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Search for Methanotrophic Producers of Exopolysaccharides

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Abstract—Bacteria that produce exopolysaccharides (EPS) and use methane as the only source of carbon were selected by studying a collection of methanotroph strains: *Methylococcus capsulatus* E 494, 874, and 3009; *M. thermophilus* 111p, 112p, and 119p; *Methylobacter ucrainicus* 159 and 161; *M. luteus* 57v and 12b; *Methylobacter* sp. 100; *Methylomonas rubra* 15 sh and SK-32; *Methylosinus trichosporium* OV3b, OV5b, and 4e; *M. sporium* 5,12, A20d, and 90v; and *Methylocystis parvus* OVVP. Mesophilic methanotroph strains with the ribulose monophosphate way of C_1-compound assimilation synthesized EPS more actively than bacteria operating the serine cycle. The dynamics of EPS synthesis by methanotrophs during chemostat cultivation was studied.

Microbial exopolysaccharides (EPS) are actively used in various industries because EPS change the rheology of aqueous systems and at low concentrations form gels of various densities. The colloid and adhesive properties of EPS and their effects on liquid rheology are used in the food, pharmaceutical, textile, oil, and other industries. The necessity of a new source of raw material for EPS production led to an active search for new producers that would be able to utilize nonedible raw material. It is known that methanotrophs can synthesize EPS from methane [1–5]. EPS synthesized by methanotrophs attract much attention not only due to their traditional industrial use but also due to the prospective application of these biopolymers to binding dust and decreasing the level of methane in coal mines [6, 7].

In this work, the most active producers of EPS stored in the collection of methanotroph strains were selected, and their EPS-synthesizing activity was studied during chemostat cultivation using natural gas as the source of carbon.

MATERIALS AND METHODS

Study objects. Strains of obligate methanotroph bacteria *Methylococcus thermophilus* 105p, 112p, and 119p; *Methylococcus capsulatus* 3009; *Methylobacter ucrainicus* 159 and 161; *Methylobacter luteus* 12b and 57v; *Methylomonas rubra* 15sh and SK-32; *Methylosinus trichosporium* 4e; *Methylosinus sporium* 90v (isolated and described by us earlier in [8, 9]); *Methylosinus trichosporium* OV3b and OV5b; *Methylosinus sporium* 5 and 12; *Methylocystis parvus* OVVR (supplied by R. Vittenbary from Edinburgh University, England [9]); *Methylobacter* sp. 100 (supplied by V. Gal'chenko from Institute of Microbiology, Russian Academy of Sciences, Russia); *Methylococcus capsulatus* E494 and *Methylosinus sporium* A20d (supplied by Yu. Kheier from Central Institute of Microbiology and Experimental Therapy, Germany [9]) and *Methylococcus capsulatus* 874 (supplied by L. Gorskaya from Scientific Research Institute of Protein Synthesis, Russia) were used in this work.

Cultivation of bacteria. Methanotrophic bacteria were cultivated under batch conditions in two mineral media (K and MM) as described earlier in [10]. The K medium contained (g/l): $K_2HPO_4 \cdot 3H_2O$, 0.4; KH_2PO_4, 0.4; NaCl, 0.3; $MgSO_4 \cdot 7H_2O$, 0.4; $FeCl_3 \cdot 6H_2O$, 0.001; and $(NH_4)_2SO_4$, 0.5. The MM medium contained (g/l): $Na_2HPO_4 \cdot 12H_2O$, 7.24; KH_2PO_4, 3.54; $MgSO_4 \cdot 7H_2O$, 0.4; $FeSO_4 \cdot 7H_2O$, 0.01; and $(NH_4)_2SO_4$, 0.6. Bacteria were cultivated in flasks on a shaker (220 rpm) at 30°C for 24–72 h (50°C for strains of *M. thermophilus*).

