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EXPERIMENTAL ARTICLES

Methane Production by the Sulfate-reducing Bacterium *Desulfosarcina variabilis*

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Abstract—The sulfate-reducing bacterium *Desulfosarcina variabilis* VKM B-1694 was found to produce up to 1.62 μmol methane per mg protein when grown on different substrates. The role of methanogenesis and the physicochemical factors determining this process in sulfate-reducing bacteria are discussed.

Key words: methane, methane production, sulfate-reducing bacteria, *Desulfosarcina variabilis* VKM B-1697

Carbon dioxide (CO_2) and acetate are the main products of decomposition of organic matter by sulfate reducers [1, 2]. The ability of sulfate reducers grown on pyruvate to produce minor amounts of methane was shown by J.R. Postgate as early as in 1969 [3]. Later, his finding was confirmed by Schauder *et al.*, who also related the methanogenesis of sulfate reducers to the presence of CO dehydrogenase in these bacteria, since all the strains of sulfate reducers that possessed this enzyme produced from 0.13 to 1.80 nmole methane per 1 l of culture liquid [4].

Methanogenesis has also been reported for other microorganisms, e.g., archaea, which comprise one of the physiological groups of methanogens [5]. These bacteria produce from 0.1 to 0.5 mmol methane per mg protein [6]. The biogenic origin of naturally occurring methane is proved by the direct evaluation of bacterial methanogenesis and the isotopic composition of methane [7].

There is evidence indicating the microbial formation of methane and other hydrocarbons in marine sediments [8–10]. Recent data suggest that sulfate reducers are able to form not only short-chain but also long-chain (C_{14} to C_{25}) hydrocarbons [11].

The present study was undertaken to estimate the methanogenic ability of *Desulfosarcina variabilis* VKM B-1697 during growth on different substrates under different cultivation conditions.

MATERIALS AND METHODS

The type strain *Desulfosarcina variabilis* VKM B-1697 was obtained from the All-Russia Collection of Microorganisms (VKM). This strain was isolated by Widdel from bottom sediments supplemented with benzoate as the sole carbon source [12]. The culture grows well on lactate and pyruvate and slightly poorer on hydrogen plus carbon dioxide and hydrogen plus acetate [12]. Our recent data suggest that this strain can grow on aromatic sulfonates, on benzenesulfonate in particular (unpublished data).

The culture was grown at 33°C in Hungate tubes in a medium of the following composition (g/l): Na_2SO_4, 3.0; KH_2PO_4, 0.2; NH_4Cl, 0.3; NaCl, 13.5; $MgCl_2 \cdot 6H_2O$, 2.2; KCl, 0.5; $CaCl_2$, 0.15; yeast extract, 0.5; Na_2S, 0.025; and a solution of microelements, 1 ml [12]. As carbon sources, we used 5 mM benzoate, 20 mM lactate, 10 mM pyruvate, 2 mM benzenesulfonate, a mixture of $H_2 + CO_2$ (2 atm), and the H_2 + 10 mM acetate in the liquid phase. All the reagents used were of analytical grade. The initial pH of the growth medium was 7.2–7.4.

The purity of the culture was checked microscopically and by plating it onto control media supporting the growth of anaerobes but not of sulfate reducers. One such control medium [5] contained 25 mM methanol to prevent the growth of methanosarcinas.

Analytical methods. Methane content in the gas phase was determined on a series 304 Pye-Unicam gas chromatograph (the United Kingdom) equipped with a flame–ionization detector and a (1m × 2 mm ID) glass column packed with Porapack Q, 80–100 mesh (Fluka, Germany). The temperatures of the column, injector, and detector were 90, 150, and 180°C, respectively. The carrier gas was nitrogen at a flow rate of 20 ml/min.

