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EXPERIMENTAL ARTICLES

Methanogenic Sarcina from an Anaerobic Microbial Community Degrading *p*-Toluene Sulfonate

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Abstract—The methanogenic strain MM isolated from an anaerobic microbial community degrading *p*-toluene sulfonate showed optimal values of temperature and pH for growth equal to 37°C and 6.3–6.9, respectively. The doubling times of the isolate grown on methanol, acetate, and methylamines under the optimal conditions were 8.8, 19.1, and 10.3–28.1 h, respectively. The growth of strain MM was observed only when the cultivation medium contained casamino acids or *p*-toluene sulfonate. The G+C content of the DNA of the isolate was 40.3 mol %. This, together with DNA–DNA hybridization data, allowed the new isolate to be identified as a strain of the species *Methanosarcina mazei*. The new isolate differed from the known representatives of this species in that it was resistant to alkylbenzene sulfonates and able to demethylate *p*-toluene sulfonate when grown on acetate.

Key words: methanogenic bacteria, *Methanosarcina mazei*, anaerobic biodegradation, demethylation, aromatic compounds.

Methanogenic archaea are important members of anaerobic microbial communities degrading xenobiotics. Consuming hydrogen and acetate from the medium, methanogens enhance the metabolism of fermentative microorganisms and diminish the acidification of the medium, which, in turn, results in a more complete utilization of xenobiotics. Methanogens can utilize such substrates as H_2 + CO_2, formate, methylamines, acetate, alcohols, methylsulfides, pyruvate, methylfurfural, 2-methylindole, and 3-*S*-methylmercaptopropionate [1–5].

The aim of this work was to isolate and characterize the methanogen involved in the degradation of alkylbenzene sulfonates and to study its ability to demethylate *p*-toluene sulfonate.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. The methanogen in question was isolated from an anaerobic microbial community (derived from the granulated biomass of a UASB reactor), which was incubated over an extended period of time in a medium with *p*-toluene sulfonate. For comparative studies, several strains of methanogenic bacteria were used, among them *Methanosarcina mazei* strains S-6 VKM B-1636 (DSM 2053) and 47 VKM B-1637 and *Methanosarcina barkeri* strain MS VKM B-1635 (DSM 800).

The methanogen was isolated from an enrichment culture incubated in medium 1 [7] using the anaerobic Hungate technique [6]. The isolate was cultivated in a liquid medium containing (g/l) K_2HPO_4, 0.27; KH_2PO_4, 0.19; NaCl, 5.0; $MgCl_2 \cdot 6H_2O$, 0.5; NH_4Cl, 1.0; $CaCl_2$, 0.1; and cysteine-HCl, 0.5. The medium was supplemented with 5 ml of a vitamin solution [7], 10 ml of a trace element solution [7], 0.2 g/l casamino acids or *p*-toluene sulfonate, and one of the carbon sources (50 mM acetate, 160 mM methanol, 20 mM methylamine, 20 mM dimethylamine, and 20 mM trimethylamine). To obtain colonies, the medium was solidified by adding 20 g/l Difco agar (United States).

The growth parameters (specific growth rate and doubling time) of the isolate during its exponential growth was determined by the linear regression of the logarithm of the total amount of methane accumulated in the gas phase as a function of time.

The effect of pH on the growth of the isolate was studied using the following buffers: 50 mM MES (pH 5.0–5.8), 0.1 M phosphate (pH 6.0–7.6), 0.1 M carbonate, and 0.1 M Tris–HCl (pH 7.4–9.1). The concentration of sulfide in the medium was measured with 6 mM titanium(III) citrate as the reductant.

