Bacillus pulvifaciens sp. nov., nom. rev.

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Because the name *Bacillus pulvifaciens* Katznelson 1950 was excluded from the Approved Lists of Bacterial Names, it has lost standing in bacteriological nomenclature. Based on deoxyribonucleic acid relatedness analyses, 24 weakly catalase-positive strains of "*B. pulvifaciens*" could be separated into two genotypically distinct groups. Because the larger group of 17 isolates included strains with high levels of deoxyribonucleic acid relatedness (80 to 100%) to a reference strain originally isolated and studied by Katznelson, this group was considered to represent the species as first described. This group was phenotypically homogeneous. Moreover, it was not genetically or phenotypically related to members of the smaller deoxyribonucleic acid relatedness group or to the type strains of *Bacillus alvei*, *Bacillus larvae*, and *Bacillus laterosporus*, species that are frequently associated with diseased honeybee larvae. Based on the results of this study, the revival of the name *Bacillus pulvifaciens* is proposed. The type strain is strain NRRL B-3685.

In 1950, Katznelson described the new species "*Bacillus pulvifaciens*," a microorganism that was isolated from the dry, powdery remnants of dead honeybee larvae. Since that time taxonomic study of this species has been sparse. Proom and Knight (13) and Afrykian (1) examined the nutritional requirements of "*B. pulvifaciens*" as basis for determining its relationship to other *Bacillus* species. Gordon et al. (4) recharacterized the strains of Katznelson and found them to be phenotypically homogeneous. This species was listed in *Bergey's Manual of Determinative Bacteriology*, 8th ed., (3) with the reservation that more work was needed to establish its status as a distinct species. Presumably because the description of the species was based on only a few strains, the name "*Bacillus pulvifaciens*" was excluded from the Approved Lists of Bacterial Names (15) and, consequently, has lost standing in bacteriological nomenclature.

The unique habitat (7), nutritional requirements (13), and biochemical characteristics (4) of "*B. pulvifaciens*" suggest that it might yet be a distinct species. Therefore, the present study was undertaken to determine, by using deoxyribonucleic acid (DNA) reassociation analyses and extensive phenotypic characterization experiments, the taxonomic position of "*B. pulvifaciens*." Based on the findings, revival of the name *Bacillus pulvifaciens* is proposed.

MATERIALS AND METHODS

Bacterial strains. Of the 34 strains collected for this study, 10 were judged not to be "*B. pulvifaciens*" strains because they exhibited strong catalase activity; they were not used in this study. The apparently catalase-negative strains studied are listed in Table 1. These strains are being maintained by the Agricultural Research Service Culture Collection (NRRL) at the Northern Regional Research Center. The NRRL strain designations include the prefixes B- and NRS-. The prefix B- denotes strains that were obtained directly from individuals or strains that were isolated at the Northern Regional Research Center; the prefix NRS- designates strains from the *Bacillus* collection of N. R. Smith, which has been deposited in the Agricultural Research Service Culture Collection in toto by R. E. Gordon.

Working stocks were grown for 48 h on soil extract agar (6) slants at 30°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 expanded to include the following compounds: L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, lactose, D-mannitol, D-mannose, melibiose, L-rhamnose, D-ribose, salicin, D-sorbitol, sucrose, trehalose, and D-xylose. The organic acid utilization test included acetate, fumarate, malate, and succinate in addition to citrate and propionate. Arginine, lysine, and ornithine decomposition was determined in Moeller decarboxylase broth (10). Hydrogen sulfide production was detected by stab culturing in triple sugar iron agar (5). Urease activity was determined by the method of Edwards and Ewing (2). The method of Steel was used to test for oxidase activity (16).

DNA purification, reassociation, and G+C contents. 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 (11).

To determine the guanine-plus-cytosine (G+C) contents, the buoyant densities of DNA samples were measured by CsCl density gradient centrifugation in a Beckman model E ultracentrifuge (14). *Micrococcus luteus* (synonym, "*Micrococcus lysodeikticus*") DNA, which was purchased from Sigma Chemical Co., St. Louis, Mo., served as an internal standard.