Benzoate in the culture liquid, from which hydrogen sulfide was preliminarily purged with CO_2, was determined on a high-pressure liquid chromatograph (Laboratorni Pristroje, Czech Republic) equipped with a 15-cm-length column packed with Separon CGC C_{18} (7 μm) (Tessek, Czech Republic). The mobile phase was ammonium phosphate buffer (pH 2.6) and methanol in a proportion of 85 : 15, supplied at a rate of 1 ml/min. Benzoate was detected at a wavelength of 254 nm.

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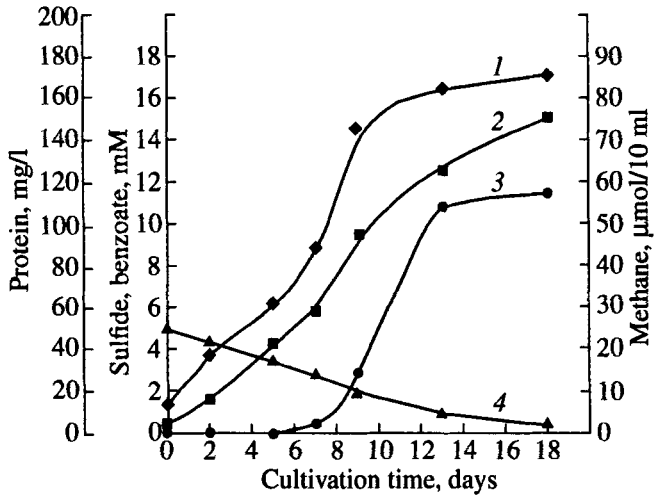

Methane production by *D. variabilis* grown on benzoate: (*1*) protein; (*2*) sulfide; (*3*) methane; and (*4*) benzoate.

Bacterial growth was monitored by measuring the protein content of the biomass by the method of Bradford [13]. Preliminarily, culture samples were centrifuged at 10000 *g* for 10 min, and precipitated cells were hydrolyzed in 1 M NaOH at 100°C for 10 min in a water bath.

Sulfate reduction was evaluated from the increase in the hydrogen sulfide concentration determined by the method of Cline [14].

RESULTS AND DISCUSSION

Methane production by the sulfate-reducing bacterium. *D. variabilis* grown on benzoate was accompanied by the consumption of the substrate, the reduction of sulfate, and the accumulation of biomass (figure). The production of methane in detectable amounts began on the 7th day of bacterial growth, when benzoate content decreased twofold and the biomass increased about sevenfold. In the following days, the methane content increased to comprise 56.8 μmol per 10 ml of culture liquid by the end of cultivation.

The relationship between methanogenesis and substrate concentration. The fact that methane formation began after the amount of substrate per unit of biomass had decreased more than tenfold could indicate that methanogenesis was stimulated by substrate deficiency. To prove this assumption, we varied the initial concentration of benzoate in the growth medium and found that, within the range of benzoate concentrations from 1 to 5 mM, the amount of methane produced by the bacterium per unit biomass was lower the greater the initial substrate concentration in the medium (Table 1). At the initial benzoate concentrations from 5 to 12 mM, methane production was virtually constant (0.37–0.38 μmol/mg protein) and decreased insignificantly (to 0.33 μmol/mg protein) with an extension of cultivation time. For comparison, when the initial benzoate concentration was 1 mM, the methane production was two times more intense (0.68 μmol/mg protein).

Table 1. Methane production by *D. variabilis* grown on different initial concentrations of benzoate

Benzoate concentration, mM	Protein yield, mg/l	Methane production, μmol/mg protein
1	144	0.68
5	252	0.38
12	272	0.37

It may be suggested that methane begins to accumulate only when the concentration of methane precursors is too low to be involved in constructive metabolism.

Methane production by bacterium grown on different carbon substrates. As is evident from Table 2, methane production was maximum (1.33 and 1.62 μmol/mg protein) when *D. variabilis* was grown on $H_2 + CO_2$ and benzenesulfonate, respectively. Such a high rate of methanogenesis on these substrates can be related not only to their specific properties but also to their relatively low concentrations.