Antibiotic sensitivity. The susceptibility of the isolate to antibiotics was assayed using the medium containing (mg/ml) benzylpenicillin, 2.5; vancomycin, 2; cephalosporin, 2; kanamycin, 2; erythromycin, 1;

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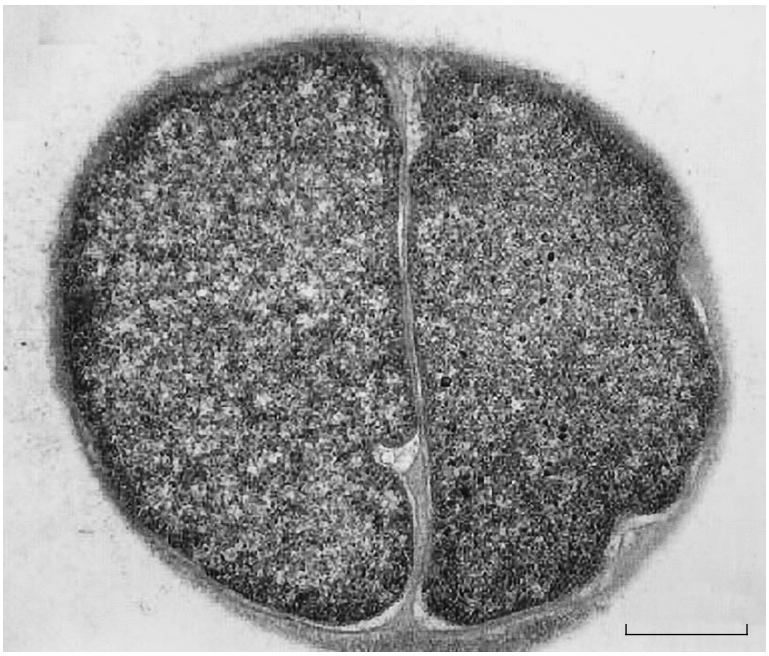

Fig. 1. A thin section of a cell aggregate of strain MM. The scale bar represents 0.5 μm.

chloramphenicol, 0.01; polymyxin, 0.01; and bacitracin, 0.01.

Cell morphology was studied using a Lumam phase-contrast microscope at a magnification of 90 × 15.

Electron microscopy. Thin sections were fixed in a 1.5% solution of glutaraldehyde in 50 mM cacodylate buffer (pH 7.2) at 4°C for 1 h, washed thrice in the same buffer, and refixed in a 1% solution of OsO_4 in the buffer at 20°C for 3 h. The sections were dehydrated in a series of alcohol solutions of increasing concentration, embedded in Epon 812 epoxy resin, mounted on a grid, and contrasted in a 3% solution of uranyl acetate in 70% ethanol and then with lead citrate [8] at 20°C for 4–5 min. The sections were examined in a JEM100 electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

Detergent susceptibility. The susceptibility of logarithmic-phase cells to lysis induced by SDS and sodium dodecylbenzene sulfonate (SDBS) was assessed by incubating the cells in the presence of different concentrations of the detergents, according to [9]. After incubation for 10 min, aliquots (1 ml) of the cell suspensions were centrifuged at 8000 *g* for 10 min, and protein in the supernatant was quantified by the Bradford method.

Immunological and molecular biological procedures. Immunological studies were carried out by the method of indirect immunofluorescence using the bank of antisera to methanogenic bacteria described by Bezrukova *et al.* [10].

DNA was isolated by the Marmur method [11]. The G+C content and hybridization of DNA were measured from renaturation rates [12] using a Pye-Unicam SP1800 spectrophotometer (United Kingdom).

Analytical methods. Methane and methanol were analyzed using a Pye-Unicam 304 gas chromatograph equipped with a (1 m × 2 mm ID) glass column packed with Porapak Q, 80–100 mesh (Fluka, Germany). The temperatures of the column, injector, and flame-ionization detector were 90, 150, and 180°C, respectively. The carrier gas was nitrogen at a flow rate of 20 ml/min. Acetate was analyzed using the same chromatograph equipped with a (2 m × 2 mm ID) glass column packed with Chromosorb W/AW-DMCS, 100–200 mesh (Fluka). The pH of samples was adjusted to 4.0 with

Table 1. The effect of some substances on the doubling time of strain MM grown in the mineral medium with methanol as the carbon source

Substance added	Doubling time, h
None	54.0
$H_2 + CO_2$	32.4
Yeast extract, 2 g/l	60.0
Coenzyme M, 50 mg/l	26.1
Casamino acids, 2 g/l	8.2
Trypticase peptone, 2 g/l	41.3
Liquid from a sewage digestor, 10 vol %	27.3
Rumen fluid, 10 vol %	50.2

Note: The data presented in the table are the means of triplicate experiments.

