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EXPERIMENTAL ARTICLES

Antioxidant Systems of Moderately Thermophilic Methanotrophs *Methylocaldum szegediense* and *Methylococcus capsulatus*

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Abstract—Moderately thermophilic methanotrophs *Methylocaldum szegediense* O-12 and *Methylococcus capsulatus* Bath exhibit activities of antioxidant protection enzymes: glutathione peroxidase, superoxide dismutase, and cytochrome *c* peroxidase. The cells of methanotrophs grown at optimal temperatures (57 or 45°C, respectively) produce reactive oxygen species more actively than those grown at suboptimal temperatures, and exhibit higher activities of the membrane-associated cytochrome *c* peroxidase. Glutathione, glutathione peroxidase, and glucose-6-phosphate dehydrogenase levels in *Md. szegediense* O-12 increased in response to lowering of the cultivation temperature. By contrast, glutathione accumulation in cells of *Mc. capsulatus* Bath and the activity of glutathione peroxidase at a suboptimal temperature (29°C) were lower than at the optimal one. The role of the multilevel system of antioxidant protection in the adaptation of methanotrophs to temperature fluctuations is discussed.

Key words: thermophilic and thermotolerant methanotrophs, *Methylocaldum szegediense, Methylococcus capsulatus*, ROS.

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Methanotrophs are a group of gram-negative aerobic bacteria, structurally and functionally specialized in utilizing methane as the sole energy and carbon source. These bacteria have been found in a variety of ecosystems, including biotopes with elevated temperatures, where their presence is limited by the solubility of methane and oxygen [1–3]. Methanotrophs isolated from high-temperature ecosystems are represented by thermophilic (*Methylocaldum szegediense* and *Methylothermus thermalis*) or thermotolerant strains (*Methylococcus capsulatus, Methylocaldum gracile*, and *Methylocaldum tepidum*), as well as thermoacidophilic obligate methanotrophs of the phylum *Verrucomicrobia* [3]. Although the optimal temperatures for growth of known thermophilic methanotrophs reach 55°C, these bacteria have been detected not only in the ecosystems with constantly high temperatures (geothermal springs), but also in relatively low-temperature biotopes, where the temperature is subject to significant variations, such as municipal and industrial sewage (*Mc. capsulatus*) [3], fields and waste from animal farms (*Methylocaldum*) [4], and lake sediments (*Methylothermus*) [5]. Accordingly, thermophilic and thermotolerant methanotrophs are capable of adaptation to significant temperature variations in natural econiches.

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Oxidative stress is among the most important consequences that the cells experience under changing environmental conditions, including elevated temperature. Oxidative stress is characterized by increased levels of reactive oxygen species (ROS), which include superoxide anion ($^{\bullet}O_2^-$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide. Excessive accumulation of these ROS may lead to mutations, damage, and even death of cells. ROS levels are sustained within physiological limits due to the functioning of specialized antioxidant protection systems, which include catalase, superoxide dismutase (SOD), and peroxidase, as well as a number of other components [6, 7]. However, it is not known which enzymatic systems are involved in the regulation of ROS levels in the cells of methanotrophs at different temperatures.

The goal of the present work was to detect antioxidant systems in a moderately thermophilic strain *Methylocaldum szegediense* O-12 and in a thermotolerant strain *Methylococcus capsulatus* Bath, growing at optimal or suboptimal temperatures.

MATERIALS AND METHODS

Objects of investigation. In the present research, two strains were used. Moderately thermophilic *Methylocaldum szegediense* O-12 was isolated from the waste

of animal farms in the precincts of Pushchino [13], and thermotolerant *Methylococcus capsulatus* Bath was provided by Prof. C.J. Murrell, University of Warwick, United Kingdom.

Culturing of the methanotrophs. *Mc. capsulatus* Bath was grown on a mineral medium "P" [2]. *Md. szegediense* O-12 was cultured on a mineral medium Pm, similar in composition to "P", except for addition of 1 mg/l of $CuSO_4 \cdot 5H_2O$ (pH of the medium was 7.2) in 750-ml flasks containing 100 ml of the medium. The inoculum was added to the medium at a ratio of 1 : 10. The flasks were filled with a methane–air mixture (1 : 1) and shaken at 100 rpm on a Clim-O-Shake rotary shaker (Switzerland) at 45 or 57°C for *Md. szegediense* O-12 and at 29 or 45°C for *Mc. capsulatus* Bath.