RESULTS AND DISCUSSION

According to the DNA reassociation analyses (Table 2), the "*B. pulvifaciens*" strains studied could be segregated into two genetically distinct groups. High DNA relatedness values of 80 to 100% demonstrated that 16 of the 24 strains studied were highly related genetically to reference strain NRRL B-3685. Because the reference strain was a strain that was originally studied by Katznelson, this group (group I) was considered to represent the taxon as it was originally described (7). High DNA relatedness values of 83 to 100% were noted between another group I strain, strain NRRL NRS-1284, and the remaining members of the group. This observation suggests that intragroup DNA relatedness values may be consistently high. Further analyses revealed high DNA relatedness values of 75 to 100% between strain NRRL B-3676 and six other strains (these seven strains were designated group II). High DNA relatedness levels between strain NRRL B-14155 and other group II organisms suggest-

TABLE 1. Strains used in this study

Laboratory no.	Received as strain:	Source ^a	Strain history
<i>"B. pulvifaciens"</i>			
NRRL B-3676	Bac 227	1	Isolated from marine mud
NRRL B-3685	NRS-1508	2	J. W. Rouatt from H. Katznelson 670
NRRL B-3686	NRS-1509	2	J. W. Rouatt from H. Katznelson 752
NRRL B-3687	NRS-1510	2	J. W. Rouatt from H. Katznelson 754
NRRL B-3688	ATCC 13537	3	G. B. Landerkin 670
NRRL B-4061	Laramie 7	4	Isolated from natural infection
NRRL B-4062	Laramie 9	4	Isolated from natural infection
NRRL B-4063	Laramie 11	4	Isolated from natural infection
NRRL B-14152	1	5	No information available
NRRL B-14153	2	5	No information available
NRRL B-14154	3	5	No information available
NRRL B-14155	4	5	No information available
NRRL B-14156	5	5	No information available
NRRL NRS-1283	NRS-1283	2	H. Katznelson 113, isolated from powdery scale
NRRL NRS-1284	NRS-1284	2	H. Katznelson 221, isolated from powdery scale
NRRL NRS-1285	NRS-1285	2	H. Katznelson 351, isolated from powdery scale
NRRL NRS-1683	NRS-1683	2	M. Gilliam 1a, isolated from dead honeybee larvae
NRRL NRS-1684	NRS-1684	2	M. Gilliam 2, isolated from dead honeybee larvae
NRRL NRS-1685	NRS-1685	2	M. Gilliam 3, isolated from dead honeybee larvae
NRRL NRS-1686	NRS-1686	2	M. Gilliam 4, isolated from dead honeybee larvae
NRRL NRS-1687	NRS-1687	2	M. Gilliam 6, isolated from dead honeybee larvae
NRRL NRS-1688	NRS-1688	2	M. Gilliam 7, isolated from dead honeybee larvae
NRRL NRS-1689	NRS-1689	2	M. Gilliam 8a, isolated from dead honeybee larvae
NRRL NRS-1690	NRS-1690	2	M. Gilliam 4a, isolated from dead honeybee larvae
<i>B. alvei</i> NRRL B-383 ^T	NRS-662	2	A. Lochhead 127, isolated from diseased honeybees
<i>B. larvae</i> NRRL B-2605 ^T	ATCC 9545	3	E. C. Holst 846, isolated from diseased honeybees
<i>B. laterosporus</i> NRRL NRS-314 ^T	NRS-314	2	ATCC 64

^a 1, G. J. Bonde, Institute for Hygiene, University of Aarhus, Denmark; 2, N. R. Smith *Bacillus* collection maintained by R. E. Gordon, Rutgers University, New Brunswick, N.J.; 3, American Type Culture Collection, Rockville, Md.; 4, J. D. Hitchcock, U.S. Honeybee Research Station, Laramie, Wyo.; 5, H. deBarjac, Laboratoire de Lutte Biologique, Institut Pasteur, Paris, France.

ed that the intragroup relatedness among these strains was high.

Low DNA relatedness values of 7 to 36% indicated that strains NRRL B-3685 and NRRL NRS-1284 were not genetically related to group II strains or to the type strains of recognized species that are frequently associated with diseased honeybee larvae, namely, *Bacillus alvei* Cheshire and Cheyne 1885 strain NRRL B-383^T (T = type strain), *Bacillus larvae* White 1906 strain NRRL B-2605^T, and *Bacillus laterosporus* Laubach 1916 strain NRRL NRS-314^T (Table 2). Group II strains also appeared to be genetically unrelated to the recognized species.

The group I and II strains were phenotypically homogeneous for the following shared characteristics: they were gram positive and motile; they produced ellipsoidal spores in swollen sporangia; they grew anaerobically and in 0.001% lysozyme, but not in 5% NaCl; they did not grow at 55°C; they hydrolyzed casein; they utilized acetate, fumarate, malate, and succinate, but not citrate or propionate; they fermented D-fructose, D-galactose, D-glucose, maltose, D-mannitol, D-mannose, D-ribose, and trehalose without gas production; they produced very weak catalase activity when they were grown on TGY agar (6); they did not decompose egg yolk lecithin, starch, lysine, ornithine, or tyrosine; they did not produce acetylmethylcarbinol, H₂S, or indole; and they caused no change in litmus milk.

