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Anaerobic Bacteria Involved in the Degradation of Aromatic Sulfonates to Methane

V. A. Shcherbakova, K. S. Laurinavichyus, N. A. Chuvil'skaya, Ya. V. Ryzhmanova, and V. K. Akimenko

Skryabin Institute of the Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow oblast, 142292 Russia

e-mail: shcherb@ibpm.pushchino.ru

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Abstract—An anaerobic microbial consortium that degrades benzene- and *p*-toluenesulfonate to form methane and fatty acids has been produced. Pure cultures of three strains of anaerobic spore-forming bacteria *Clostridium* spp., as well as the sulfate-reducing bacteria *Desulfovibrio* sp., were isolated and characterized. Phylogenetic analysis of 16S rRNA gene sequences of strains showed that pure cultures of clostridia strains 14, 24, and 21 are close to *Clostridium lituseburense* DSM 797^T, *C. sartagoforme* DSM 1292^T, and *C. pascui* DSM 10365^T, and the sulfate-reducing strain SR1 is genotypically closer to *Desulfovibrio aminophilus* ALA-3^T. Preliminary characterization of isolated bacteria makes it possible to assume that these are new species of the genera *Clostridium* and *Desulfovibrio*, the distinctive feature of which is the ability to incorporate aromatic sulfonates in their metabolisms.

Keywords: anaerobic consortium, aromatic sulfonates, genera of bacteria *Clostridium*, *Desulfovibrio*, benzene- and *p*-toluenesulfonates

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INTRODUCTION

Recent studies have shown that the transformation of aromatic compounds, which can successfully proceed not only under aerobic but also under anaerobic conditions, poses a real threat to human health even at minimal concentrations [1]. The upflow anaerobic granular sludge blanket (UASB) bioreactor technology that uses granules of an active community of microorganisms that degrade pollutants to form methane have continuously been improved and developed over two decades [2]. However, the development and use of anaerobic technology for the treatment of aromatic compounds containing wastewater is hindered by the lack of information on the microorganisms transforming these compounds under conditions facilitating methanogenesis and on the regularities of this process. We obtained a consortium of benzene- and *p*-toluenesulfonate-degrading microorganisms, from which the pure culture of three strains *Clostridiums* sp., two methanogenic bacteria, and a sulfate-reducing bacterium (SRB) *Desulfovibrio* sp. were isolated and partially characterized [3, 4]. The aim of this work was to study the physiological, biochemical, and phylogenetic characteristics of strains of primary and secondary anaerobes involved in the degradation of lower homologs of alkylbenzenesulfonates to form methane.

MATERIALS AND METHODS

Preparation of Anaerobic Consortium of Anaerobic Microorganisms. Granules of the UASB reactor, which was operated with wastewater from papermaking production (the city of Syktyvkar), were disrupted by forcing a suspension of the granules through the needle of a medical syringe and inoculated into 125 mL flasks containing 50 mL of phosphate-buffered basal medium (PBBM) with the following composition (g/L): K_2HPO_4–0.29; KH_2PO_4–0.29; NaCl–1.0; $MgCl_2 \cdot 6H_20$–0.2; NH_4CI–1.0; $CaCl_2$–0.1; cysteine-hydrochloride– 0.5; vitamin solution [5]–5 mL; microelements solution [5]–10 mL; benzenesulfonate (BS) and *p*-toluenesulfonate (TS)–0.4; yeast extract–1.0. The flasks were incubated anaerobically at 29 or 37°C. The association was considered stable after eight passages with full utilization of aromatic sulfonates.

Cultivation of Bacteria. Pure cultures of bacteria were maintained and cultured according to techniques and methods of anaerobic culturing techniques [6]. To maintain a pure culture and to obtain a biomass of clostridia, a medium with the following composition was used (g/L): NH_4C1–1.0; K_2HNPO_4–0.4; $MgCl_2 \cdot 6H_20$–0.1; cysteine hydrochloride–0.5; $Na_2S \cdot 9H_20$–0.5; microelements solution [5]–10 mL; yeast extract–5.0; bactotryptone–2.0; resazurin–0.002; and TS–0.2–0.4. Glucose was further included in the medium for strain 24