Visualization of the peroxide-decomposing activity in the cells of *Md. szegediense* O-12. Cells grown at 45 or 57°C were centrifuged at 6000 *g* for 10 min and resuspended in a 0.05 M cacodylate buffer. The control samples were heated for 5 min at 95°C. The cells (~0.1 g) were fixed with 0.1% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.5) for 1 h at 4°C, then washed with the same buffer and transferred into 10 ml of the incubation mixture containing 0.05 Tris–HCl buffer (pH 9), 25 mg of 3,3-diaminobenzidin tetrahydrochloride, and 0.1 ml of 3% H_2O_2. A reaction mixture without H_2O_2 was used as an additional control. The mixtures were incubated at room temperature in the dark, and then the cells were washed with 0.05 M cacodylate buffer and fixed with osmium tetroxide [9]. Ultrathin sections were prepared using a Reichert ULTRACUT System ultramicrotome (Austria) and examined with a Jeol JEM 100B electron microscope (Japan), as earlier described [10].

Analytical investigations. Potential activities of ^{14}C-methane uptake by whole cells were determined by the radioactive tracer technique [11]. The total glutathione content (GSH + GSSG) was determined by thionitrobenzoate production from 5,5'-dithio-*bis*(2-nitrobenzoic acid) (DTNB) in the presence of SH groups at 412 nm [12]. Fractionation of the cell components was performed as described in [13].

The ROS content was assayed by a modified spectrofluorometrical method [14]. The exponential-phase cells were concentrated by centrifugation of 1 ml of the suspension ($OD_{600} = 2$) at 6000 *g* for 10 min. The pellet was transferred to penicillin vials containing 5 ml of methane in the gas phase and incubated for 1 h on the Clim-O-Shake rotary shaker (Switzerland) at the relevant temperatures. Then, after addition of 2,7-dichlorofluorescein to the final concentration of 100 μM, the vials were blown through with 5 ml of methane and incubated on the rotary shaker for another 30 min. The fluorescence intensity of the suspensions was measured with a Hitachi 850 spectrofluorophotometer in quartz cells with an optical path length of 1 cm at the wavelengths of excitation and emission of 480 and 521 nm, respectively. The fluorescence values obtained were recalculated per the intracellular protein content, the latter determined by Lowry assay [15].

The quantity of products that react with thiobarbituric acid (TBA) was assayed by spectrophotometry. The biomass (0.5 g) was treated successively with 1 ml of 20% trichloroacetic acid (TCA) and 2 ml of 46 mM TBA; the mixture was boiled in a water bath for 30 min, cooled, and subjected to photometrical analysis at 532 nm [16]. The fluorescence values thus obtained were recalculated per the intracellular protein content.

Enzyme activity measurements. Catalase activity was determined by the decrease in H_2O_2 content at 240 nm (E = 42.6/M cm), in the reaction mixture (1 ml) containing 0.05 M potassium phosphate buffer (pH 7), 14 mM H_2O_2, and protein extract (1 mg of protein) [17]. Glutathione peroxidase activity was determined by NADPH oxidation at 340 nm in the reaction mixture (1 ml) containing 0.01 M Tris–HCl buffer (pH 7.6), 0.2 mM NADPH, 0.5 mM EDTA, 2 mM reduced glutathione (GSH), 1 U of GSH reductase, 150 μM H_2O_2, and the extract [17]. The glutathione reductase activity was determined by NADPH oxidation at 340 nm in the reaction mixture (1 ml) containing 0.01 M phosphate buffer (pH 7.2), 0.5 mM EDTA, 5 μM FAD, 2.2 mM oxidized glutathione (GSSG), and the extract. The reaction mixture was transferred to a quartz cell and, after 5-min incubation, 0.2 mM NADPH was added [17]. Cytochrome *c* peroxidase activity was determined by a reaction with 4-aminoantipyrine (AAP), ammonium-2,2'-azino-*bis*(3-ethylbenzthiazoline-6-sulfonate) (ABTS), or 2,6-dimethoxyphenol (DMP). Peroxidase activity with AAP was measured at 510 nm (E = 13.16 mM) in a reaction mixture containing 0.2 M phosphate buffer (pH 7.0), 1.7 mM H_2O_2, 0.17 M phenol solution with 2.5 mM 4-AAP, and the extract. Activity with ABTS was measured at 436 nm (E = 29 mM), in the reaction mixture containing 50 mM Tris–HCl buffer (pH 7.5), 1.7 mM H_2O_2, 0.5 mM ABTS, and the extract. Activity with DMP was measured at 468 nm (E = 14.8 mM) in the reaction mixture containing 50 mM Tris–HCl buffer (pH 7.5), 1.7 mM H_2O_2, 0.5 mM DMP, and the extract [18]. Peroxidase activity was also measured after electrophoretical separation of the proteins of the extract in a thin PAG (polyacrilamide gel) layer [19], according to [20]. Superoxide dismutase (SOD) activity was determined spectrophotometrically at 560 nm, by determining the inhibition of the nitro-blue tetrazolium (NBT) reduction by xanthine oxidase, in the reaction mixture containing 50 mM Tris–HCl buffer (pH 8), 0.056 mM NBT, 0.05 mM xanthine dissolved in 0.004 N NaOH, 0.1 mM disodium EDTA, 20 μM KCN, and 0.08 U of xanthine oxidase. Fifty-percent inhibition of NBT reduction was designated as a unit of SOD activity. Also, SOD was detected after electrophoretical separation of proteins in 10% (PAG), according to the recommendations given in [21]. The protein concentrations in the extracts were determined using a modified Lowry assay [22].

