Taxonomy of Psychrophilic Strains of Bacillus

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The morphological and physiological characteristics of 20 isolates of psychrophilic Bacillus were compared with 29 strains representing nine species of mesophilic Bacillus and 2 strains of Sporosarcina ureae to determine the taxonomic position of the psychrophiles. The psychrophiles formed four distinct groups which were sufficiently different from the mesophiles to warrant their designation as new species of Bacillus. The names B. psychrosaccharolyticus, B. insolitus, B. globisporus, and B. psychrophilus are proposed for the new species.

There is a wide variety of types of bacteria which may be defined as psychrophilic, i.e., they grow well at 0 C (11). Psychrophilic representatives of at least 17 genera have been reported. They include rods, cocci, and vibrios, and they may be gram-positive or gram-negative, aerobic, anaerobic, or facultative (1, 2, 4, 11, 12). Recently, we described the isolation of 90 psychophilic strains of Bacillus, and also the growth kinetics, growth at subzero temperatures, and effect of low temperatures on spore formation and spore germination of several of the isolates (5). The possibility existed that some, or all, of the psychrophilic isolates were new species. To determine the taxonomic position of the psychrophiles, a detailed study was made of the morphological and physiological properties of 20 of the psychrophilic isolates and, for comparison, several mesophilic species of Bacillus and Sporosarcina ureae. Several new species were established for the psychrophiles.

Materials and Methods

Cultures. Twenty psychrophilic Bacillus isolates were selected for detailed taxonomic investigation after preliminary studies of the colonial morphology and cell size, as well as shape, size, and position of the spores of the 90 isolates. The selected strains appeared to be representative.

Strains of known mesophilic species of Bacillus, with morphological characteristics similar to those of the psychrophiles, were obtained from Ruth E. Gordon, Institute of Microbiology of Rutgers University, to serve as controls for the various physiological tests and for establishment of differences and similarities between the two groups. The mesophilic species were B. pantothenticus strains NRS 1317-1322, B. circulans strains ATCC 61, 4513, 4515, 4516, 4530, 7049, 8384, 9995, and 11034, B. sphaericus strains NRS 349, 350, 1089, 1090, ATCC 7063, 12300, and 12844, B. rottans strains NRS 633 and ATCC 4978, B. loehnisii strain NRS 672, B. freudenreichii strain ATCC 7053, B. cascanensis strain ATCC 12327, B. aneurinolyticus strain ATCC 12856, and B. aminovorans strain ATCC 7046. In addition, S. ureae strains 860 and 752 were obtained from R. E. McDonald, Cornell University, because of a morphological similarity between this organism and some of the isolates.

Methods. To determine the taxonomic position of the isolates, the methods of Smith, Gordon, and Clark (8, 9) were used. In addition, for fermentation studies, their basal medium (9), modified to include 1% of substrate and phenol red indicator, was used. These changes provided more rapid and definitive results. All tests with the psychrophiles were made at least once at 15 and 25 C, and several times at 20 C. Results were the same at all temperatures unless otherwise indicated. All tests on the mesophiles were conducted at the same time as those on the psychrophiles, but with incubation of the bacilli at 30 C and S. ureae at 25 C. To insure that the results were comparable, the tests for both groups were made on media from the same batch. Analyses for cytochrome oxidase and phenylalanine deaminase were made with Patho-Tech (Warner-Chilcott Laboratories, Richmond, Calif.) differential test papers on 1-, 3-, and 5-day-old cultures. Phenylalanine deaminase was determined also by the method of Ewing, Davis, and Reavis (3). Proteus mirabilis and P. vulgaris served as positive controls.

Hucker's modification of the Gram stain (10) and, the Schaeffer-Fulton modification of Wirtz's spore stain (10) were used to determine the size and shape of the cells and spores. Flagella were stained by Gray's method (10) on cover slips which had been heated previously to 385 C for 2 hr in a muffle furnace. Measurements of cells and spores were made with a microscope fitted with a filar micrometer.

All photomicrographs were made with a Polaroid MP-3 Industrial Camera on Kodak Contrast Process Ortho film.

Results

Fifteen of the isolates formed four distinct groups, and the five remaining isolates differed from these groups and from each other. Because
only one isolate of each of the latter was studied, it was decided to postpone their taxonomic allocation until additional strains become available. The four groups were sufficiently different from known species of Bacillus to warrant their designation as new species. A description of these new species and the derivation of their names are as follows.

Description of B. psychrosaccharolyticus sp. n.

