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EFFECT OF TEMPERATURE ON GROWTH OF OBLIGATELY PSYCHROPHILIC BACTERIA

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Effect of temperature on the growth of five obligately psychrophilic bacteria and behavior of *Curtobacterium psychrophilum* strain 27–O–b at elevated temperatures were studied. Most of obligate psychrophiles showed lower growth activities than a facultative psychrophile at lower temperature. Arrhenius plots of the growth of most of obligate psychrophiles were linear from a temperature little below their optimum growth temperature to 0°. However, there was a deviation in Arrhenius plots of *Cytophaga antarctica* strain 16–O–d and a facultative psychrophile, *Pseudomonas fluorescens* strain 76–O–a. Therefore, it seems unreasonable to characterize psychrophiles only from the points of growth activities at lower temperature and/or a pattern of the Arrhenius plot. When *C. psychrophilum* was transferred to elevated temperatures after preincubation at the optimum growth temperature of 9°, it exhibited faster growth for the first few hours than at 9°, and then the growth ceased.

Recently, a considerable number of microorganisms, which have maximum growth temperatures of 20° or less, have been isolated from the oceans and polar regions. Several investigators have tried to define this unique group of microorganisms, psychrophiles. INGRAHAM (*1*) reported that psychrophiles could be defined on the basis of the value of temperature characteristic. On the other hand, STOKES (*2*) defined obligate and facultative psychrophiles as follows: "Both can grow macroscopically within 1 week at 0°, and those which have optimum growth temperature below 20° are obligate and those above 20° are facultative." The present author isolated nine obligately psychrophilic bacteria, according to the definition of STOKES, including four new species, from samples collected in Antarctica (*3*).

Growth is an overall reaction of microorganisms, and all changes in intra-

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cellular reactions are reflected in this phenomenon. Therefore, the effect of temperature on the growth of these bacteria and their behavior at elevated temperatures are considered to be useful for defining and understanding these microorganisms.

MATERIALS AND METHODS

Organisms. Five strains of obligately psychrophilic bacteria were used. They were *Curtobacterium psychrophilum* strain 27–O–b, *Cytophaga antarctica* strain 13–O–d, *Cytophaga xantha* strain 5–O–c, *Spirillum pleomorphum* strain 22–O–d, and *Micrococcus cryophilus* strain 72–O–d. The descriptions of these bacteria have been given in our previous paper (*3*). A facultative psychrophile, *Pseudomonas fluorescens* strain 76–O–a, isolated from a sample from Antarctica was also employed for comparison.

Medium. The medium used was composed of 1% peptone (Difco), 0.5% yeast extract (Difco) and 0.3% glucose, and pH was adjusted to 7.2.

Measurement of growth. Bacteria were grown in L-tubes (18 mm in diameter) which contained 7 ml of the medium. One L-tube was usually inoculated with 0.1 ml of the pre-culture at the middle exponential phase at 9° of bacteria tested. L-tubes were incubated in a water bath with shaking of 60 oscillations per minute. The water baths were controlled to appropriate temperatures with a difference of $\pm 0.2°$. Growth was measured as an increase in the absorbance at 660 nm by Spectronic 20 (Bausch-Lomb, U.S.A.). The specific growth rate, k, was calculated from a linear portion of the graph obtained according to the formula:

$$k = \frac{2.303\,(\log x_2 - \log x_1)}{t_2 - t_1}$$

where x_1 and x_2 are absorbances at times t_1 and t_2, respectively.

Viable cell count. The viable cell count was determined by the plate spread method. The culture broth was appropriately diluted with a 1% peptone solution. Five plates were prepared for each dilution. After incubation at 9° for 2 weeks, the number of colonies was recorded. The viable cell count was the average of counts of 5 plates.

Lag time. In this paper, lag time is defined as the time required for the start of the exponential growth phase when the same size of seed was used. It was graphically determined as the time of a starting point of a linear portion of the exponential growth phase in the growth curve.

