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# Isolation and growth temperature of psychrophiles

**Haruo SARUYAMA, Toshiro OCHIAI, Yasuhiro TAKADA, Hidetoshi OKUYAMA and Shoji SASAKI**

An attempt was made to isolate psychrophilic bacteria from a variety of source materials: Sample A, mosses of tundra in Alaska; Sample B, deep-frozen fish, dust and dirt in a cold-room; and Sample C, snow and water in a snow valley at the Taisetsu Mountains: Sixteen strains of psychrophilic bacteria and 5 strains of psychrophilic yeasts from Sample A, 55 strains of bacteria from Sample B, and 18 strains of bacteria and 3 strains of yeasts from Sample C were isolated. Determination of the maximum temperatures for growth of these isolates showed that all isolates were facultative psychrophiles. Some of them were studied taxonomically.

Temperature is one of the most important environmental factors affecting the biological functions. Among the studies on the bacterial functions at extremes of temperature, there are only a few studies concerning psychrophily in contrast with thermophily.

Psychrophile has been defined in many ways by microbiologists (SCHMIDT-NIELSEN, 1902; HUCKER, 1954; INGRAHAM, 1958; INGRAHAM and STOKES, 1959; BAXTER and GIBBONS, 1962; INGRAM. 1965 and so on). According to INGRAHAM and STOKES (1959), psychrophiles were subdivided to obligate psychrophiles and facultative psychrophiles: the former is defined as those bacteria that grow rapidly at 0°C and also grow most rapidly at temperature below 20°C, and the latter is capable of growth at 0°C and the optimum temperatures of them are similar to those of mesophiles. Recently, MORITA (1975) defined obligate psychrophile and facultative psychrophile as psychrophile and psychrotroph, respectively. After all, psychrophile means "cold-loving organism" and can grow well at near 0°C where mesophilic bacteria can not grow.

To investigate psychrophily, we attempted firstly to isolate psychrophiles from a variety of source materials. In this paper, experimental results of the maximum temperatures for growth of the isolates and taxonomic studies of some isolates are described.

## Materials and Methods

*Source Materials*: Mosses which were collected at Pointbarrow and Inuvik in Alaska in July, 1974, by Dr. A. SAKAI, dried and transported to

Sapporo, were used as Sample A. Sample B was deep-frozen fish, dust and dirt in a cold-room at the Package-center of the Sapporo Civil Co-operation, which were not permitted to warm up. Sample C was snow and water from a snow valley at the Taisetsu Mountains in Hokkaido, which were collected in August, 1975.

*Isolation*: An appropriate amount of each of the samples was sunk into or suspended in sterilized water, stirred, and settled. Then 0.2 m*l* of the supernatant was spread on the ordinary nutrient agar plate containing 1% each of peptone and meat-extract or Sabouraud's agar plate, which were previously chilled at 0°~2°C. Each of the visible colonies developed during 1 to 2 weeks of incubation at 2°C was picked and streaked on a chilled agar plate. These procedures were repeated until pure cultures were obtained.

*Measurement of Growth*: Degrees of growth at the temperatures shown in Table 1 were determined conveniently by increase of the cell mass on the nutrient agar slants.

To estimate the growth rates at different temperatures, 0.06 m*l* of the preculture (20°C, 24 hr) was inoculated into the test tube containing 6 m*l* of the nutrient medium and incubated at 0°, 10°, 20° and 30°C with vigorous aeration by a magnetic stirrer. At intervals, 0.2 m*l* of the culture was poured into 1.8 m*l* of the same medium and immediately after mixing the turbidity was measured at 600 nm with a Hitachi Perkin-Elmer Type 139 spectrophotometer. In the case of incubation at 0°C, increased size of inoculum was used so as to be about 0.5 of the optical density at zero time.

Growth rate was calculated from the formula

$$k = \frac{2.303\,(\log x_2 - \log x_1)}{t_2 - t_1}\, hr^{-1},$$

where $x_1$ and $x_2$ are optical densities at time $t_1$ and $t_2$, respectively.

