Deoxyribonucleic Acid Homologies Among Strains of *Bacillus sphaericus*

VIRGINIA K. KRYCH,¹ JOHN L. JOHNSON,² AND ALLAN A. YOUSTEN¹

Microbiology Section, Biology Department,¹ and Anaerobe Laboratory,² Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

The genetic relatedness of 62 strains of *Bacillus sphaericus*, 4 strains of *B. pasteurii*, 6 strains of *B. brevis*, and 1 strain each of *B. globisporus* and *B. aminovorans* was examined by deoxyribonucleic acid (DNA) homology. Five homology groups were identified, and they contained all but 12 of the *B. sphaericus* strains. Intergroup homologies ranged from about 20 to 40% except for groups III and IV, which had 50 to 60% intergroup homology. Although a few strains within some groups had 63 to 69% homology to the reference strain, most strains were in the 70 to 90% range. Homology group I contained the type of strain of *B. sphaericus*, ATCC 14577, and three other strains. Homology group II was subdivided into groups IIA and IIB, the former containing all of the strains found to be pathogenic for mosquito larvae. Although none of the physiological characteristics or combination of characteristics examined allowed the complete differentiation of strains into the appropriate homology groups, the strains of group I could be distinguished from those of group IIA-IIB. The strains of group IIA could be distinguished from all others by mosquito pathogenicity. The *B. pasteurii* strains formed a distinct homology group, although the guanine plus cytosine contents of their DNAs fell within the range of 34 to 37 mol% found for the 45 strains of *B. sphaericus* examined for this property. The *B. brevis* strains possessed a higher guanine plus cytosine content, and their DNAs were unrelated to the DNAs of the *B. sphaericus* reference strains tested.

Although *Bacillus sphaericus* has been studied by a number of workers since its original description by Neide in 1904 (19), the boundaries around this species continue to be "diffuse and arbitrary" (9). Most efforts to clarify its taxonomic position have depended heavily upon the shape and position of the spore in the sporangium, a few nutritional characteristics, the inability to ferment a number of sugars, deamination of phenylalanine, growth in broth supplemented with 7% NaCl or lysozyme, decomposition of tyrosine, and production of urease (2, 3, 6, 8, 10, 15, 23). The distinction between *B. latter* has been characterized by its high level of urease activity and by a growth requirement for urea or ammonia in an alkaline medium (8, 9, 10). Many strains of *B. sphaericus* are also urease positive. *B. loehnisii* has been proposed to include intermediate strains which do not complete the decomposition of 2% urea in 48 h and which are less dependent than *B. pasteurii* on ammonia in an alkaline medium (8). In addition, there are some less-well-known species such as *B. globisporus* and *B. aminovorans* which are similar to *B. sphaericus* but which have slightly different biochemical properties or temperature ranges for growth.

The possibility of human pathogenicity among strains of *B. sphaericus* has been raised by the reports of Allen and Wilkinson (1) and Farrar (7). Pathogenicity for mosquito larvae has also been shown (14, 22), although it is unknown how widespread either human or mosquito larval pathogenicity is among strains of the species.

In the investigation reported here, we examined the relationships among strains of *B. sphaericus* and their relationship to some similar species. This has involved the determination of the guanine plus cytosine (G+C) content of the deoxyribonucleic acid (DNA) and the DNA homology among the strains. In addition, the strains were tested for their pathogenicity to mosquito larvae.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study and their sources are listed in Table 1.

Media and growth conditions. All of the strains were grown in nutrient broth (Difco) supplemented with 0.05% yeast extract (Difco), 5×10^{-5} M MnCl_2 , 7×10^{-4} M CaCl_2 , and 10^{-3} M MgCl_2 (NYSM). For growth of *B. pasteurii*, NYSM broth was supplemented with 2.0% urea before autoclaving. Cells used as an inoculum were grown in static tubes of NYSM