RESULTS

Growth patterns of psychrophiles at various temperatures

As shown in Figs. 1 to 6 and in Table 1, all the obligate psychrophiles tested showed almost the same growth pattern, and their optimal growth temperatures

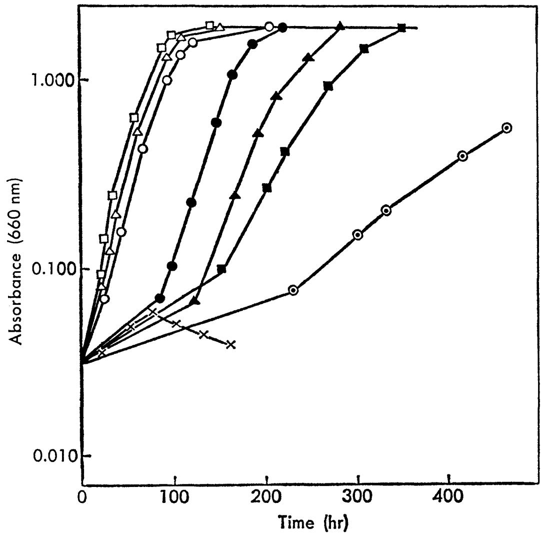

Fig. 1. Effect of temperature on growth of *Curtobacterium psychrophilum*.
×—×, 18°; ○—○, 15°; △—△, 12.5°; □—□, 9°; ●—●, 7.5°; ▲—▲, 3°; ■—■, −0.4°; ⊙—⊙, −2°.

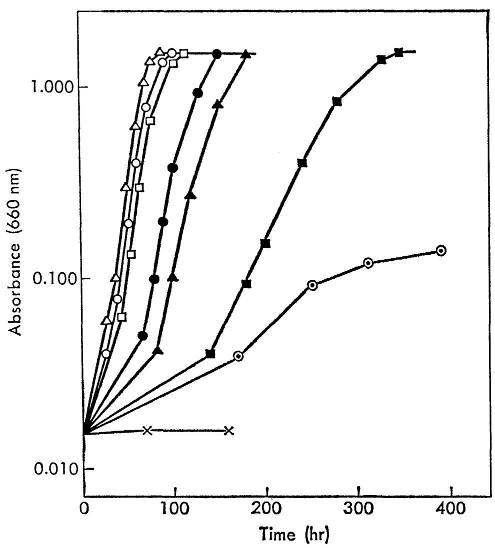

Fig. 2. Effect of temperature on growth of *Cytophaga antarctica*.
×—×, 20°; ○—○, 15°; △—△, 12.5°; □—□, 9°; ●—●, 5°; ▲—▲, 3°; ■—■, −0.4°; ⊙—⊙, −2°.

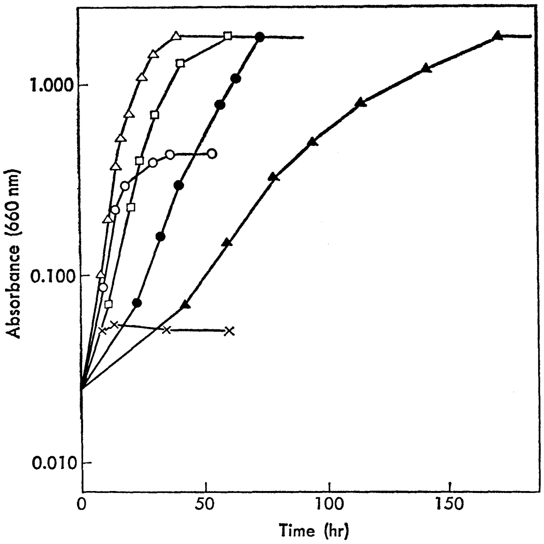

Fig. 3. Effect of temperature on growth of *Cytophaga xantha*.
×—×, 25°; ○—○, 20°; △—△, 15°; □—□, 9°; ●—●, 5°; ▲—▲, 0°.

