Characterization of *Bacillus brevis* with Descriptions of *Bacillus migulanus* sp. nov., *Bacillus choshinensis* sp. nov., *Bacillus parabrevis* sp. nov., and *Bacillus galactophilus* sp. nov.

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Thirty-five *Bacillus brevis* strains obtained from culture collections, including protein-producing isolates, were taxonomically studied by using numerical analysis, DNA base composition, and DNA-DNA hybridization. Six DNA relatedness groups were represented, and these groups correlated well with clusters based on the numerical analysis. The *B. brevis* strains were separated into *B. brevis sensu stricto*, four new species, and an unidentified species of the genus *Bacillus*. *Bacillus migulanus* sp. nov., *Bacillus choshinensis* sp. nov., *Bacillus parabrevis* sp. nov., and *Bacillus galactophilus* sp. nov. are proposed.

The heterogeneity of *Bacillus brevis* has been indicated by the wide range of maximum growth temperatures (10) and the results of numerical classification (13, 26, 27) and classification based on API test data (17). The protein producers identified as *B. brevis* (45) had guanine-plus-cytosine (G+C) contents ranging from 47 to 55 mol%. These observations suggest that this taxon is genetically heterogeneous. Recently, Nakamura (23) reported that the phenotypic heterogeneity of *B. brevis* was due to variability introduced by the presence of genetically unrelated strains. Previously, Udaoka (44) and Takagi et al. (37) reported the bacterial production of large amounts of proteins in culture fluids; many of the bacteria involved appeared phenotypically to be *B. brevis* (37, 44). These strains showed either weak or no detectable proteolytic activity in the culture supernatant (37). *B. brevis* 47 and HPD31 have been employed successfully as host bacteria for extracellular production of foreign proteins, such as bacterial thermophilic α -amylase (38) and human epidermal growth factor (49). Additional unique characteristics of *B. brevis* that have been reported are gramicidin production (9) and molluscicidal activity (31).

In order to clarify the taxonomic positions of 14 *B. brevis* strains from culture collections and of 21 protein-producing isolates, we examined their phenotypic characteristics and processed the data obtained by using standard numerical analysis. In addition, the relationships between clusters resulting from the numerical analysis were evaluated by DNA-DNA hybridization.

As a result, the above-mentioned 35 strains assigned to *B. brevis* were divided into six independent species, four of which are proposed below as the new species *Bacillus migulanus*, *Bacillus choshinensis*, *Bacillus parabrevis*, and *Bacillus galactophilus*.

MATERIALS AND METHODS

Bacterial strains. *B. brevis* strains and reference strains obtained from culture collections and other sources and the protein-producing strains described previously (37, 44) were

used in this study. The sources and histories of these strains are listed in Table 1. Working stocks were cultivated on T2 agar (44) for 24 h at 30 or 37°C and were stored at a room temperature.

Characterization. Unless indicated otherwise, the methods described by Gordon et al. (10) were used for characterization of organisms. Acid production from carbohydrates was determined for D-glucose, L-arabinose, D-fructose, D-galactose, D-xylose, maltose, lactose, sucrose, trehalose, glycerol, D-mannitol, and starch. The methods described by Komagata (14) were used for hydrolysis of urea, Tween 60, and Tween 80; utilization of nitrate, ammonium, and citrate; the oxidase reaction; fermentation of glucose; and production of hydrogen sulfide. Hydrolysis of DNA was determined by using Bacto DNase test agar (Difco Laboratories, Detroit, Mich.). Resistance to antibiotics was determined by growth on T2 agar supplemented with antibiotics (Table 2). Growth inhibition by the strains tested was investigated by using *Escherichia coli* JCM 1649^T (T = type strain) and *Bacillus subtilis* JCM 1465^T.

Chromosomal DNA preparation. Chromosomal DNAs were isolated from bacterial cells cultured overnight in T2 medium at 30 or 37°C by the method of Saito and Miura (30). The prepared DNA had A_{235}/A_{260} and A_{280}/A_{260} ratios of less than 0.55.

DNA base composition. DNA base composition was determined by the method of Tamaoka and Komagata (39), with some modifications. DNAs were heated at 100°C for 10 min and hydrolyzed with 4 U of nuclease P1 (Yamasa Shoyu Co., Chiba, Japan) per ml at 50°C for 2 h. After treatment with bacterial phosphatase (Takara Shuzo Co., Kyoto, Japan), the samples were analyzed by high-performance liquid chromatography with an apparatus equipped with a Microbondasphere C₁₈ column (3.9 by 150 mm; Waters, Nihon Millipore Ltd., Tokyo, Japan). Deoxyribonucleosides were eluted with a mobile phase consisting of 200 mM NH₄H₂PO₄ (pH 4.0) supplemented with 2.5% acetonitrile.

Numerical analysis. Phenetic relationships among the strains tested were determined by a cluster analysis, using 57 differential characteristics (Table 2). Similarity among strains was estimated by using the simple matching coefficient.