Table 3 lists the salient characteristics for differentiating group I strains from group II strains and from the previously recognized species *B. alvei*, *B. larvae*, and *B. laterosporus*. A conspicuous characteristic of the strains of both group I and group II was the inability to produce strong catalase activity; a dissecting microscope was needed to detect

evolution of gas bubbles. Gordon et al. (4) also noted that "*B. pulvifaciens*" decomposed hydrogen peroxide very slowly. Therefore, these groups superficially resembled each other and *B. larvae*, which shows no catalase activity. Growth in 3% NaCl, pH values of 4.9 to 5.3 in Voges-Proskauer broth, inability to grow at pH 5.6 or 5.7, and failure to ferment L-arabinose and D-xylose differentiated group I strains from group II strains, which did not grow in 3% NaCl, produced pH values of 6.6 to 7.4 in Voges-Proskauer broth, grew at pH 5.6, and fermented L-arabinose and D-xylose. Other carbohydrates fermented only by group II strains were cellobiose, lactose, melibiose, D-sorbitol, and sucrose (data not shown in Table 3). Furthermore, the G+C contents (44 to 46 mol%) of group I strains were significantly different from the G+C contents (54 mol%) of group II organisms. Group II organisms may be strains of a new species; further studies will be done to confirm this hypothesis. Growth after serial transfer in nutrient broth, growth at 20°C, and G+C contents of 44 to 46 mol% distinguished group I strains from *B. larvae*, a species which failed to grow after repeated transfer in nutrient broth and at 20°C and had a G+C content of 50 mol%.

Although suggesting some relationship of group I strains to *B. larvae*, the very weak catalase activity presumptively eliminated classification of group I organisms as *B. alvei* or *B. laterosporus*. Furthermore, to be classified as *B. alvei*, the group I strains had to have the following characteristics: they had to hydrolyze starch, produce acetylmethylcarbinol, show urease activity, not ferment D-mannitol, and not decompose arginine. The group I strains failed to meet these criteria. The ability to decompose arginine but not tyrosine, the inability to produce parasporal bodies, and G+C con-

TABLE 2. DNA relatedness of "*B. pulvifaciens*" strains

Strain	% Reassociation with DNA from strain: ^a			
	NRRL B-3685	NRRL NRS-1284	NRRL B-3676	NRRL B-14155
Group I				
NRRL B-3685	(100) ^b	100	33	26
NRRL B-3686	95	100	26	18
NRRL B-3687	80	89	17	26
NRRL B-3688	100	100	22	12
NRRL B-4061	93	100	19	7
NRRL B-4062	99	92	27	28
NRRL B-4063	90	90	22	20
NRRL B-14152	100	100	17	10
NRRL B-14153	100	93	15	20
NRRL B-14154	100	100	3	21
NRRL NRS-1283	100	94	19	7
NRRL NRS-1284	100	(100)	25	20
NRRL NRS-1285	89	83	25	25
NRRL NRS-1683	100	96	26	13
NRRL NRS-1684	100	96	19	20
NRRL NRS-1687	100	97	18	17
NRRL NRS-1690	93	100	10	22
Group II				
NRRL B-3676	33	25	(100)	80
NRRL B-14155	26	20	80	(100)
NRRL B-14156	33	30	100	91
NRRL NRS-1685	31	30	75	95
NRRL NRS-1686	23	25	81	99
NRRL NRS-1688	23	18	75	100
NRRL NRS-1689	25	29	99	100
Type strains				
<i>B. alvei</i> NRRL B-383	7	15	3	9
<i>B. larvae</i> NRRL B-2605	36	25	27	20
<i>B. laterosporus</i> NRRL NRS-314	25	20	3	15

^a Reassociation values are averages of two determinations; the maximum difference noted between determinations was 7%.

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

tents of 44 to 46 mol% distinguished the group I strains from *B. laterosporus*.