TABLE 1. *Strains used in the study*

Strain	Source
<i>B. sphaericus</i>	
ATCC 14577	1, 3
ATCC 10208	1, 2
NRS 967	1, 2
NCTC 9602	Dr. Tipper, University of Mass
1593	3; Mosquito larvae, Indonesia
SSII-1	3; Mosquito larvae, India
1404	3; Mosquito larvae, Philippines
Kellen K	3; Mosquito larvae, California
Kellen Q	3; Derived from Kellen K
1881	3; Mosquito larvae, El Salvador
1691	3; Mosquito larvae, El Salvador
ATCC 7055	1, 2, <i>B. fusiformis</i>
ATCC 7054	1, 2, <i>B. fusiformis</i>
ATCC 7063	1, 2, <i>B. serositidis</i>
ATCC 12300	1, 2
NRS 718	2; Soil, N. R. Smith
NRS 1191	2, 4
NRS 1192	2, 4
NRS 1194	2, 4
NRS 1196	2, 4
NRS 1200	2, 4
NRS 1201	2, 4
NRS 156	2; F. Mauer, Army Medical Center, Washington, D.C.
NRS 592	1, 2
NRS 593	2; National Institutes of Health
NRS 719	1, 2
NRS 800	1, 2
NRS 810	2; J. R. Porter, Iowa University
NRS 1023	2, E. Steinhaus
NRS 1187	2, 4
NRS 1193	2, 4
NRS 1195	2, 4
NRS 1197	2, 4
NRS 1223	1, 2
NRS 1692	2, 5; T. Gibson, strain 6, <i>B. pycnoticus</i>
ATCC 4525	1, 2
ATCC 4978	1, 2, <i>B. rotans</i>
ATCC 12123	1, 2
P1	D. Tipper, University of Massachusetts
NRRL B4297	L. Nakamura; Peoria, Ill.
NRS 400	1, 2
NRS 717	1, 2
NRS 1090	2; L. Margolena, U.S. Department of Agriculture
NRS 1307	1, 2
NRS 1693	2, 5; T. Gibson, strain 10, <i>B. pycnoticus</i>
ATCC 245	1, 2
ATCC 13805	2
NRS 1198	2, 4
NRS 1199	2, 4
NRS 1184	1, 2
1537-4B	3; Mosquito larvae, Nigeria
NRS 250	1, 2
NRS 344	2, AMNH741, W. W. Ford
NRS 349	2
NRS 1089	1, 2
NRS 1186	2, 4
NRS 1188	2, 4
NRS 1611	2; L. G. K. Carr from water
NRS 1691	2, 5; T. Gibson, strain 4, <i>B. pycnoticus</i>
NRS 1694	2, 5; T. Gibson, strain 13, <i>B. pycnoticus</i>

TABLE 1—continued

Strain	Source
NRRL B183	L. Nakamura, Peoria, Ill.
NRRL B1876	L. Nakamura, Peoria, Ill.
<i>B. pasteurii</i>	
ATCC 11859	1, 2
NRS 929	1, 2
NRS 673	2; T. Gibson
NRS 674 = ATCC 6452	1, 2
<i>B. aminovorans</i>	
NRS 341 = ATCC 7046	1, 2
<i>B. globisporus</i>	
NRS 1533 = ATCC 23301	1, 2
<i>B. brevis</i>	
ATCC 8185	1, 2
ATCC 8246	1, 2
ATCC 10027	1, 2
ATCC 10068	1, 2
ATCC 11031	1, 2
NRS 1610	2; M. S. Shane
<i>Bacillus</i> sp.	
NRS 1513	2
NRS 1514	2
NRS 1529	2

^a Sources: 1, refer to The Genus *Bacillus*, Agriculture Handbook 427, U.S. Dept of Agriculture; 2, immediate source, R. Gordon, Rutgers University; 3, immediate source, S. Singer, Western Illinois University; 4, H. W. Reuszer, Army Deterioration Laboratory; 5, J. R. Norris, Meat Research Institute, Bristol, England.

broth at 30°C for 24 h. For DNA isolations, the organisms were grown at 33°C in 1 liter of NYSM broth in a 2-liter Erlenmeyer flask. They were shaken at 200 rpm in a New Brunswick model G25 shaker. For bioassays, cells were grown at 33°C for 14 h in 15 ml of NYSM broth in 125-ml Erlenmeyer flasks which were shaken as above.

Cell lysis and DNA isolation. Bacterial cells from one liter of late-logarithmic-phase culture were suspended in 20 ml of distilled water in a 125-ml ground glass-stoppered flask. Lysozyme (1 mg/ml) was added, and the cells were incubated at 33°C for 30 min. Sodium chloride and ethylenediaminetetraacetic acid buffer, pH 8.0 (0.15 and 0.01 M final concentrations), and pancreatic ribonuclease (50 µg/ml) were added, and the cells were lysed by the addition of sodium lauryl sulfate to a final concentration of 1.0%. For those strains of *B. sphaericus* which did not lyse well with the lysozyme and sodium lauryl sulfate treatment, penicillin G (0.8 mg/ml) was added to the culture in the late logarithmic phase, and cultivation was continued for 2 h. The cells treated with penicillin lysed well after lysozyme and sodium lauryl sulfate treatment.

DNA was isolated from the lysates by the hydroxylapatite procedure described by Johnson (13). The unlabeled DNA preparations were prepared for the homology experiments by sonicating them at 90 W for two 30-s time periods with the needle probe of a Virsonic cell disrupter. The DNA preparations were

then denatured, and any contaminating ribonucleic acid was hydrolyzed by the addition of NaOH to a final concentration of 0.25 M. The solution was incubated at 50°C for 15 min, and samples were cooled in ice and neutralized with HCl. The preparations were dialyzed overnight against 0.02 M NaCl-10⁻³ M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.0). The final concentration of each DNA preparation was adjusted to 0.6 mg/ml by addition of 0.02 M NaCl-10⁻³ M HEPES buffer (pH 7.0).