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Laboratory no.	Strain Designation ^a	Source ^b	History ^c
<i>B. brevis</i> strains			
1	ATCC 9999 ^T	ATCC	NCTC 7096 from R. Synge from Moscow, USSR, gramicidin S-producing strain
2	NRRL NRS-606	NRRL	J. R. Porter from G. Bredemann
3	HP926	Our isolate	Isolated from soil
4	HPD31	Our isolate	H. Takagi et al., protein-producing strain
5	HPD52 ^T	Our isolate	H. Takagi et al., protein-producing strain
6	HP1501	Our isolate	H. Takagi et al., protein-producing strain
7	444	Our isolate	S. Udaka, protein-producing strain
8	JCM 2503 ^T	JCM	DSM 30 from ATCC 8246 from N. R. Smith strain 604 from J. R. Porter from NCTC 2611 from W. W. Ford strain 27B
9	NRRL NRS-614	NRRL	J. R. Porter from G. Bredemann
10	ATCC 11031	ATCC	N. R. Smith strain 799 from C. Lamanna strain B4
11	481	Our isolate	S. Udaka, protein-producing strain
12	ATCC 35690	ATCC	Z. Kurylo-Borowska strain Vm4, edein A- and B-producing strain
13	47	Our isolate	S. Udaka, protein-producing strain (= JCM 6285)
14	144	Our isolate	S. Udaka, protein-producing strain
15	899	Our isolate	S. Udaka, protein-producing strain
16	NRRL NRS-1212	NRRL	H. W. Reuszer Army strain 1133
17	HP033	Our isolate	H. Takagi et al., protein-producing strain
18	HP026	Our isolate	H. Takagi et al., protein-producing strain
19	HP1201	Our isolate	H. Takagi et al., protein-producing strain
20	ATCC 8185	ATCC	N. R. Smith strain 751 from R. J. Dubos strain BG, gramicidin-producing strain
21	IFO 12334 ^T	IFO	ATCC 10027 from N. R. Smith strain 605 from J. R. Porter from G. Bredemann
22	IFO 12374	IFO	CCM 1463 from NCIB 7577 from NCTC 7577 from T. Gibson strain 108
23	NRRL NRS-616 ^T	NRRL	J. R. Porter from G. Bredemann
24	HP1301	Our isolate	H. Takagi et al., protein-producing strain
25	IFO 12333	IFO	ATCC 8186 from R. J. Dubos strain OKA
26	IFO 12335	IFO	ATCC 10068 from WRRL, gramicidin-producing strain
27	HP030	Our isolate	H. Takagi et al., protein-producing strain
28	HP1304	Our isolate	H. Takagi et al., protein-producing strain
29	HP032	Our isolate	H. Takagi et al., protein-producing strain
30	HP1302	Our isolate	H. Takagi et al., protein-producing strain
31	HP601	Our isolate	H. Takagi et al., protein-producing strain
32	HP602	Our isolate	H. Takagi et al., protein-producing strain
33	HP603	Our isolate	H. Takagi et al., protein-producing strain
34	HP1502	Our isolate	H. Takagi et al., protein-producing strain
35	HP037	Our isolate	H. Takagi et al., protein-producing strain
Other <i>Bacillus</i> strains			
36	<i>B. subtilis</i> JCM 1465 ^T	JCM	IAM 12118 from ATCC 6051 from H. J. Cohn Marburg strain
37	<i>B. sphaericus</i> JCM 2502 ^T	JCM	CCM 2120 from R. E. Gordon
38	<i>B. laterosporus</i> JCM 2496 ^T	JCM	CCM 2116 from R. E. Gordon
39	<i>B. licheniformis</i> JCM 2505 ^T	JCM	CCM 2145 from R. E. Gordon
40	<i>B. coagulans</i> JCM 2257 ^T	JCM	IAM 1115 from ATCC 7050 from N. R. Smith strain 609 from J. R. Porter from W. Hammer
41	<i>B. macerans</i> JCM 2500 ^T	JCM	CCM 2012 from R. E. Gordon
42	" <i>B. aneurinolyticus</i> " ATCC 11376	ATCC	K. Arima, thiaminase-producing strain
43	" <i>B. aneurinolyticus</i> " ATCC 12856	ATCC	Y. Ito from R. Kimura (= IAM 1077)
44	<i>B. alvei</i> IFO 3343 ^T	IFO	IMAB B-3-4 from ATCC 6344 from N. R. Smith strain 662 from A. G. Lochhead strain 127
45	<i>B. badius</i> ATCC 14574 ^T	ATCC	R. E. Gordon from N. R. Smith strain 663 from Henry strain 110 from M. Batchelor
46	" <i>B. freudenreichii</i> " ATCC 7053	ATCC	N. R. Smith strain 671 from T. Gordon strain 68
47	<i>B. thiaminolyticus</i> JCM 7538	JCM	R. Azuma strain 461 from cattle (= NIAH 1025)
48	<i>B. thiaminolyticus</i> JCM 7539	JCM	Osaka University Medical School (= IFO 3115)
49	<i>B. thiaminolyticus</i> JCM 7540	JCM	K. Arima (= IFO 1034)
50	<i>B. thiaminolyticus</i> JCM 8360 ^T	JCM	AHU 1393

^a Names in quotation marks are not on the *Approved Lists of Bacterial Names* (32) or in the *Index of the Bacterial and Yeast Nomenclatural Changes* (20) and had not been validly published as of 1 April 1992.

