Bacillus psychrophilus sp. nov., nom. rev.

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Presumably because the name "*Bacillus psychrophilus*" Larkin and Stokes 1967 was considered to be a subjective synonym of *Bacillus globisporus*, it was not included on the Approved Lists of Bacterial Names and, therefore, has no standing in bacteriological nomenclature. The low deoxyribonucleic acid relatedness values of 8 to 36% found in the present study between "*B. psychrophilus*" and *B. globisporus* strains suggested that these two organisms are not closely related genetically. Moreover, the ability of "*B. psychrophilus*" strains to ferment D-mannitol, D-ribose, trehalose, and D-xylose, to reduce nitrate to nitrite, and to grow at 30°C and in 3% NaCl distinguished them phenotypically from *B. globisporus* strains. Based on the results of this study, revival of the name *Bacillus psychrophilus* is proposed. The type strain is strain NRRL NRS-1530.

In 1967, Larkin and Stokes described two new phenotypically similar psychrophilic species, *Bacillus globisporus* and "*Bacillus psychrophilus*" (8). The similarities were such that using numerical taxonomy methods, Gyllenberg and Laine (5) were not able to distinguish between the two species on the basis of their biochemical characteristics. Studying only the type strains, Ruger and Richter (12) found that the two species were very similar in the qualitative chemical compositions of their cell walls and in the guanine-plus-cytosine (G+C) contents of their deoxyribonucleic acids (DNAs); in the opinion of these authors the two species exhibited only minor differences in their biochemical and physiological characteristics. Based on their own observations and on those of others, Ruger and Richter concluded that the existence of the species "*B. psychrophilus*" was unwarranted and, hence, that the name was a later subjective synonym of *B. globisporus* (12). Presumably because of this conclusion, the name "*B. psychrophilus*" was excluded from the Approved Lists of Bacterial Names (14) and, consequently, lost standing in bacterial nomenclature.

I felt that the differences in the biochemical and physiological characteristics of the two taxa and the recent observation by Ruger (11) of low DNA relatedness between the type strains of *B. globisporus* subsp. *globisporus* and "*B. psychrophilus*" might be indicative of two separate species. Therefore, the present study was undertaken to clarify by DNA reassociation experiments the genetic relationship of strains identified as *B. globisporus* and "*B. psychrophilus*." My results indicated that the two groups are not closely related genetically and are phenotypically distinguishable. Based on these findings, the revival of the name *Bacillus psychrophilus* is proposed.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. The strains used in this study were *B. globisporus* NRRL NRS-1519, NRRL NRS-1521, NRRL NRS-1527, NRRL NRS-1532, and NRRL NRS-1533^T (T = type strain) and "*B. psychrophilus*" NRRL NRS-1515, NRRL NRS-1524, NRRL NRS-1525, NRRL NRS-1526, NRRL NRS-1528, and NRRL NRS-1530^T (former type strain). The Agricultural Research Culture Collection (NRRL) at the Northern Regional Research Center maintains these organisms, which were received from the N. R. Smith (NRS) *Bacillus* collection held by R. E. Gordon, Rutgers University, New Brunswick, N.J. The above-mentioned strains were designated *B. globisporus* T26A,

T38B, W8, W17, and W25 and "*B. psychrophilus*" T5A, W3, W5A, W5B, W10A, and W16A, respectively, by J. L. Stokes, Department of Bacteriology and Public Health, Washington State University, Pullman, when they were deposited in the N. R. Smith *Bacillus* collection.

Working stocks were grown for 48 h on soil extract agar (4) slants at 25°C and were stored at 4°C.

Characterization. Unless stated otherwise, the methods of Gordon et al. (4) were used to characterize the strains. The carbohydrate test was modified to use 1% carbohydrate and 0.001% phenol red as the indicator and was expanded to include the following substrates: L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, lactose, D-mannitol, D-mannose, melibiose, L-rhamnose, D-ribose, salicin, sorbitol, sucrose, trehalose, and D-xylose. In addition to citrate, the organic acid utilization test included acetate, fumarate, malate, and succinate. The method of Breuil and Gounot (1) was used to assess the hydrolysis of Tween 80. The decomposition of arginine, lysine, and ornithine was determined in Moeller decarboxylase broth (9). Hydrogen sulfide production was detected by stab culturing in triple sugar iron agar (6). Urease activity was detected by the method of Edwards and Ewing (3). The method of Steel (15) was used to test for oxidase.

DNA purification, reassociation, and G+C content. The procedures used for preparing highly purified DNA samples by hydroxyapatite chromatography and the methods used for estimating the extent of DNA reassociation by spectrophotometric measurement of renaturation rates have been described previously (10).

To determine the G+C contents, the buoyant densities of DNA samples were measured by CsCl density gradient centrifugation in a Beckman model E ultracentrifuge (13). *Micrococcus luteus* (synonym, "*Micrococcus lysodeikticus*") DNA, which was purchased from Sigma Chemical Co., St. Louis, Mo., served as an internal standard.

