Isolation and Identification of Psychrophilic Species of *Bacillus* from Milk

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Forty isolates from 97 raw milk samples (heated to 80 C for 10 min and stored at 4 to 7 C for 3 to 4 weeks) were sporeforming, aerobic, gram-positive or gram-variable, rod-shaped bacteria. Fifteen isolates that were identified had characteristics similar to species of *Bacillus*, except that they had lower growth temperature ranges, were gram-variable, and were somewhat different in sugar fermentations. Four isolates grew well within 2 weeks at 0 C, but they grew faster at 20 to 25 C. These psychrophilic sporeforming bacteria, the importance of which is discussed, are considered to be variant strains of mesophilic bacilli adapted to low temperatures.

Most psychrophilic bacteria isolated from natural sources are heat-sensitive, gram-negative, nosporeforming rods (3, 5, 10, 19, 22). However, Larkin and Stokes (11) and Sinclair and Stokes (15) isolated psychrophilic species of *Bacillus* and *Clostridium* from soil, mud, and water, and Marshall and Ohye (12) isolated a gram-negative psychrophilic *Bacillus* from sub-Antarctic soil. The first report of psychrophilic sporeforming bacteria in food was that of Grosskopf and Harper (Abstr., J. Dairy Sci. 52:897, 1969), who reported isolating some from milk.

We have found species of *Bacillus* capable of growing at 7 C or less to be present in 25 to 35% of raw milk samples. This paper reports their isolation and identification.

MATERIALS AND METHODS

Samples. Ninety-seven raw milk samples were collected from several areas of California. The samples were refrigerated during transit to the laboratory and tested immediately. The samples (200 ml of each) were held at 80 C for 10 min in a water bath, cooled, stored at 4 to 7 C, and examined for microbiological spoilage after 3 to 4 weeks.

Media. The following media (from BBL) were used: Trypticase soy agar (TSA), Trypticase soy broth (TSB), nutrient broth, nutrient agar, and AK sporulation medium. Sporulation was determined with each of the above media, with nutrient agar plus 0.05% MgSO₄ and 0.05% MnSO₄ and with soybean agar (17).

Microscopic examination. Cell morphology, cell arrangement, cell size, motility, presence of spores, and presence of cell granules were determined by phase microscopy. Stained mounts were used for determining the Gram reaction and spore formation (18).

Identification tests. Isolates were identified according to Bergey's Manual. Analyses for biochemical and microbiological characteristics were done by the procedures of Smith et al. (17).

Measurement of growth. For determining ability to grow at different temperatures, the isolates were streaked on plates of TSA, and the plates were incubated at predetermined temperatures from 4 to 50 C. Before the streaking, the plates were preheated or pre-cooled to the incubation temperature. Plates were discarded if colonies did not develop in 1 week. Isolates that grew at 4 C were tested for growth within 2 weeks at 0 C in TSB in an antifreeze-water bath thermostatically controlled at 0 ± 1 C.

Experiments for determining specific growth rates were conducted in a G76 gyrotary water-bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.) adjusted to predetermined temperatures (5 to 35 C) and an agitation rate of 120 rev/min. A 250-ml Erlenmeyer flask containing 50 ml of TSB was inoculated with 0.2 ml of culture (grown at 10 C for 2 to 3 days in TSB), and the culture was transferred periodically (inoculum, 0.1 ml) until it reached the steady-state of growth (13, 14). Growth was determined by periodically measuring optical density at 600 nm with a Beckman spectrophotometer (model DB) with 5-cm cuvettes.

RESULTS

About 25% of 50 milk samples collected in the summer spoiled in 3 to 4 weeks in the refrigerator (4 to 7 C). This percentage was about 35% for 47 samples collected in the winter. Portions of the spoiled samples were plated on TSA, and the plates were incubated at 21 C for 2 to 3 days. Colonies were transferred to TSA slants, incubated at 21 C, and stored in a refrigerator.

Forty cultures isolated from morphologically different colonies by the above procedure were found to be sporeforming, aerobic, gram-positive.
Table 1. Growth characteristics of the isolates

| Group | No. of isolates | Minimal tempa (°C) | Maximal tempb (°C) | Spore formationc |
|-------|-----------------|--------------------|--------------------|-----------------|
| A     | 4               | 0c                 | 32–35              | Poor            |
| B     | 11              | 5 to 7             | 40–45              | Good            |

a Minimal (except 0°C) and maximal growth temperatures were determined on Trypticase soy agar. Tests at 0°C were in Trypticase soy broth in an antifreeze-water bath at 0 ± 1°C.

b Spore formation was tested in a variety of media.

c Lowest temperature tested.

Fifteen isolates that were morphologically different and indicated by preliminary experiments to have low minimal growth temperatures were selected for further study and identification. These isolates could be grouped into two different groups on the basis of minimal growth temperatures (Table 1). Those in group A were able to grow at temperatures as low as 0°C, whereas those in group B had minimal growth temperatures of 5 to 7°C. More recent experiments in broth have indicated a doubling time of about 30 hr at 0°C for the isolates of group A. Although the isolates of group A grew at 0°C within 2 weeks, they did not sporulate well at any temperature tested (3 to 25°C). In contrast, the isolates of group B, which had higher minimal growth temperatures, sporulated well at each temperature tested.

The growth kinetics of one isolate (RH3) of group A are shown in Fig. 1. Growth was slow at 5°C, in comparison to higher temperatures, and growth did not occur at 35°C. Specific growth rates and doubling times at different temperatures for this isolate are given in Table 2. The doubling times at 5°C for the other three isolates of group A (TS3, TS4, and RH22) were 6.75, 6.75, and 9.8 hr, respectively.

