

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

Tahar Mechichi,¹ Marc Labat,¹ Bharat K. C. Patel,² Tony H. S. Woo,² Pierre Thomas³ and Jean-Louis Garcia¹

Author for correspondence: Jean-Louis Garcia. Tel: +33 4 9182 8572. Fax: +33 4 9182 8570.
e-mail: garcia@esil.univ-mrs.fr

¹ Laboratoire ORSTOM de Microbiologie des Anaérobies, Université de Provence, CESB-ESIL case 925, 163 Avenue de Luminy, 13288 Marseille Cedex 9, France

² School of Biomolecular and Biomedical Sciences, Faculty of Science, Griffith University, Nathan 4111, Brisbane, Queensland, Australia

³ Département de Biologie, Université de la Méditerranée, 13288 Marseille cedex 9, France

A strictly anaerobic, spore-forming bacterium (3.0–5.0 × 0.4–0.8 μm), designated strain SR3^T (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 SR3^T 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 SR3^T 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 SR3^T as *Clostridium methoxybenzovorans* sp. nov. The type strain is SR3^T (= DSM 12182^T).

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 thermoautotrophica* (formerly *Clostridium thermoautotrophicum*) (Collins *et al.*, 1994; Wiegel *et al.*, 1981), *Moorella thermoacetica* (formerly *Clostridium thermo-*

The GenBank accession number for the 16S rDNA sequence of strain SR3^T is AF067965.

aceticum) (Collins *et al.*, 1994; Fontaine *et al.*, 1942) and *Acetobacterium woodii* (Balch *et al.*, 1977). *o*-Demethylation also occurs in natural ecosystems and has been demonstrated for strains isolated from digestive tracts of termites (Grech-Mora *et al.*, 1997), subsurface sediments (Liu & Suflita, 1993), aggregated forest soils (Kuhner *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 oil 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 oil 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 SR3^T was isolated from these enrichment cultures and is the subject of this paper. *Clostridium aerotolerans* (DSM 5434^T), *Clostridium celerecrescens* (DSM 5628^T) and *Clostridium xylanolyticum* (DSM 6555^T) 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 (l⁻¹): 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% (w/v) NaHCO₃ and 0.05 ml 2.5% (w/v) Na₂S·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 1.6% agar) were picked. This procedure was repeated several times until only one type of colony (white) was observed. Routine culturing of strain SR3^T was achieved in basal medium containing 5 mM syringic acid.

Cellular studies. Light and electron microscopy were performed as previously described (Fardeau *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, cellobiose), 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-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-trimethoxyphacetophenone) 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)], 20 mM alcohols (methanol, ethanol, glycerol), 20 mM each of betaine, methylamine, trimethylamine, sarcosine, dimethylglycine, methanethiol and dimethylsulfide, and 2 g peptides l⁻¹ (Casamino acids, peptone, gelatin) were injected into Hungate tubes that contained 5 ml pre-sterilized basal medium, from pre-sterilized and pre-reduced concentrated stock solutions. Concentrated stock solutions were prepared, neutralized if necessary, rendered anaerobic by gassing with O₂-free N₂ and sterilized by filtration (pore size 0.2 µm; Millipore). Polysaccharides (cellulose and xylan) were weighed directly into tubes before the medium was dispensed to give a final concentration of 0.1% (w/v). Autotrophic growth was tested using H₂/CO₂ (20:80%, v/v) at a final pressure of 2 bar. An increase in OD₅₈₀ in tubes containing added substrates, compared to control tubes lacking a substrate, was considered to be positive growth.

C. aerotolerans, *C. celerecrescens* and *C. xylanolyticum* were tested for their ability to grow on syringate, vanillate and 3,4,5-trimethoxybenzoate at a final concentration of 5 mM in basal medium.

Electron acceptors. Sulfate, thiosulfate, sulfite, nitrate, elemental sulfur and fumarate were tested as electron acceptors at a final concentration of 10 mM in basal medium containing 5 mM syringate.

Analytical methods. Anaerobic Hungate tubes were inserted directly into the cuvette holder of a Shimadzu model UV

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 0.0025 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.* (1989). Non-methylated lambda DNA (Sigma) was used as the standard.

DNA extraction and amplification of 16S 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 mix contained 1–20 ng genomic DNA, 1 µM of each primer, 5 µl × 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 mix 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 SR3^T, was studied further.