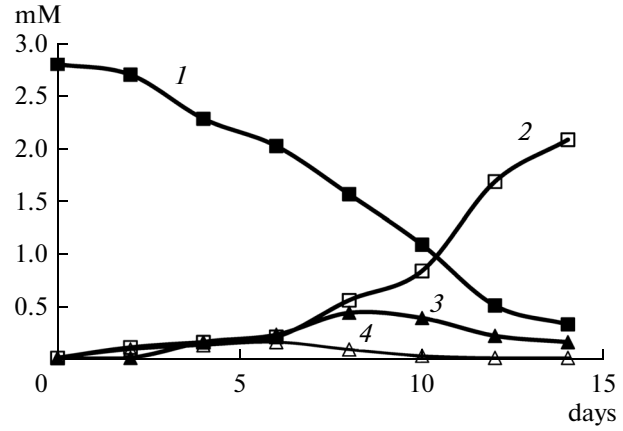

Fig. 1. Formation of methane from *p*-toluenesulfonate by an anaerobic association of microorganisms (mM): *1*–methane, *2*–BS, *3*–TS, *4*–toluene.

(5.0 g/L); histidine (2 g/L) was included for strain 14, and glucose and toluene (0.5 mL/L) were included for strain 21. The SRB strain SR1 was cultured on Widdel and Pfennig's medium [7] with lactate (4 g/L) as an electron donor. All isolated strains, 14, 24, 21 and SR1, were deposited in the All-Russia Collection of Microorganisms (VKM) under the accession numbers B-2201, B-2202, B-2279, and B-2200, respectively.

The cell morphology was studied in a phase contrast using a light microscope Opton ICM 405 (Germany) with a magnification of 100 × 3.2. The physiological characteristics of isolates were studied according to conventional methods [8–9].

Desulfoviridin was determined according to the Postgate's method [9], and sulfide was determined colorimetrically [10].

The fatty acid and methane content in the gas phase were determined using a Pye-Unicam 304 gas chromatograph (United Kingdom) as described previously [3]. Sodium benzenesulfonate (BS), p-toluenesulfonate (TS), and toluene were analyzed by high pressure reverse-phase liquid chromatography. For this, samples of the culture fluid with a volume of 0.5 mL were centrifuged at 8000 *g* for 5 min and the supernatant was analyzed at isocratic mode on a high pressure liquid chromatographer manufactured by Laboratome Pristroje (Czech Republic) with a 15 cm glass column filled with Separol SGXC C_{18} (7 μm) (Tessek, Czech Republic). When determining the BS and TS, the mobile phase consisted of phosphate buffer (pH 6.7) and methanol at 85 : 15, as well as acetonitrile, *n*-butanol, and water in a ratio of 52 : 8 : 40 when the toluene was determined. The flow rates of the mobile phase were 1.5 and 1.0 mL/min, respectively. The analyzed compounds were detected at 254 nm.

Determination of 16S rRNA Gene Nucleotide Sequences. DNA isolation from the bacterial biomass was performed by a method described previously [11]. Universal primers 27F, 1429R, and 515-533F were used to amplify a fragment of the 16S rRNA genes. Polymerase chain reaction (PCR) was performed using a thermocycler Tertsik (DNA Technology, Russia). PCR fragments were synthesized at the following time-temperature regimes: initial denaturation at 94°C for 3 min; subsequent 30 cycles at 94°C for 20 s, at 55°C for 10 s, and at 72°C for 1 min 30 s; final polymerization at 72°C for 3 min. The reaction mixture (25 μL) contained 1 × buffer for Taq-polymerase (Fermentas, Lithuania), 10–50 ng of template DNA, 50 micromoles of each deoxyribonucleotide triphosphates (dNTP) (Fermentas, Lithuania), 10 pmol of respective primers (Synthol, Russia), 2.5 mM $MgCl_2$, and 1 unit of Taq-polymerase (Sileks, Russia). Isolation and purification of fragments from agarose gels were performed using a reagent kit (Sileks, Russia) according to the manufacturer's recommendations. DNA sequencing was performed at the Inter-Institute Center for Collective Use, Genom, of the Institute of Molecular Biology, Russian Academy of Sciences (http://www.genome-centre.narod.ru/) with the ABIPRISM® BigDye™ Terminator v.3.1 reagent kit (Applied Biosystems, United States), followed by analysis of the reaction products on an ABIPRISM 3730 automated DNA sequencer (Applied Biosystems, United States) according to the protocol supplied with the kit.

