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Eubacterium aggregans sp. nov., a New Homoacetogenic Bacterium from Olive Mill Wastewater Treatment Digestor

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A strictly anaerobic, homoacetogenic, Gram-positive, non spore-forming bacterium, designated strain SR12^T (T = type strain), was isolated from an anaerobic methanogenic digestor fed with olive mill wastewater. Yeast extract was required for growth but could also be used as sole carbon and energy source. Strain SR12^T utilized a few carbohydrates (glucose, fructose and sucrose), organic compounds (lactate, crotonate, formate and betaine), alcohols (methanol), the methoxyl group of some methoxylated aromatic compounds, and H_2+CO_2. The end-products of carbohydrate fermentation were acetate, formate, butyrate, H_2 and CO_2. End-products from lactate and methoxylated aromatic compounds were acetate and butyrate. Strain SR12^T was non-motile, formed aggregates, had a G+C content of 55 mol % and grew optimally at 35°C and pH 7.2 on a medium containing glucose. Phylogenetically, strain SR12^T was related to *Eubacterium barkeri*, *E. callanderi*, and *E. limosum* with *E. barkeri* as the closest relative (similarity of 98%) with which it bears little phenotypic similarity or DNA homology (60%). On the basis of its phenotypic, genotypic, and phylogenetic characteristics, we propose to designate strain SR12^T as *Eubacterium aggregans* sp. nov. The type strain is SR12^T (= DSM 12183).

Introduction

A large number of anaerobic acetogenic and sulfate-reducing bacteria are involved in the degradation of one-carbon compounds such as methoxyl groups from aromatic compounds, methanol, formate, CO, H_2, CO_2, betaine, sarcosine, and methylamine. Acetate is the main end product produced from these substrates. However, in some strains acetate and buty-rate or acetate, butyrate, and formate can be formed. Methoxyl groups from aromatic compounds

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can serve as growth substrate for many acetogenic bacteria such as *Acetobacterium carbinolicum* [1], *A. malicum* [2], *A. woodii* [3], *Butyribacterium methylotrophicum* [4], *Clostridium formicoaceticum* [5], *C. pfennigii* [6], *Moorella thermoacetica* (formerly *C. thermoaceticum*) [7], *Eubacterium limosum* [8], *E. callanderi* [9], *Sporomusa termitida* [10], and *S. silvacetica* [11]. Most of these strains belong to the Gram-positive bacteria with low G+C content. *Acetobacterium woodii*, *M. thermoacetica*, *E. limosum*, and *S. termitida* use a wide range of one carbon compounds in contrast to *E. callanderi*, which is only able to use methoxyl groups from aromatic compounds (ferulate, sinapate, syringate, trimethoxycinnamate, and vanillate). We report on the isolation and the characterization of a new homoacetogenic bacterium, *Eubacterium aggregans*. sp. nov. which specifically degrades methoxylated aromatic compounds.

Materials and Methods

Source of strains

Strain SR12^T was isolated from enrichment cultures from an anaerobic methanogenic digestor fed with olive mill wastewater. Liquid samples were collected anaerobically from the digestor using N_2-flushed syringes and inoculated into the basal medium. *Eubacterium barkeri* (DSM 1223) used as a reference culture was purchased from the DSMZ-Deutsche Sammlung von Microorganismen und Zelkulturen GmbH, Braunschweig, Germany. The reference culture was routinely grown using glucose containing basal medium.

Culture media

The basal medium used for enrichment, isolation, and cultivation was prepared using anaerobic techniques [12–14]. It contained (per liter of deionized water): 1 g of NH_4Cl, 0.3 g of K_2HPO_4, 0.3 g of KH_2PO_4, 0.6 g of NaCl, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of KCl, 0.5 g of cysteine–HCl, 1 g of yeast extract (Difco Laboratories), 1.5 ml of trace element solution [15], and 1 mg of resazurin. pH was adjusted to 7 with 10 M KOH solution, the medium was then boiled under a stream of O_2-free N_2 gas and cooled to room temperature. Five-milliliters aliquots of basal medium were dispensed into Hungate tubes, degassed under N_2–CO_2 (80:20% v/v) and subsequently sterilized by autoclaving at 110°C for 45 min. Prior to culture inoculation, 0.2 ml of 5% (w/v) $NaHCO_3$ and 0.05 ml of 2.5% (w/v) $Na_2S \cdot 9H_2O$ were injected from sterile stock solutions into the tubes.

Enrichment and Isolation

A 0.5-ml aliquot of sample was inoculated into Hungate tubes containing 5 ml of basal medium and 5 mM syringic acid. Tubes were incubated at 37°C. To obtain pure cultures, the enrichment was subcultured several times under the same growth condition and subsequently serially diluted ten-fold in roll tubes containing basal medium, 5 mM syringic acid and 1.6% agar. Single colonies that developed were picked up and this procedure was repeated several times until only one type of colony was observed.

