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EXPERIMENTAL ARTICLES

Growth of Mesophilic Methanotrophs at Low Temperatures

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Abstract—The optimal growth of mesophilic methanotrophic bacteria (collection strains of the genera *Methylocystis*, *Methylomonas*, *Methylosinus*, and *Methylobacter*) occurred within temperature ranges of 31–34°C and 23–25°C. None of the 12 strains studied were able to grow at 1.5 or 4°C. Representatives of six methanotrophic species (strains *Mcs. echinoides* 2, *Mm. methanica* 12, *Mb. bovis* 89, *Mcs. pyriformis* 14, *Mb. chroococcum* 90, and *Mb. vinelandii* 87) could grow at 10°C (with a low specific growth rate). The results obtained suggest that some mesophilic methane-oxidizing bacteria display psychrotolerant (psychrotrophic) but not psychrophilic properties. In general, the Rosso model, which describes bacterial growth rate as a function of temperature, fits the experimental data well, although, for most methanotrophs, with symmetrical approximations for the optimal temperature.

Key words: methanotrophs, collection cultures, mesophiles, psychrotolerants, mathematical models.

Extremophilic microorganisms, especially psychrophilic bacteria, have attracted much attention of the researchers over the last two decades. Huge terrestrial areas, as well as the ocean water thickness, are permanently under low temperatures. Low temperature conditions are also characteristic of Arctic zones, zones of mountain ice, Alpine lakes and deep meromictic lakes, and deep mountain caves [1]. In addition to permanently cold zones, seasonal temperature variations occur in many ecosystems, particularly tundra bogs of circumpolar zones in Eurasia and North America.

Microorganisms growing at low temperatures (psychrophiles and psychrotolerants) play a substantial role in the global carbon, nitrogen, and sulfur biogeochemical cycles [1, 2]. Psychrophiles can be found in many physiological (systematic) groups of microorganisms, for example, among methanotrophic bacteria, which have a significant effect on the flux of methane (both buried and newly formed) into the atmosphere. Methanotrophic bacteria in soils serve as a sink for atmospheric methane.

As one of the greenhouse gases, methane may influence the climate on Earth to the same extent as CO_2 [3, 4]. Bacterial methane oxidation was reported to occur in many low-temperature ecosystems, such as groundwaters [5], tundra soils [6, 7], and Antarctic lakes [8]. Psychrophilic methane-oxidizing bacteria have been isolated. *Methylobacter psychrophilus* Z-0021 [9], isolated from the tundra soil of Polar Ural, grows within a temperature range from 3.5 to 20°C. *Methylosphaera hansonii* inhabits a saline meromictic lake in Antarctica and can grow at 0°C (the optimal growth temperature is 10 to 13°C, and the maximal growth temperature is 21°C). The description of this new genus was based on the 16S rRNA sequence analysis and certain phenotypic features distinguishing this bacterium from other methanotrophs. It should be noted that the data from 16S rRNA sequencing placed *Methylosphaera hansonii* in the same cluster as methanotrophic endosymbionts of molluscs from the Gulf of Mexico [10].

It is still unclear whether or not the aforementioned organisms are true psychrophiles (cryophiles) or they are psychrotolerant (psychrotrophic) methanotrophs. For instance, in *Mb. psychrophilus* Z-0021, the activity of both primary and intermediate metabolism enzymes decreased as the temperature was lowered from 30 to 5°C [11]. The psychrotrophic methanotroph *Methylomonas scandinavica* described recently [12] can grow within a temperature range from 5 to 30°C, with optimal growth occurring at 15°C. This species is quite similar to the mesophilic methanotrophs *Methylomonas rubra* and *Methylomonas methanica* in many phenotypic traits.

In various permanently or temporarily cold ecological niches, bacterial methane oxidation was found to occur [6, 8, 13–16]. Mesophilic methanotrophs were identified by means of the immunofluorescent method in permanently cold (1–3°C) bottom sediments and the near-bottom water of the Black Sea and Pacific and Atlantic Oceans [13, 14], in tundra bogs of eastern Siberia [6], Antarctic lakes [8], and landfilled solid wastes [15]. Thus, the adaptation of mesophilic methanotrophs to low temperatures cannot be excluded.

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In this work, we studied the ability of collection cultures of mesophilic methanotrophic bacteria to grow at low temperatures.