Preparation of labeled DNA. A 10-ml amount of NYSM broth was supplemented with 250 µCi of [³H] deoxyadenosine (New England Nuclear Corp.). Bacteria were grown in this broth with shaking for 8 h at 33°C. Cells were harvested by centrifugation, and the DNA was isolated by the hydroxylapatite method as previously described. After elution from hydroxylapatite, the DNA was sheared by two passages through a French pressure cell and dialyzed overnight against 0.02 M NaCl. The DNA preparations were alkaline hydrolyzed by the addition of NaOH to a final concentration of 0.25 M and concentrated by ethanol precipitation; the precipitates were resuspended in 0.02 M NaCl-10⁻³ M HEPES buffer, pH 7.0. The specific activity of the DNA preparations ranged from 7,000 to 22,000 cpm/µg.

DNA homology experiments. DNA homology experiments were performed by a variation of the S1 nuclease procedure as described by Crosa et al. (5).

The reassociation mixtures contained 10 μ l (0.1 or 0.05 μ g) of labeled DNA, 50 μ l (30 μ g) of unlabeled DNA (either homologous, heterologous bacterial, or native fragmented salmon sperm DNA), and 50 μ l of 0.88 M NaCl- 10^{-3} M HEPES buffer (pH 7.0). Just before each experiment, the amount of labeled DNA needed for the experiment was denatured by heating in a boiling-water bath for 5 min. Reaction vials containing native salmon sperm DNA were used to measure the amount of self-renaturation of labeled DNA during the incubation period. The reaction vials were incubated for 16 h at 60°C.

After incubation, a 100- μ l sample was removed from each vial and transferred into a 13- by 100-mm tube for S1 nuclease digestion. In addition to the sample of reassociation mixture, each digestion tube contained 1.0 ml of 0.02 M sodium acetate-0.3 M NaCl-0.5 mM ZnCl₂-5% glycerol buffer (pH 4.6), 25 μ g of fragmented, denatured salmon sperm DNA, and 50 μ l of S1 nuclease (Calbiochem, Behring). The enzyme preparation was diluted such that twice the amount of enzyme was added than was needed to digest totally 30 μ g of denatured DNA in 1 h. After incubation of the preparation for 1 h at 50°C, an equal volume of 10% trichloroacetic acid was added to each tube. After cooling the preparation in a refrigerator for at least 1 h, the precipitates were collected on nitrocellulose filters (BA85, Schleicher and Schuell, Inc.). The membranes were dried, and the radioactivity was measured by means of a liquid scintillation counter and a toluene-based scintillation fluid.

Thermal stability. The extent of base pair mismatching was estimated by determining the thermal stability of heterologous duplexes. The reaction mixtures contained 0.12 ml of labeled DNA (10 μ g/ml), 0.6 ml of unlabeled DNA (0.6 mg/ml), and 0.6 ml of 0.88 M NaCl- 10^{-3} M HEPES buffer (pH 7.0) in 13 by 100-mm screw-capped tubes. The mixtures were incubated at 60°C for 16 h, and, after cooling, 1.32 ml of 50% (vol/vol) formamide was added. The tubes were placed in a water bath at 45°C, and the temperature was increased in increments of 5°C at 10-min intervals. At the end of each interval, a 200- μ l sample was removed and placed in a tube for S1 digestion as previously described. The S1-resistant, acid-precipitated DNA was collected by filtration and counted for radioactivity. The cumulative counts up to and including each sample were plotted versus the temperature at which that sample was removed. This resulted in the typical melting point profile. The $T_{m(e)}$ (temperature at which 50% of the duplexes became S1 sensitive) was determined from the midpoint of the curve. The $\Delta T_{m(e)}$'s were calculated by subtracting the $T_{m(e)}$'s of the heteroduplexes from the $T_{m(e)}$ of the reference strain homoduplexes.

Determination of DNA base composition. The G+C contents of the DNA preparations were calculated from their thermal melting points (T_m) by means of the method of Marmur and Doty (17). *Escherichia coli* B DNA served as the standard.