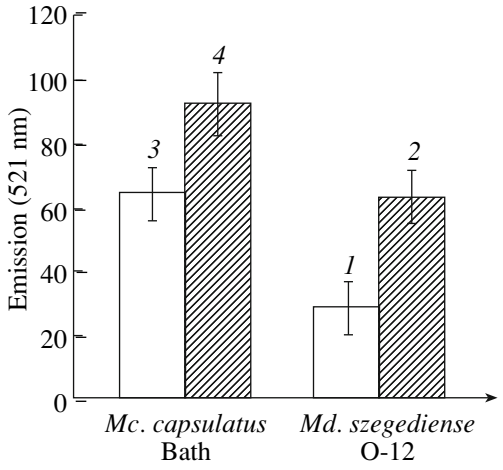

Fig. 1. Fluorescence intensity in the cells of methanotrophs *Md. szegediense* O-12 grown at 45°C (*1*) or 57°C (*2*), and *Mc. capsulatus* Bath, grown at 29°C (*3*) or 45°C (*4*), in the presence of 100 μM 2,7-dichlorofluorescein diacetate. The fluorescence was registered at wavelengths of excitation and emission of 480 nm and 521 nm, respectively.

RESULTS

The rates of methane oxidation and assimilation by *Md. szegediense* O-12 suspensions were two- to three-fold higher at the incubation temperature of 57°C (8 and 1.4 nmol mg^{-1} of dry cells min^{-1}), than at 45°C (3.5 and 0.5 nmol mg^{-1} of dry cells min^{-1}). Generally, the rates of methane oxidation corresponded to the growth rates of the culture at these temperatures (0.066 and 0.027 h^{-1}, respectively). The rates of methane oxidation and assimilation by the cells of *Mc. capsulatus* Bath reached a maximum at 45°C (11 and 4 nmol mg^{-1} of dry cells min^{-1}, respectively), which is significantly higher than the corresponding values at 29°C (0.92 and 0.22 nmol mg^{-1} of dry cells min^{-1}). The growth rates of *Mc. capsulatus* Bath at these temperatures were 0.077 and 0.048 h^{-1}, respectively.

ROS levels in the cells of methanotrophs were assayed using a dye of 2,7-dichlorofluorescin diacetate, which penetrates into the cells and fluoresces when activated by ROS [23]. The fluorescence of exponentially growing cells of *Md. szegediense* O-12 and *Mc. capsulatus* Bath was respectively 2.2-fold and 1.4-fold more intensive at the temperatures close to optimal (57 and 45°C, respectively) than the fluorescence of the cells growing at low temperatures (Fig. 1). Accordingly, an increase of the cultivation temperatures was accompanied by an increase in ROS production, with a distinct correlation between the rate of ROS production and the growth rates of cultures and rates of methane oxidation and assimilation.

The content of lipid peroxides was determined in the cells of methanotrophs, by the TBA reaction [16]. The relative quantity of products reacting with TBA was higher in the cells of *Mc. capsulatus* Bath growing at the optimal temperature (45°C), and in cells of *Md. szegediense* O-12 at a suboptimal temperature (45°C) (Fig. 2).