MATERIALS AND METHODS

Methanotrophic bacteria from the UNIQEM collection (V.F. Gal'chenko, Institute of Microbiology, Russian Academy of Science), both thermotolerant (*Methylococcus capsulatus* 10529) and mesophilic (*Methylomonas methanica* 12, *Methylobacter bovis* 89, *Mb. chroococcum* 90, *Mb. vinelandii* 87, *Methylosinus trichosporium* 20, *Ms. sporium* 5, *Methylocystis minimus* 42, *Mcs. methanolicus* 10, *Mcs. pyriformis* 14, *Mcs. echinoides* 2, and *Mcs. parvus* OBBP) were used in this study.

Bacteria were cultivated in Hungate tubes at 1.5, 4, 10, 15, 20, and 30°C in liquid P mineral medium [17]. The inocula were grown in tubes with glass caps containing agarized P medium, in a methane–air (1 : 1) atmosphere at 30°C (except for *Methylococcus capsulatus*, which was grown at 37°C). The cells washed from the solid medium surface were suspended in 100 ml of liquid P medium and inoculated into Hungate tubes (5 ml into each tube). The gas phase in the tubes was aseptically replaced with a methane–air (1 : 5) mixture through a sterile setting with a Millipore filter (0.2 μm pore size). Culture growth was assessed by measuring for optical density at 600 nm.

The specific growth rates of bacterial cultures were calculated from the formula

$$\mu = \frac{\ln X - \ln X_0}{t - t_0},$$

where X and X_0 are the values of the culture optical density at the times t and t_0.

To determine optimal, maximal, and minimal growth temperatures for the methanotrophic bacteria, the experimental results were approximated according to the Rosso model (1), which relates the maximal specific growth rate μ_{max} at a temperature T to the optimal specific growth rate μ_{opt} at a temperature T_{opt} and to the minimal, optimal, and maximal temperatures (T_{min}, T_{opt}, and T_{max}) [18]:

$$\mu_{max}(T) = \begin{cases} 0, & T < T_{min} \\ \mu_{opt}\tau(T), & T_{min} < T < T_{max}, \\ 0, & T > T_{max} \end{cases} \qquad (1)$$

where

$$\tau(T) = \frac{(T - T_{max})(T - T_{min})^2}{(T_{opt} - T_{min})[(T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)]}$$

The least-squares method was used to calculate T_{min}, T_{opt}, and T_{max} by minimizing the sum of squares of the deviations Q:

$$Q = \sum_{i=1}^{n} (\mu^i_{max} - \mu_{opt}\tau(T_i))^2,$$

where μ^i_{max} is the maximal specific growth rate of the culture at temperature T_i, μ_{opt} is the optimal specific growth rate of a given culture; and n is the number of experimental points.

The accuracy of the approximation was assessed from the standard deviation σ expressed in percents:

$$\sigma = \frac{\sqrt{\frac{Q_{min}}{n-p}}}{\mu_{opt}} \times 100\%,$$

where n is the number of experimental points and p is the number of parameters.

RESULTS AND DISCUSSION

All the mesophilic methane-oxidizing bacteria studied grew at 15, 20, and 30°C, and only six cultures (species) could grow at 10°C. None of the cultures could grow at 1.5 or 4°C (incubation time, 4 months).

Visual analysis of the growth curves revealed that the methanotrophs differed in their growth temperatures and could be subdivided into four groups (Fig. 1). Groups A and B comprise species incapable of growing at 10°C and lower temperatures and differing in the parameters of their growth at 15–30°C. *Methylococcus capsulatus* 10529 and *Methylocystis minimus* 42 (group A) essentially developed worse at 15 and 20°C than at 30°C. The growth curves for the group B methanotrophs recorded at 20 and 30°C differed insignificantly. These methanotroph species can be conventionally described as follows: group A [[30°]–[20°–15°]] and group B [[30°–20°]–[15°]].

The cultures capable of growing at 10°C can also be divided into two groups. The group D microorganisms, especially *Mm. methanica* 12 and *Mb. bovis* 89, show better growth at 20 and 15°C than at 30°C. In group C, *Mcs. echinoides* 2 can be described by the formula [30°–20°–15°–10°], whereas both *Mcs. pyriformis* 14 and *Mb. chroococcum* 90, by the formula [[30°–20°–15°]–[10°]]. In the group D, the *Mm. methanica* and *Mb. vinelandii* can be described by the formula [[20°–15°–30°]–[10°]], and *Mb. bovis* 89 by the formula [[20°–30°–15°]]–[10°]].