Phenotypic tests. With the exceptions described below, phenotypic tests were carried out by the methods of Gordon et al. (10). Urease activity was determined with the medium described by Christensen (4) supplemented with 0.05% wt/vol yeast extract (Difco)

and also by the method described by Gordon et al. (11). Deamination of phenylalanine was tested by pipetting 4 or 5 drops of 10% (wt/vol) FeCl₃ over cultures grown 7 to 21 days on phenylalanine (Difco) agar slants. A greenish coloration of the agar indicated formation of phenylpyruvic acid from phenylalanine. The oxidase test was performed by placing a moistened test disk (Difco) on a colony grown on NYSM agar for 48 h. Oxidase was present if the colony turned red to purple. For the detection of catalase, 4 to 5 drops of 3% H₂O₂ were added to 24- to 48-h-old NYSM broth cultures, and the appearance of bubbles was observed. All strains were examined for motility by phase-contrast observation of a drop of 24-h-old NYSM broth culture. Acetylmethylcarbinol production was tested by adding 0.5 ml of α -naphthol solution (5%, wt/vol, in ethanol) and 0.5 ml of 40% (wt/vol) KOH to cultures grown for 14 days in MR-VP broth (Difco). Tubes were allowed to stand for 30 min, and the appearance of a red color indicated formation of acetylmethylcarbinol. Three drops of alcoholic methyl red were added to duplicate tubes, and acid production was indicated by a red color. Aerobic reduction of nitrate was tested by adding sulfanilic acid and α -naphthylamine to a culture of nitrate broth (Difco) supplemented with 0.05% (wt/vol) yeast extract (Difco). A pink to red color indicated the presence of nitrite. Zinc powder was added to those tubes which were negative to detect possible reduction beyond nitrite. Casein hydrolysis within 10 days was detected by a clearing around the colony on NYSM agar (Difco) plates containing 10% (wt/vol) skim milk (Difco). A similar clearing was observed on plates supplemented with 2.0% (wt/vol) corn starch if amylase was present. Gelatin hydrolysis was tested by growing cultures in tubes of NYSM broth containing 12% (wt/vol) gelatin.

Bioassays. Pathogenicity for second instar larvae of the mosquito *Culex pipiens quinquefasciatus* was determined as previously described (18). Strains were judged to be pathogenic if larval death greater than control death occurred when larvae were incubated with approximately 10^8 bacteria per ml for 4 days.

RESULTS

G+C content of the DNA. The G+C contents of the DNAs from most of the strains used in this study are given in Tables 2 and 4. These values ranged from 34 to 37 mol%, with the majority of strains at 35 or 36 mol%. The strains of *B. pasteurii* and *B. aminovorans* had G+C values in the same range and could not be distinguished from *B. sphaericus* on this basis. *B. brevis*, a species forming an elliptical spore but having somewhat similar metabolic reactions to those of *B. sphaericus*, had higher G+C values: 46 or 47% (three strains) to 52 to 55% (three strains). The apparent heterogeneity among the *B. brevis* strains was not investigated further, but these strains were readily distinguishable from those of *B. sphaericus*.

DNA homologies among strains of *B. sphaericus*. When DNA homologies were de-

TABLE 2. *B. sphaericus* DNA homology groups

Unlabeled DNA from strain	G+C content of DNA (mol%)	% Homology to DNA of reference strains						<i>P. pas- teurii</i> ATCC 11859
		ATCC 14577	1593	ATCC 7055	NRS 592	NRS 400	NRS 1198	
Group I								
ATCC 14577 ^a	36	100	23	20	22	22	20	6
ATCC 10208	36	91	25	21	21	23	17	3
NRS 967	36	89	25	17	16	13	10	4
NCTC 9602	36	77	25	20	21	20	21	3
Group IIA								
1593	35	19	100	60	40	47	24	2
SSII-1	35	20	83	60	40	47	23	4
1404-924B	35	19	86	57	37	ND ^b	20	4
Kellen K	35	19	82	59	40	43	21	3
Kellen Q	35	19	79	61	41	ND	24	2
1881	ND	19	88	64	38	ND	23	ND
1691	ND	19	80	60	37	41	24	ND
Group IIB								
ATCC 7055	36	16	64	100	43	43	22	4
ATCC 7054	ND	23	63	93	43	ND	21	ND
ATCC 12300	35	21	66	93	37	ND	20	ND
NRS 718	36	17	62	81	38	38	ND	4
NRS 1191	36	18	66	78	40	ND	20	2
NRS 1196	36	14	62	76	41	50	22	1
ATCC 7063	35	21	62	76	39	ND	ND	5
NRS 1194	36	21	60	75	42	ND	26	2
NRS 1200	35	20	64	74	43	46	30	6
NRS 1201	35	17	63	72	38	ND	ND	ND
NRS 1192	36	18	62	69	39	43	ND	4
NRS 156	35	14	63	68	41	45	21	2
Group III								
NRS 592	36	18	42	44	100	52	24	5
ATCC 12123	35	21	37	36	88	56	26	ND
P1	36	19	41	35	88	54	ND	4
NRS 800	35	16	41	34	86	54	21	3
NRS 1692	ND	23	37	ND	84	ND	ND	ND
NRS 593	36	18	35	32	83	43	19	4
NRS 1195	35	22	41	37	83	ND	ND	2
NRS 1193	34	18	43	33	81	55	ND	2
NRS 1197	36	17	42	36	80	51	ND	4
NRS 1187	35	17	42	47	79	61	45	2
NRS 1023	35	18	34	42	78	62	27	ND
NRRL B4297	35	18	43	41	79	64	32	3
NRS 810	35	21	41	36	77	64	21	ND
NRS 719	35	18	41	43	76	55	0	5
ATCC 4525	35	23	38	37	76	57	26	ND
Group IIII								
ATCC 4978 ^c	36	19	39	29	70	64	25	ND
NRS 1223	36	20	36	45	63	56	23	ND
Group IV								
NRS 400	36	18	41	37	43	100	24	ND
NRS 717	37	18	40	37	49	100	23	4
NRS 1529 ^d	ND	23	42	37	47	98	9	ND
ATCC 13805	35	18	38	32	50	97	25	5
ATCC 245	36	21	39	39	48	94	23	ND
NRS 1090	36	16	45	41	41	93	22	3
NRS 1693	ND	22	43	ND	52	86	ND	ND
NRS 1307	37	19	41	41	63	66	38	ND

