Deoxyribonucleic Acid Homology and Taxonomy of the Genus *Bacillus*

TATSUJI SEKI, CHI-KWAN CHUNG,[†] HIDETADA MIKAMI,^{††} AND YASUJI OSHIMA

Department of Fermentation Technology, Osaka University, Yamadakami, Suita-shi, Osaka 565, Japan

The taxonomic relationships among 56 strains of 16 species of the genus *Bacillus* were studied by deoxyribonucleic acid (DNA)-DNA hybridization. In general, no significant DNA homology was detected between two strains of different species, except for a group of species consisting of *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. pumilus* and for another group of species including *B. cereus* and *B. thuringiensis*. Species of the former group were related, but they were independent of each other as their DNA homologies were 19% or less. The DNA homology indexes of three strains of *B. thuringiensis* to *B. cereus* T, so far tested, showed high DNA homologies (54 to 80%). This fact indicates that *B. thuringiensis* should be an identical species to *B. cereus*. The intraspecific DNA homology indexes of 16 strains of *B. pumilus* were 51% or more to strain IFO 12092 as the standard, and those of 10 strains of *B. coagulans* were 76 to 113% to strain ATCC 7050 as the standard. Thus, the species identification of *B. pumilus* and *B. coagulans* by the conventional taxonomic method was well in accord with the DNA homology data. On the other hand, significant heterogeneities were suggested among the strains of *B. circulans* and among those of *B. sphaericus* by the DNA homology data of the three and five strains so far tested, respectively. Although *B. lentus* was described to be closely related to *B. firmus* in *Bergey's Manual* (8th ed., 1974), the interspecific DNA homology index between these two species was 3%. It was concluded that these two species are independent.

According to the description by Smith et al. (13), a wide variety of *Bacillus* species were divided into three groups with respect to their sporangium and spore characteristics. The species belonging to group 1 form indefinitely swollen sporangia, whereas the other species showing swollen sporangia are included in the second and third groups. Of the latter two groups, group 3 consists of species forming characteristic spherical spores. In a previous communication (12), the taxonomic relationships among 52 test strains of 8 species of *Bacillus* belonging to group 1, except for one strain of *B. brevis*, were studied by deoxyribonucleic acid (DNA)-DNA hybridization and by the transformability of the auxotrophic and antibiotic resistance genetic markers. Results of species identification by DNA-DNA hybridization data for *B. licheniformis* and *B. megaterium* showed good agreement with those obtained by the conventional taxonomic method according to *Bergey's Manual*, 8th ed. (5), and by the methods of Gordon et al. (6) and Smith et al. (13). However, the strains of

B. subtilis identified by the conventional taxonomic method were divided into two groups, in which *B. subtilis* 168 and *B. amyloliquefaciens* KA-63 are representative of each group, by DNA-DNA hybridization data and by transformation ability. This communication is a follow-up to the previous one (12) and deals with the taxonomic relationships between various additional species of *Bacillus* distributed in groups 1, 2, and 3 by the DNA-DNA hybridization technique.

MATERIALS AND METHODS

Strains. The strains used are listed in Table 1. Fifty-two strains were obtained from authentic sources and six strains of *B. coagulans* were newly isolated from soil. *Escherichia coli* K-12 was also used as a test strain. The taxonomic significance of the test strains was critically examined according to *Bergey's Manual*, 8th ed. (5), by the method of Gordon et al. (6). All strains, except for 11 strains of *B. coagulans*, were stored at room temperature on Difco nutrient agar slants containing potato extract (Difco, 0.5%) and supplemented with 200 µg of thymine per ml if necessary. The 11 strains of *B. coagulans* were stored at 4°C on PGY agar slants composed of polypeptone (Daigo Eiyo, 1%), glucose (1%), yeast extract (Oriental Yeast, 1%) (pH 7.0), and agar (2%) supplemented with 200 µg of thymine per ml if necessary. Five thymine

[†] Present address: Chong Kun Dang Corporation, no. 410 Shin Do Rim-Dong, Young Deung Po-ku, Seoul, Republic of Korea.