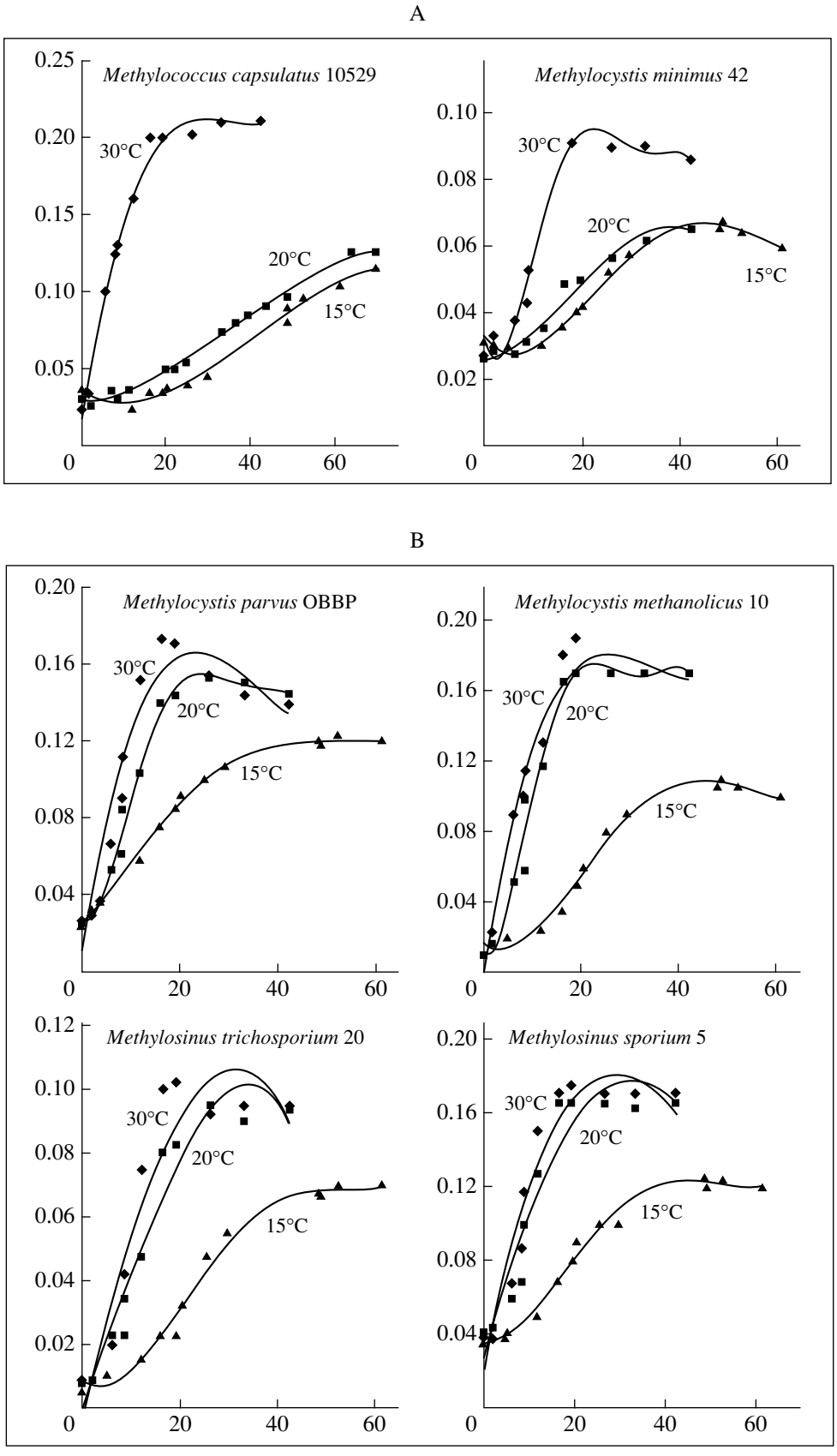

Fig. 1. Growth curves of the collection strains of methanotrophs. The ordinate shows optical density. The abscissa shows time in days. Here and in Fig. 2, A–D indicate methanotroph groups (see the text).