TABLE 2—continued

Unlabeled DNA from strain	G+C content of DNA (mol%)	% Homology to DNA of reference strains						
		ATCC 14577	1593	ATCC 7055	NRS 592	NRS 400	NRS 1198	<i>B. pas- teurii</i> ATCC 11859
Group V								
NRS 1198	35	20	24	24	30	30	100	6
NRS 1199	35	23	13	28	27	32	80	4
NRS 1184	35	25	13	26	24	ND	74	ND
Ungrouped Strains								
1537-4B	ND	25	25	ND	30	ND	54	ND
NRS 1186	36	21	27	22	25	31	48	2
NRRL B1876	35	22	32	ND	24	ND	47	ND
NRS 1089	36	23	24	ND	23	ND	43	ND
NRS 1533 ^e	ND	25	24	4	32	6	42	ND
NRS 250	34	21	24	22	29	50	33	1
NRS 1611	34	20	32	20	26	41	33	2
NRRL B183	36	30	26	ND	20	ND	15	ND
NRS 344	35	15	38	36	32	39	18	ND
NRS 1188	36	4	4	4	5	7	6	2
NRS 1694	34	7	8	ND	8	ND	4	5
NRS 1691	ND	7	6	ND	10	ND	7	ND
NRS 349	ND	8	7	8	10	18	4	ND
NRS 341 ^f	36	7	8	ND	5	ND	3	3
NRS 1513	ND	3	9	ND	4	ND	2	3
NRS 1514	ND	3	7	ND	6	ND	3	ND

^a Type strain.^b ND, Not determined.^c *B. rotans*.^d *B. species*.^e *B. globisporus*.^f *B. aminovorans*.

terminated, a considerable amount of heterogeneity was found among the strains previously identified as *B. sphaericus*. Five distinct DNA homology groups have been identified, and one of these, group II, has been divided into two subgroups. Sixteen additional strains did not fit into any of the homology groups, although in some cases their homology relationships are incomplete (Table 2).

DNA homology group I (Table 2) represents the species *B. sphaericus* since it includes ATCC 14577, the type strain of the species. Only three other strains showed a high percentage of homology to this reference strain.

DNA homology group II (Table 2) was divided into two subgroups, with reference strains 1593 (group IIA) and ATCC 7055 (group IIB). Subgroup IIA was clearly distinguishable from subgroup IIB, since all strains of IIA had 79% or greater homology to strain 1593 but no greater than 64% homology to the IIB reference strain ATCC 7055. The strains placed in IIB had homologies ranging down to 68% for ATCC 7055 while having homologies up to 66% for the group IIA reference strain 1593. The grouping of strains NRS 1192 and NRS 156 was somewhat

equivocal. This problem was resolved by determining the difference in thermal stability of homologous and heterologous duplexes $T_{m(e)}$ for the questionable strains (Table 3). The $\Delta T_{m(e)}$ of the heteroduplexes formed between DNA preparations from strains of the two homology subgroups had values that ranged between 6.7 and 7.5°C while the $\Delta T_{m(e)}$ for heteroduplexes within a subgroup was no greater than 2.5°C (Table 3). These results indicated a significant difference in the arrangement of nucleotides between the strains of the two subgroups. Subgroup IIA could be readily distinguished from subgroup IIB and from all other homology groups by the fact that only the seven strains belonging to this subgroup were found to be pathogenic for mosquito larvae.

The largest of the DNA homology groups was group III (Table 2). This group contained 17 strains of *B. sphaericus*. Homology values within this group ranged from 88 down to 63%. This group is closely related to group IV in that the DNA preparations had an average homology of 57% to the group IV reference strain, NRS 400. *B. rotans*, described by Roberts (20) as a strain of *Bacillus* having mobile colonies, was

found to belong to this group.