This species is represented by three strains, T15A, T25B, and T27B.

Morphology: The rods, on nutrient agar, are 0.6 to 1.0 μ by 1.5 to 3.5 μ with rounded or pointed ends. The usual size is 0.9 to 1.0 μ by 2.5 to 3.0 μ. They occur singly or in pairs. The protoplasm is granular or foamy when lightly stained. On glucose agar the cells are larger and vacuolated. They are motile at 20 C with peritrichous flagella (Fig. 1). Gram-positive.

Spores, on nutrient agar (Fig. 1), are 1.0 to 1.2 μ by 1.5 to 1.8 μ. Ellipsoidal, central to paracentral, and often filling most of the sporangium. Some formed laterally with remnants of sporangium thicker on one side. Spores are produced at 0 C and germinate at 0 C.

The sporangia are definitely swollen.

Gelatin agar streak plate: Wide zone of hydrolysis.

Nutrient agar colonies: White, raised, mucoid, and irregular. Entire to undulate margin. No motile microcolonies.

Nutrient agar slants: Growth moderate, white, and glistening.

Glucose agar slants: Growth the same as on nutrient agar.

Glucose nitrate agar slants: Scant, if any, growth.

Proteose-peptone acid agar slants: No growth.

Soybean agar slants: Growth greater than on nutrient agar. Sporulation poorer.

Trypticase Soy Agar (BBL) slants: Growth better than on nutrient agar.

Nutrient broth: Light to moderate turbidity, with sediment. A pellicle may be formed.

Fig. 1. (A) Flagella of Bacillus psychrosaccharolyticus strain T25B after 24 hr of growth on Trypticase Soy Agar, and (B) spores of B. psychrosaccharolyticus strain T27B after 8 days of growth on Nutrient Agar. (C) Flagella of B. insolitus strain T16B after 24 hr of growth on Trypticase Soy Agar, and (D) spores of B. insolitus strain W16B after 8 weeks of growth on Trypticase Soy Agar. Incubation of all cultures was at 20 C. X 2,400.
NaCl broth: Growth in 2%. No growth in 4%. Litmus milk: Initially slightly alkaline and then acidic in 3 weeks.

Milk agar streak plate: Narrow zone of hydrolysis.

Potato: No growth.

Indole production: Negative.

Fermentation tests: Acid but no gas (with ammonium salts as source of nitrogen) from glucose, lactose, sucrose, mannitol, arabinose, xylene, and glycerol.

Starch hydrolysis: Positive. No crystalline dextrins produced.

Acetymethylcarbinol production: Negative.

pH of glucose broth cultures: 5.2 to 6.0 after 6 days.

Citrate utilization: Negative.

Methylene blue reduction: Negative.

Nitrate reduction to nitrite: Positive. No gas produced in nitrate broth under anaerobic conditions.

Urease: Negative.

Phenylalanine deamination: Negative.

Cytochrome oxidase: Positive.

Relation to oxygen: Aerobic, facultatively anaerobic. Growth occurs in glucose broth under anaerobic conditions. Final pH 6.2.

Catalase: Positive.

Temperature relations: Maximum, 30 C. Minimum, 0 C.

Source: Soil and lowland marshes.

The name given to this organism was derived from the Greek noun psychros, meaning cold, the Greek noun sacchar, meaning sugar, and the Greek adjective lyticus, meaning able to loosen. In modern taxonomic Latin, lyticus is used as an adjective meaning digesting. Thus, B. psychrosaccharolyticus is a psychrophilic sugar-digesting organism. This name was chosen because the organism rapidly fermented all the sugars tested. The type strain selected for this species is B. psychrosaccharolyticus strain T25B.

B. psychrosaccharolyticus most closely resembles B. circulans among the mesophilic species of Bacillus. However, the psychrophile is able to grow at 0 C, whereas the minimal growth temperature for B. circulans is usually 10 to 15 C. At the other end of the growth temperature scale, B. psychrosaccharolyticus has a maximal growth temperature of 30 C and that of B. circulans is at least 10 C higher. In addition, the cells of B. psychrosaccharolyticus differ from those of B. circulans in that they are much larger, granular in appearance when lightly stained, and vacuolated when grown on glucose agar. These three characteristics are generally associated with the B. megaterium-B. cereus subgroup in group I of Bergey's Manual of Determinative Bacteriology. Also, the cells of B. psychrosaccharolyticus are gram-positive, whereas those of B. circulans are usually gram-negative.

Description of B. insolitus sp. n. This species is represented by two strains, W16B and T16B.