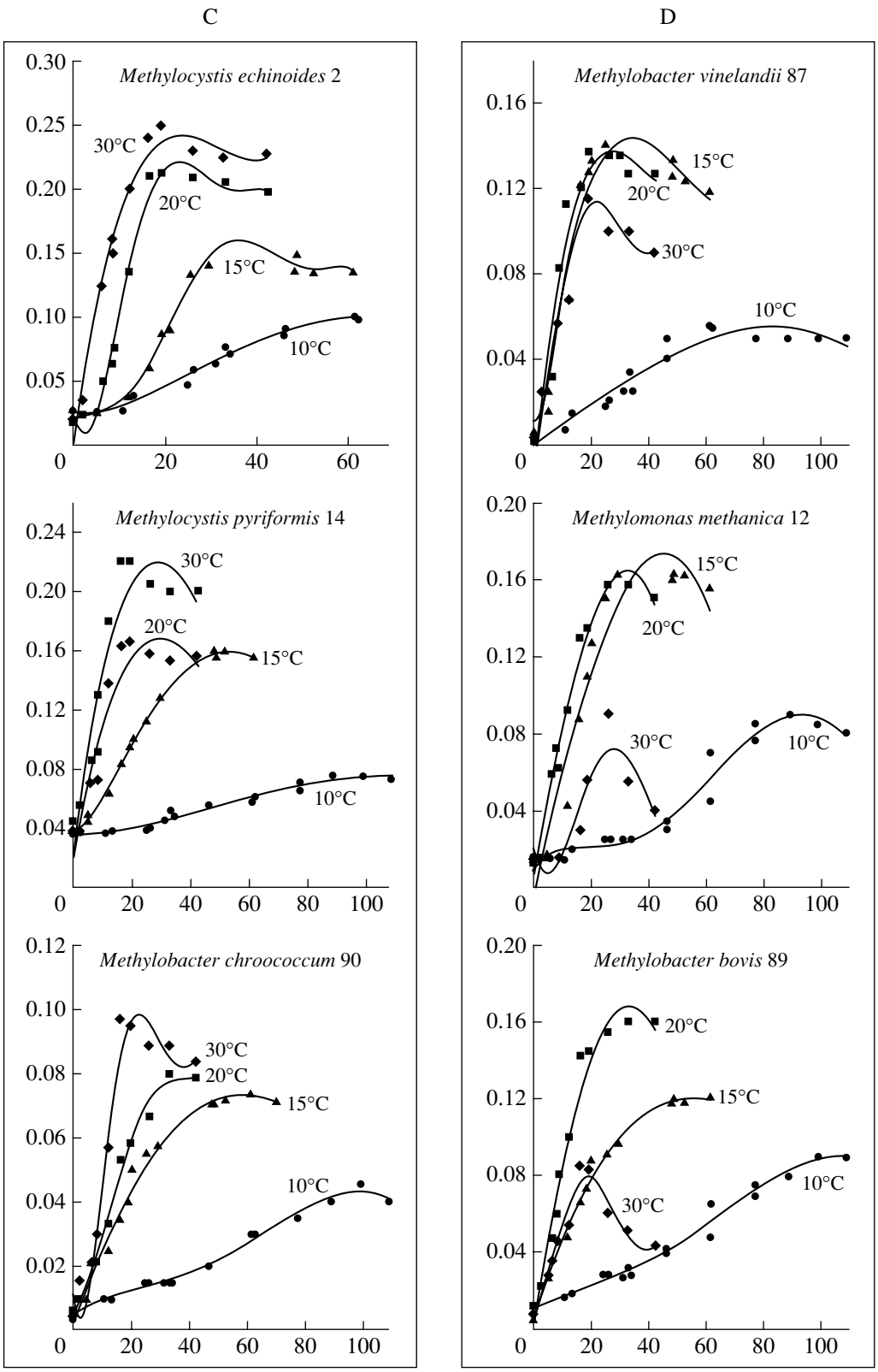
C
D
Methylocystis echinoides 2
30°C
20°C
15°C
10°C
Methylocystis pyriformis 14
30°C
20°C
15°C
10°C
Methylobacter chroococcum 90
30°C
20°C
15°C
10°C
Methylobacter vinelandii 87
15°C
20°C
30°C
10°C
Methylomonas methanica 12
15°C
20°C
30°C
10°C
Methylobacter bovis 89
20°C
15°C
30°C
10°C

Fig. 1. (Contd.)