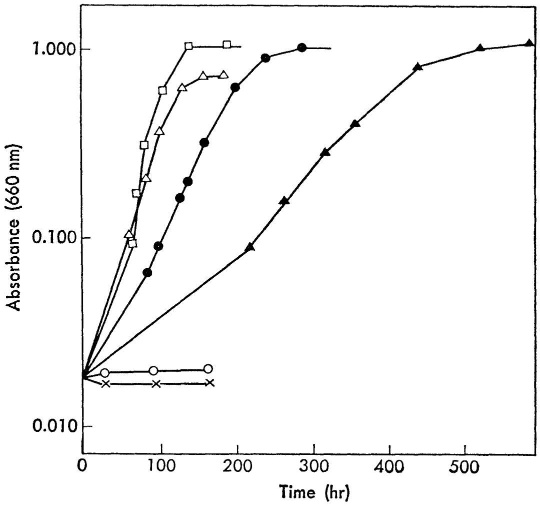

Fig. 4. Effect of temperature on growth of *Spirillum pleomorphum*.
×—×, 25°; ○—○, 20°; △—△, 15°; □—□, 9°; ●—●, 5°; ▲—▲, 0°.

were between 9° and 15°, but a facultative psychrophile, *Pseudomonas fluorescens* strain 76-O-a, grew well at 27° and even at 0°.

Lag time of obligate psychrophiles, with the exception of *C. xantha* and *M. cryophilus,* was far longer than those of *P. fluorescens* at low temperatures.

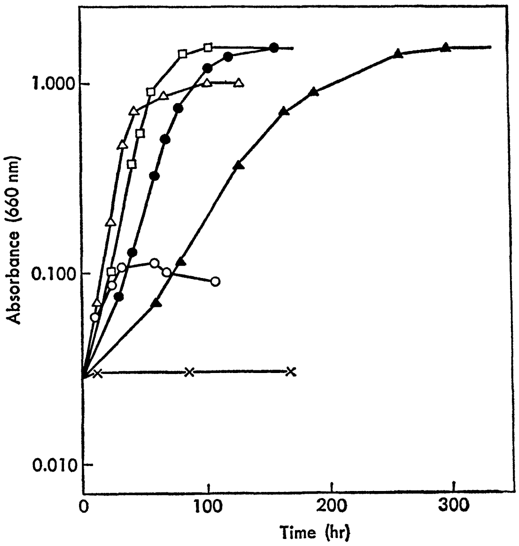

Fig. 5. Effect of temperature on growth of *Micrococcus cryophilus*.
×—×, 25°; ○—○, 20°; △—△, 15°; □—□, 9°; ●—●, 5°; ▲—▲, 0°.

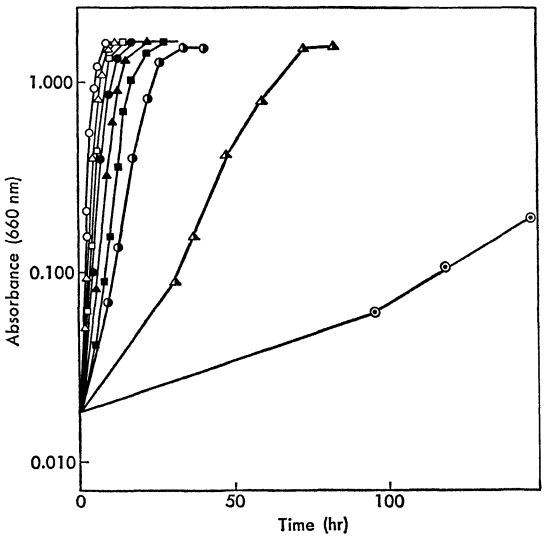

Fig. 6. Effect of temperature on growth of *Pseudomonas fluorescens*.
○—○, 27°; △—△, 24°; □—□, 21°; ●—●, 18°; ▲—▲, 15°; ■—■, 12°; ◑—◑, 9°; ◭—◭, 3°; ⊙—⊙, 0°.

Table 1. Lag times of psychrophiles.

Culture	Lag time (hour) at			
	15°	9°	5°	0°
C. psychrophilum	20	20	120*	150**
C. antarctica	40	40	70	140
C. xantha	8	10	20	40
S. pleomorphum	55	55	90	210
M. cryophilus	10	20	30	70
P. fluorescens	5	10	30*	100

Definition of lag time is described in materials and methods.
*, at 3°; **, at −0.4°.

Furthermore, at lower temperatures, the specific growth rate of *P. fluorescens* was almost equal to that of *C. xantha,* which could grow most rapidly among these obligate psychrophiles.