Substrate utilization

Experiments were performed in duplicate with inocula that had been subcultured under the same test conditions at least once. The following substrates were tested: 20 mM carbohydrates (glucose, fructose, xylose, sorbose, galactose, myo-inositol, sucrose, lactose, cellobiose, mannitol, and ribose) and 10 g/L xylan and cellulose, 20 mM organic acids (formate, fumarate, pyruvate, crotonate, malonate, and succinate), 5 mM monomethoxylated aromatic compounds (2-, 3- and 4-methoxybenzoates, 2-, 3- and 4-methoxybenzaldehydes, 2-methoxyphenol), 5 mM dimethoxylated aromatic compounds (2,3-, 2,4-, 2,5-, 2,6-, 3,4- and 3,5-dimethoxybenzoates, 3,4- dimethoxybenzaldehyde), 5 mM trimethoxylated aromatic compounds (3,4,5-trimethoxycinnamate, 3,4,5-trimethoxybenzoate), 5 mM mixed meth- oxylated/hydroxylated aromatic compounds (4-hydroxy-3,5-dimethoxybenzoate (syringate), 4-hydroxy-3,5-dimethoxycinnamate (sinapate), 3-hydroxy-4- methoxybenzoate, 4-hydroxy-3-methoxybenzoate (vanillate), 4-hydroxy-3-methoxycinnamate (ferulate), 3-hydroxy-4-methoxycinnamate, 3-hydroxy-4-methoxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde (vanillin), and 4-hydroxy-3-methoxycinnamylalcohol), 20 mM alcohols (methanol, ethanol, and glycerol), and 20 mM betaine. Substrate stock solutions (100 ×) were prepared, neutralized if required, sterilized by filtration (pore size 0.2 µm, Millipore), rendered anaerobic by gassing with O_2-free N_2, and injected into Hungate tubes that contained presterilized media. The N_2 in Hungate tubes was replaced with H_2+CO_2 (20:80%) (final pressure of 2 bars gas phase) for some experiments after inoculation. An increase in optical density (OD) of 0.1 at 580 nm in tubes containing the added substrate over control tubes lacking the added substrate, was considered as positive for growth. Carbon source degradation and production of volatile fatty acid, as determined by high-performance liquid chromatography (HPLC), were also used as indicators of growth.

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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.

pH, Temperature, and NaCl concentration ranges

The pH of the pre-reduced anaerobic basal medium was adjusted by injecting 5% $NaHCO_3$, 5% Na_2CO_3, or 0.1 M HCl to give a pH range between 5 to 9.5. Appropriate amounts of NaCl was weighed directly into Hungate tubes prior to dispensing of 5 ml of the medium to give the desired concentration (range 0 to 40 g/L). The temperature range for growth was tested from 15 to 45°C.

Light microscopy and electron microscopy

Light microscopy and electron microscopy observations were performed as described previously [16].

Analytical methods

Growth was measured at 580 nm by inserting anaerobic Hungate tubes directly into the cuvette holder of a Shimadzu Model UV 160A spectrophotometer. Aromatic compounds were quantified using a ConstaMetric 200 LDC-Analytical High Performance Liquid Chromatograph (HPLC) equipped with a C_{18} Symmetry 5-μm particle size column 250 by 4.6 (inside diameter) mm (Waters Chromatography), a Shimadzu SPD-6A UV detector at 240 nm and a CR-6A Shimadzu integrator. The column temperature was maintained at 35°C. An isocratic mobile phase of 30:69.5:0.5 (v:v:v) acetonitrile/distilled water/acetic acid was used at a flow rate of 0.6 mL/min. The volume of the injection loop was 20 μL. Volatile fatty acids, ethanol, glycerol, and carbohydrates were measured using HPLC (Spectra Series 100 model, Thermo Separation Products) equipped with an Aminex HPX-87X 300 by 7.8-mm (inside diameter) column (Bio-Rad Laboratories) and connected to a differential refractometer (RID-6A Shimadzu). Analysis was performed using a CR-6A Shimadzu integrator. The mobile phase was 0.005 M H_2SO_4 at a flow rate of 0.5 mL/min, and the column temperature was 35°C. The volume of the injection loop was 20 μL.

Determination of G+C content

The G+C content of DNA was determined at DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. The DNA was isolated and purified by chromatography on hydroxyapatite, and the G+C content was determined by HPLC [17]. Non-methylated lambda DNA (Sigma) was used as the standard.

DNA–DNA hybridization

DNA was isolated by the method of Cashion *et al.* [19]. DNA–DNA hybridization was performed at the DSMZ and carried out as described by De Ley *et al.* [20], with the modification described by Escara and Hutton [21] and Huss *et al.* [22] using a Gilford System model 2600 equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program by Jahnke and Bahnweg [23] and Jahnke [24].