Table 2. The effect of some substances on the growth parameters of strain MM incubated in the mineral medium with casamino acids

Substance	Growth rate, h^{-1}	Doubling time, h
Methanol	0.079	8.8
Acetate	0.036	19.2
Methylamine	0.025	28.1
Dimethylamine	0.034	20.2
Trimethylamine	0.067	10.3
$H_2 + CO_2$	–	–

Note: The data presented in the table are the means of triplicate experiments.

orthophosphoric acid. The temperature of the column was raised from 80 to 175°C at a rate of 6°C/min. The injector and detector were kept at 150 and 180°C, respectively. The carrier gas was CO_2.

Benzene sulfonate and *p*-toluene sulfonate were analyzed by reversed-phase HPLC. For this, aliquots (0.5 ml) of the culture liquid were centrifuged at 8000 *g* for 5 min, and the supernatant was analyzed using a Laboratorni Pristroje liquid chromatograph equipped with a 15-cm SGXC C18 (7 μm) column (Tessek, Czech Republic). The mobile phase was 100 mM phosphate buffer (pH 6.7) with 15% methanol at a flow rate of 1.5 ml/min. The substances eluted from the column were monitored at 254 nm.

RESULTS AND DISCUSSION

Isolation of strain MM. The strain was isolated from an enrichment culture obtained in medium I with acetate as the growth substrate. The isolation was carried out by the roll tube method of Hungate with penicillin and streptomycin as selective agents. The strain was maintained in a liquid mineral medium containing 50 mM acetate (or 150–300 mM methanol), casamino acids, and/or *p*-toluene sulfonate.

Morphological characteristics. The colonies of strain MM grown for 10 days were white, granular in appearance, and had a diameter of up to 1 mm. When incubated in a liquid medium, the strain grew as small aggregates of, as a rule, four cells. We were unable to obtain the growth of the isolate in the form of individual cocci. A thin section of a cell aggregate is shown in Fig. 1. In many properties, the cells of strain MM resembled methanosarcinas. The cell wall was gram-positive and had a thickness of 40–50 nm. The division of cell aggregates was nonuniform. Many cells contained electron-opaque inclusions (likely polyphosphates), which are typical of methanosarcinas [13].

Nutritive requirements and physiological properties. The effect of various organic substances on the growth of strain MM on methanol is shown in Table 1. Casamino acids stimulated the growth of strain MM most efficiently, as is evident from the decrease in the doubling time from 54 to 8.2 h. In the medium without casamino acids, strain MM lost the ability to grow after 2–3 serial passages. The measurement of the growth parameters of strain MM in the casamino acid–containing medium on substrates typical of methanogens showed that it was unable to utilize $H_2 + CO_2$ (Table 2), the most appropriate growth substrates being methanol (t_d = 8.8 h) and trimethylamine (t_d = 10.3 h). The strain could not grow on formate, lactate, glucose, yeast extract, butyrate, ethanol, and propionate (data not presented). The strain could grow at temperatures from 10 to 50°C (optimum temperature 37°C), at pH from 5.5 to 7.7 (optimum pH 6.6) (Fig. 2a), and at salinities from zero to 0.3 M NaCl (optimum salinity 0.15 M NaCl) (Fig. 2c). The optimal concentration of sulfide in the cultivation medium was 0.09–0.13 mM (Fig. 2b).

Susceptibility to lysis. Cell lysis induced by detergents is a specific feature of methanogens of the genera *Methanococcus*, *Methanomicrobium*, *Methanolobus*, *Methanohalophilus*, and *Methanoplanus* [9]. The induction of cell lysis by SDS suggests that the cell wall is of a protein nature. Among the species of the genus *Methanosarcina*, such lysis was observed for *M. acetivorans* and *M. mazei*. It should be noted that these two species grow not only in the form of cell aggregates but also as individual cocci.