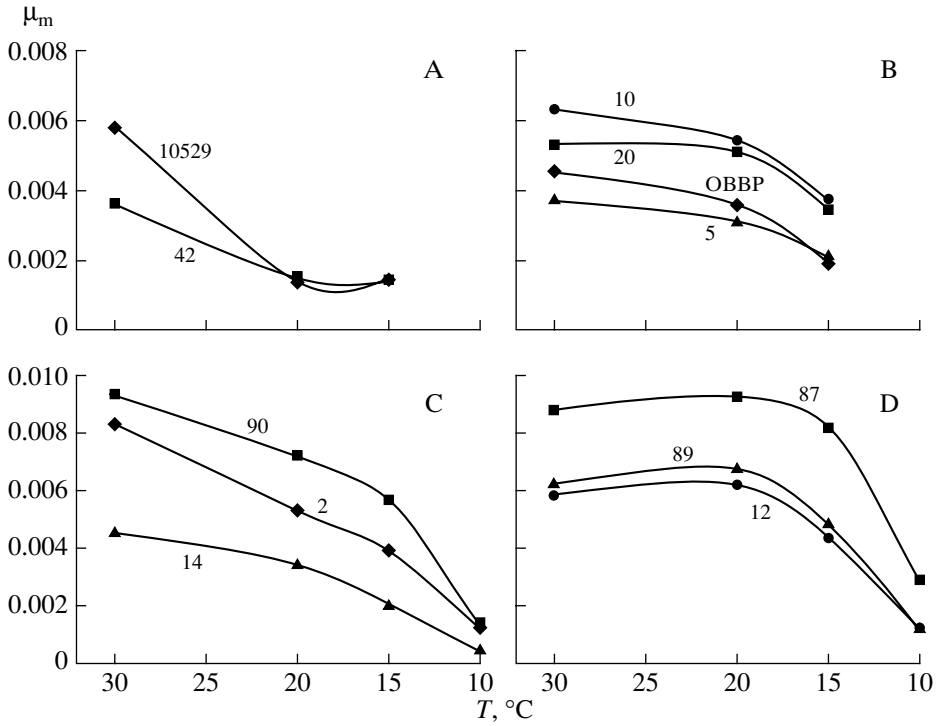

Fig. 2. Mean specific growth rates of methanotrophs as a function of temperature. Here and in Figs. 3 and 4, the figures over the curves are strain designations.

The *mean specific growth rates* of methanotrophs (μ_m) in the exponential growth phase were calculated for all the temperatures studied. According to this parameter, the bacteria proved to group identically to the foregoing clustering (cf. groups A–D in Figs. 1 and 2). However, taking into account that the μ_m values cannot be used for the calculation of the *optimal specific growth rates* (μ_{opt}), we tested the results obtained with the available mathematical models of microbial growth as a function of temperature.

The growth of microorganisms is known to be determined by temperature-dependent chemical reactions.

Temperature parameters describing growth of some methanotrophic bacteria

Organism	T_{min}	T_{opt}	T_{max}	Standard deviation σ, %
Ms. trichosporium 20	9	23	38	29
Mm. methanica 12	5	24	34	12
Mb. vinelandii 87	6	24	42	23
Mcs. minimus 42	9	25	41	30
Mcs. echinoides 2	0	31	41	2
Mcs. pyriformis 14	7	32	54	2
Ms. sporium 5	9	33	57	20
Mcs. parvus OBBP	10	34	59	13

The Arrhenius law, which was originally proposed to describe the growth rate as a function of temperature, poorly fits the experimental data. Ratkowsky suggested a formula of a linear relationship between growth rate and temperature [19]:

$$\sqrt{r} = b(T - T_0),$$

where r is growth rate constant, b is the regression coefficient, T is absolute temperature, and T_0 is the apparent minimal temperature, which is peculiar to the given microorganism.

However, this model does not adequately describe the effect of temperature on the bacterial growth, especially at temperatures extreme for the given microorganism. Therefore, Ratkowsky developed a new empirical model of nonlinear regression [20]:

$$\sqrt{r} = b(T - T_{min})\{1 - \exp[c(T - T_{max})]\},$$

where T_{min} and T_{max} are temperatures at which bacterial growth no longer occurs, and b and c are the regression coefficients.