DNA extraction and amplification of 16S rRNA genes

DNA was extracted from the isolate as described previously [25]. The universal primers Fd1 and Rd1 were used to obtain a PCR product of approximately 1.5 kb. A 50-μL reaction contained 1–20 ng of genomic DNA, 1 μM of each primer, 5 μL of 10 × buffer, 200 μM of dNTP, 3.5 mM of $MgCl_2$, and 2.5 U of *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 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 using the following conditions. A 10-μL reaction containing 35 ng of PCR product, 4 μL of cycle sequencing reaction mix, 3.2 pmol of primer [25], and 2.5 μg of 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, extension at 60°C for 3 min.

Sequence alignments and phylogenetic inferences

The new sequence data that was generated was assembled, aligned to an almost full length consensus 16S rRNA gene sequence assembled and checked for accuracy manually using the alignment editor, ae2 [26]. The sequence was compared with other sequences in the GenBank database [27] using BLAST [28], and in the Ribosomal Database Project, version 5.0 using SIMILARITY_RANK and SUGGEST_TREE [26]. Reference sequences most related to our newly generated sequences were extracted from these databases and aligned. Sequences for *Acetobacterium woodii, A. malicum, A. paludosum, A. fimetarium, A. bakii* and *E. callanderi* (accession numbers X96954, X96957, X96958, X96959, X96960, and X96961, respectively), were extracted from EMBL. Positions of sequence and alignment uncertainty were omitted from the analysis. Pairwise evolutionary distances based on 1198 unambigious nucleotides were computed using dnadist (Jukes and Cantor option) and neighbor-joining programs that form part of the PHYLIP suite of programs [29]. TREECON was used extensively for bootstrap analysis [30].

Results

Enrichment and isolation

After 2 weeks of incubation at 37°C, a positive growth was obtained in the enrichment culture as indicated by syringate degradation and acetate production. Pure cultures were checked microscopically for culture purity and for aromatic compound degradation. A culture designated strain SR12^T was used for further characterization studies.

Cellular morphology

Cells of strain SR12^T were rod shaped measuring 2 to 3 μm long by 0.4 and 0.8 μm wide. Spores were not seen in late exponential growth phase and for cultures grown under different conditions. However, growth occurred in fresh medium after inoculation of cultures that had been incubated for 10 min at 80°C, 90°C, but not at 100°C indicated cellular thermal resistance and the probability that resistant bodies were present. Cells were non-motile and no flagella were observed in negative stained preparations using electron microscopy (Fig. 1a). The cells stained Gram-positive and electron micrographs of thin sections of the cells showed the presence of a thick bilayered cell wall structure resembling Gram- positive type cell walls (Fig. 1b). Cells of strain SR12^T formed extensive aggregates (Fig. 1c), but were freed by vigorous vortexing. Examination of ultrathin section of cells identified numerous projections from the cell wall surface resembling extracellular glucocalix matrix, which is most likely to be responsible for cell aggregation (Fig. 1d).

Temperature, pH, and NaCl concentration ranges

The temperature range for growth was 20 to 42°C, with an optimal growth occurring at 35°C. The pH range for growth was between pH 6.0 and 9.0 with an optimum of pH 7.2. NaCl concentration range for growth was between 0 to 35 g/L with no growth occurring at 40 g/L.

Substrate utilization

Yeast extract was required for growth and could be used as sole carbon and energy source. Strain SR12^T used a limited number of carbohydrates including glucose (20 mM), fructose (20 mM), and sucrose (20 mM); fatty acids including lactate (20 mM), formate (20 mM), and crotonate (20 mM); one-carbon compounds including methanol (20 mM), H_2–CO_2 (2 bar), and betaine (20 mM). Strain SR12^T did not utilize xylose (20 mM), sorbose (20 mM), galactose (20 mM), myo-inositol (20 mM), lactose (20 mM), ribose (20 mM), mannitol (20 mM), cellobiose (20 mM), xylan 10 g/L, cellulose 10 g/L, fumarate (20 mM), pyruvate (20 mM), succinate (20 mM), malonate (20 mM), glycerol (20 mM), and ethanol (20 mM).

