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SHORT COMMUNICATIONS

Synthesis of Melanin by a Moderately Thermophilic Methanotroph *Methylocaldum szegediense* Depends on Cultivation Temperature

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The production of melanin-like pigments is a common feature of moderately thermophilic methanotrophs of the genus *Methylocaldum* (*M. szegediense*, *M. gracilis*, and *M. tepidum* [1, 2]. Melanins are negatively charged hydrophobic high-molecular weight pigments formed in the course of oxidative polymerization of aromatic (phenol and/or indole) compounds. Although melanin formation has been shown in different pro- and eukaryotes and the relation of this process to protection against various stress factors has been shown [3], their nature, the mechanisms of their synthesis, and the role of these pigments in heat adaptation of methanotrophs are still unknown. This fact has prompted us to study the physiological and biochemical bases of melanization in the moderately thermophilic methanotroph *M. szegediense* O-12.

M. szegediense O-12 was cultivated at 42°C or 57°C in the atmosphere of methane and air (1 : 1) on P medium [4]. The aromatic compounds were extracted from the freeze-dried culture liquid with ethyl acetate [5], separated by thin-layer chromatography (TLC), and analyzed using a Finnigan MAT Model 8430 mass-spectrometer (Germany). The melanin particles were isolated by treating the cells with lysozyme and guanidine isothiocyanate and by boiling in 6.0 N HCl [6]; they were analyzed by the method of electron paramagnetic resonance (EPR) on a SEPR-5 spectrometer (Rubin Design Office, Russia). Cell-free extracts were prepared as described previously [4]. The activity of tyrosine-α-ketoglutarate aminotransferase was measured by detection of tyrosine-depending formation of glutamate from α-ketoglutarate using TLC. Homogentisate-1,2-dioxygenase and maleylacetoacetate isomerase were determined by the known method [7]. Tyrosine decarboxylase was detected by the rate of $^{14}CO_2$ formation from u^{14}C-tyrosine; carbon dioxide was absorbed with phenethylamine [8].

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The moderately thermophilic methanotroph *M. szegediense* O-12 showed optimal growth at 55–57°C; the culture liquid was stained light yellow and the colonies grown on agarized medium were cream-hued. At 42°C, active culture growth started after a lag period of three days and the culture liquid became brownish yellow. The colonies grown on a solid medium at 42°C gradually became dark brown, but the pigment did not diffuse into the agar. Thus, the production of a brown pigment by *M. szegediense* O-12, which was associated with cell growth and metabolism, was induced by decrease of cultivation temperature.

M. szegediense O-12 cells grown at 57°C were almost completely dissolved when treated with 4 M guanidine isothiocyanate and boiled in 6 N HCl. In the case of the cells grown at 42°C, such treatment resulted in formation of a black pellet, which was insoluble in water, concentrated HCl, or 5 N NaOH, as well as in butanol, chloroform, acetone, hexane, benzene, and toluene, but was slightly soluble in methanol and dimethyl sulfoxide. Besides, the pellet was partially soluble in 1 N NaOH (the solution became light brown). The pigment particles exhibited peroxide-degrading activity; their incubation with 3% H_2O_2 led to the appearance of gas bubbles. These properties are typical of some melanins, the compounds for which tyrosine is a precursor [3]. The EPR method revealed the presence of free radicals in the pigment composition, which pointed to its melanin nature (Fig. 1) and corresponded to the spectra of microbial melanins [6].

Using TLC and visualization by UV, several compounds were found in the culture liquid of *M. szegediense* O-12 grown at 42°C; one of them (R_f = 0.29) was identified as 4-hydroxyphenyl acetic acid (Fig. 2).

Cell-free extracts of *M. szegediense* O-12 showed the activity of tyrosine-α-ketoglutarate aminotransferase, as well as homogentisate-1,2-dioxygenase and maleylacetoacetate isomerase. The activities of the second and third enzymes were much higher in the cells

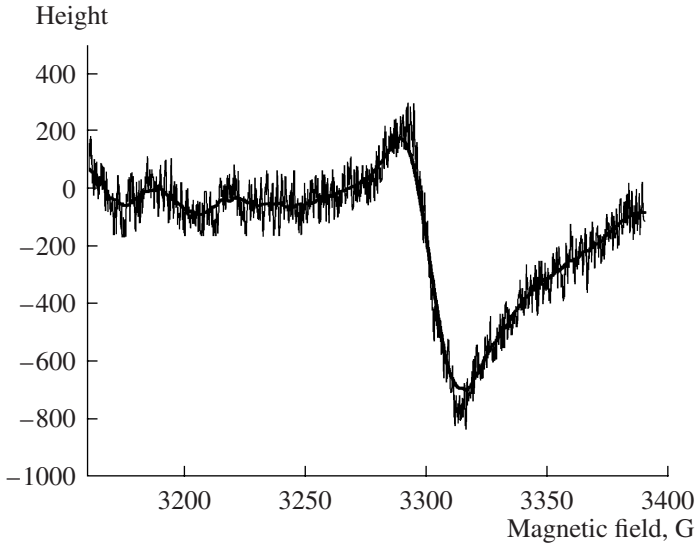

Fig. 1. The EPR spectrum of melanin isolated from *M. szegediense* O-12 cells.