DNA homology group IV (Table 2) contained seven strains with high homology to the reference strain NRS 400. Strain NRS 1529 had previously been identified merely as *Bacillus* sp. Strain NRS 1307 was placed in homology group IV because of its slightly higher homology to NRS 400 than to NRS 592.

Homology group V was the smallest of those identified, and it contained only three strains (Table 2). The four strains of *B. pasteurii* examined comprised a very homogeneous homology group having little homology to any of the *B. sphaericus* reference strains (Table 4). None of the *B. sphaericus* strains showed homology to the *B. pasteurii* reference strain, ATCC 11859. The DNA from six strains of *B. brevis* was tested for homology to the DNA from the reference strains for homology groups I, IIA, and III and the *B. pasteurii* group. Little homology was detected, as would have been predicted from the different G+C values found for these bacteria.

Phenotypic characteristics. A limited number of tests for physiological characteristics was carried out in an attempt to separate the

DNA homology groups. The data summarized in Table 5 indicate that no group of phenotypic traits allowed complete separation of all the homology groups. Group I was distinguishable from groups IIA and IIB by its utilization of citrate and by its failure to produce urease or to grow in broth supplemented with 7% NaCl. Group IIA was distinguishable from all other groups in that it contained strains which were pathogenic for mosquito larvae. The other characteristics examined were either constant among all the strains or were variable among the members of the homology groups. Five strains of *B. pasteurii* (the four listed in Table 4 as well as NRS 675) failed to grow on NYSM agar unless this was supplemented with urea, and thus they were readily distinguishable from all of the strains of *B. sphaericus* which grew on unsupplemented NYSM agar.

DISCUSSION

The genetic heterogeneity found among the strains of *B. sphaericus* studied is not particularly surprising in view of the phenotypic variation which has been described for this species. Also, Seki et al. (21) had observed that only one of four strains of *B. sphaericus* which they examined had high DNA homology to the type strain of the species. We found only three strains to have high homology with the type strain ATCC 14577 (homology group I). This group appears to be less related to the other homology groups than the other groups are to each other. Strains from the other *B. sphaericus* groups had homologies ranging from 14 to 26% to the type strain.

Homology group IIA-IIB contains strains which are phenotypically very similar (Table 5) and which bear a definite genetic relationship. Nonetheless, there is an easily recognizable gap in percent homology between most of the strains of subgroups IIA and IIB. The two strains which were in a questionable position, NRS 1192 and NRS 156, were confirmed in their IIB position by the determination of the thermal stability of their DNA heteroduplexes. The bacteria in

TABLE 3. Thermal stability of DNA heteroduplexes constructed between group II strains

Strain	$\Delta T_{m(e)}$ of DNA duplexes	
	1593	ATCC 7055
Group IIA		
1593	0	ND ^a
SSII-1	0.7	6.7
1881	0.7	ND
Group IIB		
ATCC 7055	7.2	0
ATCC 12300	ND	0
NRS 718	7.45	0.75
NRS 1200	7.5	2.0
NRS 156	7.0	2.3
NRS 1192	7.0	2.25
NRS 1194	ND	1.8
NRS 1196	ND	2.5

^a ND, Not determined.

TABLE 4. *B. pasteurii* DNA homology group

Unlabeled DNA from strain	G+C content of DNA (mol%)	% Homology to DNA of reference strains:						<i>B. pasteurii</i> ATCC 11859
		ATCC 14577	1593	ATCC 7055	NRS 592	NRS 400	NRS 1198	
ATCC 11859 ^a	37	2	6	0	2	0	0	100
NRS 929	37	0	6	0	3	4	ND ^b	99
NRS 673	37	2	5	7	2	4	ND	94
NRS 674	37	0	2	ND	3	ND	ND	97

^a Type strain.

^b ND, Not determined.

TABLE 5. *Physiological characteristics of the DNA homology groups*

Trait	% of strains positive					
	Group I	Group IIA	Group IIB	Group III	Group IV	Group V
Mosquito pathogenicity	0	100	0	0	0	0
Urease production	0	100	92 ^a	61 ^a	0	66 ^a
Utilization of citrate	100	0	0	22 ^b	57 ^c	0
Growth with 7% NaCl present	0	100	100	78 ^d	71 ^d	66 ^d
Casein hydrolysis	75 ^e	100	83 ^e	23 ^f	71 ^e	100
Gelatin liquefaction	100	100	92 ^g	89 ^g	100	100
Oxidase, motility, catalase, growth in presence of 5% NaCl	100	100	100	100	100	100
Amylase, nitrate reduction, methyl red-Voges-Proskauer test, indole, utilization of L-tyrosine, growth with 10% NaCl present, acid production from glucose or lactose	0	0	0	0	0	0
Deamination of phenylalanine	100	100	66 ^h	89 ^h	86 ^h	100
Growth with lysozyme present (10 µg/ml)	25 ⁱ	100	75 ^j	6 ⁱ	28 ⁱ	33 ⁱ

^a Negative strains—IIB: ATCC 7063; III: NRS 1187, 810, 719, 4525, 4978, 1223, NRRL 4297; V: 1184.