^{††} Present address: Research Laboratory, Toyama Chemical Co., Ltd., 2-4-1 Shimo-okui, Toyama 930, Japan.

TABLE 1. *List of strains used*

Strain	Remarks ^a
<i>B. subtilis</i>	
168 <i>trpC</i>	← S. Yuki ← J. L. Farmer and F. Rothman
168 <i>trpC thyAB</i>	← S. Yuki ← J. L. Farmer and F. Rothman
<i>B. subtilis</i> subsp. <i>niger</i> OUT 8111	← IFO 3108 ← Research Laboratories, Takeda Chemical Industries Ltd., Osaka, Japan
<i>B. amyloliquefaciens</i> ^b KA-63	OUT stock culture ← G. Terui (Department of Fermentation Technology, Osaka University, Suita, Japan)
<i>B. licheniformis</i> IFO 12107	← ATCC 9945a ← C. B. Thorne, CD-2
<i>B. pumilus</i>	
IFO 3813	← NCTC 8241, strain Mill Hill (ATCC 14884)
IFO 12086	← NCIB 2595 ← NCTC ← ATCC 4520 ← W. W. Ford, 9
IFO 12087	← NCIB 7576 ← NCTC 7576 ← T. Gibson, 33
IFO 12088	← NCIB 8081 ← ATCC 6632 ← N. R. Smith, 247
IFO 12089	← NCIB 8600 ← CDA 658
IFO 12090	← NCIB 8738 ← A. T. Fuller (Medical Research Council, London)
IFO 12092	← NCIB 9369 ← ATCC 7061 ← N. R. Smith, 272
IFO 12093	← CCM 77 ← L. Hájek (Central Research Institute of Plant Production, Prague-Ruzyne, C.S.S.R.)
IFO 12094	← CCM 340 ← O. Mráz (Department of Microbiology, Faculty of Veterinary, Medical School, Brno University, C.S.S.R.)
IFO 12097	← CCM 386 ← M. Kocur (University of J. E. Purkyne, Brno, C.S.S.R.)
IFO 12099	← CCM 615 ← M. Kocur (University of J. E. Purkyne, Brno, C.S.S.R.)
IFO 12100	← CCM 1697 ← G. Ivánovics (Institute of Microbiology, Medical University, Szeged, Hungary) ← N. R. Smith, 736
IFO 12101	← CCM 1725 ← G. Ivánovics (Institute of Microbiology, Medical University, Szeged, Hungary)
IFO 12102	← CCM 2218 ← NCIB 8982
IFO 12110	← NRRL B-1489 ← N. R. Smith, 236 (ATCC 6631)
IFO 12111	← NRRL B-1875 ← N. R. Smith, T-2003
<i>B. firmus</i>	
ATCC 14575* ^c	← R. E. Gordon ← N. R. Smith, 613 ← J. R. Porter ← G. Bredeman ← W. Werner
IFO 3330	← ATCC 8247 ← N. R. Smith, 854 ← J. R. Porter ← G. Bredeman, A
<i>B. lentus</i> ATCC 10840*	← N. R. Smith, 670 ← T. Gibson, 165
<i>B. coagulans</i>	
ATCC 7050*	← N. R. Smith, 609 ← J. R. Porter ← B. W. Hammer
IFO 3557	← T. Harada (Institute of Scientific and Industrial Research, Osaka University, Suita, Japan)
IFO 3886	← O. Nakayama, P22 (Research Institute of Fermentation, Yamanashi University, Kofu, Japan)
IFO 3887	← O. Nakayama, A22 (Research Institute of Fermentation, Yamanashi University, Kofu, Japan)
IFO 12583	← IAM 1115
C1	Isolated from soil
C2	Isolated from soil
C3	Isolated from soil
C4	Isolated from soil
C5	Isolated from soil
C6	Isolated from soil
<i>B. megaterium</i> IAM 1030	
<i>B. cereus</i>	
T	← H. O. Halvorson
T <i>thy</i>	← H. O. Halvorson
ATCC 14579*	← R. E. Gordon ← T. Gibson, 971 ← W. W. Ford, 13
IAM 1229	
ATCC 13824	← NCIB 2600 (<i>B. cereus</i> subsp. <i>fluorescens</i>) ← W. W. Ford