Interestingly, the bacterium produced methane when grown on benzoate or lactate but not on their mixture. As noted above, methane may accumulate in the culture only if its precursors are not involved in cell anabolism. Probably, this is not the case during the growth of the bacterium on benzoate plus lactate.

Our previous experiments with samples of bottom sediments from the Indian Ocean showed that sediment microflora can produce hydrocarbons with chain lengths from C_1 (methane) to C_5 (pentane) [10]. The original samples contained sulfate reducers and no hydrocarbons [10]. The maximum production of methane was observed when selective medium for sulfate reducers was inoculated with anoxic mud samples (Table 3). Thus, the data obtained in the present study agree well with those obtained in the previous work: (1) methane was produced in selective media for sulfur reducers and (2) better production of methane was observed in the case of nutritionally deficient selective media. We believe that, depending on the species composition, the abundance in situ, and the availability of necessary substrates, sulfate reducers can essentially contribute to the production of natural gaseous hydrocarbons.

The ability of pure cultures of sulfate reducers to produce methane in trace amounts [3, 4] has been known for a long time. The question that arises here is as follows: Are the amounts of methane that can be produced by pure cultures of sulfate reducers and archaea, as classical methanogens, comparable? So far, it has been conceived that the role of sulfur reducers in methanogenesis is only the supply of substrates to methano-

Table 2. Methane production by *D. variabilis* grown on different substrates

Substrate	Methane production, μmol/10 ml	Protein yield, mg/l	Methane production, μmol/mg protein
Benzoate, 5 mM	56.8	172	0.33
Lactate, 20 mM	57.8	165	0.35
Pyruvate, 10 mM	0	147	0
Benzoate, 5 mM + lactate, 20 mM	0	202	0
$H_2 + CO_2$	69.0	52	1.33
H_2 + acetate, 10 mM	3.6	51	0.07
Benzenesulfonate, 2 mM	68.0	42	1.62

Table 3. Production of gaseous hydrocarbons on various media inoculated with marine mud

Station	Sampling depth, cm	Medium*	Hydrocarbon production, 10^{-6} ml/cm^3 gas phase			
			CH_4	C_2H_6	C_5H_{12}	iC_5H_{12}
3357	10–20	1	5.0	0	0	0
		2	0.2	0	0	0
		3	187.3	43.0	0	0
		4	–	–	0	0
		5	6.2	3.4	0	0
		6	384000	–	295	30.0
3364	0–7	1	65.0	0	0	0
		2	2.8	0	0	0
		3	2.0	3.8	0	0
		4	1846	0	0	0
		5	–	–	0	0
		6	160000	–	0	330
3365	10–20	1	10.0	0	0	0
		2	1.0	0	0	0
		3	14.4	18.8	0	0
		4	126	0	0	0

* The media used in this work: (1) mineral medium [16] with H_2; (2) mineral medium [15] with 5 g/l acetate; (3) agar medium with 10 g/l dextrose, 20 g/l Difco casitone, and reducers ("Anaerobic agar," Difco); (4) Pfenning medium [16] with 2 g/l cellulose; (5) the mixture of media 3 and 4; (6) medium B of Postgate [17] deficient in lactate (0.5 g/l).

gens [1]. In the present work, we showed that *D. variabilis* can produce methane in amounts of up to 70 μmol/10 ml culture (1.62 μmol/mg protein), i.e., in much greater amounts than it was reported by Schauder *et al.* [4] for desulfosarcinas (19 nmol/100 ml culture) and other sulfate-reducing bacteria (13–180 nmol/100 ml culture). The typical concentrations of carbon substrates in nature are several orders of magnitude lower than in laboratory nutrient media for sulfate reducers, which may also be a factor stimulating methanogenesis by these bacteria.

Thus, the present study showed that sulfate reducers may play an important part in methanogenesis accompanying the decomposition of organic matter in nature.

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