^b ATCC, American Type Culture Collection, Rockville, Md.; NRRL, Microbial Properties Research, National Center for Agricultural Utilization Research, Peoria, Ill.; JCM, Japan Collection of Microorganisms, Saitama, Japan; IFO, Institute for Fermentation, Osaka, Osaka, Japan.

^c NCTC, National Collection of Type Cultures, Central Public Health Laboratory Service, London, United Kingdom; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; CCM, Czechoslovak Collection of Microorganisms, Masaryk University, Brno, Czechoslovakia; NCIB, National Collection of Industrial Bacteria, NCIMB Ltd., Aberdeen, Scotland, United Kingdom; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; WRRL, Western Utilization Research and Development Division, U.S. Department of Agriculture, Albany, Calif.; NIAH, National Institute of Animal Health, Ibaraki, Japan; AHU, Department of Agricultural Chemistry, Hokkaido University, Hokkaido, Japan.

TABLE 2. Phenotypic characteristics of *B. brevis* and the new species

Characteristic	<i>B. migulanus</i> (DNA group A; <i>n</i> = 2) ^a	DNA group B (<i>n</i> = 1)	<i>B. choshinensis</i> (DNA group C; <i>n</i> = 2)	<i>B. brevis</i> (DNA group D; <i>n</i> = 14)	<i>B. parabrevis</i> (DNA group E; <i>n</i> = 14)	<i>B. galactophilus</i> (DNA group F; <i>n</i> = 2)
Cell width of >0.5 µm	100 ^b	100	100	71	79	50
Cell length of >3.0 µm	100	100	100	79	50	50
Sporangia swollen	100	100	100	100	100	100
Spherical spores	0	0	0	0	0	0
Spore position terminal	100	100	100	93	100	100
Colonies pale yellow	100	100	100	71	50	100
Gram positive	100	100	100	93	88	50
Anaerobic growth	0	0	0	0	0	0
Catalase	100	100	100	100	100	100
Oxidase	100	100	100	93	93	50
Nitrate reduction	100	100	0	79	100	0
Voges-Proskauer reaction	0	0	0	0	0	0
pH of Voges-Proskauer broth of >7.0	100	100	100	100	100	100
Utilization of:						
Citrate (Koser)	0	100	100	64	29	0
Citrate (Christensen)	0	0	100	79	79	50
Nitrate	0	0	0	0	0	0
Ammonium	100	100	100	86	100	100
Hydrolysis of:						
Casein	0	0	0	100	100	100
Gelatin	0	0	0	100	100	100
DNA	100	0	50	100	100	100
Starch	0	0	0	0	0	0
Tween 60	0	0	0	100	100	100
Tween 80	0	0	0	71	93	100
Urea	0	0	0	0	0	0
Growth in the presence of:						
2% (wt/vol) NaCl	100	100	0	71	79	100
5% (wt/vol) NaCl	0	0	0	0	0	0
Growth at:						
pH 5.5	100	0	0	0	14	0
pH 9.0	100	0	0	36	21	0
15°C	0	0	100	79	36	0
50°C	100	100	0	0	79	100
Acid produced from:						
D-Glucose	0	0	100	93	100	100
L-Arabinose	0	0	0	0	0	0
D-Fructose	100	100	50	86	0	100
D-Galactose	0	0	0	29	0	100
Maltose	0	0	0	79	93	100
Lactose	0	0	0	0	0	0
Sucrose	0	0	0	64	29	0
D-Xylose	0	0	0	0	0	0
Trehalose	0	0	50	86	100	50
Glycerol	100	0	0	93	93	100
D-Mannitol	0	0	50	100	100	100
Starch	0	0	0	0	0	0
Gas produced from:						
D-Glucose	0	0	0	0	0	0
L-Arabinose	0	0	0	0	0	0
D-Xylose	0	0	0	0	0	0
D-Mannitol	0	0	0	0	0	0
Fermentation of glucose	0	0	0	0	0	0
Litmus reduction	0	0	0	7	7	0
Resistance to:						
Ampicillin (10 µg/ml)	0	0	50	100	7	0
Chloramphenicol (10 µg/ml)	0	0	100	86	0	0
Neomycin (50 µg/ml)	100	100	0	93	36	50
Erythromycin (20 µg/ml)	0	0	0	7	0	0
Tetracycline (30 µg/ml)	100	0	0	0	0	0
Gramicidin (50 µg/ml)	100	100	100	100	93	100
Growth inhibition of:						
<i>E. coli</i>	0	0	0	14	79	50
<i>B. subtilis</i>	100	100	0	71	81	50
S layer protein	0	100	100	100	100	100

^a *n* is the number of strains studied.^b Percentage of positive strains.