Morphology

Cells of strain SR3^T were rod-shaped and 3.0–5.0 × 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 SR3^T 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 SR3^T 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 SR3^T required yeast extract for growth.

Strain SR3^T, but not *C. celerecrescens*, *C. aerotolerans*, *C. xylanolyticum* 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 SR3^T was able to degrade all methoxylated monoaromatic compounds tested including mono-

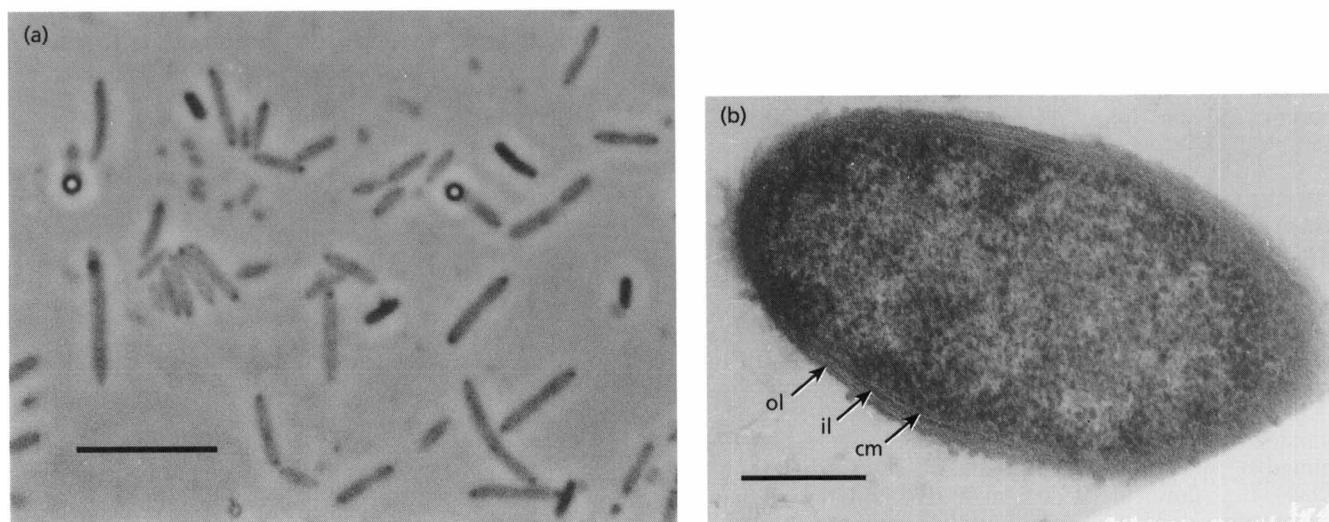


Fig. 1. (a) Phase-contrast micrograph of strain SR3^T showing terminal spores; bar, 10 μm. (b) Electron micrograph of an ultrathin section of strain SR3^T showing the cytoplasmic membrane (cm), the inner layer (il) and the outer layer (ol); bar, 0.2 μm.

Table 1. Fermentation end products of strain SR3^T

Degraded substrate*	Product formed					
	Formate	Acetate	Butyrate	Ethanol	H ₂	CO ₂
Methoxy group†	—	+	+	—	+	+
Glucose	+	+	—	+	+	+
Fructose	—	+	—	+	+	+
Sorbose	+	+	—	+	+	+
Galactose	+	+	—	+	+	+
<i>myo</i> -Inositol	—	+	—	+	+	+
Sucrose	—	+	—	+	+	+
Lactose	+	+	—	+	+	+
Cellobiose	+	+	—	+	+	+
Methanol	—	+	+	—	—	—
Lactate	—	+	+	—	—	—
H ₂ /CO ₂	+	+	—	—	—	—
Betaine	—	+	—	—	—	—
Sarcosine	—	+	—	—	—	—
Dimethyl glycine	—	+	—	—	—	—
Dimethyl sulfide	—	+	—	—	—	—
Methanethiol	—	+	—	—	—	—
Casamino acids	—	+	—	—	—	—
Peptone	—	+	—	—	—	—

* All substrates were used at a final concentration of 20 mM except for peptone and Casamino acids (2 g l⁻¹) 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.