Our visual and microscopic observations showed that the incubation of the new isolate and the type strain of *M. mazei* in the presence of 0.1% SDS induced cell lysis. The quantitative estimation of the degree of cell lysis by measuring the concentration of protein in the supernatant after the removal of cell debris showed that the degree of lysis of both strains in the presence of 0.025 to 0.05% SDS was low (the concentration of protein in the supernatant did not exceed 25 mg/l), but considerably increased (especially in the case of strain MM) in the presence of 0.1% SDS (Fig. 3a).

As was found by Khalil et al. [14], the detergent SDBS at concentrations higher than 20 mg/l stops the growth of the methanosarcina *M. barkeri* and induces damage to the polysaccharide envelope of cell packages. Since strain MM was isolated from the medium containing *p*-toluene sulfonate, it might be relatively resistant to detergents from the class of linear alkylbenzene sulfonates. This assumption was confirmed experimentally (Fig. 3b). As can be seen from this figure, the type strain S-6 underwent substantial lysis in the presence of 0.05% SDBS, whereas the new isolate was fairly resistant to this concentration of SDBS. A notable increase in the degree of cell lysis in strain MM was observed only in the presence of 0.125% SDBS. These data suggest that the cells of strain MM acquired resistance to linear alkylbenzene sulfonates suffering the selective pressure of *p*-toluene sulfonate during the incubation of the anaerobic microbial community from which this strain was isolated.

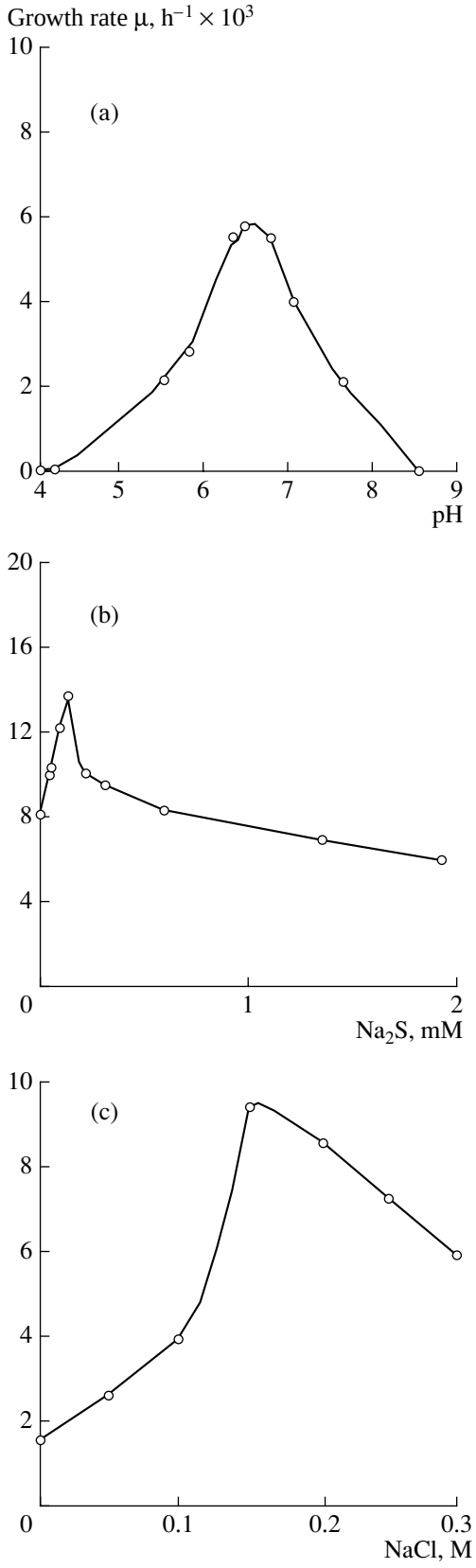

Fig. 2. The effect of (a) pH, (b) Na_2S, and (c) NaCl on the growth rate of strain MM.