*Determinative Methods*: All the methods were those described by HARRIGAN and MCCANCE (1966) and HASEGAWA (1976).

## Results

*Isolates from Sample A*: After incubation at 2°C for more than 2 weeks, on the basis of morphology and colony type, different colonies were picked and streaked on the agar plates. Resultant cultures were certified their purity microscopically and stored at 2°C as the stock culture. As the microbes which could grow well at 2°C for 5 to 10 days, 13 strains of bacilli, 3 cocci and 5 yeasts were isolated from the dried mosses (Table 1A). Of

these bacilli, 11 strains were Gram-positive, spore-forming aerobic bacilli, and seemed to belong in the genus *Bacillus*. All strains of cocci were Gram-positive, spore-forming tetrad or octad cocci, so they should be placed in the genus *Sporosarcina*. Degrees of growth at 24°, 30° and 37°C of these isolates were determined to know whether they are obligate psychrophiles or not. As shown in Table 1A, yeasts and 5 bacilli could not grow at 30°C

TABLE 1 Isolates from the Samples A, B and C.

| Sample | | Gram | Spore | Growth at 24° | 30° | 37° | Number of isolates | Remarks |
|---|---|---|---|---|---|---|---|---|
| A | Bacilli | + | + | ++[2] | − | − | 4 | P-10, I-12 etc. |
| | | | | + | + | − | 2 | |
| | | | | + | ++ | ± | 1 | |
| | | | | ++ | ++[3] | ++[1] | 4 | I-13 etc. |
| | Bacilli | − | − | ++ | − | − | 1 | I-9 |
| | | | | ++ | ++ | ++ | 1 | |
| | Cocci | + | + | ++ | ++ | ++[2] | 3 | |
| | Yeast | | | + | − | − | 5 | |
| C | Bacilli | − | − | ++ | − | − | 2 | |
| | | | | ++ | ± | − | 1 | |
| | | | | ++ | ++[5] | − | 8 | include 2 coccobacilli |
| | | | | ++ | ++ | ± | 2 | |
| | Bacilli | + | − | ++ | − | − | 2 | coccobacilli |
| | | | | ++ | ± | − | 1 | |
| | | | | ++ | ++ | − | 1 | |
| | Bacilli | + | + | + | − | − | 1 | |
| | Yeast | | | ++ | − | − | 3 | |
| | | | | 20° | 24° | 30° | | |
| B | Bacilli | − | − | ++[1] | ++[1] | − | 3 | G-3 etc. |
| | | | | ++[7] | ++[7] | ± | 9 | |
| | | | | ++[3] | ++[3] | + | 4 | E-3 etc. |
| | Bacilli | + | ± | ++ | ++ | − | 2 | G-4 etc. |
| | Cocci | − | − | ± | ± | − | 1 | |
| | | | | + | + | − | 1 | |
| | | | | ++ | ++ | ± | 1 | |

Symbols of growth: −, not grow; ±, growth was uncertain; + (++), grow (grow well). Superscripts mean true number of isolates suitable for each symbol, e.g., ++[3], 3 isolates are ++ and the other +.