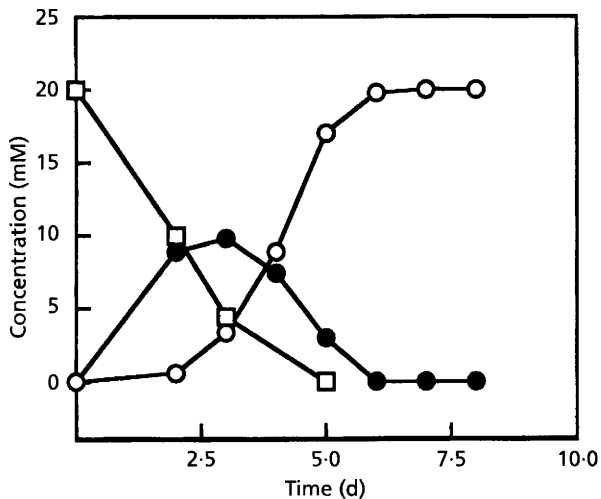


Fig. 2. Degradation of syringate by strain SR3^T. The basal medium contained 5 mM syringate (□); the intermediate product formed was 5-hydroxyvanillate (●), which was further degraded to gallate (○).

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-trimethoxyphacetophenone) 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-methoxycinnamylalcohol).

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 SR3^T also used glucose, fructose,