Specific o-demethylation of methoxylated aromatic compounds by strain SR12^T

o-demethylation of methoxylated aromatic compounds by strain SR12^T was specific for the *ortho* position but not to the *meta* position of the methoxyl group. Strain SR12^T demethylated various methoxylated aromatic compounds with at least one *ortho*-positioned hydroxyl group (2-methoxyphenol, 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), or methoxyl group (3,4-, 2,3-dimethoxybenzoates, 3,4-dimethoxybenzaldehyde, 3,4,5-trimethoxycinnamate, 3,4,5-trimethoxy benzoate). Methoxylated aromatic compounds with

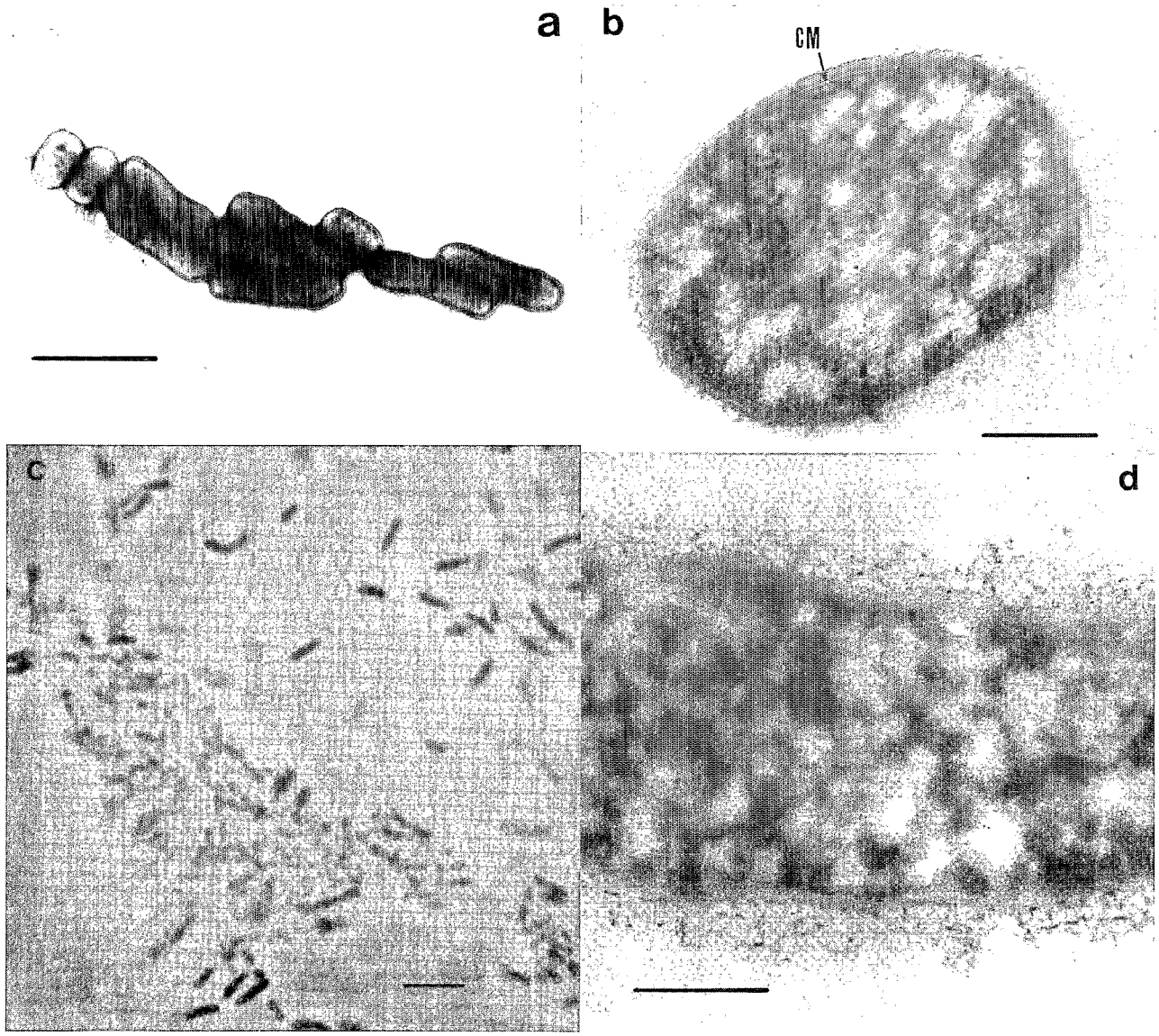

Figure 1. (a) Negatively stained cells of strain SR12^T, Bar = 2 μm; (b) transmission electron micrograph of the cell wall ultrastructure of strain SR12^T showing the presence of cytoplasmic membrane (CM) and a thick electron dense cell wall, Bar = 0.2 μm; (c) phase contrast micrograph of aggregating cells at the exponential growth phase of strain SR12^T, Bar = 5 μm; (d) transmission electron micrograph of a thin section of strain SR12^T showing numerous projections from the cell wall surface resembling extracellular glucocalix matrix which would be responsible for the aggregation, Bar = 0.2 μm.

carboxyl group *ortho*-positioned (2-methoxybenzoate, 2,6-dimethoxybenzoate, 2,5-dimethoxybenzoate), or with *meta*-positioned methoxyl group (3,5-dimethoxybenzoate), or without any *ortho*-positioned methoxyl or hydroxyl group (3- or 4-methoxybenzoates and 3- or 4-methoxybenzaldehydes) were not used.

