrap analysis (100 data sets) were computed using programs that form part of the PHYLIP package (Felsenstein, 1993). Only values greater than 90% were considered significant and are therefore reported. Scale bar, 10 nucleotide substitutions per 100 nucleotides.

*Clostridium methoxybenzovorans* sp. nov.

methoxylated compounds (2-, 3-, 4-methoxybenzoates, 2-, 3-, 4-methoxybenzaldehydes, 2-methoxyphenol), dimethoxylated compounds (2,3-, 2,4-, 2,5-, 2,6-, 3,4-, 3,5-dimethoxybenzoates, 3,4-dimethoxybenzaldehyde), trimethoxylated aromatics (3,4,5-trimethoxycinnamate, 3,4,5-trimethoxybenzoate, 3,4,5-trimethoxyphenylacetate, 3,4,5-trimethoxyphenylpropionate, 3,4,5-trimethoxyphenol, 1,3,5-trimethoxybenzene, 3,4,5-trimethoxyphacetonphone), and mixed methoxylated/hydroxylated compounds (4-hydroxy 3,5-dimethoxybenzoate, 4-hydroxy 3,5-dimethoxycinnamate, 3-hydroxy 4-methoxybenzoate, 4-hydroxy 3-methoxybenzoate, 4-hydroxy 3-methoxycinnamate, 3-hydroxy 4-methoxycinnamate, 3-hydroxy 4-methoxybenzaldehyde, 4-hydroxy 3-methoxybenzaldehyde, 4-hydroxy 3-methoxycinnamyl alcohol).

Aromatic compounds possessing an aldehydic group were oxidized to their corresponding carboxylic derivatives. With all the aromatic compounds tested and even after 1 month incubation, no ring cleavage was observed. Strain SR3T also used glucose, fructose,
sorbose, galactose, myo-inositol, sucrose, lactose, cellobiose, methanol, lactate, H_2/CO_2, peptone, Casamino acids, betaine, sarcosine, dimethylglycine, methanethiol and dimethylsulfide, but not xylose, formate, fumarate, pyruvate, crotonate, malonate, succinate, ethanol, glycerol, gelatin, methylamine, trimethylamine, xylan or cellulose. Table 1 shows the fermentation end products formed from various substrates. Acetate was produced from the utilization of methoxylated aromatic compounds, carbohydrates, methanol, lactate, H_2/CO_2, betaine, sarcosine, dimethylglycine, methanethiol and dimethylsulfide. In addition, ethanol was detected from carbohydrate utilization; butyrate was detected from methoxylated aromatic compounds, methanol and lactate utilization; and formate was detected from glucose, sorbose, galactose, lactose, cellobiose and H_2/CO_2 utilization.

Strain SR3^T required yeast extract for growth. Yeast extract at 0.1 and 0.3% was optimal for growth on syringic acid as the carbon source, with marginal growth occurring with 0.02%. The growth rate of strain SR3^T on syringic acid was much slower (doubling time 24 h) than that on glucose (doubling time 4 h). During growth on syringic acid, the intermediate compound 5-hydroxyvanillate acid was produced after a lag of 2 d, which was concomitantly degraded to gallic acid (Fig. 2). Sulfate, thiosulfate, sulfite, nitrate, elemental sulfur or fumarate could not be used as electron acceptors.

16S rRNA sequence analysis

Using twelve primers, we determined an almost complete sequence consisting of 1514 bases of the 16S rRNA gene of strain SR3^T (corresponding to Escherichia coli positions 17–1539). Phylogenetic analysis revealed that strain SR3^T was a member of the low-G+C-containing Gram-positive branch as defined by Collins et al. (1994) and grouped with members of cluster XIVa. The closest relatives were C. celerecrescens, C. aerotolerans, C. xylanolyticum and C. sphenoides (mean similarity of 98%). Fig. 3 shows a dendrogram generated by the neighbour-joining method (Felsenstein, 1993) from the Jukes & Cantor evolutionary similarity matrix (Jukes & Cantor, 1969).

DISCUSSION

Strain SR3^T is an obligate anaerobe isolated from a sample taken from a methanogenic pilot-scale digester fed with olive mill wastewater. The olive mill wastewater is a rich source of aromatic compounds resulting from olive cell wall degradation during the industrial process. High concentrations and a wide range of hydroxylated and methoxylated aromatic compounds are present in the olive mill wastewater, including 4-hydroxybenzoate, 4-hydroxycinnamate, 4-hydroxyphenylacetate, 3,4-dihydroxybenzoate, 3,4-dihydroxycinnamate, 3,4,5-trihydroxybenzoate, 4-hydroxy 3-methoxybenzoate, 4-hydroxy 3,5-dimethoxybenzoate, 2-methoxyphenol, 3,4-dimethoxybenzoate and 3,4-dimethoxyphenylalcohol (Capasso et al., 1995). It was therefore not surprising that several different strains capable of demethylating aromatic compounds were isolated from the same enrichment culture. However, we currently do not know how dominant these microbes are and how efficient they are at demethylating aromatic compounds in situ. In addition, the methoxyl group serves as a carbon and energy source for strain SR3^T and it is possible that the resulting aromatic rings of these hydroxylated derivatives are cleaved by other strains in situ. Strain SR3^T is also a strictly anaerobic Gram-positive bacterium and cannot grow in a medium in which resazurin has turned pink due to the presence of traces of oxygen. It is also a spore former and therefore can survive the fluctuating conditions of the digester. Strain SR3^T withstood pasteurization indicating that heat-resistant spores were present. In medium containing yeast extract but no carbon source many spores were produced.