RESULTS AND DISCUSSION

High DNA reassociation values of 80 to 100% measured among all six strains studied indicated that the organisms identified as "*B. psychrophilus*" belong to a genetically homogeneous group (Table 1). In contrast, of the five *B. globisporus* strains studied, only three (NRRL NRS-1527, NRRL NRS-1532, NRRL NRS-1533^T) gave high reassociation values. Since these three strains included the type strain, this group was considered to represent *B. globisporus*

TABLE 1. DNA relatedness among *B. globisporus* and "*B. psychrophilus*" strains

Strain	% DNA reassociation with: ^a										
	<i>B. globisporus</i> strains					<i>"B. psychrophilus"</i> strains					
	NRRL NRS- 1519	NRRL NRS- 1521	NRRL NRS- 1527	NRRL NRS- 1532	NRRL NRS- 1533 ^T	NRRL NRS- 1515	NRRL NRS- 1524	NRRL NRS- 1525	NRRL NRS- 1526	NRRL NRS- 1528	NRRL NRS- 1530 ^T
NRRL NRS-1519	(100) ^b	25	13	19	2	12	10	16	17	25	26
NRRL NRS-1521		(100)	15	21	30	16	18	4	9	6	11
NRRL NRS-1527			(100)	97	98	36	31	27	10	17	8
NRRL NRS-1532				(100)	90	25	20	16	16	14	24
NRRL NRS-1533 ^T					(100)	20	18	13	15	26	23
NRRL NRS-1515						(100)	100	110	99	100	100
NRRL NRS-1524							(100)	88	96	83	100
NRRL NRS-1525								(100)	100	85	80
NRRL NRS-1526									(100)	93	95
NRRL NRS-1528										(100)	94
NRRL NRS-1530 ^T											(100)

^a Reassociation values are averages of two determinations; the maximum difference between determinations was 8%.

^b The values in parentheses indicate that by definition the reassociation value was 100%.

sensu stricto. The DNA reassociation values between strains NRRL NRS-1519 and NRRL NRS-1521 and between either one of these two strains and strain NRRL NRS-1527, NRRL NRS-1532, or NRRL NRS-1533^T or any of the "*B. psychrophilus*" strains were low (less than 30%). Hence, the two odd strains are not genetically related to each other or to the "*B. psychrophilus*" strains. Finally, the low DNA relatedness values of 8 to 36% observed between *B. globisporus* sensu stricto and "*B. psychrophilus*" strains indicated strongly that the two groups are not closely related genetically (Table 1).

The two species shared the following phenotypic characteristics: formed round spores in swollen sporangia; were motile; produced catalase, oxidase, and urease; were aerobes and grew anaerobically only in the presence of glucose; utilized acetate, fumarate, malate, and succinate, but not citrate; fermented D-fructose, D-galactose, D-glucose, and maltose; did not grow in the presence of lysozyme, in the presence of 5 and 7% NaCl, and at pH 5.6; did not produce arginine-, lysine-, or ornithine-degrading enzymes; did not hydrolyze casein, starch, or Tween 80; decomposed gelatin; did not synthesize acetylmethylcarbinol, indole, hydrogen sulfide, or egg yolk lecithinase; effected only minor changes in litmus milk and the pH of Voges-Proskauer broth; and had G+C contents ranging from 42.8 to 44.1 mol%. The G+C values obtained in this study were higher than the range (39.7 to 40.6 mol%) reported by Ruger and Richter (12), who used the thermal melting procedure. Interestingly, discrepancies have frequently been observed between the G+C contents of *Bacillus* species determined by the thermal melting and buoyant density methods (2). The higher values obtained by the buoyant density method have been attributed in part to the presence of unusual bases (2).

The salient characteristics that differentiated the two species are given in Table 2. The *B. globisporus* group appeared to be phenotypically heterogeneous because it included two genetically unrelated strains, NRRL NRS-1519 and NRRL NRS-1521. With these strains included, the *B. globisporus* strains varied in their ability to grow at 30°C, to hydrolyze starch and casein, and to ferment different carbohydrates. In contrast, the *B. globisporus* sensu stricto strains formed a homogeneous group consisting of physiologically inactive members. The "*B. psychrophilus*" strains not only were phenotypically homogeneous but were also physiologi-

cally active. The ability to grow at 30°C and in the presence of 3% NaCl, the ability to reduce nitrate to nitrite, and the ability to ferment D-mannitol, D-ribose, trehalose, and D-xylose differentiated strains of "*B. psychrophilus*" from strains of *B. globisporus* sensu stricto. On sugar substrates such as D-fructose, D-galactose, D-glucose, and maltose, "*B. psychrophilus*" effected pH changes after 2 to 3 days of fermentation, but *B. globisporus* sensu stricto caused pH changes only after 7 to 14 days of fermentation. Finally, the cells of "*B. psychrophilus*" tended to be thinner than those of *B. globisporus* sensu stricto (Table 2).