Morphology: The rods, on nutrient agar, are 1.0 to 1.5 μ by 1.6 to 2.7 μ. Many cells are spherical, or nearly so. They occur singly or in pairs. They stain uniformly with an occasional shadow or ghost form present. On enriched media, such as Trypticase Soy Agar, the cells are rod shaped, 0.7 to 0.9 μ by 2.4 to 5.3 μ. Spherical cells are nonmotile, whereas rod-shaped cells are motile at 5 and 20 C by means of peritrichous flagellation (Fig. 1). Gram-positive.

Spores, on nutrient agar, are round to cylindrical, 0.8 to 1.4 μ in diameter when round, and 1.0 to 1.2 μ by 1.3 to 2.0 μ when cylindrical. On Trypticase Soy Agar (Fig. 1), they are oval to cylindrical, 0.7 to 1.0 μ by 1.1 to 2.4 μ, and usually terminal. Spores are produced at 0 C and germinate at 0 C.

The sporangia are not appreciably swollen. Sporulation slow on nutrient agar. Slow also on Trypticase Soy Agar, but more spores are formed. Gelatin agar streak plate: No hydrolysis. Growth only with large inoculum.

Nutrient agar colonies: Small, soft, off-white, and irregular. Undulate margin. No motile microcolonies.

Nutrient agar slants: Growth scant to moderate, soft, and white.

Glucose agar slants: Growth the same as on nutrient agar.

Glucose nitrate agar slants: Growth poorer than on nutrient agar.

Proteose-peptone acid agar slants: No growth.

Soybean agar slants: Growth better than on nutrient agar. Slight darkening of the medium.

Tyrosine agar slants: Growth the same as on nutrient agar. Pigment is not produced.

Trypticase Soy Agar slants: Growth better than on nutrient agar. Slight darkening of the medium.

Nutrient broth: Light to moderate turbidity, with sediment.

NaCl broth: Growth variable in 2%. No growth in 4%.

Litmus milk: No change.

Milk agar streak plate: No growth.

Potato: No growth.

Indole production: Negative.

Fermentation tests: No acid or gas (with ammonium salts or organic sources of nitrogen) from glucose, lactose, sucrose, mannitol, arabinose, xylene, and glycerol.
Starch hydrolysis: Negative. No crystalline dextrins produced.
Acetylthiocholine production: Negative.

pH of glucose broth cultures: Unchanged after 6 days.

Citrate utilization: Negative.
Methylen blue reduction: No growth.

Nitrate reduction to nitrite: Negative. No gas produced in nitrate broth under anaerobic conditions.

Urease: Negative.
Phenylalanine deamination: Negative.

Cytochrome oxidase: Positive.
Relation to oxygen: Strictly aerobic. No growth in glucose broth under anaerobic conditions.

Catalase: Positive.

Temperature relations: Maximum, 25 C. Optimum, about 20 C. Minimum, below 0 C.

Source: Marshy and normal soil.

The name given to this organism was derived from the Latin adjective insolitus, meaning unusual. This refers to the unusual ability of the organism to appear often coccaloid on nutrient agar and bacillary on Trypticase Soy Agar. In addition, its flagellation, which consists of one polar and one subpolar flagellum when grown at 5 or 20 C, is unusual for members of this genus since most motile strains of Bacillus have a large number of flagella arranged peritrichously. The type strain selected for this species is B. insolitus strain W16B.

This organism appears to be unlike any known species of Bacillus. Its unusual morphology and its negative reactions in almost all of the routine physiological tests serve to characterize the species. Its morphology on nutrient agar is suggestive of S. ureae, but it differs from S. ureae in its inability to utilize citrate as a sole source of carbon or to reduce nitrate to nitrite, its motility in the absence of urea, and its lack of urease. Perhaps the most important difference between these two organisms is that B. insolitus forms rod-shaped cells with ellipsoidal spores on enriched media, such as Trypticase Soy Agar, whereas S. ureae is coccaloid on all media.

Description of B. globisporus sp. n. This species is represented by five strains, W8, W17, W25, T26A, and T38C.

Morphology: The rods, on nutrient agar, are 0.8 to 1.1 μ by 1.3 to 6.4 μ with rounded ends. The usual size is 0.9 to 1.0 μ by 2.5 to 4.0 μ. They occur singly or in pairs. They stain uniformly. They are motile at 20 C and have peritrichous flagella (Fig. 2). Gram-positive or gram-variable.