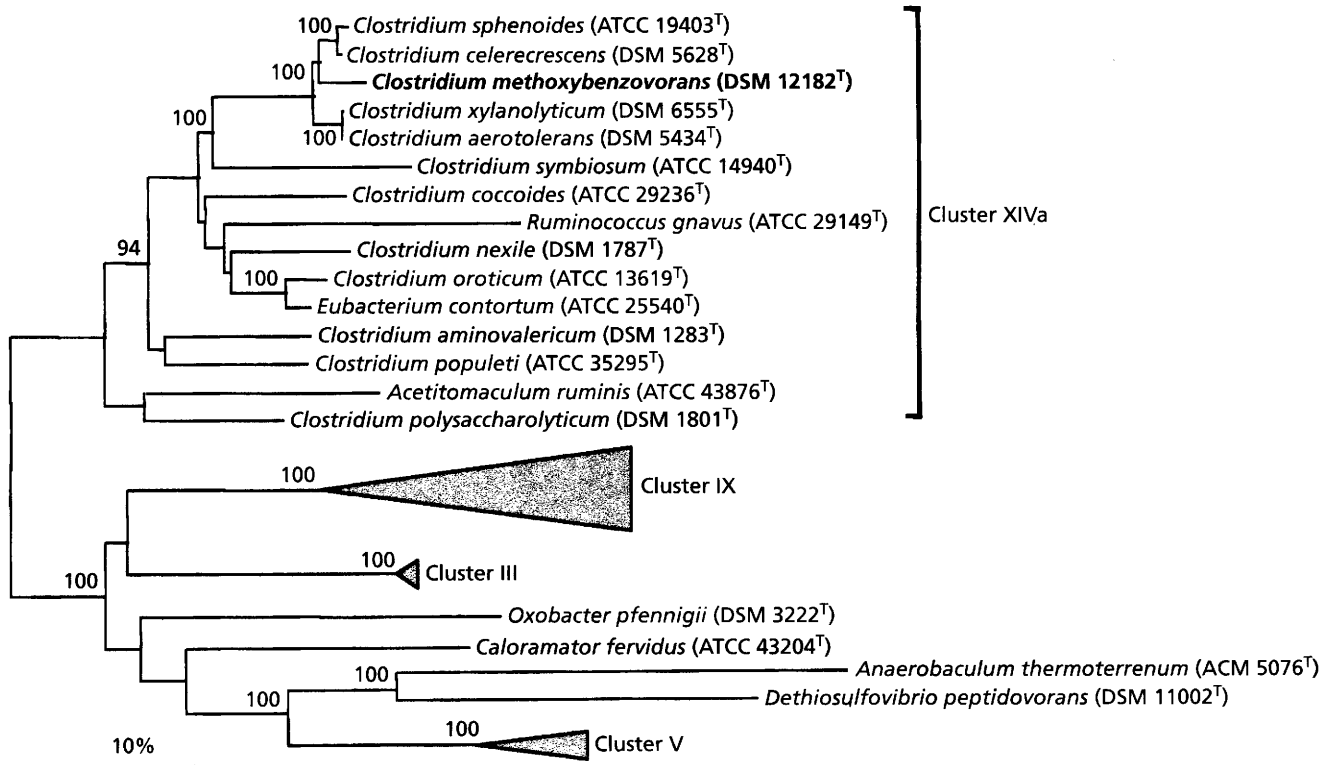


Fig. 3. Unrooted phylogenetic dendrogram based on 16S rRNA sequence data indicating the position of *Clostridium methoxybenzovorans* strain SR3^T within the radiation of representatives of the low-G+C-containing Gram-positive bacteria. All the sequences used in the analysis, with the exception of sequences of *Anaerobaculum thermoterrenum* and *Dethiosulfovibrio peptidovorans* (GenBank accession nos U50711 and U52817, 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 thermocopriae*), cluster III (*Acetovibrio cellulolyticus* and *Clostridium aldrichii*) and cluster IX (*Megasphaera elsdenii* and *Clostridium quercicolum*). 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.

sorbose, galactose, *myo*-inositol, sucrose, lactose, cellobiose, methanol, lactate, H₂/CO₂, 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, H₂/CO₂, 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 H₂/CO₂ 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-hydroxyvanillic 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 *E. 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 *Moorella 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. thermoaceticum*) (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 SR3^T

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 SR3^T (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.

Character	1	2	3	4	5
Optimum pH	7.4	7.0	7.0	7.0	NR
Optimum temperature (°C)	37	35	38	35	37
G + C content (mol %)	44	38	40	40	41
Substrates utilized:					
Cellulose	–	+	–	NR	NR
Xylan	–	NR	+	+	NR
Glucose	+	+	+	+	+
Cellobiose	+	+	+	+	+
Arabinose	–	+	+	+	+
Xylose	–	+	+	+	+
Methoxylated aromatic compounds*	+	–	–	–	–
Major end product from glucose fermentation:†					
Acetate	+	+	+	+	+
Ethanol	+	–	–	–	+
Formate	+	–	+	+	–
Lactate	–	+	+	+	–
Isobutyrate	–	+	+	+	–

* 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-trimethoxyphenacetophenone, 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-methoxycinnamyl-alcohol. Data from this study

† Data from this study.

pfennigii (Collins *et al.*, 1994; Krumholz & Bryant, 1985), *Butyribacterium methylotrophicum* (Zeikus *et al.*, 1980) and *Eubacterium limosum* (Genthner *et al.*, 1981) have the same fermentation end product profile from methoxyl groups as strain SR3^T does. However, both these strains do not sporulate. Strain SR3^T appears to be one of the most versatile bacteria in terms of its ability to demethylate a wide range of methoxylated aromatic compounds without ring cleavage. It would be tempting to conclude that strain SR3^T is the most versatile amongst members of the *Clostridium* group as it is able to demethylate a wider range of methoxylated compounds than any other known species of this group, but such conclusions cannot be drawn as most aromatic-degrading strains have not yet been tested exhaustively for this property.

Further evidence that strain SR3^T is a member of the order *Clostridiales* comes from 16S rDNA sequence analysis in which strain SR3^T clustered closely with *C. celerecrescens*, *C. aerotolerans*, *C. xylanolyticum* and *C. sphenoides* of cluster XIVa with a similarity of 98%.

Stackebrandt & Goebel (1994) have suggested that if the 16S rDNA similarity is greater than 97%, then phenotypic and genotypic differentiation should be used for species differentiation. There are numerous characteristics that differentiate strain SR3^T from its close relatives (Table 2). These include the higher G + C content (44 mol % as opposed to 38–41 mol %), the ability to utilize methoxylated aromatic compounds and the different end product profile from sugar fermentation. Based on this evidence, strain SR3^T is sufficiently different from other taxonomically validated *Clostridium* species and therefore we propose to designate it *Clostridium methoxybenzovorans* sp. nov. The type strain is strain SR3^T (= DSM 12182^T).

Description of *Clostridium methoxybenzovorans* sp. nov.