Continuous cultivation was performed in an AK-10 fermenter (working volume, 4 l). Aeration was performed by feeding 0.3 l/min natural gas mixture (98% methane, 1% ethane, and 1% other hydrocarbons) and air (0.7 l/min). Cultivation was performed at a concentration of dissolved oxygen (pO_2) of 10% air saturation level, pH 6.7, and 30°C. Ammonium nitrogen was used as a bacterial growth-limiting factor (diluted by the medium at a rate of 0.05 h^{-1}). Microelements were added to the mineral K medium containing (μg/l): $CuSO_4 \cdot 5H_2O$, 200; H_3BO_3, 10; $MnSO_4 \cdot 4H_2O$, 10; $ZnSO_4 \cdot 7H_2O$, 70; and $Na_2MoO_4 \cdot 2H_2O$, 10. The concentration of mineral components in the medium introduced into the fermenter increased proportionally to the concentration of biomass. The culture liquid pH in the fermenter was adjusted automatically with 3% NaOH. The purity of methanotrophic strains grown fermenter was monitored by inoculating glucosepotato and meat–

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Table 1. Synthesis of exopolysaccharides by methanotrophs during batch cultivation in K and MM media

Methanotroph	The strain	EPS synthesis, g/g dry cells in the media	
		K medium	MM medium
Ribulose monophosphate way of methane assimilation			
Methylobacter ucrainicus	159	0.16	0.22
	161	0.05	0.06
Methylobacter luteus	12b	0.17	0.24
	57c	0.11	0.13
Methylobacter sp.	100	0.31	0.37
Methylococcus thermophilus	105p	0.19	0.38
	112p	0.21	Is not determined
	119p	0.32	Is not determined
Methylococcus capsulatus	874	0.30	0
	E494	0.19	0
	3009	0.12	0
Methylomonas rubra	SK32	0.26	Is not determined
	15sh	0.30	0.35
Serine way of methane assimilation			
Methylosinus trichosporium	OB3b	0.02	0.01
	OB5b	0.01	Is not determined
	4e	0.01	0.01
Methylosinus sporium	5	0.02	0.01
	12	0	Is not determined
	A20d	0.01	0.01
	90c	0	0.01
Methylocystis parvus	OBBP	0.02	0.03

Table 2. Production of EPS by methanotroph under chemostat cultivation

Methanotroph	EPS-synthetic activity, g/g dry cells	Viscosity of the culture liquid, mPa s
Methylobacter luteus 12b	0.17 ± 0.03	350 ± 40
Methylobacter ucrainicus 159	0.55 ± 0.05	110 ± 30
Methylobacter sp. 100	0.30 ± 0.05	170 ± 30
Methylomonas rubra 15sh	0.06 ± 0.03	290 ± 35

peptone agar media. The absence of microbial growth in these media indicated the absence of contaminating bacteria.

Characterization of bacterial growth and EPS synthesis. The biomass growth was determined by an increase in the optical density (D_{540}) of the cell culture followed by calculation per unit weight of oven-dried cell biomass using a calibration curve.

The amount of synthesized EPS was determined as the concentration of carbohydrates by the reaction with phenol and sulfuric acid as described in [11]. The rate of EPS synthesis in bacteria was expressed as the amount of EPS that was produced by 1 g of bacterial biomass (gram EPS per gram dry biomass).

The concentration of ammonium nitrogen in the culture liquid was determined by the Nessler method [12].

Isolation of EPS. EPS were isolated as described in [13]. A twofold to threefold dilution of the culture liquid with distilled water was performed. Then NaCl at a final concentration of 0.01 M was added, and the cells were harvested by centrifugation (12 000*g*, 40 min). The supernatant was dialyzed against distilled water for three days, then concentrated under vacuum (50°C) to the initial volume, and EPS were extracted by organic solvents (ethanol and isopropyl alcohol). The precipitated EPS obtained were washed with pure solvent and dried at room temperature. The amount of carbohydrates in EPS was determined by the reaction with phenol and sulfuric acid. The cinematic viscosity of EPS solutions was determined in a VPJ-4 glass capillary viscometer (Soyuznauchpribor, Russia) at 20°C.