TABLE 1—CONTINUED

Strain	Remarks ^a
<i>B. thuringiensis</i>	
subsp. <i>aizawai</i>	← K. Aizawa (Institute of Biological Control, Faculty of Agriculture, Kyushu University, Fukuoka, Japan), serotype 1
subsp. <i>kurstaki</i>	← K. Aizawa (Institute of Biological Control, Faculty of Agriculture, Kyushu University, Fukuoka, Japan), serotype 3a, 3b
subsp. <i>thuringiensis</i>	← K. Aizawa (Institute of Biological Control, Faculty of Agriculture, Kyushu University, Fukuoka, Japan), serotype 7
<i>B. circulans</i>	
ATCC 4513*	← W. W. Ford, 26
IFO 3329	← ATCC 9966 ← FDA PCI-221
IFO 3342	← Instituto de Microbiologia Agrícola, Buenos Aires, Argentine, B-3-6 ← ATCC 7049 ← N. R. Smith, 358 ← W. W. Ford, 26
<i>B. laterosporus</i> ATCC 64*	← AMC 797 ← AMNH 797 ← W. W. Ford, 29 ← C. A. Laubach
<i>B. polymyxa</i> ATCC 842*	← A. J. Kluyver (N. R. Smith, 1105)
<i>B. brevis</i>	
ATCC 8246*	← N. R. Smith, 604 ← J. R. Porter ← NCTC 2611 ← W. W. Ford, 27B
IFO 3864	← ATCC 9999 ← NCTC 7096 ← R. L. M. Synge
IFO 12333	← ATCC 8186 ← R. E. Gordon
<i>B. sphaericus</i>	
ATCC 14577*	← R. E. Gordon ← T. Gibson, 1013 ← Král collection (Marburg strain) ← M. Wund ← E. Neide
IFO 3341	← IMAB B-3-5 ← N. R. Smith, 344
IFO 3525	← ATCC 10208 ← N. R. Smith, 966
IFO 3526	← ATCC 4525 ← W. W. Ford, 25 (N. R. Smith, 348)
IFO 3528	← ATCC 7055 ← N. R. Smith, 350
<i>B. pantothenicus</i> ATCC 14576*	← R. E. Gordon ← N. R. Smith, 1321 ← H. Proom, CN3028
<i>Escherichia coli</i> K-12	Our stock culture

^a Abbreviations of the authentic culture collections were as follows: AMC, Walter Reed Army Medical Center, Washington, D.C.; AMNH, American Museum of Natural History, New York, N.Y.; ATCC, American Type Culture Collection, Rockville, Md.; CCM, Czechoslovak Collection of Microorganisms, University of J. E. Purkyne, Brno, Czechoslovakia; CDA, Canadian Department of Agriculture, Ottawa, Canada; FDA, Food and Drug Administration, Washington, D.C.; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; NCIB, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, England; NRRL, Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Ill.; OUT, Department of Fermentation Technology, Osaka University, Suita, Japan.

^b This strain was designated as *B. subtilis* KA-63 in a previous communication (12).