Phylogenetic Analysis. Preliminary analysis of 16S rRNA gene fragment nucleotide sequences was performed with the software package BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were edited and aligned with the software package Clustal_X [12]. A phylogenetic tree was constructed based on the 16S rRNA gene nucleotide sequences of strains 21, 14, and 42 and the nearest related species of the genus *Clostridium*, with the neighbor-joining algorithm "nearest neighbors" implemented in the software package MEGA 4 [13, 14]. The scale bar was 2 substitutions per 100 nucleotides (Fig. 2). The registration number in the GenBank database in the figures is in parentheses. Data from the bootstrap analysis (expressed as a percentage of 1000 replicas) are indicated at the branch points. The sequences of 16S rRNA genes were placed in GenBank under accession numbers FJ606756–FJ606759.

RESULTS AND DISCUSSION

Degradation of *p*-Toluenesulfonate by a Consortium of Anaerobic Bacteria. With the use of different physicochemical conditions (E_h and pH) and electron acceptors, more than 30 anaerobic enrichment cultures were produced; they were characterized by the ability to degrade BS and TS to varying degrees. Figure 1 shows the dynamics of TS degradation and the formation of methane by an anaerobic microbial community incubated for 14 days at 37°C. As seen from Fig. 1, while reducing the concentration of TS by 85% in the gas phase, 31.2 mmol/L of methane was formed. Com-