Fermentation end products

Fermentation end products from glucose utilization were H_2, CO_2, formate and butyrate; from sucrose and fructose were H_2, CO_2, acetate and butyrate; from methanol and betaine were acetate; from lactate and crotonate were formate, acetate and butyrate; and from H_2+CO_2 were formate and acetate (Table 1). Acetate, butyrate, and the corresponding hydroxylated aromatic compounds were the end products from the fermentation of 2-methoxyphenol, 3,4-, 2,3-dimethoxybenzoates, 3,4-dimethoxybenzaldehyde, 3,4,5-trimethoxycinnamate, 3,4,5-trimethoxybenzoate, 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,

Table 1. Major end products from different carbon sources fermentation by strain SR12^T

Substrates	End products				
	CO_2	H_2	Formate	Acetate	Butyrate
Glucose	+	+	+	−	+
Fructose	+	+	−	+	+
Sucrose	+	+	−	+	+
Methanol	−	−	−	+	−
Lactate	−	−	−	+	+
Crotonate	−	−	−	+	+
Formate	+	−		+	−
Betaine	−	−	−	+	−
H_2–CO_2	−	−	+	+	−
Methoxyl groups[a]	−	−	−	+	+

+: compound is produced; −: compound is not produced.

[a] 2-methoxyphenol, 3,4-dimethoxybenzoates, 3,4-dimethoxybenzaldehyde, 3,4,5-trimethoxycinnamate, 3,4,5-trimethyoxybenzoate, 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-3-3-methoxycinnamylalcohol.

3-hydroxy-4-methoxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde, and 4-hydroxy-3-methoxycinnamylalcohol. Aromatic compounds possessing an aldehydic group were oxidized to their corresponding carboxylic group.

Electron acceptors utilization

Sulfate, thiosulfate, sulfite, nitrate, elemental sulfur, or fumarate could not be used as electron acceptors in basal medium containing 5 mM syringate as the electron donor.

G+C content and DNA–DNA relatedness. The G+C content of strain SR12^T was 55 mol % (HPLC). The level of DNA–DNA relatedness between strain SR12^T and *Eubacterium barkeri* was 59.5%.

16S rRNA sequence analysis

Using 12 primers, we determined an almost complete sequence consisting of 1509 bases of the 16S rRNA gene of strain SR12^T (corresponding to *E. coli* position 14 to 1539 [31]). Phylogenetic analysis revealed that strain SR12^T was a member of cluster XV, the low G+C containing Gram-positive branch [32]. The closest relative were *Eubacterium barkeri* (similarity average of 98%) and *E. callanderi* and *E. limosum* (similarity average of 95%). Figure 2

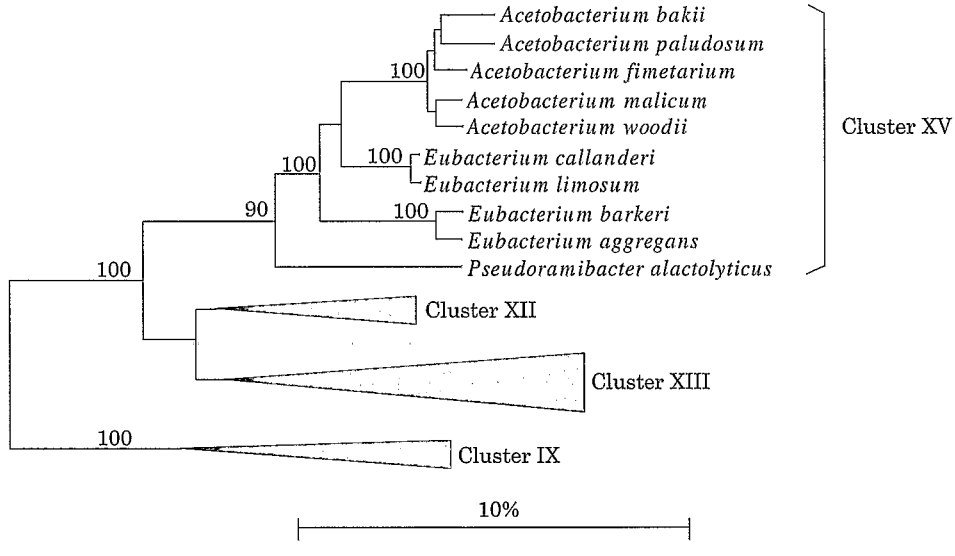

Figure 2. Phylogenetic dendrogram based on 16S rRNA sequence data indicating the position of *Eubacterium aggregans* strain SR12^T within the radiation of representatives of the low G + C containing Gram-positive bacteria. The clusters are defined based on the guidelines described by Collins *et al.* [32]. Sequences for *Acetobacterium woodii, A. malicum, A. paludosum, A. fimetarium, A. bakii,* and *Eubacterium callanderi* (accession numbers X96954, X96957, X96958, X96959, X96960, and X96961, respectively) were extracted from EMBL. Other sequences were obtained from the Ribosomal Database Project, version 5.0 [26]. Bootstrap values, expressed as a percentage of 100 replications, are shown at branching points. Only values above 90% were considered significant and reported. Scale bar indicates 10 nucleotide substitution per 100 nucleotides.

represents a dendrogram generated by the neighbor-joining method [29] from the Jukes & Cantor evolutionary distance matrix [33].