^c The type strain or neotype strain in *Bergey's Manual*, 8th ed. (4), is marked with an asterisk (*).

auxotrophic mutants were isolated from *B. pumilus* IFO 12092, *B. firmus* ATCC 14575, *B. coagulans* ATCC 7050, *B. circulans* ATCC 4513, and *B. sphaericus* ATCC 14577 by the enrichment method, using trimethoprim (Sigma) in Spizizen minimal medium (15) containing 200 or 2 µg of thymine per ml as described previously (12). For the isolation and cultivation of thymine mutants of *B. pumilus* IFO 12092, *B. firmus* ATCC 14575, *B. coagulans* ATCC 7050, and *B. circulans* ATCC 4513, the minimal medium was further supplemented with 1 µg of biotin per ml; 1 µg of biotin and 10 µg of thiamine per ml; 1 µg of biotin, 1 µg of thiamine, and 10 µg of nicotinic acid per ml; or 1 µg of thiamine and 10 µg of nicotinic acid per ml, respectively, whereas *B. sphaericus* ATCC 14577 could grow on the enrichment medium without addition of vitamins.

Media and growth conditions for cells in the preparation of DNA. For the cultivation of *B. brevis* ATCC 8246 and *B. polymyxa* ATCC 842, PGY medium without agar was used. For cultivation of 3 strains of *B. circulans* and 11 strains of *B. coagulans*, PGY medium supplemented with 1% Casamino Acids (Difco, vitamin-free) was used. For cultivation of two strains of *B. firmus* and *B. pantothenicus* ATCC 14576, brain heart infusion (Difco, 3.7%) medium supplemented with 1% Casamino Acids (pH 7.0) was used. All the other strains were cultivated in nutrient broth (Difco, 1.6%). The cultivation temperature was 37°C for all strains except *B. coagulans*, for which it was 48°C.

For the preparation of [³H]DNA, cells of the thymine-requiring mutants were cultivated in Spizizen minimal medium (15) containing 1% Casamino Acids

and 2 μ g of [*meth*-³H]thymine per ml (1.25 μ Ci/ μ g; The Radiochemical Centre Ltd., Amersham, England), and supplemented with 200 μ g of L-tryptophan per ml and the vitamins described above, if necessary.

DNA preparation and DNA-DNA hybridization. In general, DNA was extracted and purified by the method of Marmur (11). Since cells of *B. cereus*, *B. thuringiensis*, and *B. sphaericus* did not lyse on treatment with lysozyme and sodium lauryl sulfate, 3 mg of penicillin G (Meiji Seika Co., Ltd.) per ml was added to the cultures at the late logarithmic phase, and cultivation was continued for a further 2 to 3 h. The cells treated with penicillin easily lysed and gave a good yield of DNA upon treatment with lysozyme and sodium lauryl sulfate, combined.

The DNA-DNA hybridization was conducted on a nitrocellulose membrane filter by the method described previously (12). One milliliter of DNA solution containing 25 μ g of DNA in 1 $\times$ SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) was denatured by adding 1 ml of 0.3 M NaOH solution and allowing it to stand for 10 min at room temperature; the solution was then neutralized with 1 M HCl. The denatured DNA solution was adjusted to 6 $\times$ SSC and passed through a nitrocellulose membrane filter (25 mm in diameter, 0.45- μ m pore size; Sartorius Membranfilter GmbH, Federal Republic of Germany), and the filter fixed with DNA was dried for 2 h at room temperature and for 2 h in a vacuum (<20 torr) oven at 80°C. The DNA-DNA hybridization was performed by gently shaking the DNA disk at 37°C for 22.5 h in a capped small glass vial containing 1 ml of 6 $\times$ SSC-formamide (50%) solution with 0.5 or 1 μ g of denatured ³H-labeled input DNA prepared from an authentic standard strain. The amount of input DNA bound to the disk DNA was estimated by counting the radioactivity of the membrane filter, using a liquid scintillation counter (Beckman model LS-250) as described previously (12).