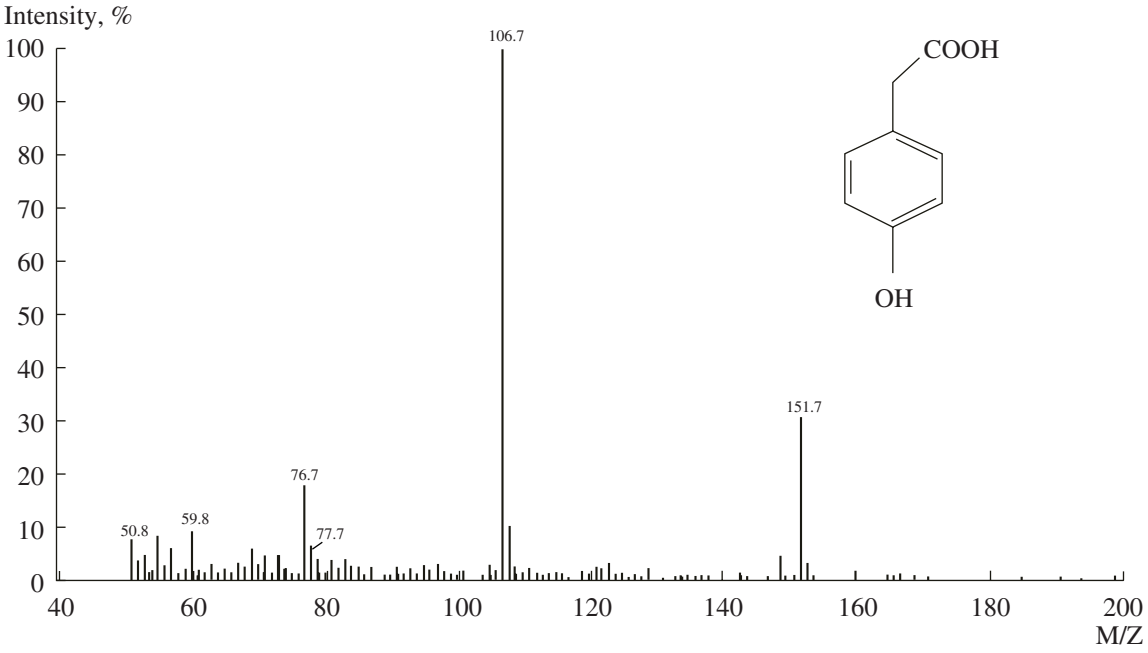

Fig. 2. The mass-spectrum of 4-hydroxyphenyl acetic acid isolated from the culture liquid of *M. szegediense* O-12. The peaks of the fragments correspond to the standard peaks given in the database [9].

grown at 57°C (4.4 and 8 nmol/min/mg of protein, respectively) as compared with the cells grown at 42°C (0.1 and 0.5 nmol/min/mg of protein). Thus, in *M. szegediense* O-12 grown at the optimal temperature, the homogentisate pathway of tyrosine degradation through fumarate and acetoacetate is functioning (Fig. 3) [10]. Obviously, the imbalance of homogentisic acid catabolism due to a drop of cultivation temperature results in its accumulation and polymerization into melanin.

Moreover, the methanotroph under study grown at 57°C exhibited tyrosine decarboxylase activity (8 nmol/min/mg of protein), which increased twofold at 42°C. Tyrosine decarboxylation and subsequent transformations of the reaction product (tyramine) are most likely to promote extra formation of hydroxyphenyl acetate and homogentisate, which can transform into melanin via spontaneous oxidative polymerization. The reasons why the temperature has an effect on

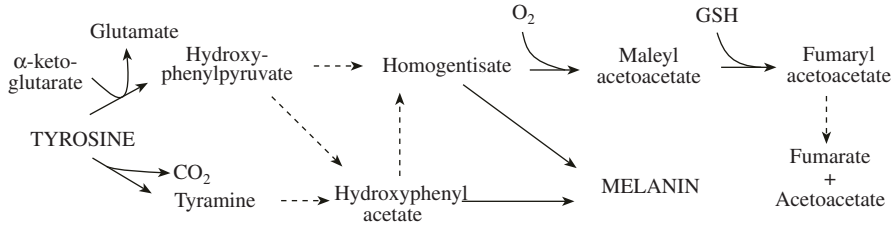

Fig. 3. The relationship between tyrosine catabolism and melanin synthesis in *M. szegediense* O-12.

expression of the enzymes of aromatic amino acid catabolism still need clarification.

Thus, for the first time it has been shown that cells of *M. szegediense* O-12 synthesize melanin at suboptimal growth temperature (42°C). Melanin is supposed to be formed of tyrosine degradation intermediates, which are accumulated in response to the decrease of methanotroph cultivation temperature.

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