Nucleotide sequence accession number

The 16S rRNA gene sequence of strain SR12^T has been deposited in the GenBank database under accession number AF013898.

Discussion

Strain SR12^T is a Gram-positive obligate anaerobe that is rod-shaped, non-motile, and saccharolytic. The main end products of glucose fermentation are butyrate, formate, H_2 and CO_2. Phylogenetically strain SR12^T is a member of cluster XV of the low G+C containing Gram-positive *Clostridium* and related genera [32]. The closest relative is *E. barkeri* (similarity value of 98%), with *E. limosum* and *E. callanderi* equidistant from strain SR12^T at a similarity value of 95%. However, strain SR12^T differs significantly both phenotypically and genotypically from its closest relative, *E. barkeri* (Table 2). The DNA–DNA relatedness value between the two strains was 59.5%. *Eubacterium barkeri* has a G+C content of 48% (determined by DSMZ by HPLC), and optimum growth temperature of 30°C, forms terminal spores, and uses mannitol, pyruvate, glycerol, and ribose. In contrast, strain SR12^T has a G+C content of 55 mol %, an optimum growth temperature of 35°C, and does not utilize mannitol, pyruvate, glycerol, and ribose. In addition, strain SR12^T uses sucrose and methoxylated aromatics, *E. barkeri* does not. The DNA homology between the two strains is also low (60%). Although strain SR12^T is an aromatic *o*-demethylating bacterium and similar to *E. callanderi* and *E. limosum*, there are several differentiating phenotypic and genotypic characteristics (Table 2). The most significant difference is the G+C content of the DNA of the latter which is 47 mol % as opposed to strain SR12^T which is 55 mol %. Strain SR12^T also differs from *E. callanderi* in its ability to use one carbon compounds and in the fermentation end products produced from methoxyl groups, which are acetate and butyrate for SR12^T but

Table 2. Characteristics differentiating strain SR12^T from its closest relatives

Characteristics	Strain SR12^T[a]	*E. barkeri*[b]	*E. callanderi*[c]	*E. limosum*[d]
G+C%	55	48	47	47
pH optimum	7.2	nr[e]	7	nr
pH range	6–9	nr	6–8.5	nr
Temperature optimum °C	35	30	37	37
Temperature range °C	20–42	25–45	25–43	30–45
NaCl range	0–4%	0–6.5%	nr	nr
Morphology	Rods, Gram +, non spore forming	Rods, Gram +, spore forming	Rods, Gram +, non spore forming	Rods, Gram +, non spore forming
Habitat	Methanogenic digestor fed with olive mill wastewater	River mud, human feces	Anaerobic digestor fed with contents wood fiber-to-alcohol fermentation plant	Human feces, rumen, intestinal contents of man, rat, poultry, fish
Substrate utilization				
one carbon compounds	Methanol, formate, betaine, methoxyl groups, H_2+CO_2	nr	Methoxyl groups	Methanol, formate, betaine, methoxyl groups, choline, CO, H_2+CO_2
other substrates	Glucose, fructose, sucrose, lactate, crotonate	Nicotinate, glucose, fructose, ribose, mannitol, sorbitol, puryvate	Glucose in presence of acetate	Adonitol, arabitol, erythritol, fructose, glucose, isoleucine, lactate, mannitol, methoxyl groups
non utilized substrates	Ribose, mannitol, sorbitol, puryvate, galactose, mannose, arbinose, lactose, cellobiose	Sucrose, sorbose, galactose, mannose, arabinose, lactose, cellobiose	Cellobiose, maltose, cellulose, galactose, xylose mannose, lactose	Arabinose, galactose, mannose, cellobiose, sucrose, maltose, lactose
End products from fermentation of				
methoxyl groups	Acetate, butyrate	not utilized[a]	Formate, acetate, butyrate	Acetate, butyrate
carbohydrates	Acetate, formate, butyrate, H_2, CO_2	Butyrate, lactate, H_2, CO_2	Lactate, H_2	Acetate, butyrate, lactate, H_2, CO_2

[a] This work.
[b] From Cato *et al.* [36].
[c] From Montfort *et al.* [9].
[d] From Bache *et al.* [3].
[e] nr, not reported.

formate, acetate, and butyrate for *E. callanderi*. Strain SR12^T does not produce lactate though *E. limosum* can. In addition, *E. callanderi* and *E. limosum* are clearly phylogenetically distinct from strain SR12^T as shown by the large evolutionary distance separating them (5%).