Arrhenius plot

The Arrhenius equation is applied to the effect of temperature on chemical reactions. When the specific growth rate is substituted for a reaction rate in the Arrhenius equation, the temperature characteristic (μ) is obtained which is analogous to activation energy. The Arrhenius plots for organisms studied are shown in Figs. 7 and 8. On the basis of these results, obligate psychrophilic bacteria were divided into two groups. *C. psychrophilum, C. xantha, Sp. pleomorphum,* and *M. cryophilus* are included in the first group as shown in Fig. 7. The Arrhenius plots for these bacteria were linear from a temperature below the optimum growth temperature to −2°, the minimum temperature studied. The other group was composed of only one organism, *C. antarctica,* as shown in Fig. 8. In the Arrhenius plot of this strain, a deviation was observed at about 5°. This was also observed in the Arrhenius plot for *P. fluorescens,* which is a facultative psychrophile, but, its deviation occurred at about 9°.

Effect of elevated temperatures on C. psychrophilum

In order to study the effect of elevated temperature on a psychrophile, *C. psychrophilum,* which has the lowest maximum growth temperature among obligate psychrophilic bacteria tested, was used. At temperatures above 18°, ordinary growth did not occur, but, when the bacterium was transferred to 20°, 25°, and 30° from the optimum growth temperature of 9°, the appreciable growth was observed for 10 to 20 hr. These results are shown in Fig. 9 and in Table 2. In Table 2, stage I was in the lag phase, stage II just in the exponential phase, and stages III and IV in the exponential phase. k and k' are increases in the absorbance (660 nm) at 9° and a new temperature in 1 hr just after being transferred, respectively. The k'/k value indicates how fast the organism grows at a new temperature, com-

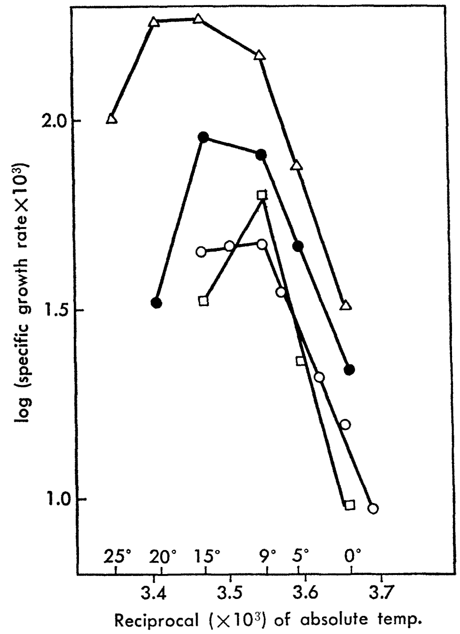

Fig. 7. Arrhenius plots of four obligately psychrophilic bacteria.
○—○, *Curtobacterium psychrophilum*; △—△, *Cytophaga xantha*; □—□, *Spirillum pleomorphum*; ●—●, *Micrococcus cryophilus*.

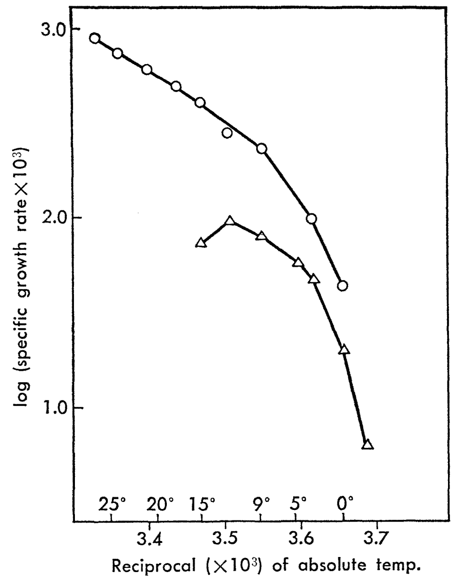

Fig. 8. Arrhenius plots of *Cytophaga antarctica* and *Pseudomonas fluorescens*.
○—○, *Pseudomonas fluorescens*; △—△, *Cytophaga antarctica*.