Spores, on nutrient agar (Fig. 2), are 1.0 to 1.1 μ in diameter. Round, subterminal to terminal, and sometimes slightly lateral. Spore wall usually thick and easily stained. Remnants of sporangium may adhere to the spore. Immature spores are sometimes ellipsoidal, later becoming round. Spores are produced at 0 C and germinate at 0 C.

The sporangia are definitely swollen.

Gelatin agar streak plate: Wide zone of hydrolysis.

Nutrient agar colonies: Off-white, raised, and irregular. Lobate to undulate margin. No motile microcolonies.

Nutrient agar slants: Growth moderate, smooth, white, and glistering.

Nutrient agar slants at pH 6.0: Scant, if any, growth.

Glucose agar slants: Growth the same as on nutrient agar.

Glucose nitrate agar slants: Scant, if any, growth.

Proteose-peptone acid agar slants: No growth.

Soybean agar slants: Growth better than on nutrient agar. Sporulation the same as on nutrient agar.

Tyrosine agar slants: Growth the same as on nutrient agar. Pigment is not produced.

Trypticase Soy Agar slants: Growth better than on nutrient agar.

Nutrient broth: Moderate turbidity, with sediment.

NaCl broth: Growth in 2%. Growth variable in 4%. No growth in 7%.

Litmus milk: Slightly alkaline with a small curd in 3 weeks.

Milk agar streak plate: Medium zone of hydrolysis.

Potato: No growth.

Indole production: Negative.

Fermentation tests: Acid but no gas (with ammonium salts as source of nitrogen) from glucose, lactose, sucrose, and glycerol. No acid from mannitol, arabinose, and xylose. No acid from lactose with organic nitrogen source.

Starch hydrolysis: Negative. No crystalline dextrins produced.

Acetylthiocholine production: Negative.

pH of glucose broth cultures: Unchanged after 6 days.

Citrate utilization: Negative.
Methylen blue reduction: Positive. May or may not be reoxidized in 45 days.

Nitrate reduction to nitrite: Negative. No gas produced in nitrate broth under anaerobic conditions.

Urease: Positive.

Phenylalanine deamination: Negative.
Cytochrome oxidase: Positive.

Relation to oxygen: Aerobic, facultatively
FIG. 2. Flagella (A) and spores (B) of Bacillus globisporus strain W25. Flagella (C) and spores (D) of B psychrophilus strain W16A. Flagella stains are from 24-hr-old cultures on Trypticase Soy Agar, and spore strains are from 4-day-old cultures on Nutrient Agar. Incubation of all cultures was at 20 °C. × 2,400.
anaerobic. Growth occurs in glucose broth under anaerobic conditions. The pH is unchanged.

**Catalase:** Positive.

**Temperature relations:** Maximum, 25 C. Optimum, between 20 and 25 C. Minimum, below 0 C.

**Source:** Soil and river water.

The name of this organism was derived from the Latin noun *globis*, meaning a round body, and the Greek noun *spora*, meaning seed. In modern taxonomic Latin, *spora* is used to indicate a spore. Thus, *globisporus* means round-spored, and refers to the round spores produced by this organism. The type strain selected for this species is *B. globisporus* strain W25.

This species most closely resembles *B. pantothenticus* among the mesophilic species, but it differs from that organism in its ability to grow well on glucose nutrient agar and soybean agar, in its ability to produce urease and to ferment lactose, and in its ability to hydrolyze starch or grow in the presence of more than 4% NaCl, or to lower the pH of glucose broth. In addition, the maximal and minimal growth temperatures of *B. globisporus* are about 20 C lower than those for *B. pantothenticus*.

**Description of B. psychrophilus sp. n.** This species is represented by five strains, W3, W5, W10A, W16A, and T5A.

**Morphology:** The rods, on nutrient agar, are 0.8 to 0.9 \( \mu \) by 1.2 to 4.5 \( \mu \), with rounded ends. They occur singly or in pairs. They stain uniformly with an occasional shadow or ghost form. They are motile at 20 C with peritrichous flagella (Fig. 2). Gram-positive.

Spores, on nutrient agar (Fig. 2), are 0.9 to 1.3 \( \mu \) in diameter. Round, subterminal to terminal, and sometimes slightly lateral. Remnants of sporangium may adhere to the spore. Immature spores are sometimes ellipsoidal, later becoming round. Spores are produced at 0 C and germinate at 0 C.

The sporangia are definitely swollen.