If the Ratkowsky model is put in the form of equation (2),

$$\mu(T) = (b(T - T_{min})\{1 - \exp[c(T - T_{max})]\})^2. \quad (2)$$

It can be easily seen that it exhibits properties characteristic of most of the models describing the dependence of the bacterial growth rate on temperature [20]. The coefficients b and c are specific for each microor-

ganism and cannot be used for comparison. Correlations between different parameters are possible [18]. Under the conditions of our experiment, it makes no sense to use a four-parameter model to approximate four pairs of values.

Among the numerous models of microbial growth as a function of temperature [18–24], we have chosen the Rosso model (1) [18] on two counts. First, since our experimental data were scarce (only four pairs of values for each strain), the preference was to be given to a model with a minimum number of parameters; second, we needed a model in which the parameters would not show strong structural correlations, which could impede their calculation. The Rosso model fits these requirements even with the small number of experimental points. In addition, whereas with the Ratkowsky model (2), the *b* and *c* coefficients are difficult to assess [20], the T_{min}, T_{opt}, and T_{max} values in the Rosso model, can be estimated from the actual properties of microorganisms (the space of logic feasibilities).

In the Rosso model, the optimum in the $\mu(T)$ curve is shifted towards the maximum temperature at which the microorganism can grow [18, 20, 24]. This shape of the curve was characteristic of *Mcs. echinoides* 2 and *Mm. methanica* 12 (Fig. 3). However, in other methanotroph species, the relationship between the specific growth rate and temperature exhibited a different pattern. Thus, *Mb. chroococcum* 90 had a growth temperature maximum at 18–20°C. *Mcs. methanolicus* 10, *Mb. bovis* 89, and *Mc. capsulatus* 10529 showed two maxima, which cannot be described by the Rosso model. Therefore, the growth temperature was calculated for only eight methanotroph species (table).

The results of extrapolation showed that the strains of methanotrophs studied can be divided into two groups with the temperature optimum at 23–25°C and 31–34°C (table, Fig. 4). In general, the approximation made fit the experimental data well. However, it should be taken into account that the values calculated for the optimal and maximal temperatures were mainly obtained by extrapolation because of the shortage of experimental data. Some discrepancies between the calculated values and the results of the experiments should be emphasized. For example, the methanotrophs *Ms. trichosporium* 20 and *Mcs. minimus* 42 did not grow at 10°C, although the calculated minimum growth temperature for these bacteria was 9°C. The high values of the mean square deviations (29 and 30%, respectively) may account for this inconsistency.

Most of the approximations obtained were symmetric with respect to the optimal temperature, whereas the Rosso model predicts an asymmetric curve with a steeper decline in the region of maximal temperatures. Unfortunately, it is unclear whether or not this was a behavior typical of the methanotrophs studied or a result of an experimental error, which was quite possible, because each strain was characterized by only four pairs of values.

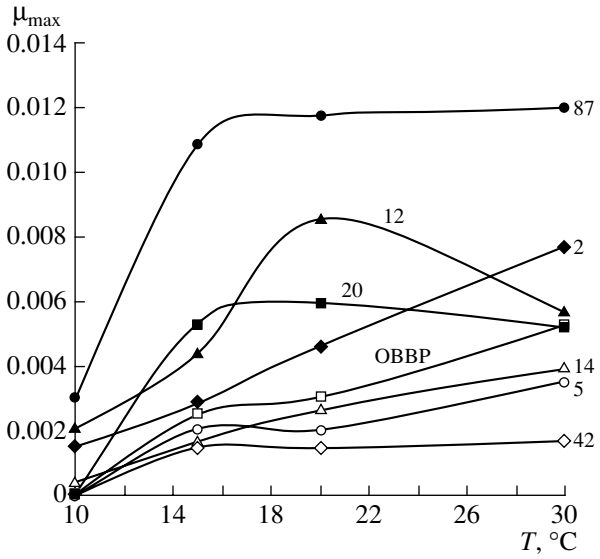

Fig. 3. Maximum specific growth rates of methanotrophs as a function of temperature.