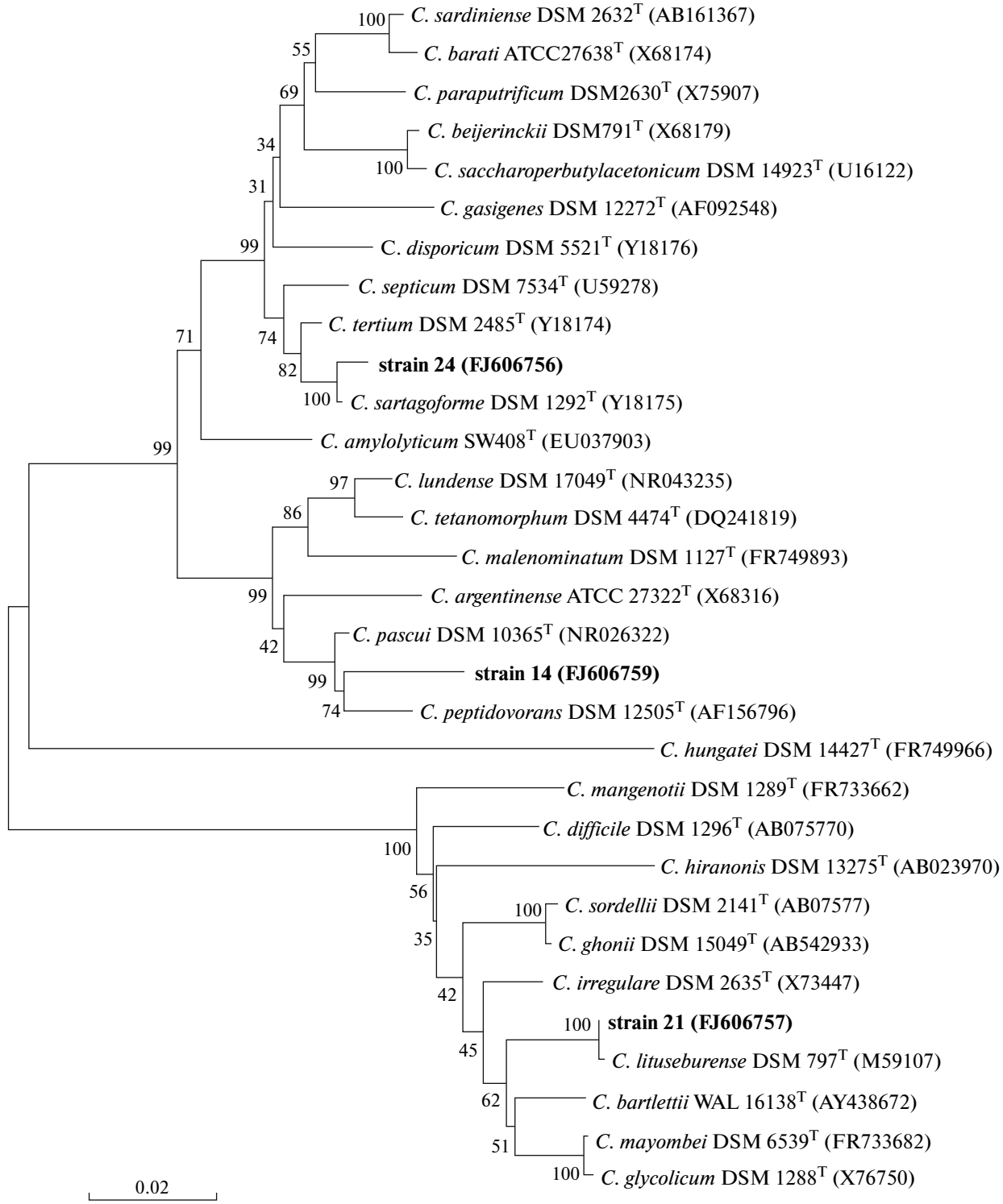

Fig. 2. Phylogenetic tree showing the position of the new strains of *Clostridium* sp., based on the analysis of 16S rRNA genes sequences.

plete degradation of TS took 25–28 days; however, the nature of the intermediates accumulation did not change.

Incubation of the resulting association with BS under the same conditions led to the formation of 18.2 mmol/L methane while reducing the initial concentration of BS to 48%. Under the same conditions during incubation of the association in a medium which did not contain sulfonates, 4.5 mmol/L methane was formed, and incubating cells of the commu-

Table 1. Formation of fatty acids by methanogenic microbial community

Product, g/L	Time, days						
	0	1	2	3	4	6	14
Benzenesulfonate							
Acetate	0	0	0.47	0.65	0.73	0.58	0.46
Propionate	0	0	0.05	0.13	0.10	0.10	0.10
Butyrate	0	0	0.05	0.06	0.06	0.06	0.06
Isobutyrate	0	0	0	0	0	0	0
Valerate	0	0	0.03	0	0.03	0	0
Hexanoate	0	0	0	0	0	0	0
p-Toluenesulfonate							
Acetate	0.10	0.53	0.62	0.70	0.68	0.65	0.43
Propionate	0.0	0.0	0	0	0.05	0.07	0.08
Butyrate	0.02	0.06	0.06	0.06	0.06	0.07	0.07
Isobutyrate	0.02	0.05	0.05	0.05	0.06	0.07	0.07
Valerate	0	0	0	0	0	0.03	0.04
Hexanoate	0	0	0	0	0	0	0.03

nity that were killed by autoclaving in the presence of TS decreased the initial concentration of BS and TS no more than 4% (data are not shown). According to our research, BS and toluene were detected in the culture medium in the process of TS degradation (Fig. 1). They were subsequently also consumed by microorganisms' community. During the development of the community, methane was formed and fatty acids were accumulated. They are essential intermediates of organic matter degradation to methane. The formation of acetate, propionate, butyrate, and isobutyrate was a characteristic feature of the growth of the community, both with BS and TS (Table 1). The content of acetate in the culture broth was much higher than the concentrations of other fatty acids. Its accumulation occurred in the first 3–4 days (0.7–0.73 g/L) and further decreased. We attribute this decrease to the development of acetate-consuming microflora.

The ability of the community to decompose the studied compounds persisted for at least 7–8 passages if the medium contained vitamins and yeast extract (1 g/L). Microscopic analysis of the obtained community showed that 9–10 microorganisms were involved in the degradation of BS and TS, five of which were successfully isolated in pure culture by seeding on selective media via the method of dilution and plating on solid medium to produce colonies [4].

Characteristics of Anaerobic Microorganisms Isolated from Microbial Community Degrading Aromatic Sulfonates to Methane. The acidogenic stage of organic substance anaerobic degradation to methane is carried out by primary and secondary anaerobes to form a wide range of lower fatty acids and defines the rate of the whole process [15]. We have identified three strains of spore-forming anaerobes and a nonsporulating strain of SRB.