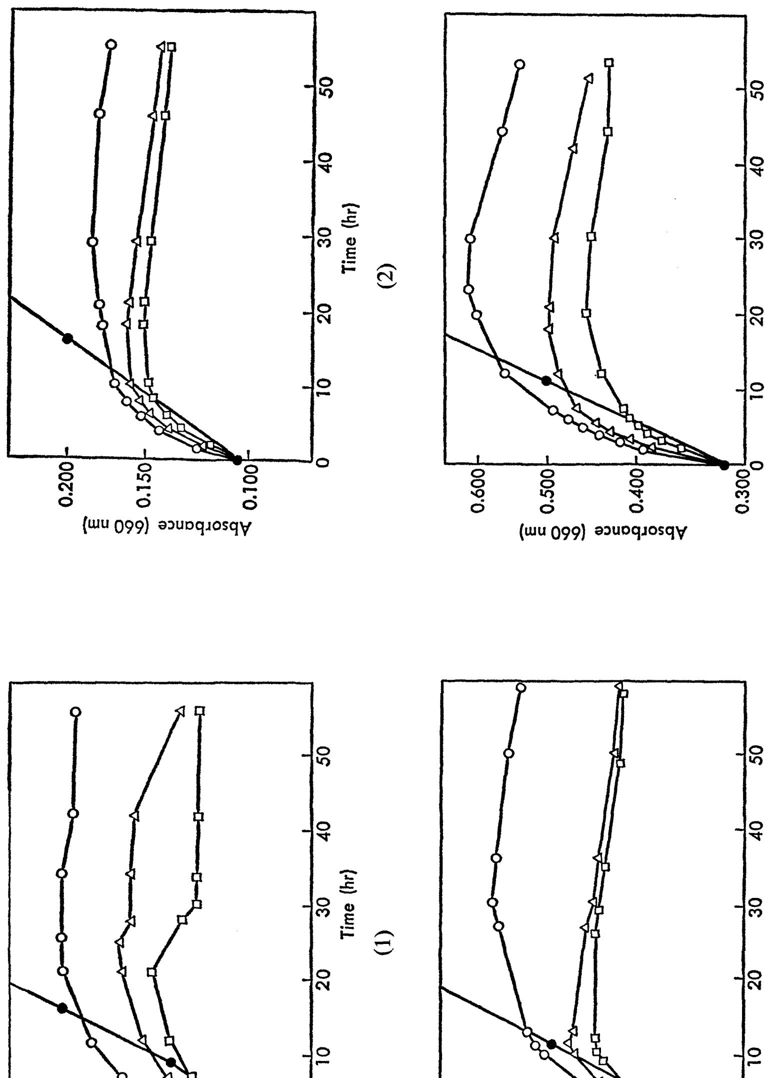

Fig. 9. Transfer of *Curtobacterium psychrophilum* from 9° to 20°, 25°, and 30°.
(1) transfer at stage I, (2) transfer at stage II, (3) transfer at stage III, (4) transfer at stage IV.
●—●, 9°; ○—○, 20°; △—△, 25°; □—□, 30°.

Table 2. Changes in growth of *Curtobacterium psychrophilum* when it was transferred from 9° to 20°, 25°, and 30°.

Stage		Temp.		
		20°	25°	30°
I	T	0.051	0.050	0.051
	C	0.080	0.070	0.068
	I	1.6	1.4	1.4
	k'/k	2.02	1.86	1.36
II	T	0.105	0.104	0.105
	C	0.182	0.160	0.152
	I	1.8	1.6	1.5
	k'/k	2.05	2.07	1.3
III	T	0.200	0.198	0.199
	C	0.385	0.301	0.280
	I	1.9	1.5	1.4
	k'/k	2.67	2.69	1.29
IV	T	0.323	0.319	0.318
	C	0.610	0.495	0.458
	I	1.9	1.5	1.4
	k'/k	2.65	2.67	1.70

T is absorbance (660 nm) when the organism was transferred from 9° to a new temperature.

C is absorbance (660 nm) when growth ceased at a new temperature.

I is a ratio C/T.

k and k' are increases in absorbance (at 9° and a new temperature) in an hour just after being transferred, respectively. The value k'/k indicates how fast the organism grows at a new temperature in comparison with at 9°.