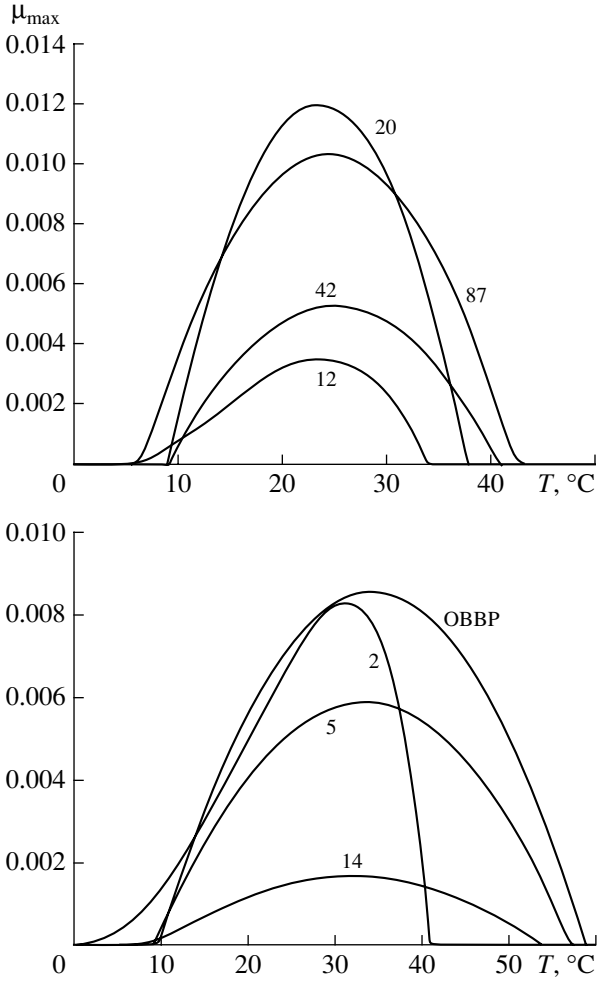

Fig. 4. Approximated curves of the dependence of the specific growth rate on temperature (calculated according the Rosso model).

According to the Morita definition [1], psychrophiles are microorganisms characterized by active growth at 0°C, optimal growth at about 15°C, and an absence of growth at temperatures higher than 20°C. Psychrotrophs (facultative psychrophiles, or psychrotolerants) are microorganisms that can grow at low temperatures and exhibit optimal growth at 20–30°C [1]. Different authors differently interpret the term *low (chill) temperatures*; however, there is a general consensus that they are in the range from 0 to 15°C. According to the growth curves (Fig. 1) and the calculated temperature parameters of growth (table), some methanotrophs (those whose optimal growth occurs at 31–34°C) belong to true *mesophiles* (groups A and B). In contrast, the members of groups C and D can be defined as *psychrotrophs*: (1) they can grow at temperatures lower than 15°C (T_{min} 5–9°C) and (2) they show optimal growth at temperatures lower than 30°C, namely within a range from 23 to 25°C. Some mesophilic methanotrophs exhibit psychrotolerant (psychrotrophic) properties: these are *Mcs. echinoides* 2, *Mm. methanica* 12, *Mb. bovis* 89, *Mcs. pyriformis* 14, *Mb. chroococcum* 90, and *Mb. vinelandii* 87 capable of growing at 10°C.

Mesophilic methanotrophs have been previously detected in sphagnums of eastern Siberia (Kolyma) by means of the immunofluorescent method [6]. In these permanently cold ecological niches, methanotrophs were revealed in conglomerates consisting of bacteria and the half putrefied remains of sphagnum located between undegraded moss filaments. On the surface of these conglomerates, bacteria exhibited a positive reaction with immune sera against mesophilic methanotrophs of the genera *Methylobacter* and *Methylomonas*. Vasil'eva *et al*. [7] have recently found that degrading hyaline cells of sphagnum contain oval bacteria with developed intracytoplasmic membrane structures. The enrichment cultures of the methanotrophs obtained by these authors showed active growth at 4°C on acid medium (pH 4.5). In their morphology, the isolated methane-oxidizing bacteria resembled the members of the genus *Methylobacter*. This suggests that in the permanently cold ecosystems (*psychroecosystems*), mesophilic methanotrophs and psychrophiles and psychrotolerants are equally often encountered.

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