Clostridium methoxybenzovorans (me.tho.xy.ben.zo' vo.rans. Fr. méthyl the methyl radical; Gr. n. oxy's acid; Fr. n. benzoin frankincense of Java; Ger. n.

benzoësäure resin obtained from the tree *Styrax benzoin*; L. v. *vorare* to devour; M.L. neut. adj. *methoxybenzovorans* 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 × 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 methanol, methoxylated aromatics are fermented to acetate and butyrate, and betaine, sarcosine, dimethylglycine, methanethiol and dimethylsulfide are fermented to acetate. Strain SR3^T 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 anaerobic methanogenic pilot-scale digester fed with olive mill wastewater. The type strain is SR3^T deposited in DSMZ (= DSM 12182^T).

ACKNOWLEDGEMENTS

The financial assistance in part to B.K.C.P. from the Australian Research Council is gratefully acknowledged. We thank M.-L. Fardeau and B. Ollivier for suggestions and continuous support for this work.

REFERENCES

- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Andreesen, J. R., Gottschalk, G. & Schlegel, H. G. (1970). *Clostridium formicoaceticum* nov. spec., description and distinction from *C. aceticum* and *C. thermoaceticum*. *Arch Microbiol* **72**, 154–174.
- Andrews, K. T. & Patel, B. K. C. (1996). *Fervidobacterium gondwanense* sp. nov., a new thermophilic anaerobic bacterium isolated from nonvolcanically heated geothermal waters of the Great Artesian Basin of Australia. *Int J Syst Bacteriol* **46**, 265–269.
- Balch, W. E., Schoberth, S., Tanner, R. S. & Wolfe, R. S. (1977). *Acetobacterium*, a new genus of hydrogen-oxidizing, carbon dioxide-reducing, anaerobic bacteria. *Int J Syst Bacteriol* **27**, 355–361.
- Benson, D., Lipman, D. J. & Olstell, J. (1993). GenBank. *Nucleic Acids Res* **21**, 2963–2965.
- Breznak, J. A., Switzer, J. M. & Seitz, H.-J. (1988). *Sporomusa termitida* sp. nov., an H₂/CO₂-utilizing acetogen isolated from termites. *Arch Microbiol* **150**, 282–288.
- Capasso, R., Evidente, A., Schivo, L., Orru, G., Marcialis, M. A. & Cristinzio, G. (1995). Antibacterial polyphenols from olive mill wastewaters. *J Appl Bacteriol* **79**, 393–398.
- Cato, E. P., George, W. L. & Finegold, S. M. (1986). Genus *Clostridium*. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1141–1200. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.
- Daniel, S. L., Wu, Z. & Drake, H. L. (1988). Growth of thermophilic acetogenic bacterium on methoxylated aromatic acids. *FEMS Microbiol Lett* **52**, 25–28.
- Dehning, I., Stieb, M. & Schink, B. (1989). *Sporomusa malonica* sp. nov., a homoacetogenic bacterium growing by decarboxylation of malonate and succinate. *Arch Microbiol* **151**, 421–426.
- Fardeau, M.-L., Cayol, J.-L., Magot, M. & Ollivier, B. (1993). H₂ oxidation in the presence of thiosulfate, by a *Thermoanaerobacter* strain isolated from an oil-producing well. *FEMS Microbiol Lett* **113**, 327–332.
- Fardeau, M.-L., Ollivier, B., Patel, B. K. C., Magot, M., Thomas, P., Rimbault, A., Rocchiccioli, F. & Garcia, J.-L. (1997). *Thermotoga hypogea* sp. nov., a xylanolytic, thermophilic bacterium from an oil-producing well. *Int J Syst Bacteriol* **47**, 1013–1019.
- Felsenstein, J. (1993). PHYLIP (Phylogenetic Inference Package) version 3.51c. Distributed by the author. Department of Genetics, University of Washington, Seattle, WA, USA.
- Fontaine, F. E., Peterson, W. H., McCoy, E., Johnson, M. J. & Ritter, G. J. (1942). A new type of glucose fermentation by *Clostridium thermoaceticum* n. sp. *J Bacteriol* **43**, 701–715.
- Genthner, B. R. S., Davis, C. L. & Bryant, M. P. (1981). Features of rumen and sewage sludge strains of *Eubacterium limosum*, a methanol and H₂-CO₂-utilizing species. *Appl Environ Microbiol* **42**, 12–19.
- Grech-Mora, I., Fardeau, M.-L., Patel, B. K. C., Ollivier, B., Rimbault, A., Prensier, G., Garcia, J.-L. & Garnier-Sillam, E. (1997). Isolation and characterization of *Sporobacter termitidis* gen. nov., sp. nov., from the digestive tract of the wood-feeding termite *Nasutitermes lujae*. *Int J Syst Bacteriol* **46**, 512–518.
- Heider, J. & Fuchs, G. (1997). Microbial anaerobic aromatic metabolism. *Anaerobe* **3**, 1–22.
- Hungate, R. E. (1969). A roll-tube method for the cultivation of strict anaerobes. *Methods Microbiol* **136**, 194–198.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Krumholz, L. R. & Bryant, M. P. (1985). *Clostridium pfennigii* sp. nov. uses methoxyl groups of monobenzenoids and produces butyrate. *Int J Syst Bacteriol* **35**, 454–456.
- Kuhner, C. H., Frank, C., Gribhammer, A., Schmittroth, G. A., Göbner, A. & Drake, H. (1997). *Sporomusa silvacetica* sp. nov., an acetogenic bacterium isolated from aggregated forest soil. *Int J Syst Bacteriol* **47**, 352–358.
- Liesack, W., Back, F., Kreft, J. U. & Stackebrandt, E. (1994). *Holofaga foetida* gen. nov., sp. nov., a new homoacetogenic bacterium degrading methoxylated aromatic compounds. *Arch Microbiol* **162**, 85–90.
- Liu, S. & Suflija, J. M. (1993). H₂-CO₂-dependent *o*-demethylation activity in subsurface sediment from an isolated bacterium. *Appl Environ Microbiol* **59**, 1325–1331.
- Lux, M. F., Keith, E., Hsu, T. & Drake, H. L. (1990). Biotransformation of aromatic aldehydes by acetogenic bacteria. *FEMS Microbiol Lett* **67**, 73–78.