^b Positive strains—NRS 592, 593, 1023, 1197.

^c Negative strains—NRS 400, 717, 1529.

^d Negative strains—III: NRS 1692, 4525, 1307, 1223; IV: NRS 245, 1693; V: NRS 1198.

^e Negative strains—I: NRS 9602; IIB: NRS 1191, ATCC 7063; IV: NRS 717, 1529.

^f Positive strains—NRS 1193, 810, 719, 1307.

^g Negative strains—IIB: ATCC 7063; III: NRS 810, 719.

^h Negative strains—IIB: NRS 718, 1196, ATCC 7054, 7063; III: NRS 1187, 719; IV: NRS 400.

ⁱ Positive strains—I: ATCC 14577; III: NRS 1023; IV: ATCC 245, NRS 1090; V: NRS 1198.

^j Negative strains—IIB: NRS 1191, 156, ATCC 7063.

group IIA are particularly interesting since all seven strains were shown to be pathogenic for mosquito larvae, a trait not possessed by the strains in group IIB. The IIA strains have been shown to be pathogenic by virtue of a cell-bound toxin of unidentified composition (18). All of the mosquito pathogens had been isolated from dead mosquito larvae (22), although nonpathogenic strains are also occasionally isolated from this source (S. Singer, personal communication). Since none of the strains which were obtained from other sources was a mosquito pathogen, it is possible that the conditions present in the mosquito larval gut represent a unique habitat suitable to the development and successful maintenance of these pathogens. Strain ATCC 7063 (*B. serositidis*), a human clinical isolate (16), was placed in group IIB. It was not closely related to NRS 592, which was also of clinical origin. ATCC 7063, NRS 592, and NRS 593, another isolate of clinical origin, did not demonstrate pathogenicity by subcutaneous injection of mice or by intraperitoneal or intracerebral injection of rats (John Shadduck, personal communication).

The largest number of the strains studied belong to homology group III, with reference strain NRS 592. The placement of ATCC 4978 into this group confirms the judgment of Gordon et al. (10) that this strain, originally called *B. rotans*, is related to *B. sphaericus*. Homology

groups III and IV are closely related. In the lower range of homologies within each group, it becomes difficult to distinguish the group to which the strains belong. We have assigned these strains simply on the basis of the highest homology values. Perhaps these strains, e.g., ATCC 4978, NRS 1223, and NRS 1307, constitute an additional homology group. This could be determined by using one of them as a reference strain.

Twelve strains of *B. sphaericus* could not be placed into any of the five homology groups based upon the data available. Four of these strains—NRS 1188, 1694, 1691, and 349—had so little relationship to any of the reference strains that it would seem advisable to review their phenotypic properties. These strains may be more closely related to another species. The type strain of *B. globisporus*, NRS 1533, had 42% homology to the group V reference strain, NRS 1198. *B. globisporus* 1533 grew well at 22°C but failed to grow at 32°C in contrast to the *B. sphaericus* strains which grew well at the higher temperature. The type strain of *B. aminovorans*, NRS 341, showed no relationship to *B. sphaericus*.

None of the *B. sphaericus* strains for which data were obtained showed any relationship to the type strain of *B. pasteurii*, ATCC 11859. The strains of *B. pasteurii* were unable to grow in NYSM broth without the addition of urea,

and the four strains examined in this study were genetically very homogenous. Despite certain similarities to *B. sphaericus*, there should be no doubt that these strains constitute a distinct species.

The DNA from *B. brevis* showed little homology to those *B. sphaericus* groups to which it was compared or to the DNA of *B. pasteurii*. These bacteria also appear to constitute a distinct species.

The phenotypic characteristics determined in this study were not adequate to allow the placement of a specific *B. sphaericus* strain into the appropriate homology group. The only exception to this conclusion is that pathogenicity for mosquito larvae is a property possessed exclusively by members of homology group IIA. Unfortunately, the chemical nature of the toxin(s) responsible for this pathogenicity and the genetic stability of the toxin(s) have not yet been sufficiently well studied for us to recommend the use of this trait as the basis for the establishment of a new species. Each of the homology groups identified in this study contains strains having a sufficiently high level of genetic relatedness to justify speciation (12). However, the present inability to distinguish these strains by readily determined phenotypic tests suggests that it would be premature to propose the establishment of new species. We propose, therefore, that these bacteria continue to be named *B. sphaericus*, at the same time recognizing that they represent genetically distinctive groups.