Spore-Forming Species. Two strains designated as 14 and 24 were isolated by serial dilution in a medium with TS, and we succeeded in isolating strain 21 using only a medium with the addition of toluene. The isolated strains were obligate anaerobes, did not reduce sulfate and nitrate, and did not require NaCl for their growth. These features, along with the presence of endospores, indicated that the isolates belong to the genus *Clostridium* [16]. Cells of spore-forming strains 14, 42, and 21 are Gram-positive, motile, straight or slightly curved rods with rounded ends with a size of 0.8–1.1 × 3.8–5.8, 0.5–0.6 × 2.0–8.0 and 1.2–1.5 × 5.5–8.5 μm, respectively, with different positions of spores. The isolates were mesophiles and neutrophils.

Strains 24 and 21 were able to use a wide range of carbohydrates and certain complex organic substrates as a source of carbon and energy. Strain 14 did not ferment carbohydrates; it was characterized by growth with glutamic acid, histidine, and tyrosine. The products formed by the new bacteria in PYG medium (strains 24 and 21) and histidine-containing medium (strain 14) were studied. It should be noted that the formation of fatty acids by strains 24 and 14, which are dominated by acetate and butyrate or isobutyrate, was accompanied by the formation of gaseous products. Strain 21 did not form hydrogen and carbon dioxide during fermentation (Table 1). The content of GC pairs in the DNA of strains 14, 42, and 21 was 30.3, 27.9, and 36.1 mol %, respectively.

Phylogenetic Analysis. We obtained partial sequences of 16S rRNA genes of strains 14, 24, and 21 sized 1151, 1435, and 1407 nucleotides, respectively. The comparison of the obtained nucleotide sequences

Table 2. Some physiological and biochemical characteristics of the isolated strains *Clostridium* sp. and closely related species

Strain	Substrate	The use of TS	Fermentation products in the PYG medium	G + C, mol %
14	TS, histidine, glutamine, tyrosine	Ferments	Acetate, isobutyrate, propionate, CO_2, H_2	30.3
C. pascui DSM 10365^T	Histidine, glutamate	Does not use	Acetate, butyrate, ethanol, H_2	27.0
24	Sugars	Used as a source of sulfur	Acetae, isobutyrate, CO_2, H_2	27.9
C. sartagoforme DSM 1292^T	Sugars	Does not use	Acetae, formiate, butyrate, lactate, H_2	28.0
21	Sugars + phenylalanine	Used as a source of sulfur	Acetate, propionate, butyrate, isobutyrate, valerate	36.1
C. lituseburense DSM 797^T	Sugars, phenylalanine, tyrosine, tryptophan	Does not use	Butyrate, acetate, isovalerate, formiate, propionate, ethanol, methanol	27.0

showed that strains 14 and 42 fell in cluster I *Clostridium sensu stricto* (Fig. 2) and were close to *S. sartagoforme* DSM 1292^T and *S. pascui* DSM 10365^T with similarity of 98.7 and 96.4%. The closest neighbor of the strain 21 was *S. lituseburense* DSM 797^T with a similarity of 99.1% of the clostridial cluster XI and was included in a new family, *Peptostreptococcaceae* [17].

Genotypically closely related clostridia had similar spectra of the used substrates and the products formed on the PYG medium (Table 2).

The Use of *p*-Toluenesulfonate by the Isolated Clostridia. We hypothesized that the isolated clostridial cultures can participate in the first stages of degradation of TS or BS. The ability to degrade TS was found in strain 14. Figure 3 shows the dynamics of TS degradation during the growth of strain 14 on the PBBM medium containing TS (Fig. 3, curve *1*) and in the same medium containing TS and histidine (Fig. 3, curve *2*). As seen from Fig. 3, the presence of histidine promoted complete degradation of TS for 14 days. It should be noted that the spectrum of the resulting fatty acids differed: propionate was formed in the absence of histidine, in addition to acetate and isobutyrate and CO_2 and hydrogen.

In the absence of other sulfur sources, strains 24 and 21 could use TS. Toluene at 0.30 and 0. 34 mmol was formed, respectively, per one mmol of consumed per TS. In control experiments with dead cells of clostridia and when other sulfur sources were used (cysteine, sulphate), toluene was not detected in the culture liquid.