RESULTS

Intraspecific DNA homologies in *B. pumilus* and *B. coagulans*. The DNA homology data were obtained from the reactions between the ³H-labeled input DNA prepared from *B. pumilus* IFO 12092 and the unlabeled disk DNAs from the other 15 strains of *B. pumilus*. It was confirmed by conventional taxonomic studies that these 16 strains are *B. pumilus* as their cell diameters were smaller than 0.9 μ m, and they could neither hydrolyze starch nor reduce NO₃ to NO₂ (6). Their DNA homology indexes were 51% or more to the homologous reaction of strain IFO 12092 (Table 2). Lovett and Young (10) made a similar observation that DNA prepared from each of seven strains of *B. pumilus* showed 58 to 99% homology to that of the reference strain, NRRL B-3275. To determine the DNA homologies of *B. pumilus* with the other species, ³H-labeled input DNA prepared from strain IFO 12092 was further tested with the disk DNAs of the other 15 different species (25 strains), and the disk DNAs from

strains IFO 12086, IFO 12092, IFO 12094, and IFO 12101 were tested with input DNA from each of the other six species. The results showed significantly lower values (1 to 8%) of DNA homology than the above intraspecific values (see Table 5), indicating that the 16 strains of *B. pumilus* are of a single species and that the conventional taxonomic criteria are effective for species identification.

To study the DNA homologies between different strains of *B. coagulans*, six strains were newly isolated from soil. Their morphological and physiological characteristics by conventional taxonomic study clearly indicated that the six newly isolated strains were *B. coagulans*, as were the five authentic strains. The DNA homology indexes of the 10 strains of *B. coagulans* to strain ATCC 7050 as the standard were 76% or more (Table 3), whereas no significant DNA

TABLE 2. *Intraspecific DNA homology among the strains of B. pumilus*^a

Strain	Homology to strain IFO 12092 (%)
IFO 3813	78
IFO 12086	80
IFO 12087	68
IFO 12088	81
IFO 12089	82
IFO 12090	58
IFO 12092	100
IFO 12093	78
IFO 12094	97
IFO 12097	74
IFO 12099	57
IFO 12100	63
IFO 12101	76
IFO 12102	92
IFO 12110	51
IFO 12111	76

^a The homology index is expressed as percentage of input DNA bound to a certain disk DNA relative to the homologous reaction.

TABLE 3. *Intraspecific DNA homology among the strains of B. coagulans*^a

Strain	Homology to strain ATCC 7050 (%)
ATCC 7050	100
IFO 3557	98
IFO 3886	90
IFO 3887	113
IFO 12583	99
C1	95
C2	76
C3	86
C4	89
C5	91
C6	98

^a The homology index is expressed as percentage of input DNA bound to a certain disk DNA relative to the homologous reaction.

homology was observed when the reference strain of *B. coagulans*, ATCC 7050, was tested against the other species (see Table 5). Thus, all 11 strains, 6 newly isolated and 5 authentic ones, should be an identical species, *B. coagulans*. Smith et al. (13) located *B. coagulans* on the line dividing the species belonging to group 1 and group 2 of *Bacillus*, because the sporangia of *B. coagulans* sometimes swelled with oval spores. Although four strains of *B. coagulans* having slightly swollen sporangia were compared with the other seven strains of *B. coagulans* showing unswollen sporangia, we could not detect any relationship between the shape of sporangia and the values of DNA homology.

Identity of the species *B. cereus* and *B. thuringiensis*. Since close similarity has been observed in morphological and physiological characteristics (6), fatty acid composition of cells (7, 8), and the flagellar antigen (9) between some strains of *B. thuringiensis* and *B. cereus*, it has been claimed that these two species should be included in the same species. However, in insect pathology, strains of *B. thuringiensis* are distinguishable from those of *B. cereus* by their ability to produce crystalline parasporal bodies (8). According to the data of DNA competition studies by Somerville and Jones (14), closely related DNA homology between the strains of these two species was suggested. To investigate the above possibility, we tested DNA homologies between four strains of *B. cereus* and three strains of *B. thuringiensis* by using *B. cereus* strain T as the standard (Table 4). All of the test strains were of authentic origin. All of the strains of *B. cereus* showed 79% or higher homology to the standard strain, and the homologies of the three strains of *B. thuringiensis* to the same standard were 54 to 80%. Since these values are significantly high, it may be concluded that these two species, *B. cereus* and *B. thuringiensis*, should be the same single species, e.g., *B. cereus*, and the designation of *B. thuringiensis* should be regarded as one variant of *B. cereus*. On the other hand, it was clear that these strains are distinct from the other species of *Bacillus* so far tested as the DNA homology indexes were low (1 to 5%) (see Table 5).