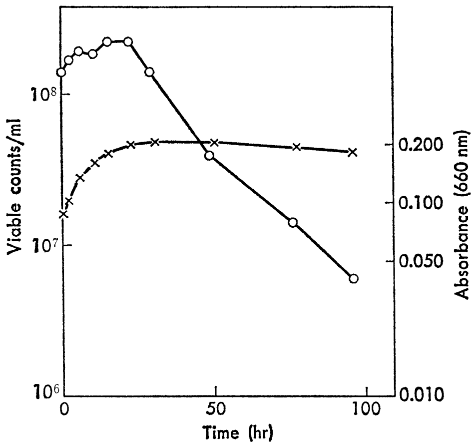

Fig. 10. Changes in viable counts and absorbance (660 nm) of *Curtobacterium psychrophilum* after being transferred from 9° to 20°.
○—○, viable counts; ×—×, absorbance (660 nm).

pared with growth at 9°. A change in the viable count is shown in Fig. 10. At 20°, after growth ceased, the viable count first began to decrease, then the absorbance at 660 nm decreased slowly. On the basis of these results, it was concluded as follows: When *C. psychrophilum* was transferred to temperatures above the maximum growth temperature after preincubation at 9°, it was able to grow faster for a while than at 9°. The absorbance at 660 nm exhibited about a 2-fold increase at the most. The higher the temperature to which the culture was transferred, the smaller the k'/k value and the increase in growth. The behavior of the cells at elevated temperatures varied according to the stage at which the cells were transferred. The cells in the exponential phase were able to grow faster and more than cells in the lag phase. When the culture was transferred to 20°, a faster growth than at the optimum growth temperature, cessation, death, and cell-lysis occurred in turn.

DISCUSSION

The Arrhenius plot and the temperature characteristic (μ) have been used to characterize psychrophiles in two aspects. First, INGRAHAM (*1*), in his comparative study of psychrophiles and mesophiles, showed that the μ value for psychrophiles was lower than that for mesophiles. On the basis of these results, he proposed that this low μ value was an important attribute of psychrophiles.

HANUS and MORITA (*4*), however, tried to obtain μ values of three taxonomically related bacteria which had different temperature ranges for growth, namely, *Vibrio marinus* MP–1 (opt. temp. 15°, max. temp. 20°), *Vibrio marinus* PS–207 (opt. temp. 24°, max. temp. 30°), and *Vibrio metschnekovii* (opt. temp. 37°, max. temp. 45°). They found that *V. marinus* MP–1 and PS–207 had almost the same values, and that *V. metschnekovii* had a somewhat lower μ value. Therefore, they pointed out that it was not reasonable to correlate the μ value with the temperature range for microbial growth, though microorganisms might have a constant μ value under given conditions. The results of SHAW (*5*) and BAIG and HOPTON (*6*) have supported this view.

Another significance of the Arrhenius plot in characterization of psychrophiles was proposed by HARDER and VELDKAMP (*7*). They compared Arrhenius plots of a facultative psychrophile and of an obligate psychrophile, which has a maximum growth temperature lower than 20°. It was found that a deviation occurred at about 3° in the Arrhenius plot of the facultative psychrophile, while a linearity was maintained from a temperature below the optimum growth temperature to −2° in that of the obligate psychrophile. They suggested that this deviation was the difference between obligate and facultative psychrophile.

As described in this paper, however, an obligate psychrophile, *C. antarctica,* exhibited a deviation at about 5° in its Arrhenius plot. Furthermore, as reported here, low maximum growth temperature is not correlated with the rate of growth activity at lower temperatures. Therefore, at present, only the low maximum growth temperature, 20° to 25°, should be used as the definition of psychrophile, though biochemical basis of this low maximum temperature is most important.

On the other hand, it is possible to presume factors which determine a maximum growth temperature, in terms of the behavior exhibited at supermaximum temperature after preincubation at the optimum growth temperature. From this point of view, psychrophiles are divided into two groups on the basis of data so far reported. In the case of first group, growth stops immediately and then death occurs very soon after being transferred to a supermaximum temperature (*8*). In the second group, faster growth than at the optimum growth temperature continues for a few hours, then growth ceases and death occurs (*9*, *10*). The data on *C. psychrophilum* indicate that this bacterium belongs to the second group. On the basis of these data, further biochemical study has been carried out and will be published elsewhere.

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