Even after observing that the DNA relatedness values between the type strains of *B. globisporus* and "*B. psychrophilus*" were low, Ruger still considered both to be *B. globisporus* strains because between the strains only 3 of 85 characteristics were different (11). According to Ruger (11), "*B. psychrophilus*" should be considered a synonym of *B. globisporus* until it has been established that those three characteristics distinguish all or most homologous strains in the two species. In this study I established that *B. globisporus* sensu stricto and "*B. psychrophilus*" strains belong to two separate genetically homologous groups that can be consistently differentiated on the basis of seven phenotypic characteristics. Therefore, the species name *Bacillus psychrophilus* is here revived and is used for the same taxon to which it was originally applied in accordance with Rules 27 and 28a and Provisional Rules B₂ and B₃ of the *International Code of Nomenclature of Bacteria* (7). Strain NRRL NRS-1530 is designated the type strain. A description of the type strain is given below.

Cells are rods shaped, 0.5 to 1.0 by 3.0 to 7.0 µm, as determined by phase microscopy, and occur singly and in short chains. Gram positive. Motile. Produces round endospores in swollen sporangia.

Agar colonies are nonpigmented, translucent, slightly raised, circular, entire, smooth with slightly glossy surfaces, and 1.0 to 2.0 mm in diameter.

Catalase and oxidase are produced.

Aerobic. Grows anaerobically in the presence of glucose.

Acetylmethylcarbinol, indole, and hydrogen sulfide are not produced. pH in Voges-Proskauer broth (test for acetylmethylcarbinol production), 6.9.

Nitrate is reduced to nitrite.

Soluble starch, casein, Tween 80, and egg yolk lecithin are

TABLE 2. Salient characteristics of *B. globisporus* and "*B. psychrophilus*" strains

Characteristic ^a	<i>B. globisporus</i> strains					" <i>B. psychrophilus</i> " strains					
	NRRL NRS- 1519	NRRL NRS- 1521	NRRL NRS- 1527	NRRL NRS- 1532	NRRL NRS- 1533 ^T	NRRL NRS- 1515	NRRL NRS- 1524	NRRL NRS- 1525	NRRL NRS- 1526	NRRL NRS- 1528	NRRL NRS- 1530 ^T
Cell width (μm)	≤1	≤1	1-1.5	1-1.5	1-1.5	≤1	≤1	≤1	≤1	≤1	≤1
Cell length (μm)	5-7	2-5	3-7	5-7	3-7	3-5	3-7	3-5	3-5	3-5	3-5
Growth in 3% NaCl	-	-	-	-	-	+	+	+	+	+	+
Growth at 30°C	+	+	-	-	-	+	+	+	+	+	+
Starch hydrolysis	-	+	-	-	-	-	-	-	-	-	-
Casein decomposition	-	+	-	-	-	-	-	-	-	-	-
Nitrate reduced to nitrite	-	-	-	-	-	+	+	+	+	+	+
Fermentation of:											
L-Arabinose	+	-	-	-	-	-	-	-	-	-	-
Cellobiose	+	-	-	-	-	-	-	-	-	-	-
Lactose	+	-	-	-	-	-	-	-	-	-	-
D-Mannitol	+	-	-	-	-	+	+	+	+	+	+
D-Mannose	+	-	-	-	-	-	-	-	-	-	-
Melibiose	+	-	-	-	-	-	-	-	-	-	-
D-Ribose	+	-	-	-	-	+	+	+	+	+	+
Salicin	+	-	-	-	-	-	-	-	-	-	-
Sucrose	+	-	d	d	-	+	+	+	+	+	+
Trehalose	+	-	-	-	-	+	+	+	+	+	+
D-Xylose	+	-	-	-	-	+	+	+	+	+	+

^a Symbols for characteristics other than carbohydrate fermentation: +, positive; -, negative. Symbols for carbohydrate fermentation: +, acid produced in 1 to 3 days; d, acid produced in 14 days; -, no acid produced in 14 days.

not degraded. Urea and gelatin are hydrolyzed.

Arginine-, lysine-, and ornithine-decomposing enzymes are not produced.

Citrate is not utilized; acetate, fumarate, malate, and succinate are utilized.

No growth occurs at pH 5.6 or 5.7

No change is effected in litmus milk in 7 days.

Growth in broth containing NaCl is as follows: good growth with 3% NaCl; weak and variable growth with 5% NaCl; no growth with 7% NaCl.

Optimum temperature, 25°C; maximum temperature, 30°C; minimum temperature, 0 to 3°C.

Acid but no gas is produced from D-fructose, D-galactose, D-glucose, maltose, D-mannitol, D-ribose, sucrose, trehalose, and D-xylose.

DNA buoyant density, 1.703 g/cm³; G+C content, 44.1 mol%.

Source: soil and river water (8).

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