RESULTS AND DISCUSSION

Selection of methanotrophic producers of EPS. During batch cultivation, the methanotrophic strains produced EPS at different rates (Table 1). The highest EPS-synthetic activity was found in the strain with the ribulose monophosphate (RMP) pathway of assimilation of C_1-compounds (0.05 to 0.32 g EPS per gram dry biomass) in MM medium (Table 1). However, the strains *M. capsulatus* E494, 874, and 3009 (RMP-way) displayed virtually no growth in MM medium because of a high concentration of phosphates and therefore produced no EPS. The strains *Methylobacter* sp. 100, *M. ucrainicus* 159, *M. luteus* 12b and *Methylomonas rubra* 15sh displayed the equally high EPS-synthetic activities in both media (0.2–0.3 g EPS per gram dry biomass). Methanotrophs with the serine cycle of C_1-assimilation displayed practically no EPS synthesis (Table 1).

Thus, it was shown that methanotrophs with the RMP pathway of C_1-assimilation synthesized EPS more actively compared to bacteria with the serine cycle, which is consistent with data reported in [1]. It was suggested that the productivity of EPS synthesis in methylotrophs depends on the way of carbon assimilation [1, 14]. Theoretical calculation of stoichiometry of polysaccharide formation from C_1-compounds showed

that the economic coefficient per carbon substrate (Y, g EPS/g substrate) was significantly higher (by 25–28%) in bacteria with the RMP pathway of C_1-compounds assimilation compared to bacteria with the serine cycle [14]. However, there is contradicting evidence [2, 15]. It cannot be excluded that the increased synthesis of EPS in methanotrophs with the serine cycle occurred under unfavorable and/or extreme conditions. For example, EPS synthesis in *Methylocystis parvus* OVVR was observed during assimilation of methanol after long-term adaptation to this substrate [15].

Synthesis of EPS under continuous cultivation. Under chemostat conditions, EPS-synthetic activity was studied in mesophilic methanotrophs with the RMP pathway of C_1-assimilation (Fig. 1). Ammonium nitrogen was used as the cell population growth-limiting factor because the solubility of methane (the carbon source) was very low in the liquid mineral medium (as well as water), and EPS synthesis in bacteria depends on the medium carbon/nitrogen ratio. The effect of other factors was not studied. Active synthesis of EPS was found under chemostat cultivation of *Methylobacter ucrainicus* 159, *Methylobacter* sp. 100, *M. luteus* 12b, and *Methylomonas rubra* 15sh (Table 2). The concentration of synthesized EPS was 0.5–0.8 g/l; the EPS-synthetic activity varied in a concentration range of 0.17 to 0.55 g EPS per gram dry biomass; the viscosity of the culture liquid varied from 110 to 350 mPa s. These results are consistent with data on EPS-synthesis by *Methylococcus thermophilus* 111p, a thermophilic methanotroph [1].

The three strains of the genus *Methylobacter* (12b, 100, and 159) synthesized EPS in the form of a cell-associated conglomerate that contained EPS and cells and was precipitated on the bottom of the flask 30 min after sampling the culture liquid from the fermenter. The cells with EPS were sedimented by centrifugation. The conditions allowing separation of EPS from cells were studied: heating of the culture liquid (80°C for 20 min) or alkalization (pH 12) followed by neutralization to pH 7. After this treatment of the culture liquid, it was possible to isolate EPS by the usual method described under Materials and Methods.

It was shown that the viscosity of 0.1% aqueous solutions of EPS synthesized by mesophilic methanotrophs varied from 2.2 to 4.0 mm^2/s, which is comparable to the viscosity of EPS solutions synthesized by known microbial produces in carbon raw material: the viscosity of 0.1% solution of xanthane (Sigma, USA) was 3.5 to 4.5 mm^2/s. The highest viscosity was determined in 0.1% solution of EPS synthesized by *M. rubra* 15sh. EPS synthesized by *M. rubra* 15 sh contained carbohydrates (56–62%); protein was not found in this preparation. After treatment with Cetavlon, cetyltrimethylammonium bromide, from aqueous solutions EPS was precipitated which indicated the presence of acid groups in EPS. The viscosity of the EPS solution did not change in the pH range of 5 to 10 and in the presence of some salts (KCl, NaCl, and $CuSO_4$) in the concentration range of 0.005 to 0.1 M.