FIG. 1. Dendrogram showing the phenotypic relationships among the strains studied, as determined by using the simple matching coefficient, the unweighted pair group arithmetic average algorithm, and 57 characteristics.

cient, and clustering was based on the unweighted pair group arithmetic average algorithm (34). Computation was carried out by using the NTSYS-pc program of Rohlf (28).

DNA-DNA hybridization. DNA-DNA hybridization was carried out by using photobiotin (Vectra Laboratories, Inc., Burlingame, Calif.) labeling probes in microplate wells as described by Ezaki et al. (6). The templates and biotinylated probe DNAs were used at levels of 1.0 µg per well and 20 ng per well, respectively. Hybridization was performed at 37°C for 12 h. The DNA reassociation ratios were determined by using streptoavidin-peroxidase (Zymed Laboratory, Inc., San Francisco, Calif.) and 3,3',5,5'-tetramethylbenzidine (Dojin Kagaku, Kumamoto, Japan) (12) and were estimated with a microplate reader at 620 nm. DNA from *E. coli* (type VIII; Sigma Chemical Co., St. Louis, Mo.) was used as the negative control.

Cellular fatty acid composition. Cells cultivated overnight in T2 medium at 30 or 37°C were used for cellular fatty acid analysis. Cellular fatty acid composition was determined by the method described by Suzuki and Komagata (36).

Quinone composition. Cells cultivated overnight in T2 medium at 30 or 37°C were used to determine quinone composition by the method described by Komagata and Suzuki (15).

Immunological analysis. Extracellular proteins were prepared as reported previously (5, 24). The 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out as described by Laemmli (16). Western blot (immunoblot) analysis of the extracellular proteins was performed as described by Towbin et al. (41). Rabbit antisera to the middle cell wall protein (protein MWP) of strain 47 and to the cell wall protein (protein HWP) of strain HPD31 were prepared as reported previously (24, 43).

Southern blot analysis. Southern blot analysis of restriction fragments of chromosomal DNAs was performed as described by Southern (35), and then the enhanced chemiluminescence gene detection system (Amersham International plc, Amersham, United Kingdom) was used according to the manufacturer's protocol. The 1.0-kb *HpaI*-*BglII* fragment of

TABLE 3. Levels of DNA relatedness for strains previously assigned to *B. brevis*

Laboratory no.	Strain Designation	% Reassociation with DNA from strain ^a :										DNA group
		ATCC 9999 ^T (cluster IA)	HP926 (cluster IA)	HPD31 (cluster IC)	HPD52 ^T (cluster IC)	444 (cluster ID1)	JCM 2503 ^T (cluster ID2)	47 (cluster ID2)	IFO 12334 ^T (cluster IE)	NRRL NRS-616 ^T (cluster IE)	HP037 (cluster IE)	
1	ATCC 9999 ^T	100	1	10	11	8	6	14	2	2	NT ^b	A
2	NRRL NRS-606	78	0	4	0	2	8	0	2	NT	NT	A
3	HP926	7	100	2	5	10	17	26	12	2	NT	B
4	HPD31	12	11	100	75	19	19	18	35	2	NT	C
5	HPD52 ^T	6	5	60	100	17	18	12	13	20	NT	C
6	HP1501	2	7	12	13	61	72	65	15	NT	NT	D
7	444	8	6	23	8	100	84	96	18	NT	NT	D
8	JCM 2503 ^T	6	7	19	8	95	100	89	21	6	NT	D
9	NRRL NRS-614	1	NT	15	NT	73	90	68	18	NT	NT	D
10	ATCC 11031	2	17	6	13	91	66	87	20	NT	NT	D
11	481	4	NT	18	32	77	71	82	24	NT	NT	D
12	ATCC 35690	8	20	16	3	96	90	100	18	NT	NT	D
13	47	5	9	18	11	78	53	82	15	9	NT	D
14	144	5	NT	23	20	94	77	95	22	NT	NT	D
15	899	5	5	21	4	72	87	81	14	NT	NT	D
16	NRRL NRS-1212	2	NT	17	NT	74	59	99	8	NT	NT	D
17	HP033	2	NT	8	NT	76	73	80	16	NT	NT	D
18	HP026	6	20	7	32	86	91	98	19	NT	NT	D
19	HP1201	5	NT	10	31	76	88	8	22	NT	NT	D
20	ATCC 8185	7	NT	7	NT	11	18	19	95	28	NT	E
21	IFO 12334 ^T	5	10	13	19	15	29	23	100	1	26	E
22	IFO 12374	12	15	15	10	19	31	29	95	1	34	E
24	HP1301	8	NT	4	NT	9	7	10	77	40	43	E
25	IFO 12333	7	20	19	30	10	2	7	90	29	29	E
26	IFO 12335	9	NT	19	NT	20	24	28	86	4	1	E
27	HP030	7	4	4	14	3	14	24	91	4	1	E
28	HP1304	5	NT	10	NT	22	9	24	85	NT	NT	E
29	HP032	8	9	20	16	17	9	28	87	0	16	E
30	HP1302	4	NT	15	NT	13	25	19	71	44	11	E
31	HP601	12	NT	9	13	35	19	8	83	NT	NT	E
32	HP602	3	NT	4	NT	16	23	3	71	0	5	E
33	HP603	9	NT	8	NT	13	20	3	76	24	10	E
34	HP1502	1	NT	20	NT	15	30	15	71	22	13	E
23	NRRL NRS-616 ^T	10	NT	4	NT	23	17	21	31	100	95	F
35	HP037	9	6	15	15	10	12	9	27	72	100	F