A large intracellular pool of total glutathione (GSSG + GSH) was revealed in *Mc. capsulatus* Bath. This pool was three times larger at the optimal temperature (45°C) than at the lowered temperature (29°C). By contrast, in the cells of *Md. szegediense* O-12 grown at 45°C, the total glutathione level was sevenfold higher than that at the optimal temperature of 57°C (Fig. 2). This may be an indication of the different role glutathione plays in the cells of these bacteria.

Cell extracts of the studied methanotrophs exhibited glutathione peroxidase activity (table). The latter was practically identical in *Mc. capsulatus* Bath cells grown at 29 and 45°C. However, *Md. szegediense* O-12 cells displayed higher glutathione peroxidase activity when grown at 45°C than at 57°C (table). At decreased culti-

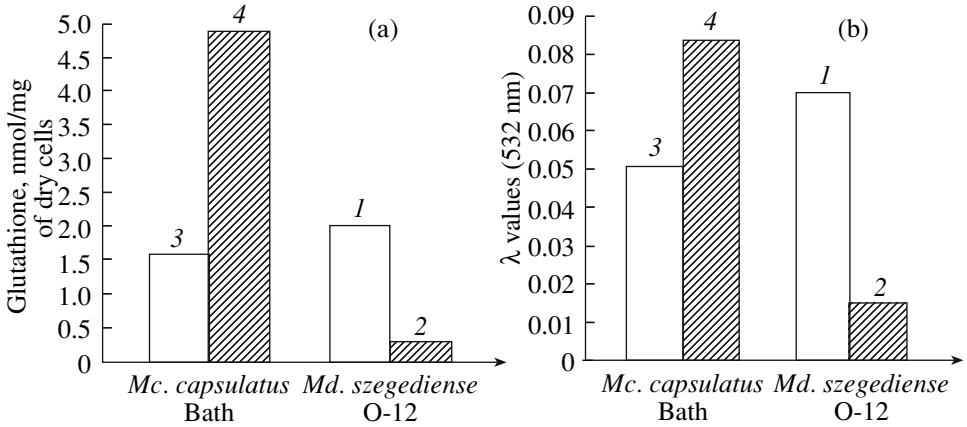

Fig. 2. Concentrations of glutathione (a) and TBA-reactive products (b), as indicators of lipid degradation in the cells of methanotrophs *Md. szegediense* O-12, grown at 45 or 57°C, and *Mc. capsulatus* Bath, grown at 29 or 45°C.

Enzyme activities and glutathione levels in the extracts of *Md. szegediense* O-12 and *Mc. capsulatus* Bath cells grown at different temperatures (nmol/min mg of protein)

Enzyme	Substrate/cofactor	*Md. segediense* O-12		*Mc. capsulatus* Bath	
		45°C	57°C	29°C	45°C
GSH peroxidase	H_2O_2, GSH	17	11	3	4
Glutathione reductase	GSSG, NADPH	2	3	1	2
Glucose-6-phosphate dehydrogenase	$NADP^+$	52	29	10	2
Catalase	H_2O_2	0	0	0	0
Superoxide dismutase	$O^{\bullet}$	2	2	1.5	1.5
Peroxidase	ABTS	7	13	47	60
″	Dimetoxyphenol	15	26	24	35
″	4-AAP	4	6	4	8
RuBisCO		5	9	3	6
Oxypyruvate reductase	NADH	2	22	25	119

vation temperatures, the activity of glucose-6-phosphate-dehydrogenase ($NADP^+$) also increased in both strains; this enzyme provides NADPH for the reduction of GSSG by glutathione reductase.

The soluble fraction of the cells of both strains was found to contain another enzyme that utilizes H_2O_2, namely peroxidase, which was active with ABTS, DMP, and 4-AAP (table). These artificial electron donors are used to determine the activity of heme-containing peroxidases. In the genome of *Mc. capsulatus* Bath, four proteins pertaining to the heme-containing peroxidases were detected. These were identified as cytochrome *c* peroxidases [24]. We showed that peroxidase activities of the two methanotrophs differed in their specificity to artificial substrates. Thus, in *Mc. capsulatus* Bath, the peroxidase activity was maximal with ABTS, and in *Md. szegediense* O-12, with DMP. This points to different properties of cytochrome *c* peroxidases in the studied methanotrophs. The peroxidase activity was higher in both methanotrophs grown at 57°C and 45°C, reaching a maximum at the measurement temperatures from 65 to 80°C.