The *o*-demethylation of aromatic compounds by strain SR12^T is similar to that observed in cell-free extracts of *Holofaga foetida* grown on 3,4,5-trimethoxybenzoate [34]. *Holofaga foetida* also demethylates various phenyl methyl ethers with at least one *ortho*-positioned methoxyl group and also contained a decarboxylase. However decarboxylation has not been observed following *o*-demethylation of aromatic compounds by strain SR12^T. The specificity of the *o*-demethylation was also described in *Moorella thermoacetica* [32,35]. However, 3,4-dimethoxybenzoate and 3,4,5-trimethoxybenzoate are not utilized by *M. thermoacetica* but utilized by strain SR12^T. In addition, 2-methoxybenzoate supports growth of *M. thermoacetica* but not that of strain SR12^T. Based on these two observations we suggest that the specificity of *o*-demethylation in these two strains is different and propose to designate strain SR12^T as *Eubacterium aggregans* sp. nov.

Description of Eubacterium aggregans *sp. nov.*

Eubacterium aggregans (ag'gre.gans. L. v. aggregare, to flock or band together; L. pres. part. *aggregans*, assembling, aggregating). Cells are non-spore forming, 2–3 μm by 0.4–0.8 μm, non-motile and stained Gram positive. The temperature range for growth is 20 to 42°C and the optimum temperature is 35°C. The pH range for growth is 6 to 9 and the optimum is 7.2. Growth occurs with NaCl concentration of 0–3.5%. Utilizes glucose, fructose, sucrose, lactate, formate, methanol, H_2+CO_2, betaine, crotonate, and methoxyl groups from the following compounds: 2-methoxyphenol, 3,4-, 3,5-dimethoxybenzoates, 3,4-dimethoxybenzaldehyde, 3,4,5-trimethoxycinnamate, 3,4,5-trimethoxybenzoate, 4-hydroxy-3,5- dimethoxybenzoate, 4-hydroxy-3,5-dimethoxycinnamate, 3-hydroxy-4-methoxybenzoate, 4-hydroxy-3-methoxybenzoate, 4-hydroxy-3-methoxycinnamate, 3-hydroxy-3-methoxycinnamate, 3-hydroxy-4-methoxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde, and 4-hydroxy-3-methoxycinnamylalcohol. No growth occurs with xylose, sorbose, galactose, myo-inositol, lactose, cellobiose, xylan, and cellulose. Isolated from a digestor loaded with olive mill wastewater. The type strain is SR12^T and has been deposited in the German culture collection as strain DSM 12183.

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References

1. Eicher B. and Schink B. (1984) Oxidation of primary aliphatic alcohols by *Acetobacterium carbinolicum* sp. nov., an homoacetogenic anaerobe. *Arch Microbiol* **140**: 147–152
2. Tanaka K. and Pfennig N. (1988) Fermentation of 2-methoxyphenol by *Acetobacterium malicum* sp. nov. and *Pelobacter venetianus*. *Arch Microbiol* **149**: 181–187
3. Bache R. and Pfennig N. (1981) Selective isolation of *Acetobacterium woodii* on methoxylated aromatic acids and determination of growth yields. *Arch Microbiol* **130**: 255–261
4. Zeikus J.G., Lynd L.G., Thompson T.E., Krzycki J.A., Weimer P.J. and Hegge P.W. (1980) Isolation and characterization of a new, methylotrophic acidogenic anaerobe, the Marburg strain. *Curr Microbiol* **3**: 381–386
5. Andreesen J.R., Gottschalk G. and Schlegel H.G. (1970) *Clostridium formicoaceticum* nov. spec.: description and distinction from *C. aceticum* and *C. thermoaceticum*. *Arch Microbiol* **72**: 154–174
6. Krumholz L.R. and Bryant M.P. (1985) *Clostridium pfennigii* sp. nov. uses methoxyl groups of monobenzenoids and produces butyrate. *Int J Syst Bacteriol* **35**: 454–456
7. Daniel S.L., Wu Z. and Drake H.L. (1988) Growth of thermophilic acetogenic bacterium on methoxylated aromatic acids. *FEMS Microbiol Lett* **52**: 25–28
8. Genthner B.R.S., Davis C.L. and Bryant M.P. (1981) Features of rumen and sewage sludge strains of *Eubacterium limosum*, a methanol and H_2-CO_2-utilizing species. *Appl Environ Microbiol* **42**: 12–19
9. Montfort D.O., Grant W.D., Clark R. and Asher R.A. (1988) *Eubacterium callanderi* sp. nov. that demethoxylates *o*-methoxylated aromatic acids to volatile fatty acids. *Int J Syst Bacteriol* **38**: 254–258
10. Breznak J.A., Switzer J.M. and Seitz H.J. (1988) *Sporomusa termitida* sp. nov., an H_2/CO_2-utilizing acetogen isolated from termites. *Arch Microbiol* **150**: 282–288
11. Kuhner C.H., Frank C., Gribhammer A., Schmittroth G.A., Göbner A. and Drake H. (1997) *Sporomusa silvacetica* sp. nov., an acetogenic bacterium isolated from aggregated forest soil. *Int J Syst Bacteriol* **47**: 352–358
12. Hungate R.E. (1969) A roll-tube method for the cultivation of strict anaerobes. *Methods Microbiol* **136**: 194–198
13. Macy J.M., Snellen J.E. and Hungate R.E. (1972) Use of syringe methods for anaerobiosis. *Am J Clin Nutr* **25**: 1318–1323
14. Miller T.L. and Wollin M.J. (1974) A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. *Appl Microbiol* **27**: 985–987
15. Widdel F. and 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
16. Fardeau M.L., Ollivier B., Patel B.K.C., Magot M., Thomas P., Rimbault A., Rocchiccioli F. and Garcia J.-L. (1997) *Thermotoga hypogea* sp. nov., a xylanolytic, thermophilic bacterium from an oil-producing well. *Int J Syst Bacteriol* **47**: 1013–1019
17. Mesbah M., Premchandran U. and 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
18. Redburn A.C. and 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

