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Isolation of Psychrophilic Species of *Bacillus*

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ABSTRACT

LARKIN, J. M. (Washington State University, Pullman), AND J. L. STOKES. Isolation of psychrophilic species of *Bacillus*. J. Bacteriol. **91**:1667–1671. 1966.—Ninety psychrophilic isolates of *Bacillus* were obtained from soil, mud, and water by selective enrichment at 0 C. They grew well at 0 C, optimally at 20 to 25 C, and failed to grow at 30 or 35 C. Their minimal and maximal growth temperatures were lower than those for mesophilic species of *Bacillus* by 10 C or more. Growth of psychrophilic isolates also occurred at −2 and −4.5 C, and both spore formation and spore germination occurred at 0 C.

A large variety of psychrophilic bacteria, i.e., those which grow at 0 C, have been isolated by various investigators from natural sources. These include strains of *Achromobacter*, *Aerobacter*, *Aeromonas*, *Alcaligenes*, *Arthrobacter*, *Corynebacterium*, *Escherichia*, *Flavobacterium*, *Klebsiella*, *Micrococcus*, *Proteus*, *Pseudomonas*, *Rhodococcus*, *Streptococcus*, and *Vibrio* (1, 4, 6, 13, 16). Also, psychrophilic strains of *Clostridium* have been isolated recently (10). It appears, however, that authentic psychrophilic strains of *Bacillus* have not been obtained. Aerobic spore-forming bacteria have been cultured from Antarctic ice, snow, mud, plant debris, and other materials (3, 8), but their ability to grow at 0 C was not determined.

The present paper describes the isolation of 90 psychrophilic isolates of *Bacillus* from soil, mud, and water, their growth kinetics, and the effect of low temperatures on spore formation and spore germination.

MATERIALS AND METHODS

Seventy-five samples of soil, mud, and water were collected in sterile jars from fields, streams, and lakes. Soil and mud samples were diluted 1:10 in sterile 0.1% peptone and heated at 80 C for 10 min to destroy vegetative cells. Water samples were heated without prior dilution. A 5-ml amount of each heated sample was inoculated into 25 ml of sterile Trypticase Soy Broth (BBL) contained in a 250-ml Erlenmeyer flask, and was incubated for 2 weeks at 0 C. The cells from all flasks which showed growth were streaked on Trypticase Soy Agar and incubated at 0 C for 2 weeks. Isolated colonies were restreaked several times to insure purity and were then transferred to slants of nutrient agar. The purity of the cultures was established also by Gram stains. In some cases, more than one colony was isolated from a single sample if the colonies appeared to be different.

The pure cultures were retested for ability to grow at 0 C and also for their maximal growth temperatures by incubating Trypticase Soy Agar and nutrient agar slant cultures at 0 to 50 C at intervals of 5 C. Prior to inoculation, the slants were precooled or preheated to approximately the incubation temperature.

Inocula for all growth rate experiments were prepared by growing the cells in 25 ml of Trypticase Soy Broth in 250-ml Erlenmeyer flasks at 20 C on a New Brunswick rotary shaker at 300 oscillations per min for 28 to 32 hr. To determine the kinetics of growth at different temperatures, 250-ml Erlenmeyer flasks containing 25 ml of Trypticase Soy Broth were used. The flasks were fitted with matched test-tube side arms which could be inserted in a Klett-Summerson photoelectric colorimeter (660-mμ filter) for repeated measurements of turbidity without loss of culture. Each flask received 0.1 ml of the above inoculum and was incubated on a rotary shaker under the same conditions as described above. Growth curves were obtained in the range of 0 to 35 C at intervals of 5 C.

Spore suspensions were prepared from sporulated nutrient agar slant cultures. The growths were removed with distilled water, and the resulting suspensions were heated at 80 C for 15 min. They were then cooled and used immediately.

The size and shape of the cells and spores were determined from Gram- and spore-stained preparations. The Schaeffer-Fulton modification of Wirtz's spore stain was used (12). Observations and measurements were made with a Bausch & Lomb light microscope fitted with a filar micrometer. Vacuolation of cells grown on glucose-agar (nutrient agar plus 1% glucose) was determined by lightly staining the cells with dilute crystal violet (11).

RESULTS

Ninety psychrophilic isolates of *Bacillus* were isolated by the previously described selective enrichment at 0 C from the 75 samples of soil, mud, and water. No more than three isolates were ob-

tained from any one sample, and no isolates were obtained from 15 samples. All 90 isolates were rod-shaped and gram-positive except for a few that were gram-variable. All of the isolates were strictly aerobic, except for three isolates which also grew anaerobically. All isolates formed spores and grew at 0 C. The 90 isolates therefore are psychrophilic species of *Bacillus*.