^a Reassociation values are averages of two to four determinations; the maximum difference found between determinations was 10%.

^b NT, not tested.

TABLE 4. DNA base compositions and levels of DNA relatedness for selected *Bacillus* strains

Strain ^a	G+C content (mol%)	% Reassociation with DNA from strain ^b :					
		ATCC 9999 ^T	HP926	HPD52 ^T	JCM 2503 ^T	IFO 12334 ^T	NRRL NRS-616 ^T
<i>B. subtilis</i> JCM 1465 ^T	44.2	14	3	3	1	5	4
<i>B. sphaericus</i> JCM 2502 ^T	36.9	8	1	8	9	1	4
<i>B. laterosporus</i> JCM 2496 ^T	42.8	21	2	16	6	19	15
<i>B. licheniformis</i> JCM 2505 ^T	46.0	5	3	2	4	10	0
<i>B. coagulans</i> JCM 2257 ^T	46.6	7	1	8	4	10	3
<i>B. macerans</i> JCM 2500 ^T	52.8	22	1	0	1	9	3
" <i>B. aneurinolyticus</i> " ATCC 12856	42.9	38	22	0	13	0	8
" <i>B. aneurinolyticus</i> " ATCC 11376	42.9	44	19	25	20	2	25
<i>B. alvei</i> IFO 3343 ^T	45.4	12	24	15	0	16	18
<i>B. badius</i> ATCC 14574 ^T	43.5	11	18	21	25	13	21
" <i>B. freudenreichii</i> " ATCC 7053	41.8	6	27	4	11	3	0
<i>B. thiaminolyticus</i> JCM 7538	53.0	5	20	19	17	13	47
<i>B. thiaminolyticus</i> JCM 7539	43.0	32	42	10	24	16	22
<i>B. thiaminolyticus</i> JCM 7540	52.5	2	16	8	1	6	4
<i>B. thiaminolyticus</i> JCM 8360 ^T	53.3	0	12	0	26	1	2

^a Names in quotation marks are not on the *Approved Lists of Bacterial Names* (32) or in the *Index of the Bacterial and Yeast Nomenclatural Changes* (20) and had not been validly published as of 1 April 1992.

^b Reassociation values are averages of two determinations; the maximum difference found between determinations was 10%.

φ-SK10 (5) containing the HWP gene of HPD31 was used as the hybridization probe.

RESULTS

Phenotypic characterization and numerical analysis. The 14 *B. brevis* strains and 21 protein producers had phenotypic characteristics that were consistent with the standard description of *B. brevis* given by Gordon et al. (10) and Claus and Berkeley (3). These 35 strains were recovered in one cluster (numerical cluster I) at a similarity level of approximately 70%, and five clusters (numerical clusters IA, IC, ID1, ID2, and IE) were recovered at similarity levels of more than 80% (Fig. 1). *Bacillus sphaericus* JCM 2502^T and *Bacillus laterosporus* JCM 2496^T were included in numerical cluster I in a broad sense, and *B. subtilis* JCM 1465^T, *Bacillus licheniformis* JCM 2505^T, *Bacillus coagulans* JCM 2257^T, and *Bacillus macerans* JCM 2500^T exhibited distant relationships to *B. brevis*.

DNA base compositions. The DNA base compositions of the 35 strains ranged from 42.5 to 53.3 mol% G+C (Fig. 1).

DNA-DNA hybridization. Ten strains belonging to numerical clusters IA to IE were selected for DNA probes. The 35 strains belonging to numerical cluster I were separated into six DNA relatedness groups (DNA groups) (Table 3). These groups correlated well with the clusters obtained by numerical analysis. DNA groups A and B included two strains and one strain belonging to numerical cluster IA, respectively. DNA group C was recovered in numerical cluster IC. DNA group D was recovered in numerical clusters ID1 and ID2, which contained the type strain of *B. brevis*. DNA groups E and F included 14 and 2 strains, respectively, of numerical cluster IE. The G+C contents for each group varied by no more than 2 mol%. Reference strains of known *Bacillus* species showed low levels of relatedness with the DNAs from six strains belonging to numerical cluster I (Table 4).