The biochemical and microbiological characteristics of those isolates of group A did not match completely any of the species of Bacillus described in Bergey's Manual. All four of these isolates were gram-variable and had growth temperature ranges considerably below those specified for species of Bacillus. One isolate, RH22, was considered to be a variant of B. subtilis since the few sporangia observed were not definitely swollen, the protoplasm of young cells grown on glucose agar were not vacuolated, growth on glucose or soybean-agar was good, and the organism hydrolyzed starch, produced nitrates from nitrates, liquefied gelatin rapidly, and grew in broth containing 4% NaCl. However, with ammonium salts as the source of nitrogen, this organism failed to ferment mannitol, sucrose, lactose, or glycerol, and it did not grow in broth containing 7% NaCl. The characteristics of another isolate, RH3, indicated that it was a variant strain of B. circulans, though there was

![Fig. 1. Effect of temperature on the growth of RH3 in Trypticase soy broth. Optical density measurements were made at 600 nm after the cultures were in the steady state of growth.](image)

Table 2. Effect of temperature on growth parameters of RH3 in Trypticase soy broth

| Temp (°C) | Growth parameter |
|----------|------------------|
|          | k/hrb | g (hr)b |
| 5        | 0.1066 | 6.5     |
| 10       | 0.1925 | 3.6     |
| 15       | 0.2887 | 2.4     |
| 20       | 0.3465 | 2.0     |
| 25       | 0.4620 | 1.5     |
| 30       | 0.4332 | 1.6     |

a Parameter k is specific growth rate per hour estimated from the equation: \( \log x = kt + \log x_0 \), where \( x \) is optical density (OD) at time \( t \) and \( x_0 \) is OD at time zero.

b Parameter g is the doubling time determined from a plot of \( \log x \) OD versus time. OD was measured at 600 nm.
good growth on soybean-agar and acid was produced from each of the above four carbon sources or arabinose or xylose. Two isolates, TS3 and TS4, resembled B. coagulans in biological and microbiological tests. They produced acid from glucose and from each of the above six carbon sources.

The characteristics of the isolates in group B, except for growth temperature ranges, matched the following species of Bacillus: brevis, circulans, cereus, coagulans, laterosporus, licheniformis, macerans, megaterium, polymyxa, pumilus, and subtilis.

DISCUSSION

Psychrophilic bacteria have been defined by Ingraham and Stokes (10) as bacteria that grow well at 0 C within 2 weeks and by Witter (22), based partly on a standard method for determining psychrophilic bacteria (1), as bacteria that grow at a relatively rapid rate at 7.2 C, i.e., that form visible colonies on plates at this temperature in 10 days. Foster et al. (6) defines them as bacteria that grow relatively rapidly at 1.7 to 10 C, the temperature normally used in commercial holding and distribution channels. Witter (22) suggested that a generation time of about 15 hr or less at 7.2 C would be a reasonable requirement for organisms that are to be called psychrophiles, and Ingraham and Stokes (10) suggested a generation time of 48 hr or less at 0 C. Two of the psychrophilic sporeformers isolated from mud and studied by Larkin and Stokes (11) had generation times of 8.5 and 11.5 hr at 5 C, and Ingraham (9) reported generation times of 7 to 10 hr at 4 C for psychrophilic pseudomonads.

Some investigators prefer to describe such bacteria as psychrotrophic rather than psychrophilic (12, 21), and there seems to be merit in using some term that indicates that they merely are able to grow at low temperatures rather than that they are cold-loving. In any event, all of the isolates we studied grew under refrigeration at 7 C or less, but they grew faster at higher temperatures (25 to 35 C). Those in group A (Table 1) were able to grow within 2 weeks at 0 C and had generation times of about 30 hr at 0 C and 6.5 to 9.8 hr at 5 C. Of the isolates we studied, at least these four fit each of the above definitions proposed for psychrophilic bacteria.

The important difference between the bacteria we isolated and known characteristics of mesophilic bacilli is that those we isolated were able to grow at lower temperatures. Mesophilic bacilli normally have not been found to grow below about 8 C, and 10 C often has been found to be their minimal growth temperature (5, 10, 19, 22).

We suspect that the organisms we isolated are variants of mesophilic bacilli adapted to lower growth temperature ranges (2, 7, 16). Simultaneously with this adaptation, there apparently developed a tendency toward poor sporulation that was particularly evident in the isolates of group A. We did not attempt to reverse this adaptation, but Grosskopf and Harper (Abstr., J. Dairy Sci 52:897, 1969) reported that the psychrophilic sporeforming bacteria they isolated could be adapted to higher growth temperature ranges and that they then were unable to grow at low temperatures.

Spoilage of pasteurized milk and milk products often results from the growth of heat-sensitive, gram-negative, nonsporeforming bacteria that enter products after pasteurization. In regard to this, failure of the isolates of group A to form spores readily on laboratory media and the fact that they were gram-variable are important. Possibly some instances have occurred in which gram-variable bacteria such as those we isolated have been mistaken for heat-sensitive species, e.g., Pseudomonas, Alcaligenes, or Aerobacter.

The doubling times of the four isolates of group A at low temperatures are not greatly different from those of some species of Pseudomonas known to be important in the spoilage of foods (4, 8, 9, 20, 22), but the isolates of group B grew more slowly. In regard to this, it is important that the organisms of group A were found in only four of 97 samples of raw milk. Obviously, it would be a mistake to relax efforts to prevent nonsporeforming psychrophilic bacteria from getting into milk and dairy products after pasteurization. However, as attempts are made to extend the shelf-life of fluid dairy products, psychrophilic sporeforming bacilli will become a greater potential problem. This is also important in regard to the use of dairy products in other foods, the development of aseptic filling, and the probability of a shift toward "sterilization" of milk and fluid milk products.

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