Two groups of *B. sphaericus*. *B. sphaericus* is characterized by its spherical spore shape (5, 6, 13). Among the five strains of *B. sphaericus* so far tested, the DNA homology indexes fell into two groups, one showing higher (98%) and the other showing lower (18%) homology to the DNA prepared from strain ATCC 14577, whereas it was clear that the strains of both the groups were distinct from the other species of *Bacillus* so far examined (Table 5). Since the relationship of these two groups in *B. sphaericus*

TABLE 4. DNA homologies among the strains of *B. cereus* and *B. thuringiensis*^a

Strain	Homology to <i>B. cereus</i> T (%)
<i>B. cereus</i>	
T	100
ATCC 14579	79
IAM 1229	88
ATCC 13824	80
<i>B. thuringiensis</i>	
subsp. <i>aizawai</i>	54
subsp. <i>kurstaki</i>	59
subsp. <i>thuringiensis</i>	80

^a The homology index is expressed as percentage of input DNA bound to a certain disk DNA relative to the homologous reaction.

is similar to that of *B. subtilis* and *B. amyloliquefaciens*, for which the DNA homology index was about 20% (12), it is possible that the strains of these two groups are different species, as they are more similar to each other than to the other species. However, no criteria have been suggested to separate these two groups in terms of the conventional taxonomic method.

Interspecific DNA homology of the genus *Bacillus*. To determine the DNA homologies among the wide variety of species in the genus *Bacillus* described above, ³H-labeled input DNAs were prepared from seven authentic standard strains, *B. subtilis* 168, *B. pumilus* IFO 12092, *B. firmus* ATCC 14575, *B. coagulans* ATCC 7050, *B. cereus* T, *B. circulans* ATCC 4513, and *B. sphaericus* ATCC 14577, and they were hybridized with the disk DNAs from 34 strains belonging to 16 species. No significant homology was indicated between the different species of *Bacillus*, except for a group of species consisting of *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. pumilus* and for another group, i.e., *B. cereus* and *B. thuringiensis*. However, most of the DNA homologies of the 16 species of *Bacillus* were higher (2% or more) than those of *E. coli* K-12 (1%) (Table 5).

In a previous study (12), we observed that *B. subtilis*, *B. amyloliquefaciens*, and *B. licheniformis* are more closely related to each other than to the other species of *Bacillus* by the study of interspecific hybridization of DNA and genetic transformation of antibiotic resistance traits. It was also suggested that *B. pumilus* IFO 12110, the only strain of *B. pumilus* that we tested in the previous study, has somewhat higher homology to the DNAs from *B. subtilis* 168 and W23, *B. amyloliquefaciens* KA-63, and *B. licheniformis* IFO 12107, as we observed homology values of 7 to 9%. This was confirmed with the other four strains of *B. pumilus*, and we observed a homology index of 6% to input