**Gelatin agar streak plate:** Wide zone of hydrolysis.

**Nutrient agar colonies:** Off-white, raised, and irregular. Lobate to undulate margin. No motile microcolonies.

**Nutrient agar slants:** Growth moderate, white, smooth, and glistening.

**Nutrient agar slants at pH 6.0:** Scant, if any, growth.

**Glucose agar slants:** Growth poorer than on nutrient agar.

**Glucose nitrate agar slants:** Growth scant.

**Proteose-peptone acid agar slants:** No growth.

**Soybean agar slants:** Growth better than on nutrient agar. Sporulation the same as on nutrient agar.

**Tyrosine agar slants:** Growth the same as on nutrient agar. Pigment is not produced.

**Tryptophan Soy Agar slants:** Growth better than on nutrient agar.

**Nutrient broth:** Moderate turbidity, with sediment.

**NaCl broth:** Growth in 2%. Growth variable in 4%. No growth in 7%.

**Litmus milk:** Slightly alkaline with a small curd in 3 weeks.

**Milk agar streak plate:** Very slow hydrolysis. Better hydrolysis by some strains at 15 C than at 20 C.

**Potato:** No growth.

**Indole production:** Negative.

**Fermentation tests:** Acid but no gas (with ammonium salts as source of nitrogen) from glucose, sucrose, mannitol, xylose, and glycerol. No acid from lactose or arabinose.

**Starch hydrolysis:** Negative with soluble starch. Some strains positive with potato starch. No crystalline dextrins produced.

**Acetylmethylcarbinol production:** Negative.

**pH of glucose broth cultures:** Unchanged after 6 days.

**Citrate utilization:** Negative.

**Methylene blue reduction:** Variable.

**Nitrate reduction to nitrite:** Positive. No gas produced in nitrate broth under anaerobic conditions.

**Urease:** Positive.

**Phenylalanine deamination:** Negative.

**Cytochrome oxidase:** Positive.

**Relation to oxygen:** Aerobic, facultatively anaerobic. Growth occurs in glucose broth under anaerobic conditions. The pH is unchanged.

**Catalase:** Positive.

**Temperature relations:** Maximum, 30 C. Optimum, about 25 C. Minimum, below 0 C.

**Source:** Soil and river water.

The name of this organism was derived from the Greek noun *psychros*, meaning cold, and from the Greek adjective *philos*, meaning loving. The type strain selected for this species is *B. psychrophilus* strain W16A.

This species most closely resembles *B. pantothenticus* among the mesophilic species, but differs from that organism in its ability to ferment mannitol and xylose, to produce urease, and to grow on soybean agar, its much larger size, and its inability to grow in the presence of more than 4% NaCl or to change the pH of glucose broth. In addition, the maximal and minimal growth temperatures of *B. psychrophilus* are about 15 C lower than those for *B. pantothenticus*.
B. psychrophilus may be differentiated easily from B. globisporus by its smaller size, higher maximal and optimal growth temperatures, poorer hydrolysis of casein, poorer growth on glucose nutrient agar, inability to ferment lactose, and ability to ferment mannitol and xylose, and reduce nitrate to nitrite.

DISCUSSION

Bergey's Manual of Determinative Bacteriology divides the genus Bacillus into three morphological groups. B. insolitus belongs to group I because the spores do not swell the sporangia, B. psychrosaccharolyticus belongs to group II because the spores are ellipsoidal and swell the sporangia, and B. globisporus and B. psychrophilus belong to group III because the spores are round and swell the sporangia. Thus, psychrophilic species belonging to each of the three morphological groups were represented in the 20 psychrophilic isolates selected for the present investigation.

Other psychrophilic sporeforming bacteria have been isolated. Sinclair and Stokes (7) reported the isolation of psychrophilic species of Clostridium from soil, mud, and sewage. Recently, Marshall and Ohye (6) isolated psychrophilic B. macquariensis from three of four soil samples obtained from Macquarie Island. It is different from the new species described in the present paper. The organism accounted for about 1% of the total viable bacteria in the samples. Also, in our previous investigation (5), 90 psychrophilic strains of Bacillus were isolated from 60 of 75 samples of soil, mud, and water. It seems probable, therefore, that aerobic and anaerobic psychrophilic sporeforming bacteria are widespread in nature.

The type strains of the four species have been deposited with the American Type Culture Collection and have been given the following accession numbers: B. psychrosaccharolyticus strain T25B (ATCC 23296), B. insolitus strain W16B (ATCC 23299), B. globisporus strain W25 (ATCC 23301), and B. psychrophilus strain W16A (ATCC 23304).

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LITERATURE CITED