Eight of the first 26 isolates grew at 30 C but not at 35 C, and the remaining 18 grew at 25 C but not at 30 C. The 64 subsequent isolates were not examined for their maximal growth temperatures. Comparison of the growth temperatures of the psychrophilic isolates with known mesophilic species of *Bacillus* (Table 1) shows clearly the marked differences in minimal and maximal growth temperatures for the two groups. Growth of the psychrophiles at 0 C was visible in two days and abundant in about 1 week. In contrast, the mesophiles failed to grow below 15 or 10 C. Likewise, the maximal growth temperatures for the psychrophiles were 25 and 30 C and for the mesophiles, 40 to 55 C. Thus, the growth temperatures for the psychrophilic isolates are lower than those for the mesophiles by 10 C or more at both ends of the growth temperature scale.

Moreover, 0 C is not the minimal growth temperature for the psychrophiles. This cardinal temperature is technically difficult to determine because of the tendency of culture media to freeze below 0 C. However, by adding 7.5% glycerol to Trypticase Soy Agar, it has been possible to obtain growth of some of the psychrophilic isolates at −2 and −4.5 C (Table 2). Growth was visible

TABLE 1. *Comparison of minimal and maximal growth temperatures of mesophilic and psychrophilic strains of Bacillus on Trypticase Soy Agar*

Organism	Minimal temp	Maximal temp
	C	C
Mesophilic strains		
Bacillus cereus NRS 942	10	50
B. subtilis NRS 949	15	55
B. circulans NRS 931	15	45
B. pumilus NRS 980	15	55
B. macerans NRS 888	15	40
B. sphaericus NRS 866	10	40
Psychrophilic isolates		
Bacillus W3	0*	30
Bacillus W4	0	25
Bacillus W6	0	30
Bacillus W16B	0	25
Bacillus W22	0	25
Bacillus W25	0	30

* Lowest temperature tested in this experiment.

TABLE 2. *Days required for visible growth of psychrophilic Bacillus isolates at subzero temperatures on Trypticase Soy Agar plus 7.5% glycerol*

Isolate	Days at	
	−2 C	−4.5 C
Bacillus W8	7	7
Bacillus W16A	7	6
Bacillus W16B	9	14*
Bacillus W25	7	6
Bacillus T3A	7	7
Bacillus T27B	—†	—†
Bacillus T75	7	6

* Trypticase Soy Agar without glycerol.
† No growth within 68 days.

usually in 6 or 7 days at these low temperatures. Quantitative data on growth rates at −2 and −4.5 C will be given later.

Types. Twenty of the psychrophilic isolates were chosen for more detailed investigation. These appeared to represent to some extent different types among the 90 cultures with respect to colonial morphology, cell size, and also shape, size, and position of the spores. Most of them exhibited swollen sporangia, and the spores appeared oval when enclosed in the cell, and spherical when free. These isolates resemble those in the *B. pantothenticus—B. sphaericus* subgroup in group III (*Bergey's Manual*). The cells of a few of the isolates exceeded 0.9 μ in diameter, appeared vacuolated when grown on glucose-agar, and formed cylindrical spores which did not swell the cells. They resemble the *B. megaterium—B. cereus* subgroup of group I (*Bergey's Manual*). The remaining isolates produced centrally located oval spores which did not swell the sporangia. The cells were less than 0.9 μ in diameter and were not vacuolated when grown on glucose-agar. These isolates may belong to the *B. subtilis—B. pumilus* subgroup in group I (*Bergey's Manual*). The 20 isolates are now under extensive investigation to determine their exact taxonomic position in the genus *Bacillus* and whether they represent new species.

Kinetics of growth. The rate and extent of growth of 10 of the 20 isolates were determined in the range of 0 to 30 C at intervals of 5 C. The data for *Bacillus* isolate W25 are plotted in Fig. 1. Growth was slowest at 0 C but was extensive with time and maximal in about 220 hr. Growth was most rapid at 20 and 25 C, and there was no growth at 30 C. The decrease in maximal growth with increase in temperature may reflect a deficiency of oxygen in the cultures, even though the