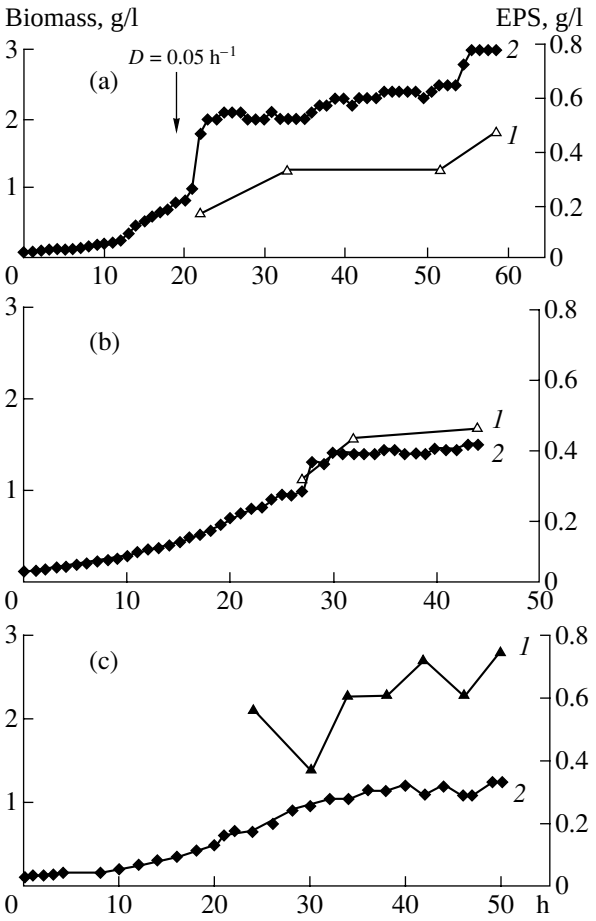

Fig. 1. Synthesis of (*1*) EPS and (*2*) biomass by bacteria of (a) *Methylobacter luteus* 12b, (b) *Methylobacter ucrainicus* 159 and (c) *Methylobacter* sp. 100 during cultivation in the chemostat mode.

The activity of EPS synthesis in the mesophilic methanotroph we studied corresponded to the same in known methanotrophic produces of EPS. It was shown in [2] that the EPS-synthetic activity in methanotrophs varied from 0.03 to 0.43 g EPS per gram dry biomass. The EPS-synthetic activity (0.17–0.55 g EPS per gram dry biomass) was higher in *Methylomonas rubra* 15sh, *Methylobacter ucrainicus* 159, *Methylobacter* sp. 100, and *M. luteus* 12 b. However, the concentration of synthesized EPS (0.5–0.8 g/l) was lower than that in *Methylococcus thermophilus* 111p [1]; *M. capsulatus* 874 [4]; and unidentified methane-using bacteria H-2 [3], which produced EPS at concentrations of 1.0, 1.64–1.84, and 1.8 g/l, respectively. It cannot be excluded that thermotolerant methanotrophs, namely, the strains H-2 [3], 874 [4], and 111p [10] (grown at 45–55°C), are the most active produces of EPS. These data allowed us to select mesophilic methanotrophic produces of EPS: *Methylomonas rubra* 15 sh, *Methylobacter ucrainicus* 159, *Methylobacter* sp. 100, and *M. luteus* 12b, which

were grown at 30°C. The viscosity (2.2–4.0 mm^2/s) of 0.1% aqueous solutions of EPS produced by these strains corresponded to the viscosity of EPS synthesized by thermotolerant methanotrophs and known industrial microbial producers of EPS.

Thus, the most active producers of EPS that use natural gas were selected: *Methylomonas rubra* 15sh, *Methylobacter ucrainicus* 159, *Methylobacter* sp. 100, and *Methylobacter luteus* 12b. These producers can be used for EPS production directly in coal mines (in fermenters using methane from coal beds [16]) and additionally used as the culture liquid containing EPS for binding dust and decreasing the level of methane in coal mines [6, 7]. In principle, these producers can also be used for obtaining EPS in the oil industry in mobile plants using oil by-product gases rich in methane and for intensification of petroleum production by pumping a methanotroph culture liquid containing EPS into abandoned oil wells, similar to the procedure that uses EPS synthesized by *Acinetobacter* sp. [17].

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