Fatty acid composition and quinone system. Fatty acid compositions and quinone systems were analyzed for the following strains: ATCC 9999^T, HP926, HPD52^T, HPD31, *B. brevis* JCM 2503^T, IFO 12334^T, and NRRL NRS-616^T. Strains HP926, HPD52, HPD31, *B. brevis* JCM 2503^T, IFO 12334^T, and NRRL NRS-616^T contained large amounts of

anteiso-C_{15:0} acid (30 to 60% of the total fatty acids) and iso-C_{15:0} acid (30 to 60%); in contrast, strain ATCC 9999^T contained large amounts of iso-C_{15:0} acid (45%), C_{16:0} acid (20%), and iso-C_{15:0} acid (5%). All strains tested had menaquinone 7 (more than 90% of the total menaquinones).

Analysis of cell wall protein. All strains identified as *B. brevis* (laboratory no. 1 through 35 [Table 1]) accumulated one or two major high-molecular-weight proteins in the culture fluid (Fig. 2A). The approximate molecular weights of the cell wall proteins observed in strains HPD31 (laboratory no. 4), HPD52^T (laboratory no. 5), 47 (laboratory no. 13), and HP033 (laboratory no. 17) are reported to be 135 × 10³ (strain HPD31), 115 × 10³ (strain HPD52^T), 150 × 10³ and 130 × 10³ (strain 47), and 150 × 10³ and 110 × 10³ (strain HP033) (5, 11, 24, 43). The immunological relationships between the extracellular proteins and the cell wall protein (HWP) of strain HPD31 are shown in Fig. 2B. The antiserum against HWP cross-reacted with the extracellular proteins produced by most of the strains belonging to numerical cluster I; the exceptions were the proteins of members of DNA group A (ATCC 9999^T [laboratory no. 1] and NRRL NRS-606 [laboratory no. 2]) and *B. sphaericus* JCM 2502^T (laboratory no. 37). The cross-reactions of the proteins with the antiserum against MWP of strain 47 were the same as those with the antibody against HWP (data not shown).

The results of hybridization between the HWP gene probe and restricted chromosomal DNAs coincided with the results of Western blot analysis and DNA relatedness studies (Fig. 3); namely, the probe hybridized with the DNA fragments of the strains belonging to numerical cluster I except the members of DNA groups A and *B. sphaericus* JCM 2502^T under stringent conditions. Therefore, these positive strains contained a sequence very similar to that of the HWP gene of strain HPD31. Moreover, the Southern blot profiles correlated well with the DNA groups.

DISCUSSION

The 35 strains assigned to *B. brevis* in numerical cluster I would be identified broadly as *B. brevis* according to phenetic classification, but on the basis of numerical data, DNA base composition, and DNA relatedness they should be

FIG. 2. Detection of S layer proteins of various *Bacillus* strains by Western blot analysis. (A) 7.5% SDS-polyacrylamide Coomassie-stained gel of extracellular proteins. (B) Western blot analysis of various strains. A 7.5% SDS-polyacrylamide gel was transferred electrophoretically to nitrocellulose sheets. Immunoreactive protein bands containing antiserum against the cell wall protein (HWP) of strain HPD31 were detected with an Immunostaining HRP Kit (Konica Co., Tokyo, Japan). The lane numbers are the laboratory numbers of the strains (Table 1). Lane M contained marker protein. Kd, kilodaltons.

separated into six independent species, which we designated species A through F. These species are consistent with the proposal of De Ley (4) that the G+C contents of members of a species should not differ by more than about ± 1 mol%. Species D included the type strain of *B. brevis*; hence, it represented *B. brevis* sensu stricto. Only 40% of the strains were correctly identified as *B. brevis*. Furthermore, species B through F have similar cellular fatty acid compositions,

and they all have a conserved specific surface layer protein. Species A clearly differs from species B through F.

B. sphaericus was allied with species A through C (Fig. 1). However, characteristics such as the form of sporangia with round spores, growth in 5% NaCl, a G+C content of 36.9 mol% (this study), and low levels of DNA relatedness (1 to 8%) (Table 4) differentiate *B. sphaericus* from species A through C. The known strictly aerobic and oval-spore-

FIG. 3. Detection of S layer protein genelike sequences of various *Bacillus* strains by Southern blot analysis. (A) Restriction map of the HWP gene of strain HPD31 (5). The probe used for hybridization is indicated by the arrow. (B) Southern blot analysis of various strains. Chromosomal DNAs were completely digested with *EcoRI*. The lane numbers are the laboratory numbers of the strains (Table 1). Fragment sizes are indicated on the left.

forming *Bacillus* species with G+C contents of 42 to 54 mol% include *B. subtilis*, *Bacillus pumilus*, *Bacillus atrophaeus*, *Bacillus badius*, *Bacillus firmus*, *Bacillus alginolyticus*, *Bacillus chondroitinus*, *Bacillus amyloliquefaciens*, *Bacillus halophilus*, and *Bacillus stearothermophilus* (Table 5). These known species are genetically and phenotypically distinct from species A through F, as shown in Tables 4 and 5. These results indicate that there is no relationship between the six independent species (species A through F) and the above-mentioned established species.