Like "*B. pulvifaciens*," the species "*Bacillus amyloliquefaciens*," *Bacillus badius*, *Bacillus brevis*, *Bacillus coagulans*, *Bacillus licheniformis*, and *Bacillus polymyxa* have G+C contents ranging approximately from 44 to 47 mol% (3, 12, 17). In contrast to "*B. pulvifaciens*," however, all of these species decompose hydrogen peroxide strongly. Fur-

thermore, two of the species, *B. badius* and *B. brevis*, degrade tyrosine and produce pH values of 8.0 to 8.6 in Voges-Proskauer broth; *B. badius* also does not ferment D-glucose or D-mannitol (4). Growth at pH 5.6 or 5.7, hydrolysis of starch, synthesis of acetymethylcarbinol, and fermentation of L-arabinose and D-xylose differentiate the four remaining species as a group from "*B. pulvifaciens*" (4, 17). Growth at 55°C, utilization of propionate, production of gas during glucose fermentation, and sporulation in nonswelling sporangia further distinguish *B. coagulans*, *B. licheniformis*, *B. polymyxa*, and "*B. amyloliquefaciens*," respectively, from "*B. pulvifaciens*" (4, 17).

The data above demonstrate that "*B. pulvifaciens*" (the group I strains) is a genotypically and phenotypically distinct taxon. Therefore, the name *Bacillus pulvifaciens* is revived and 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* (8). Strain NRRL B-3685 is designated the type strain. A description of the type strain is given below.

Bacillus pulvifaciens (pul.vi. faci.ens. L.n. pulvis powder, dust; L. pres. part. faci.ens. making; M.L. adj. pulvifaciens making powder or dust).

Rods, 0.5 to 1.0 by 2.0 to 4.0 µm. Gram positive. Motile. Ellipsoidal spores produced in swelling sporangia.

Agar colonies are nonpigmented, opaque, thin, smooth, circular, and entire and average about 1.0 mm in diameter.

Very weak catalase activity produced by cultures grown on TGY agar. Oxidase not produced.

Facultatively anaerobic.

Acetymethylcarbinol, indole, and H₂S are not produced. The pH in Voges-Proskauer (test for acetymethylcarbinol production) broth is 5.0.

Nitrate is reduced to nitrite.

Starch, urea, and egg yolk lecithin are not hydrolyzed. Casein is hydrolyzed.

Arginine dihydrolase is produced. Lysine, ornithine, and tyrosine are not degraded.

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

No growth at pH 5.6 or 5.7.

No change effected in litmus milk in 7 days.

Grows in the presence of 3% NaCl but not 5% NaCl. Growth not inhibited by 0.001% lysozyme.

Optimum growth temperature, 28°C; maximum temperature, 45°C; minimum temperature, 20°C.

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

TABLE 3. Characteristics for differentiating group I strains from group II organisms, *B. alvei*, *B. larvae*, and *B. laterosporus*

Characteristic	Group I	Group II	<i>B. alvei</i> ^a	<i>B. larvae</i> ^a	<i>B. laterosporus</i> ^a
Growth after serial transfer in nutrient bath	+ ^b	+	+	—	+
Growth at 20°C	+	+	+	—	+
Growth in 3% NaCl	+	—	—	+	+
Growth at pH 5.6 or 5.7	—	+	—	—	—
Hydrolysis of starch	—	—	+	—	—
Voges-Proskauer test	—	—	+	—	—
Reduction of nitrate to nitrite	+	v	—	v	+
Catalase	w	w	+	—	+
Oxidase	—	v	+	—	+
Arginine dihydrolase	+	v	—	+	—
Urease	—	+	+	—	—
Degradation of tyrosine	—	—	v	—	+
pH in Voges-Proskauer broth ^c	4.9–5.3	6.6–7.4	4.6–6.2	5.5–6.2	5.0–6.0

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TABLE 3—Continued

Characteristic	Group I	Group II	<i>B. alvei</i> ^a	<i>B. larvae</i> ^a	<i>B. laterosporus</i> ^a
Fermentation of:					
L-Arabinose	—	+	—	+	—
D-Glucose	+	+	+	+	+
D-Mannitol	+	+	—	v	+
D-Xylose	—	+	—	—	+
Parasporal bodies	—	—	—	—	+
G+C content (mol%)	44–46	54	46 ^d	50 ^e	40 ^f

^a Except for the information on oxidase, arginine, dihydrolase, and urease activities, the data are from reference 4. The data for the enzyme activities were obtained during this study; only the type strains were tested.

^b +, Positive; —, negative; v, varies with strain; w, very weakly positive.

^c pH after 7 days at 28°C.

^d Data from reference 9.

^e Determined in this study.

^f Data from reference 3.

DNA buoyant density, 1.703 g/cm³; G+C content, 44 mol%.

Habitat: powdery remnants of dead honeybee larvae (7).

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