The localization of peroxidase activity was revealed by a cytochemical approach, for which purpose *Md. szegediense* O-12 cells were incubated with H_2O_2 and diaminobenzidine. On ultrathin sections, the electron-dense reaction products were mostly membrane-associated (Fig. 3). In nonboiled cells, peroxidase activity was also detected under peroxide-free conditions, possibly due to the production of endogenous H_2O_2. Furthermore, the intracellular localization of the enzyme was studied by fractionating the cells of *Md. szegediense* O-12 destroyed by osmotic shock. The resulting periplasmic, cytosolic, and total membrane fractions were separated by electrophoresis in PAG in the presence of SDS (Fig. 4). Peroxidase activity was revealed primarily in the membrane and periplasmic fraction (Fig. 4), as consistent with the results of the cytochemical experiments.

Cell-free extracts of both methanotrophs exhibited SOD activities that were independent of the cultivation temperature (table). As shown by the native PAG electrophoresis, *Mc. capsulatus* Bath contained two distinct proteins with SOD activity, differing in molecular mass, whereas *Md. szegediense* O-12 exhibited an additional major band possessing this activity (Fig. 5). Two types of SOD are known, containing Mn or Fe in their active sites; the Fe-containing enzyme is inhibited by the reaction product, H_2O_2 [25]. Accordingly, to determine the SOD type, the gel was incubated in the presence of H_2O_2 and KCN (to inhibit peroxidase), prior to the activity measurement. Hydrogen peroxide inhibited