TABLE 5. DNA homologies among various strains of *Bacillus*^a

Disk DNA	Input DNA homology (%) of:						
	<i>B. subtilis</i> 168	<i>B. pumilus</i> IFO 12092	<i>B. firmus</i> ATCC 14575	<i>B. coagulans</i> ATCC 7050	<i>B. cereus</i> T	<i>B. circulans</i> ATCC 4513	<i>B. sphaericus</i> ATCC 14577
<i>B. subtilis</i>							
168	100	8	4	4	5	3	2
subsp. <i>niger</i> OUT 8110	73	8	4	— ^b	4	4	2
<i>B. amyloliquefaciens</i> KA-63	19	8	4	—	4	4	3
<i>B. licheniformis</i> IFO 12107	9	8	4	4	—	4	2
<i>B. pumilus</i>							
IFO 12086	6	80	4	—	4	4	4
IFO 12092	6	100	4	—	4	4	3
IFO 12094	6	97	4	—	5	4	3
IFO 12101	6	76	4	3	5	5	3
<i>B. firmus</i>							
ATCC 14575	3	4	100	2	4	5	3
IFO 3330	4	3	101	—	4	5	3
<i>B. lentus</i> ATCC 10840	2	2	3	2	4	3	3
<i>B. coagulans</i>							
ATCC 7050	3	4	4	100	4	4	2
IFO 3887	4	—	—	113	—	—	—
IFO 12583	4	—	—	99	—	—	—
C2	4	—	—	76	—	—	—
<i>B. megaterium</i> IAM 1030	3	4	3	—	5	4	3
<i>B. cereus</i>							
T	—	2	2	—	100	—	—
ATCC 14579	1	—	2	—	79	1	3
<i>B. thuringiensis</i>							
subsp. <i>kurstaki</i>	2	—	2	—	59	3	3
subsp. <i>thuringiensis</i>	—	2	2	—	80	—	—
<i>B. circulans</i>							
ATCC 4513	3	4	4	2	5	100	3
IFO 3329	2	3	2	—	3	2	2
IFO 3342	2	2	2	—	3	2	2
<i>B. polymyxa</i> ATCC 842	2	2	2	2	—	2	2
<i>B. laterosporus</i> ATCC 64	2	3	2	2	3	3	2
<i>B. brevis</i>							
ATCC 8246	2	3	3	3	4	3	2
IFO 3864	1	1	1	—	—	1	1
IFO 12333	2	2	2	—	2	2	2
<i>B. sphaericus</i>							
ATCC 14577	3	3	—	2	—	3	100
IFO 3341	—	3	—	—	5	—	18
IFO 3525	—	3	2	—	—	4	98
IFO 3526	2	3	3	—	5	3	18
IFO 3528	—	3	—	—	—	—	18
<i>B. pantothenicus</i> ATCC 14576	3	4	3	2	4	4	2
<i>Escherichia coli</i> K-12	1	1	1	1	1	1	1

^a The homology index is expressed as percentage of input DNA bound to a certain disk DNA relative to the homologous reaction.

^b —, Not tested.

DNA from *B. subtilis* 168. Conversely, disk DNAs prepared from two strains of *B. subtilis*, 168 and OUT 8110, from *B. amyloliquefaciens* KA-63, and from *B. licheniformis* IFO 12107 showed a homology index of 8% to the ³H-labeled input DNA from *B. pumilus* IFO 12092

(Table 5). These results strongly suggest that *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. pumilus* are distinctive species but that they are more closely related to each other than to the other 12 species.

B. lentus was originally distinguished from *B.*

firmus by the production of a distinct titratable alkalinity in urea broth and by a lack of action on gelatin and casein (4, 13). In the last edition of *Bergey's Manual* (8th ed.; 5), this species was considered to be a homologous species with *B. firmus* and was rejected from the type species of *Bacillus*. However, the DNA homology index of *B. lentus* ATCC 10840 to *B. firmus* ATCC 14575 was significantly low (3%) (Table 5). This result indicates that the species of *B. lentus* was independent from *B. firmus*. Further investigation of the DNA homology and taxonomic significance with more test strains will be interesting.