Furthermore, the addition of toluene in the culture medium stimulated growth of strain 21, reducing the doubling time from 11.2 to 6.7 hours.

Previously, a number of foreign researchers demonstrated the possibility of using alkyl- and aryl sulfonates as sources of sulfur for *Clostridium* sp. [18]. It is generally known that soil humic substance of a polyaromatic structure is largely sulfated [19]. Consequently, we can assume that the anaerobic desulfurization process is a very probable process during the degradation of humus under anaerobic conditions with the participation of bacteria of the genus *Clostridium*.

Sulfate-Reducing Strain SR1. Strain SR1 was represented by small, mobile, single vibrios, which did not form spores and were Gram-negative when stained. The isolated bacteria were strict anaerobes that withstand an NaCl content to 30 g/L in the medium. The temperature optimum for growth is 37–40°C, and the optimum pH is 7.0–7.2. Strain SR1

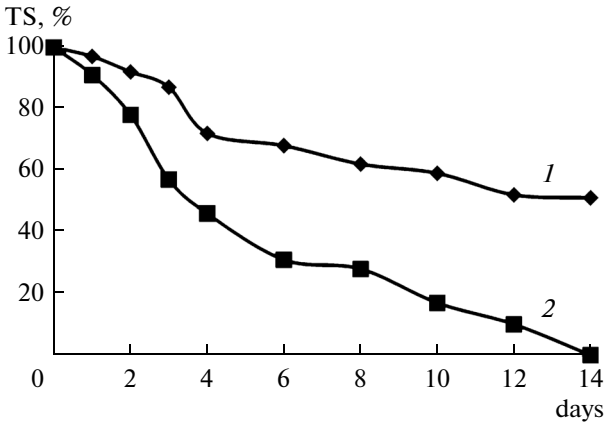

Fig. 3. Consumption of TS by strain 14 on PBBM growth medium containing (1) *p*-toluenesulfonate and (2) histidine and p-toluenesulfonate.

used lactate, pyruvate, $H_2 + CO_2$, and formiate as an electron donor in the presence of sulfate with a doubling time of cells 6.3, 6.8, 7.2, and 10.3 hours, respectively. In the absence of electron acceptors, the strain fermented pyruvate to form acetate and small amounts of valerate. The 16S rRNA gene sequence of strain SR1 had 1429 nucleotides. A search in GenBank with the BLASTn program confirmed the close relationship of the SR1 strain with members of the genus *Desulfovibrio* and, in particular, with the species *D. aminophilus* ALA-3^T, with a nucleotide sequence similarity of 97.8%. The content of G + C pairs in the DNA of strain SR1 was 66.5 mol %, which is close to the G + C content in DNA of *D. aminophilus* [20].

The genus *Desulfovibrio* is widespread in both natural and anthropogenic ecosystems. The representatives of this genus use a variety of substrates for growth and sulfide genesis: from aromatic compounds [21] to amino acids [20, 22] and halogenated hydrocarbons [23]. A characteristic feature of the new strain *Desulfovibrio* sp. is the use of TS as a terminal electron acceptor, along with the sulphate, sulfite, and S^o. The use of 2 mM TS as an electron acceptor by strain SR1 led to the formation of 1.6 mM sulfide. Therefore, we have identified SRB for the first time, which has the unique ability to use a sulfur-containing aromatic compound as electron acceptor. Considering the fundamental phenotypic differences and the fact that the level of similarity between 16S rRNA gene nucleotide sequences is less than 98.5%, we believe that the isolated bacterium is a new species of the genus *Desulfovibrio*.

Anaerobic bacteria isolated from a microbial community degrading aromatic sulfonates to methane are new bacterial taxa of genera *Clostridium* and *Desulfovibrio*. Their unique properties make these bacteria promising model organisms for studying mechanisms of the degradation of aromatic compounds under anaerobic conditions. Our studies have shown that anaerobic bacteria can not only use aromatic sulfonates as a source of sulfur or an electron acceptor but also as a carbon source for growth and metabolism. Subsequent studies will allow us to clarify the picture of the metabolic process.

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