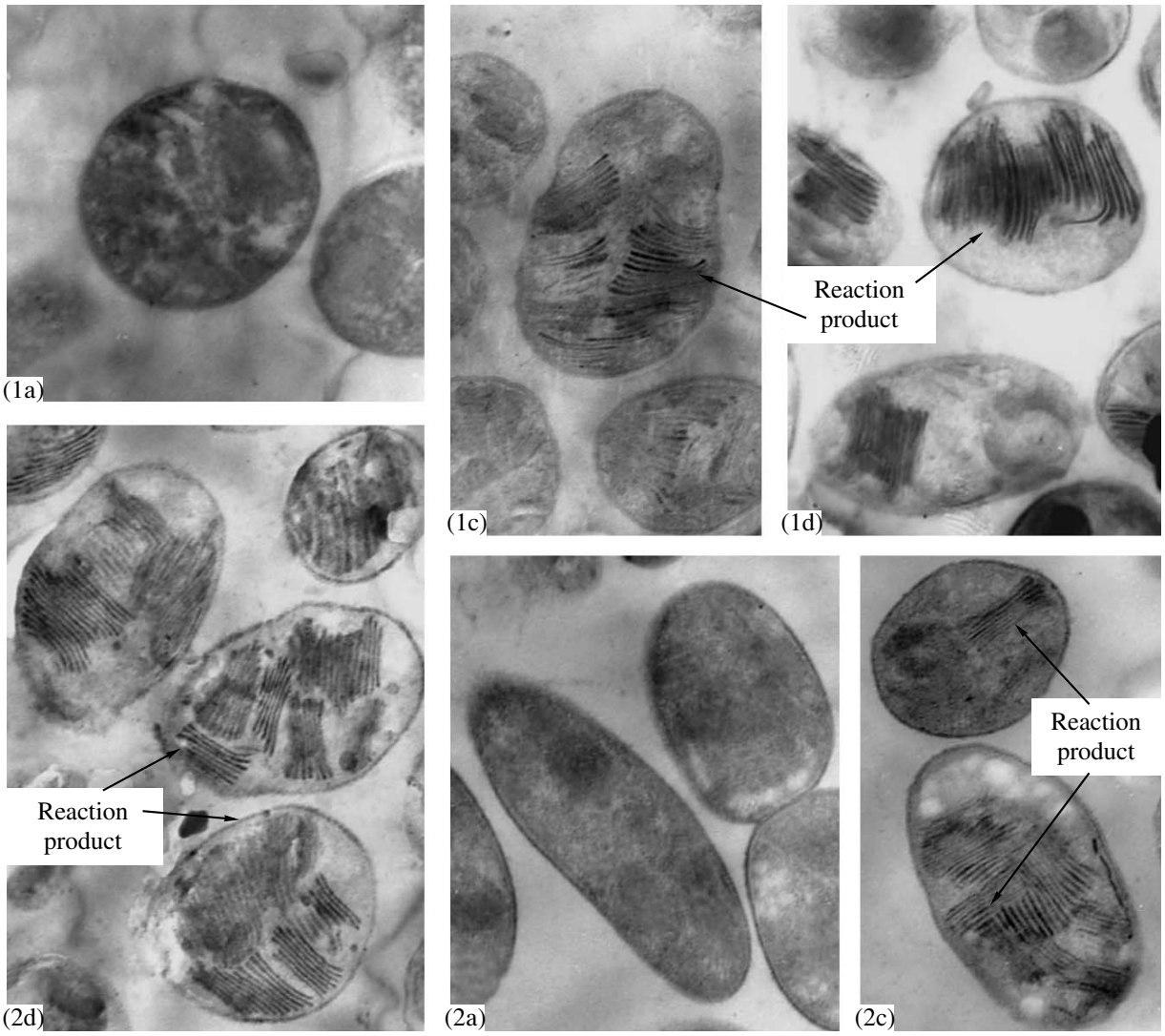

Fig. 3. Localization of peroxidase activity in the cells of *Md. szegediense* O-12 grown at 57 (1d) or 45°C (2d). Cells incubated in the absence of H_2O_2, (1c and 2c), and boiled cells, incubated in the presence of H_2O_2 (1a and 2a) were used as the controls. Magn. ×23000

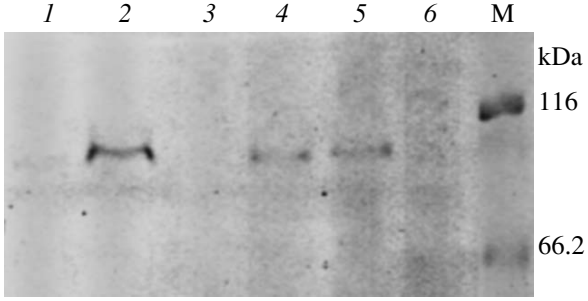

Fig. 4. Peroxide activity in different fractions of *Md. szegediense* O-12 cells grown at 57 or 45°C: periplasm, 45°C (*1*); membranes, 45°C (*2*); cytosol, 45°C (*3*); periplasm, 57°C (*4*); membranes, 57°C (*5*); cytosol, 57°C (*6*); M - protein markers. The reaction was conducted after PAG electrophoresis.

SOD activity both in *Mc. capsulatus* Bath and *Md. szegediense* O-12. Apparently, both methanotrophs have only Fe-containing SOD. The genome analysis of *Mc. capsulatus* Bath confirmed the above results; the genome bears the sequences coding for two Fe-containing SOD differing in amino acid composition: 194 (YP_114872) and 210 amino acid residues (YP_114498).

Previously, enzymological analysis of cell extracts revealed that, in response to increasing the cultivation temperature, both strains increased the production of oxipyruvate reductase (the indicator enzyme of the serine pathway) and RuBisCO (the key enzyme in the Calvin cycle, or RuBP cycle), while the activity of hexulosephosphate synthase, the first enzyme of the ribulose monophosphate cycle, remained practically unchanged (table). On the one hand, carbon assimilation via the first two pathways requires high consump-

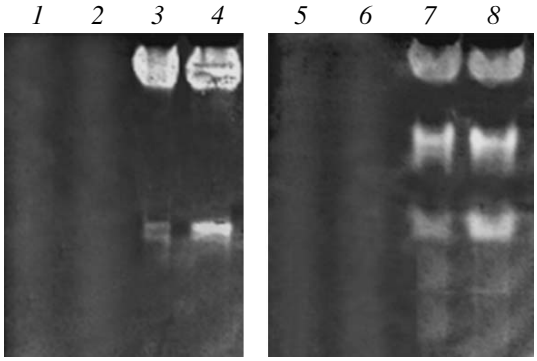

Fig. 5. Detection of SOD activity in thermophilic methanotrophs, grown at different temperatures by native PAG electrophoresis. The slots were filled with the following samples with equal protein content: boiled cell extract of *Mc. capsulatus* Bath grown at 45°C (*1*) or 29°C (*2*); cell extract of *Mc. capsulatus* Bath grown at 45°C (*3*) or 29°C (*4*); boiled cell extract of *Md. szegediense* O-12, grown at 57°C (*5*) or 45°C (*6*); cell extract of *Md. szegediense* O-12 grown at 57°C (*7*) or 45°C (*8*).

tion of NADH, so assimilation via the serine and RuBP cycles acts as an outflow channel for reducing agents. On the other hand, hydrogen peroxide and other ROS are produced at least from CH_4 oxygenation by mMMO and RuBisCO oxygenase activity [26, 27]. It is safe to assume that high rates of ROS production result from the high rates of methane oxidation and assimilation.