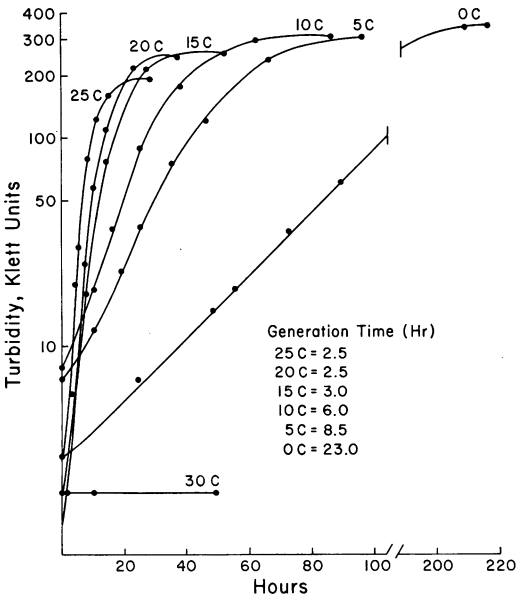

FIG. 1. *Effect of temperature on the growth of psychrophilic Bacillus isolate W25 in Trypticase Soy Broth.*

cultures were shaken (9). Similar data for *Bacillus* isolate W16B are shown in Fig. 2. The optimal temperature for most rapid growth was 20 C. There was no growth at 30 C.

To determine whether turbidity was a reliable measure of growth, the increase in turbidity, viable cells, and dry weight of *Bacillus* isolate W25 were measured simultaneously at 20 C in Trypticase Soy Broth cultures. It is evident from Fig. 3 that there is a close correlation among the three parameters. Similar results were obtained with three additional psychrophilic strains.

Growth rates at subzero temperatures are shown in Fig. 4. For this experiment, *Bacillus* isolate T3A was grown at −2 and −4.5 C in Trypticase Soy Broth to which 5 and 7.5% glycerol, respectively, were added to prevent freezing of the medium. The cultures were not shaken. The generation time at −2 C was 2 days and at −4.5 C it increased to 7 days. The relatively low cell yields at the subzero temperatures is probably due to O_2 deficiency in these unshaken cultures. It seems unlikely that the difference of 2.5% glycerol concentration at the two temperatures would markedly affect the growth rates. In any event, the data indicate that the organism can grow at subzero temperatures. It has not been possible to use temperatures below −4.5 C because the much larger amounts of glycerol required to prevent the medium from freezing inhibited growth.

Spore formation and germination at 0 C. Psychrophilic strains of *Clostridium* readily sporulate at 0 C (10). To determine whether this occurs with the psychrophilic *Bacillus* isolates, the 20

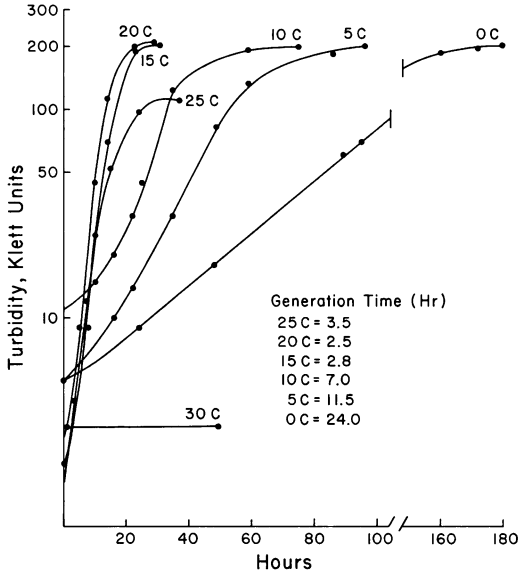

FIG. 2. *Effect of temperature on the growth of psychrophilic Bacillus isolate W16B in Trypticase Soy Broth.*

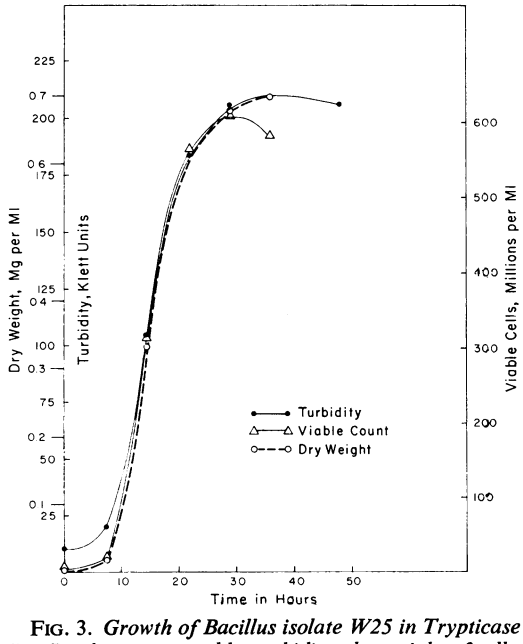

FIG. 3. *Growth of Bacillus isolate W25 in Trypticase Soy Broth as measured by turbidity, dry weight of cells, and viable counts.*