ACKNOWLEDGMENTS

This investigation received support from the Vector Biology and Control component of the VNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases. We thank Ruth Gordon for her contribution of cultures and for her advice.

REPRINT REQUESTS

Address reprint requests to: A. Yousten, Microbiology Section, Biology Department, Virginia Polytechnic Institute, Blacksburg, VA 24061.

LITERATURE CITED

- Allen, B., and H. Wilkinson. 1969. A case of meningitis and generalized Schwartzman reaction caused by *Bacillus sphaericus*. *Johns Hopkins J.* 125:8-13.
- Bornside, E. H., and R. E. Kallio. 1956. Urea-hydrolyzing bacilli. I. A physiological approach to identification. *J. Bacteriol.* 71:627-634.
- Bornside, E. H., and R. E. Kallio. 1956. Urea-hydrolyzing bacilli. II. Nutritional profiles. *J. Bacteriol.* 71:655-660.
- Christensen, W. B. 1946. Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. *J. Bacteriol.* 52:461-466.
- Crosa, J., D. Brenner, and S. Falkow. 1973. Use of a single-strand specific nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and heteroduplexes. *J. Bacteriol.* 115:904-911.
- deBarjac, H., and A. Bonnefoi. 1972. Essai de classification biochimique de 64 *Bacillus* des groupes II et III représentant II espèces différentes. *Ann. Inst. Pasteur.* 122:463-473.
- Farrar, W. 1963. Serious infections due to non-pathogenic organisms of the genus *Bacillus*. *Am. J. Med.* 34:134-141.
- Gibson, T. 1935. An investigation of the *Bacillus pasteurii* group. III. Systematic relationships of the group. *J. Bacteriol.* 29:491-501.
- Gibson, T., and R. E. Gordon. 1974. Endospore-forming rods and cocci. Family I. *Bacillaceae*, genus I. *Bacillus* (Cohn, p. 529-550. In R. E. Buchanan and N. E. Gibbons (ed.), *Bergey's manual of determinative bacteriology*, 8th ed. The Williams and Wilkins Co., Baltimore.
- Gordon, R. E., W. C. Haynes, and C. Hov-Nay Pang. 1973. The genus *Bacillus*. *Agricultural handbook no. 427*. U.S. Department of Agriculture, Washington, D.C.
- Gordon, R. E., J. Hyde, and J. Moore. 1977. *Bacillus firmus-Bacillus lentus*: a series or one species? *Int. J. Syst. Bacteriol.* 27:256-262.
- Johnson, J. L. 1973. Use of nucleic-acid homologies in the taxonomy of anaerobic bacteria. *Int. J. Syst. Bacteriol.* 23:359-375.
- Johnson, J. L. 1978. Taxonomy of the *Bacteroides*. I. Deoxyribonucleic acid homologies among *Bacteroides fragilis* and other saccharolytic *Bacteroides* species. *Int. J. Syst. Bacteriol.* 29:245-256.
- Kellen, W., T. Clark, J. Lindengren, B. Ho, M. Rogoff, and S. Singer. 1965. *Bacillus sphaericus* Neide as a pathogen of mosquitoes. *J. Invertebr. Pathol.* 7:442-448.
- Knight, B. C. J. G., and H. Proom. 1950. A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus*. *J. Gen. Microbiol.* 4:508-538.
- LaCorte, J. 1932. *Bacillus serositidis*, nova especie. *Mem. Inst. Oswaldo Cruz Rio de Janeiro.* 26:1-7.
- Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. Mol. Biol.* 5:109-118.
- Myers, P., and A. Yousten. 1978. Toxic activity of *Bacillus sphaericus* SSII-1 for mosquito larvae. *Infect. Immun.* 19:1047-1053.
- Neide, E. 1904. Botanische Beschreibung einiger sporenbildenden Bakterien. *Zentralbl. Bacteriol. Parasitenk. Infektionskr. Hyg. Abt. 2* 12:1-32.
- Roberts, J. L. 1935. A new species of the genus *Bacillus* exhibiting mobile colonies on the surface of nutrient agar. *J. Bacteriol.* 29:229-236.
- Seki, T., C.-K. Chung, H. Mikami, and Y. Oshima. 1978. Deoxyribonucleic acid homology and taxonomy of the genus *Bacillus*. *Int. J. Syst. Bacteriol.* 28:182-189.
- Singer, S. 1973. Insecticidal activity of recent bacterial isolates and their toxins against mosquito larvae. *Nature (London)* 244:110-111.
- Smith, N. R., R. E. Gordon, and J. B. Clark. 1952. Aerobic sporeforming bacteria. *Agriculture monograph no. 16*. U.S. Department of Agriculture, Washington, D.C.