O-demethylation of syringate by strain SR3^T was determined to be a two-step process in which the intermediate compound 5-hydroxyvanillate formed was subsequently degraded to gallate. Gallate was also determined to be an end product of o-demethylation of 3,4,5-trimethoxybenzoate and no further decarboxylation of gallate to pyrogallol was observed. Strain SR3^T oxidized the aldehyde group of vanillin to the carboxyl level as described for Clostridium formicoaceticum and Moorrella thermoacetica (formerly Clostridium thermoaceticum) (Collins et al., 1994; Lux et al., 1990). This suggests that strain SR3^T has a similar pathway to those reported for other o-demethylating strains.

The property of o-demethylation without ring cleavage is found amongst members of the low-G+C-containing Gram-positive bacteria which include some members of the genera Acetobacterium, Clostridium and Sporomusa (Heider & Fuchs, 1997). The Gram-positive sporulating nature of strain SR3^T indicates that it is a member of the genus Clostridium rather than Acetobacterium. As the cells of strain SR3^T are rod-shaped rather than curved, its membership to the genus Sporomusa can be excluded. Amongst Clostridium species, C. formicoaceticum (Andreesen et al., 1970), M. thermoautotrophica (formerly C. thermoautotrophicum) and M. thermoacetica (formerly C. thermoacetica) (Collins et al., 1994; Daniel et al., 1988) are able to o-demethylate aromatic compounds. However, strain SR3^T has a DNA G+C content of 44 mol%, is a mesophile and produces acetate and butyrate whereas M. thermoautotrophica and M. thermoacetica are thermophiles, have a DNA G+C content of 54–55 mol% and produce only acetate as the end product. Strain SR3^T is a mesophile and is similar to C. formicoaceticum in this respect but differs in its DNA G+C content (34 mol%) and in the production of end products (acetate only). Oxobacter
Table 2. Differentiating characteristics of strain SR3T

Growth was performed in basal medium containing 20 mM substrates at 37 °C and results were recorded after 1 week of incubation. Strains: 1, strain SR3T (data from this study); 2, C. celerecrescens (data from Palop et al., 1989); 3, C. aerotolerans (data from Van Gylswyk & Van Der Toorn, 1987); 4, C. xylanolyticum (data from Rogers & Baecker, 1991); 5, C. sphenoides (data from Cato et al., 1986). +, Substrate used as carbon source; -, substrate not used; NR, not reported.

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<tr>
<td>Optimum pH</td>
<td>74</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>NR</td>
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<tr>
<td>Optimum temperature (°C)</td>
<td>37</td>
<td>35</td>
<td>38</td>
<td>35</td>
<td>37</td>
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<tr>
<td>G+C content (mol %)</td>
<td>44</td>
<td>38</td>
<td>40</td>
<td>40</td>
<td>41</td>
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<td>Substrates utilized:</td>
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<tr>
<td>Cellulose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Xylan</td>
<td>-</td>
<td>NR</td>
<td>+</td>
<td>+</td>
<td>NR</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Xylose</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Methoxylated aromatic compounds*</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Major end product from glucose fermentation:†</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Acetate</td>
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<tr>
<td>Ethanol</td>
<td>+</td>
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<td>Formate</td>
<td>+</td>
<td>-</td>
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<td>+</td>
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<tr>
<td>Lactate</td>
<td>-</td>
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<td>+</td>
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<td>Isobutyrate</td>
<td>-</td>
<td>+</td>
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</tbody>
</table>

*2-, 3-, 4-Methoxybenzoates, 2-, 3-, 4-methoxybenzaldehydes, 2-methoxyphenol, 2,3-, 2,4-, 2,5-, 2,6-, 3,4-, 3,5-dimethoxybenzoates, 3,4-dimethoxybenzaldehyde, 3,4,5-trimethoxycinnamate, 3,4,5-trimethoxybenzoate, 3,4,5-trimethoxymethylacetate, 3,4,5-trimethoxypropionate, 3,4,5-trimethoxyphenol, 1,3,5-trimethoxybenzene, 3,4,5-trimethoxycacetophenone, 4-hydroxy 3,5-dimethoxybenzoate, 4-hydroxy 3,5-dimethoxycinnamate, 3-hydroxy 4-methoxybenzoate, 4-hydroxy 3-methoxybenzaldehyde, 3-hydroxy 3-methoxybenzaldehyde, and 4-hydroxy 3-methoxycinnamyl alcohol. Data from this study
† Data from this study.
benzosäure resin obtained from the tree Styrax benzoin; L. v. vorare to devour; M.L. neut. adj. methoxybenzovarans pertaining to the use of the organic acid methoxybenzoic acid as carbon and energy source, which is characteristic of this organism).

Rod-shaped cells, 3.0-5.0 x 0.4-0.8 μm in size, occurring singly or in pairs, Gram-positive, non-motile. Formation of spherical, terminal endospores swelling the vegetative cell. Strictly anaerobic chemo-organoheterotroph. Growth on a number of carbohydrates (glucose, fructose, sorbose, galactose, myo-inositol, sucrose, lactose, cellobiose), organic compounds (lactate, betaine, sarcosine, dimethylglycine, methanethiol, dimethylsulfide), alcohol (methanol) and all methoxylated aromatic compounds as sole source of carbon and energy. Carbohydrates are fermented to formate, acetate and ethanol, lactate is fermented to carbon and energy. Carbohydrates are fermented to methanol, methoxylated aromatics are fermented to acetate and butyrate, and betaine, sarcosine, dimethylglycine, methanethiol and dimethylsulfide are fermented to acetate. Strain SR35 has a G+C content of 44 mol% and grows optimally at 37 °C and pH 7.4 on a glucose-containing medium. Isolated from an olive mill wastewater. The type strain is SR35 (≡ DSM 121825).

ACKNOWLEDGEMENTS

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REFERENCES


Clostridium methoxybenzovorans sp. nov.