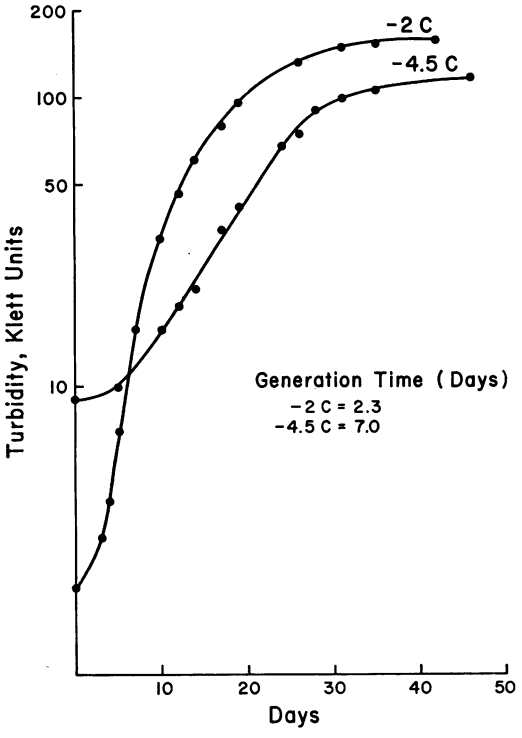

FIG. 4. *Effect of subzero temperatures on the growth of psychrophilic Bacillus isolate T3A.*

cultures were inoculated on nutrient agar slants, incubated at 0 C, and examined periodically for the presence of spores by means of spore stains. All of the isolates formed spores at 0 C.

The spores of psychrophilic strains of *Clostridium* also germinate at 0 C (10). The initial successful isolation of the psychrophilic *Bacillus* isolates from heated materials indicated that their spores can germinate at 0 C. This property, however, was reinvestigated with the pure cultures. Cell suspensions of cultures of the 20 isolates which had sporulated were heated at 80 C for 15 min to destroy vegetative cells. The suspensions were cooled to 0 C, inoculated on precooled Trypticase Soy Agar slants, and incubated at 0 C. Growth was obtained with all of the isolates. Thus, both spore formation and spore germination readily occur with the psychrophilic isolates of *Bacillus* at 0 C. Subzero temperatures have not been tested.

Germination of a spore occurs in two steps. The first, sometimes called "incipient germination" (2), is characterized by an increase in heat sensitivity, increased stainability, a change from bright to dark when observed by phase contrast, decrease in optical density, and excretion of dipicolinic acid. The second stage is the outgrowth of a vegetative cell, but does not include cell division (7). The minimal temperature at which the first stage can occur has been investigated by several workers. Williams, Clegg, and Wolf (15), using loss of heat resistance as the criterion, found a minimum of 4 C for 11 of 54 strains of *B. subtilis*. The remaining strains had a higher minimal temperature. Halvorson, Wolf, and Srinivasan (5) reported −6 C for *B. cereus* strain T. Lower temperatures were not investigated. Knaysi (7) investigated the minimal temperature for both stages with *B. cereus* C_3 and found minima of −1 and 10 C for the first and second stage, respectively. The first stage in thermophilic *B. stearothermophilus* has a minimal temperature of 14 C, although vegetative growth does not occur below 38 C (2). In general, therefore, the first stage can occur at temperatures well below the minimum for growth. The second stage, however, has a minimal temperature near the minimal growth temperature. The fact that spores of psychrophilic *Bacillus* and *Clostridium* can germinate and produce vegetative growth at 0 C indicates that outgrowth occurs at least 10 C lower than with spores of mesophiles. It also indicates that the first stage may occur at very low temperatures, although this aspect has not been investigated.

DISCUSSION

The results of numerous investigations on psychrophilic bacteria during recent years have dispelled the previously held idea that psychrophiles are predominantly strains of a few genera—*Pseudomonas*, *Flavobacterium*, and *Achromobacter*. It is now clear that psychrophilic bacteria belonging to a large number of genera exist. Morphologically, psychrophiles may be short or long rods, cocci, or vibrios. They may be gram-positive or gram-negative. They may be strictly aerobic, anaerobic, or facultative. They may be sporeformers or nonsporeformers. Psychrophilic representatives of at least 16 genera have been reported, and the present paper describes the isolation of psychrophiles of still another genus, *Bacillus*. It can probably be safely predicted that further investigations will extend the list of psychrophilic genera.

This large variety of psychrophilic bacteria, their ubiquity in nature, and their occurrence in many habitats in large numbers which may at times exceed those of mesophilic bacteria and frequently also thermophilic bacteria (14), strongly suggest that psychrophiles are of considerable importance in the various cycles of matter.

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