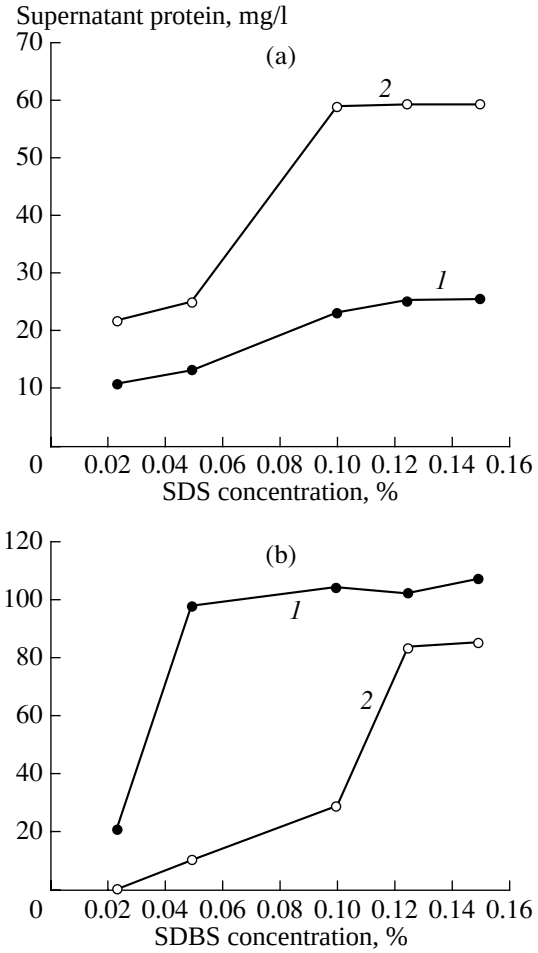

Fig. 3. The effect of (a) SDS and (b) SDBS on the degree of cell lysis in the suspensions of (*1*) *M. mazei* strain S-6 and (*2*) *M. mazei* strain MM.

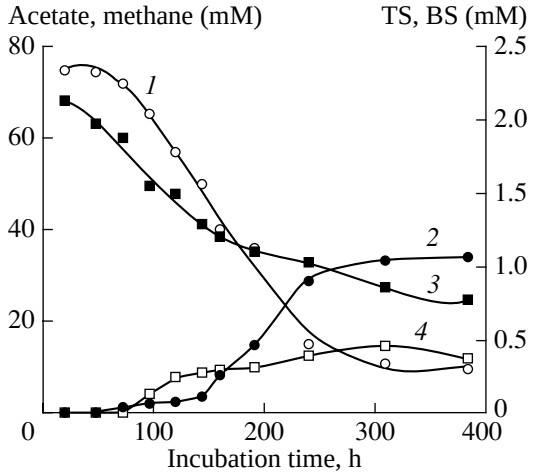

Fig. 4. The formation of methane and degradation of *p*-toluene sulfonate (TS) by cells of strain MM incubated in the acetate-containing medium: (*1*) acetate, (*2*) methane, (*3*) *p*-toluene sulfonate, and (*4*) benzene sulfonate (BS).

Table 3. The degradation of *p*-toluene sulfonate by the washed cells of strain MM grown on acetate or methanol

Experimental variant	Biomass, mg/ml	Initial TS concentration, mM	Final TS concentration, mM	TS consumed, μmol	Methane produced, μmol
Acetate-grown cells					
With TS	1.2	1.98	0.25	17.3	60.0
Without TS	0.9	0	0	0	18.0
Methanol-grown cells					
With TS	1.7	1.87	1.43	4.4	27.7
Without TS	2.0	0	0	0	36.8

Note: TS stands for *p*-toluene sulfonate.