Therefore, we propose for species A, C, E, and F the following names: *Bacillus migulanus* sp. nov., *Bacillus cho-*

shinensis sp. nov., *Bacillus parabrevis* sp. nov., and *Bacillus galactophilus* sp. nov., respectively. Species B was left unnamed because this group consists of only one strain. Although the combination of phenotypic characteristics in Table 2 rather clearly differentiated *B. brevis* and the newly named species, evaluation of G+C contents and extent of DNA-DNA hybridization were highly useful for confirming the identification of these organisms.

Crystalline protein surface layers have been found in 19 members of the genus *Bacillus* (33), including "*Bacillus aneurinolyticus*" (1), *B. brevis*, *B. sphaericus* (47), *B. licheniformis* (40), *B. stearothermophilus* (19), and *Bacillus thuringiensis* (18). The cell wall structures of *B. brevis* 47 and HP033 (identified in this study) and *B. choshinensis* HPD31 and HPD52 (identified in this study) are quite different from those of most gram-positive bacteria. The cell walls of these organisms consist of a peptidoglycan layer and two cell wall protein layers (outer wall and middle wall) in the case of strains 47 and HP033 (10, 43) or one cell wall protein layer in the case of strains HPD31 and HPD52^T (11). The cell wall layers (surface layers [S layers]) observed in these strains have a hexagonal array with lattice constants of 14.5 to 18 nm (11, 42, 48). The S-layer proteins of strains 47 and HPD31 are composed of proteins with approximate molecular weights of 130×10^3 (outer wall protein) and 150×10^3 (MWP) (strain 47) (24, 43) and 135×10^3 (HWP) (strain HPD31) (5, 11). The deduced amino acid sequence obtained from the nucleotide sequence of the HWP gene shows a high level of homology (78%) with the sequence of the MWP gene (5).

Therefore, an interesting question is how the taxonomic properties of protein-secreting bacteria correlate with their cell wall morphology. On the basis of the limited data available, many eubacterial S layers were considered to be strain-specific features (33). However, the present study showed immunologically and genetically that a specific protein of the S layer was conserved in all strains of *B. brevis*, *B. choshinensis*, *B. parabrevis*, *B. galactophilus*, species B, and *B. laterosporus* JCM 2496^T. This observation elucidates the taxonomic significance of the S layer and suggests that there is a close evolutionary relationship among the above-mentioned species.

Although they differ in their G+C contents and DNA relatedness, *B. brevis*, *B. choshinensis*, *B. parabrevis*, *B. galactophilus*, and species B have the specific S layer in common; thus, they can be designated the "*B. brevis* group." *B. brevis* and *B. laterosporus*, which have a similar specific S-layer protein, belong to the same cluster on phylogenetic trees based on 16S rRNA sequences (2, 8, 29), whereas "*B. aneurinolyticus*," whose S layer differs from that of *B. brevis* immunologically and genetically, forms a separate line of descent different from *B. brevis* (2, 8).

The strains of the *B. brevis* group have the following characteristics in common. The cells are rod shaped (0.5 to 0.9 by 2 to 4 μ m), motile, and peritrichous. Ellipsoidal spores are formed in swollen sporangia. Colonies are flat and smooth on nutrient agar. The maximum growth temperature is 45 to 55°C. The organisms are positive for catalase activity, development of an alkaline pH in Voges-Proskauer broth, and the presence of a specific S-layer protein. They are negative for the Voges-Proskauer reaction, anaerobic growth, hydrolysis of starch and urea, utilization of nitrate, growth in the presence of 5% NaCl and 30 μ g of tetracycline per ml, and acid production from L-arabinose, lactose, D-xylose, and starch. The major cellular fatty acids are

TABLE 5. Characteristics that differentiate *B. migulanus*, the *B. brevis* group, and allied *Bacillus* species^a

Taxon	Swollen sporangia	Voges-Proskauer reaction	pH in Voges-Proskauer broth	Hydrolysis of starch	Growth in the presence of 5% NaCl	Growth at 60°C	G+C content (mol%)
<i>B. migulanus</i> ^b	+	—	>7.0	—	—	—	43
<i>B. brevis</i> group ^b	+	—	>7.0	—	—	—	47–53
<i>B. subtilis</i> ^d	—	+	V	+	+	—	34–48
<i>B. pumilus</i> ^d	—	+	<6.0	—	+	—	33–45
<i>B. atrophaeus</i> ^e	+	+	<6.0	+	+	—	41–43
<i>B. badius</i> ^d	+	—	>7.0	—	+	—	44–50
<i>B. firmus</i> ^d	+	—	6 to 7	—	+	—	41–47
<i>B. alginolyticus</i> ^f	+	—	>7.0	+	—	—	47–49
<i>B. chondroitinus</i> ^f	+	—	>7.0	+	—	—	47–48
<i>B. amyloliquefaciens</i> ^g	—	+	NT	+	+	—	44–46
<i>B. halophilus</i> ^h	—	—	NT	—	+	—	52
<i>B. stearothermophilus</i> ^d	+	—	<6.0	+	V	+	44–52

^a Strictly aerobic, oval-spore-forming species with G+C contents of 42 to 54 mol%.^b Data from this study.^c +, positive; —, negative; V, variable; NT, not tested.^d Physiological data from reference 3; G+C content data from reference 7.^e Data from reference 22.^f Data from reference 21.^g Data from reference 25.^h Data from reference 46.

anteiso-C_{15:0} and iso-C_{15:0} acids. The quinone is menaquinone 7.