- Macy, J. M., Snellen, J. E. & Hungate, R. E. (1972). Use of syringe methods for anaerobiosis. *Am J Clin Nutr* **25**, 1318–1323.
- Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J. & Woese, C. R. (1996). The Ribosomal Database Project (RDP). *Nucleic Acids Res* **24**, 82–85.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Miller, T. L. & Wolin, M. J. (1974). A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. *Appl Microbiol* **27**, 985–987.
- Palop, M. L., Valles, S., Pinaga, F. & Flors, A. (1989). Isolation and characterization of an anaerobic, cellulolytic bacterium, *Clostridium celerecrescens* sp. nov. *Int J Syst Bacteriol* **39**, 68–71.
- Redburn, A. C. & Patel, B. K. C. (1993). Phylogenetic analysis of *Desulfotomaculum thermobenzoicum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. *FEMS Microbiol Lett* **113**, 81–86.
- Rogers, G. M. & Baecker, A. A. W. (1991). *Clostridium xylanolyticum* sp. nov., an anaerobic xylanolytic bacterium from decayed *Pinus patula* Wood Ships. *Int J Syst Bacteriol* **41**, 140–143.
- Stackebrandt, E. & Goebel, B. M. (1994). A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Van Gylswyk, N. O. & Van Der Toorn, J. J. T. K. (1987). *Clostridium aerotolerans* sp. nov., a xylanolytic bacterium from corn stover and from the rumina of sheep fed corn stover. *Int J Syst Bacteriol* **37**, 102–105.
- Widdel, F. & Pfennig, N. (1981). Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. Isolation of new sulfate reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov. *Arch Microbiol* **129**, 395–400.
- Wiegel, J., Braun, M. & Gottschalk, G. (1981). *Clostridium thermoautotrophicum* species novum, a thermophile producing acetate from molecular hydrogen and carbon dioxide. *Curr Microbiol* **5**, 255–260.
- Winker, S. & Woese, C. R. (1991). A definition of the domains *Archaea*, *Bacteria* and *Eucarya* in terms of small subunit ribosomal RNA characteristics. *Syst Appl Microbiol* **13**, 161–165.
- Wolin, M. J. & Miller, T. L. (1993). Bacterial strains from human feces that reduce CO₂ to acetic acid. *Appl Environ Microbiol* **59**, 3551–3556.
- Zeikus, J. G., Lynd, L. G., Thompson, T. E., Krzycki, J. A., Weimer, P. J. & Hegge, P. W. (1980). Isolation and characterization of a new, methylotrophic acidogenic anaerobe, the Marburg strain. *Curr Microbiol* **3**, 381–386.