Antibiotic susceptibility. The different susceptibility of methanogenic archaea to antibiotics is widely used for the isolation of pure cultures [7] and for the differentiation of methanogens belonging to different taxa. The new isolate was found to be resistant to penicillin antibiotics (benzylpenicillin, vancomycin, cephalosporin, and erythromycin) but very sensitive to chloramphenicol, polymyxin, and, to a considerable degree, kanamycin (data not presented).

In the cell division pattern, the structure of the cell wall, and the range of utilizable substrates, strain MM is very close to methanogenic archaea of the genus *Methanosarcina.* Furthermore, this strain is very close to the type strain S-6 of the species *M. mazei* but differs from the type strain MS of the species *M. barkeri* in a smaller size of cells and inability to utilize $H_2 + CO_2$ as a sole source of carbon and energy [15].

The nucleotide composition of DNA. The G+C content of the DNA of strain MM was found to be 40.3 mol %, which is within the range typical of *M. mazei* and *M. barkeri* (38.8 to 43.9 mol %). The degree of DNA–DNA homology between strain MM and the species *M. mazei* was 80%. These data, together with the results of morphological, cultural, and physiological studies presented above, allowed the new isolate to be identified as a new strain of the species *M. mazei.*

Immunological analysis. The new isolate, which was analyzed using the immune antisera to various species of the genus *Methanosarcina*, showed some serological affinity to *M. barkeri* (3+, 2+), *M. thermophila* TM-1 (2+), *M. thermophila* TS-2 (1+), and *M. vacuolata* Z-761 (1+), but not to *M. acetivorans* C2A and *M. thermophila* TS-1. The serological similarity between strain MM and the type strain S-6 of *M. mazei* was very high. These data confirmed the assignment of strain MM to the species *M. mazei.* The new strain *M. mazei* MM is deposited in the All-Russia Collection of Microorganisms under the name *M. mazei* MM VKM B-2199.

Demethylation of aromatic compounds by strain MM. The addition of 2 mM *p*-toluene sulfonate to the growth medium containing no casamino acids halved the doubling time of strain MM on acetate but not on methanol. The effect of *p*-toluene sulfonate on the dynamics of acetate consumption and methane production by strain MM is shown in Fig. 4. As is evident from this figure, the decrease in the concentration of *p*-toluene sulfonate from 2.1 to 0.77 mM was accompanied by the formation of about 0.4 mM benzene sulfonate and 1.1 mM methane, suggesting that the methyl group of *p*-toluene sulfonate may be used by the acetate-grown methanosarcina for methanogenesis.

To verify the assumption that strain MM may utilize *p*-toluene sulfonate as a sole carbon source, cells of this strain grown on acetate or methanol were washed free of the medium, suspended in 10 ml of fresh medium with *p*-toluene sulfonate and incubated for two weeks. Analysis showed (Table 3) that the cells grown on acetate consumed 17.3 μmol *p*-toluene sulfonate and produced 60 μmol methane (three times more than the control cells grown in the absence of *p*-toluene sulfonate). At the same time, the cells grown on methanol poorly degraded *p*-toluene sulfonate and produced methane in a smaller amount than in the control (Table 3). Accordingly, only the acetate-adapted cells of strain MM are able to degrade *p*-toluene sulfonate, forming methane.

It is known that anaerobic homoacetic acid bacteria can demethylate methoxylated phenols [16]. Demethylation likely occurs with the involvement of methyl-S-coenzyme M reductase [17]. Furthermore, it has long been known that methanogens of the genus *Methanosarcina* are able to demethylate 3-S-methylmercaptopropionate to 3-mercaptopropionate [5] and those of the genus *Methanococcus* are able to demethylate 2- and 5-furfurals [3]. Some researchers believe that sulfide may serve as an acceptor of methyl groups and that some methanogens are able to grow and produce methane on methyl- and dimethylsulfide [9]. In view of these data, it can be suggested that the metabolism of *p*-toluene sulfonate by strain MM lies in that the methyl group of *p*-toluene sulfonate is transferred to sulfide with the formation of methylsulfide, the latter being used for methanogenesis. This suggestion needs a strong experimental underpinning.

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