Description of *Bacillus migulanus* sp. nov. *Bacillus migulanus* (mi.gu.la'nus. M.L. adj. *migulanus*, referring to the German bacteriologist W. Migula, who contributed to bacterial taxonomy). Cells are rod shaped (0.5 to 1.0 by 2 to 6 µm). Gram positive. Motile and peritrichous. Ellipsoidal spores are formed in swollen sporangia. Colonies are flat, smooth, and pale yellow on nutrient agar. Growth occurs at temperatures ranging from 20 to 50°C. Grows at pH 5.5 to 9.0. Positive for catalase, oxidase, reduction of nitrate to nitrite, development of an alkaline pH in Voges-Proskauer broth, hydrolysis of DNA, utilization of ammonium, acid production from D-fructose and glycerol, and growth in the presence of 2% NaCl and 30 µg of tetracycline per ml. Negative for the Voges-Proskauer reaction; anaerobic growth; alkaline digestion of litmus milk; hydrolysis of casein, gelatin, starch, Tween 60, Tween 80, and urea; utilization of citrate and nitrate; growth in the presence of 5% NaCl; acid production from D-glucose, L-arabinose, D-galactose, maltose, lactose, sucrose, D-xylose, trehalose, D-mannitol, and starch; and the presence of a specific S-layer protein. The major cellular fatty acid components are iso-C_{15:0} and C_{16:0} acids. The quinone is menaquinone 7. The G+C content ranges from 42.5 to 43.2 mol%; the G+C content of the type strain is 42.5 mol%. The type strain is ATCC 9999 (= JCM 8504), which was isolated as a gramicidin producer.

Description of *Bacillus choshinensis* sp. nov. *Bacillus choshinensis* (cho.shi.nen'sis. M.L. adj. *choshinensis*, referring to Choshi, a city in Japan, where the organism was isolated). Strains of this species have the common characteristics of the *B. brevis* group. In addition, positive for oxidase, utilization of citrate and ammonium, and acid production from D-glucose. Negative for reduction of nitrate to nitrite; hydrolysis of casein, gelatin, Tween 60, and Tween 80; growth in the presence of 2% NaCl, at 50°C, and at pH 5.5 and 9.0; acid production from D-galactose, maltose, sucrose, and glycerol; and alkaline digestion of litmus milk. The G+C content ranges from 48.2 to 50.5 mol%; the G+C content of

the type strain is 48.2 mol%. Two strains were isolated from soils in Choshi, Chiba, Japan, as protein producers. The type strain is HPD52. Strain HPD52^T has been deposited in the Japan Collection of Microorganisms as strain JCM 8505^T.

Description of *Bacillus parabrevis* sp. nov. *Bacillus parabrevis* (pa.ra.bre'vis. Gr. prep. *para*, alongside of, like; M. L. adj. *brevis*, short; M. L. *parabrevis*, *brevis*-like, referring to *B. brevis*). Strains of this species have the common characteristics of the *B. brevis* group. In addition, positive for reduction of nitrate to nitrite; utilization of ammonium; hydrolysis of casein, gelatin, DNA, and Tween 60; and acid production from D-glucose, trehalose, and D-mannitol. Negative for acid production from D-fructose and D-galactose. The G+C content ranges from 51.3 to 53.3 mol%; the G+C content of the type strain is 51.8 mol%. The type strain is IFO 12334 (= ATCC 10027 = JCM 8506).

Description of *Bacillus galactophilus* sp. nov. *Bacillus galactophilus* (ga.lac.to.phi'lus. Gr. n *galacto*, milk, referring to galactose; Gr. adj. *philus*, loving; M. L. *galactophilus*, galactose loving). In addition to the common characteristics of the *B. brevis* group, strains of *B. galactophilus* are positive for utilization of ammonium; hydrolysis of casein, gelatin, DNA, Tween 60, and Tween 80; growth in the presence of 2% NaCl and at 50°C; and acid production from D-glucose, D-fructose, D-galactose, glycerol, and D-mannitol. Negative for reduction of nitrate to nitrite; growth at pH 5.5 and 9.0; and alkaline digestion of litmus milk. The G+C content ranges from 52.5 to 52.6 mol%; the G+C content of the type strain is 52.5 mol%. The type strain is NRRL NRS-616 (= JCM 8507).

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