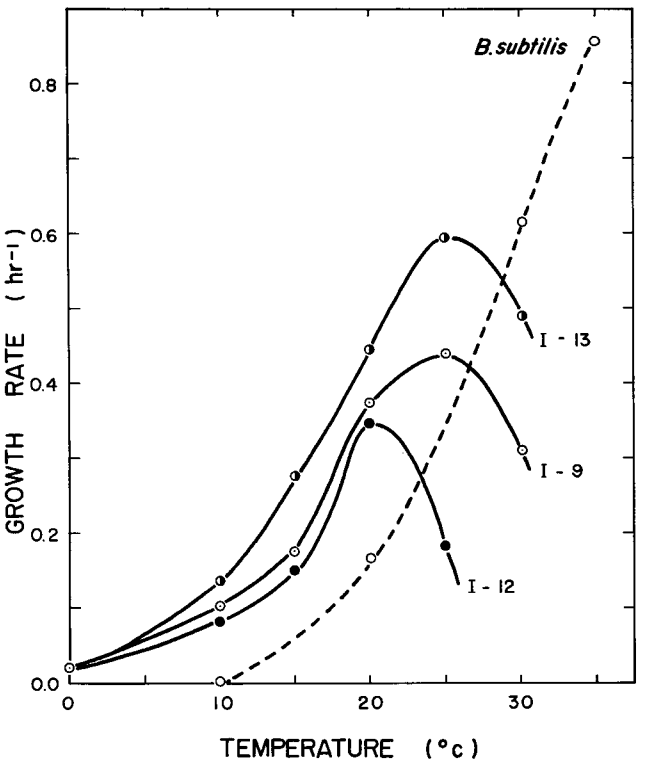


**Fig. 1.** Growth rates of psychrophilic bacteria isolated from mosses of tundra (Sample A). Growth rate was determined as described in the Materials and Methods. As a control, mesophilic *Bacillus subtilis* was used.

or above. Then 2 strains of these bacilli (I-9, I-12) and a bacillus growing extremely well at 24°C (I-13) were examined exactly for their growth rates at the temperatures between 0° and 30°C (Fig. 1). Comparing these isolates with mesophilic *Bacillus subtilis*, the growth rates of the psychrophiles at the range of lower temperatures were obviously higher than those of *B. subtilis*, but all these isolates were facultative psychrophilic bacteria.

*Isolates from Sample B*: Fifty-five isolates of psychrophilic bacteria were obtained from Sample B, but some of them might be identical strains. Accordingly, 21 strains, which might be different strains, were chosen and examined with respect to elementary properties. Of these isolates, 16 strains were Gram-negative asporogenous bacilli, 3 strains were Gram-negative asporogenous cocci, and 2 strains were Gram-positive bacilli and one of which was spore-forming bacillus (Table 1B). Growth rates at various temperatures

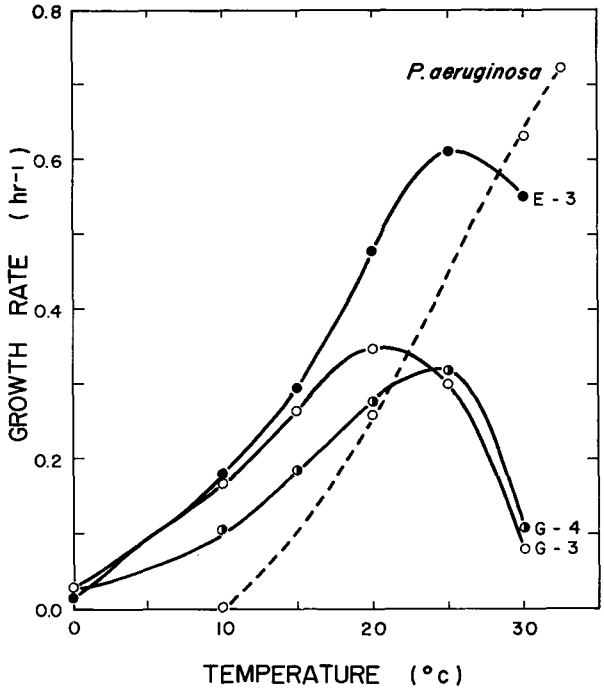


**Fig. 2.** Growth rates of isolates from a cold-room (Sample B). Conditions were the same as in Fig. 1. *Pseudomonas aeruginosa* was used as a control.

of some these isolates were examined (Fig. 2). Fig. 2 showed that these bacteria were facultative psychrophiles having the maximum temperature at above 30°C.

*Isolates from Sample C*: From snow and water at the Taisetsu Mountains, 18 strains of psychrophilic bacteria and 3 strains of yeasts were isolated. These bacteria were all bacilli or coccobacilli. Of these bacteria, 13 Gram-negative and 4 Gram-positive strains did not form endospore. Only one strain of Gram-positive bacillus formed endospore and maybe belong in the genus *Bacillus*. Although further identification and exact growth rates have not yet been determined, they were all facultative psychrophiles (Table 1C).