19. Cashion P., Holder-Franklin M.A., McCully J. and Franklin M. (1977) A rapid method for base ratio determination of bacterial DNA. *Anal Biochem* **81**: 461–466
20. De Ley J., Cattoir H. and Reynaerts A. (1970) The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**: 133–142
21. Escara J.F. and Hutton J.R. (1980) Thermal stability and renaturation of DNA in dimethylsulphoxide solutions: acceleration of renaturation rate. *Biopolymers* **19**: 1315–1327
22. Huss V.A.R., Festel H. and Schleifer K.H. (1983) Studies on the spectrometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**: 184–192
23. Jahnke K.-D. and Bahnweg G. (1986) Assessing natural relationships in the basidiomycetes by DNA analysis. *Tans Br Mycol Soc* **87**: 175–191
24. Jahnke K.-D. (1992) Basic computer program for evaluation of spectroscopic DNA renaturation data from GILFORD system 2600 spectrometer on a PC/XT/AT type personal computer. *J Microbiol Methods* **15**: 61–73
25. Andrews K.T. and 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
26. Maidak B.L., Olsen G.J., Larsen N., Overbeek R., McCaughey M.J. and Woese C.R. (1996) The ribosomal database project (RDP). *Nucleic Acids Res* **24**: 82–85
27. Benson D., Lipman D.J. and Olstell J. (1993) GenBank. *Nucleic Acids Res* **21**: 2963–2965
28. Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W. and Lipman D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402
29. Felsenstein J. (1993) PHYLIP (Phylogenetic Inference Package) version 3.51c. Distributed by the author. Department of Genetics, University of Washington, Seattle, USA
30. Van de Peer Y. and De Wachter R. (1993) TREECON: a software package for the construction and drawing of evolutionary trees. *Comput Appl Biosci* **9**: 177–182
31. Winker S. and Woese C.R. (1991) A definition of the domain *Archaea, Bacteria* and *Eucarya* in terms of small subunit ribosomal RNA characteristics. *Syst Appl Microbiol* **13**: 161–165
32. Collins M.D., Lawson P.A., Willems A., Cordoba J.J., Fernandez-Garayzabal J., Garcia P., Cai J., Hippe H. and 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
33. Jukes T.H. and Cantor C.R. (1969) Evolution of protein molecules. In Munro H.N. (ed.) *Mammalian protein metabolism*, pp 211–232. Academic Press, New York
34. Kreft J.U. and Schink B. (1997) Specificity of σ-demethylation in extracts of the homoacetogenenic *Holophaga foetida* and demethylation kinetics measured by coupled photometric assay. *Arch Microbiol* **167**: 363–368
35. Daniel S.L., Keith E.S., Yang H., Lin Y.-S. and Drake H.L. (1991) Utilization of aromatic compounds by the acetogen *Clostridium thermoaceticum*: expression and specificity of the CO-dependent σ-demethylation activity. *Biochem Biophys Res Commun* **180**: 416–422
36. Cato E.P., George W.L. and Finegold S.M. (1986) Genus *Clostridium*. In Sneath P.H.A., Mair N.S., Sharpe M.E. and Holt J.G. (eds) *Bergey's Manual of Systematic Bacteriology*, Vol. 2, pp 1141–1200. Williams & Wilkins, Baltimore, MD