Neither of the two methanotrophs exhibited catalase activity, and the corresponding genes were not found in the *Mc. capsulatus* Bath genome. The absence of catalase in the methanotrophs is quite comprehensible in view of the participation of hydrogen peroxide in the reaction catalyzed by MMO [26].

DISCUSSION

Aerobic microorganisms are subject to the effects of ROS, such as H_2O_2, the superoxide anion ($^{\bullet}O_2^-$), and hydroxyl radical ($OH^{\bullet}$), produced at certain stages of four-electron reduction of O_2 to H_2O, or due to the effect of environmental factors. The effect of elevated temperatures on microbial cells is one of the factors causing oxidative stress, indicated by the induction of the genes responsible for the scavenging of intracellular oxidants [6, 7].

We have demonstrated that in both methanotrophs, the thermophilic *Md. szegediense* O-12 and the thermotolerant *Mc. capsulatus* Bath, the rate of ROS production is higher in the cells growing at relatively high, near-optimal temperatures, as compared with cells growing at low temperatures. In our case, the high rate of ROS production correlated with more rapid methane oxidation, as well as high levels of RuBisCO, the key enzyme of the Calvin cycle. ROS production is a characteristic result of active metabolic processes in methanotrophs, since oxygen radicals perform a specific function in their cells, directly participating in methane oxidation by MMO, while the RuBisCO oxygenase activity is known to result in hydrogen peroxide formation [26, 27].

Although high growth rates of methanotrophs are accompanied by an extensive ROS production, their steady-state levels in the cells are sustained within optimal physiological limits, due to the functioning of specialized anti-oxidant protection systems: SOD, cytochrome *c* peroxidase, glutathione, and glutathione peroxidase. The activity of cytochrome *c* peroxidase increased in both methanotrophs with increasing temperature. This suggests induction of the genes coding for this enzyme, which is a major scavenger of intracellular oxidants at high temperatures.

One of the implications of the oxidative stress is damage to the biological membrane (caused, in particular, by an increase in ROS concentration). The studied methanotrophs are essentially distinct in the levels of lipid peroxide accumulation in cells. The higher degree of lipid degradation is revealed in *Md. szegediense* O-12 grown at a low temperature; by contrast, in *Mc. capsulatus* Bath, decreased cultivation temperature resulted in a decrease of lipid peroxide levels. The process of lipid peroxide removal involves primarily glutathione and glutathione peroxidase. Their activities, along with the activity of glucose-6-phosphate dehydrogenase (the enzyme that provides energy for glutathione reduction), were augmented by low cultivation temperature.

Thus, *Md. szegediense* O-12 and *Mc. capsulatus* Bath use different systems of ROS protection. At near-optimal temperatures, the cells of both methanotrophs express cytochrome *c* peroxidase, while the SOD activity remains unchanged and catalase is not detected; additionally, cells of *Md. szegediense* O-12 accumulate free glutathione and express glutathione peroxidase in response to decreased cultivation temperatures. Furthermore, this methanotroph uses another mechanism of ROS protection under low-temperature conditions, which is melanin production by oxidative polymerization of phenolic compounds. Melanin exhibits peroxide-decomposing activity [28]. Very recently, a sMMO-associated Cu-binding compound, methanobactin, which is analogous to Fe-siderophores of some bacte-

ria, was reported to participate in the dismutation of the superoxide anion to H_2O_2, as well as in the reduction of H_2O_2 to H_2O not involving formation of hydroxyl radicals [29]. Although methanobactin levels in the studied methanotrophs remain to be determined, it is not improbable that this chromopeptide plays a key role in the cell protection from oxidative stress. Bacteria of the genus *Methylocaldum* occur both in high-temperature ecosystems (thermal springs and industrial waste) and in the biotopes where the temperature is subject to significant fluctuations (waste from animal farms, rice fields, etc.). The characteristic features of *Md. szegediense* O-12 antioxidant systems are attributable to a higher thermophilicity and the necessity for adaptation to drastic temperature gradients in the natural habitats of this methanotroph.

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