*Taxonomic Studies of Several Isolates*: Among these isolates from Samples A and B, several strains having extremely good yield of growth at low temperature have been subjected for biochemical studies such as respiratory activities, oxidative phosphorylation, protein synthetic activities and so on. Taxonomic studies of these bacteria are summarized in Table 2. Strains P-10, I-12 and I-13 were Gram-positive, spore-forming aerobic (or

TABLE 2 Characteristics of several isolates

| | Reaction temperature (°C) | I-9 | P-10 | I-12 | I-13 | G-3 | G-4 | E-3 |
|---|---|---|---|---|---|---|---|---|
| Gram reaction | | − | + | + | + | − | + | − |
| Spore formation | | − | + | + | + | − | ± | − |
| Oxidase | Room temp. | + | ± | + | + | + | − | + |
| Catalase | Room temp. | + | + | + | + | − | + | + |
| Methyl red test | 10 | − | | − | − | − | − | − |
| Glucose fermentation[a)] | 10 | − | − | − | − | − | − | − |
| Glucose oxidation[a)] | 10 | + | + | ± | + | + | + | + |
| Acetoin production | 10 | − | | − | − | − | − | − |
| Gelatin liquefaction | 10 | − | + | + | − | + | + | − |
| Casein hydrolysis | 10 | − | + | + | − | + | + | − |
| Casein hydrolysis | 20 | − | + | + | − | + | + | − |
| Litmus milk acid and | 10 | + | − | − | + | − | − | + |
| coagulation | 20 | + | ± | − | + | − | − | + |
| Starch hydrolysis | 10 | | | | | − | | ± |
| Starch hydrolysis | 24 | | | | | − | | ± |

a) was determined with Hugh and Leifson's medium.

facultative anaerobic) rod-shaped bacteria and produce catalase, so these strains should be placed in the genus *Bacillus*. Strain G-4 seems to be *Bacillus* sp., but its spore-formation has not been certified. Strain G-3 could not ferment glucose and weakly oxidize glucose. Non-diffusible orange pigment of G-3 showed different absorption spectrum from those of *Xanthomonas*; the absoprtion maxima in methanol were (425), 452 and 479 nm (cf. STARR and STEPHENS, 1964). We consider that the strain G-3 should be placed in the genus *Flavobacterium*. Strains E-3 and I-9 seemed to belong in the genus *Pseudomonas*, but the possibility that it may be *Agrobacterium* was not excluded, because identification was not completed.

## Discussion

Psychrophiles may have some mechanisms other than mesophiles by which they can maintain their low-temperature lives. We are interested in research on such mechanisms, which have been studied by a few investigators but as yet have been poorly understood. To obtain psychrophiles suitable for such investigations, an attempt was made to isolate the bacteria being able to grow well at 0°C, from various source materials such as dust and dirt in a cold-room, deep-frozen fish, snow and water at a snow valley, and

mosses of tundra. In this study, a total of 89 strains of psychrophilic bacteria and 8 strains of psychrophilic yeasts were isolated. Although the above count may contain several identical strains, especially in those from Sample B, obvious differences were observed between the source materials A and B or C. Namely, isolates from Sample A were mostly Gram-positive, spore-forming psychrophiles, but most isolates from Samples B and C were not so. Sample A was collected and transported in mid-summer without controlling temperature, therefore, asporogenous and obligate psychrophilic microbes seemed to be unable to survive under those conditions.

Although we have examined to isolate psychrophiles from deep water of the Lake Shikotsu and the Lake Kuttara other than the above samples, any obligate psychrophile has not been isolated. According to MORITA (1970), psychrophiles are not rare in permanently cold environments and can be isolated easily if the correct procedure is followed, and all the obligate psychrophiles reported were isolated from the marine enviroment. In the present study, the source materials or the isolation procedures may not be suitable for isolation of obligate psychrophiles. Recently, we isolated an obligate psychrophilic bacteria from the Sea of Okhotsk in winter and description of the isolate will be published elsewhere.

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