B. pantothenicus was also rejected from the type species of *Bacillus* in *Bergey's Manual*, 8th ed. (5, 6, 13), because of its physiological similarity to *B. circulans*. However, DNA from *B. pantothenicus* ATCC 14576 showed a low homology index (4%) to that of *B. circulans* strain ATCC 4513 (Table 5). These data clearly indicate that these two strains should belong to separate species. Since the other two strains of *B. circulans*, IFO 3329 and IFO 3342, also showed low homology indexes (2%) to *B. circulans* ATCC 4513, it was strongly suggested that there is significant heterogeneity of strains in *B. circulans*, and further intensive study of this species is necessary.

DISCUSSION

In a previous paper (12), we demonstrated two different groups of strains in *B. subtilis* and claimed that *B. subtilis* subsp. *amyloliquefa-*

ciens should be separated from *B. subtilis*. The DNA homology between two strains belonging to an identical group was 59% or more, whereas that between two strains belonging to the different groups was 15 to 36%, although these two groups of strains could not be distinguished by the conventional taxonomic procedure. No transformation of the auxotrophic markers in the Marburg strain of *B. subtilis* with DNA prepared from the strains of *B. amyloliquefaciens* has been detected. With the same DNA preparation, the antibiotic resistance markers have been transformed to the Marburg strain at a low frequency and also with DNA prepared from some strains of *B. licheniformis* and *B. pumilus*. From the above observation, we tentatively formulated a definition of a species: a species is a group of strains of which the intra-specific DNA homology indexes are higher than 50%. According to this definition, the strains of *B. pumilus* tested in the present study and those of *B. coagulans* were in good accord with the species nomenclature by the conventional taxonomic method, whereas significant heterogeneities were suggested in strains of *B. circulans* and those of *B. sphaericus*. On the other hand, *B. cereus* and *B. thuringiensis* should be grouped into the same nomenclatural species, since the DNA homology indexes ranged from 54 to 88% for four *B. cereus* and three *B. thuringiensis* strains tested (Table 4), although these two species are distinct with respect to insect pathology. Similarly, difficulty was also encountered in the

FIG. 1. Diagrammatic representation of the relation between 16 species of *Bacillus*. Each branching point represents the average percent homology between each combination of two species from Table 5 and the results of a previous report (12). For the species connection, the solid lines were drawn with the average values of the direct reciprocal data by using the input ³H-labeled DNA and disk DNA for both the reference strains, whereas each broken line was drawn with data from the combination of respective disk DNA with an input ³H-labeled DNA prepared from the reference strain.

classification of *Enterobacteriaceae*. Although the genera *Escherichia*, *Shigella*, and *Salmonella* were separated with regard to their pathogenic potencies, the facts of high DNA homology (1-3) and occurrence of genetic recombination among them suggested that they should be classified into one genus or into one species. To solve the above difficulty, further intensive genetic and molecular analysis of the microorganisms is required.

Since the DNA homologies of 16 species including *B. amyloliquefaciens* were low, except for a group of species consisting of *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. pumilus*, and the two strain-groups in *B. sphaericus*, the genus *Bacillus* consists of a wide range of divergent species. No distinctive relation was observed by the study of DNA homology with respect to the strain classification of groups 1, 2, and 3 by the morphological characteristics of sporangia and spores. The relationships deduced from the DNA homology data are summarized in a dendrogram (Fig. 1), in which the DNA homology indexes between two species are represented by the branching point on the scale, and the extent of the intraspecific divergence of DNA homogeneity so far observed is shown by the black wedge-shape.

ACKNOWLEDGMENTS

We thank K. Aizawa of Kyushu University, H. O. Halvorson of Brandeis University, and the Institute for Fermentation, Osaka, for their generous gifts of the authentic strains used. We wish to thank N. Takada of our department for his taxonomic advice. We also thank K. Sato of Meiji Seika Co., Ltd., for his gift of penicillin G.

REPRINT REQUESTS

Address reprint requests to: Dr. Yasuji Oshima, Department of Fermentation Technology, Osaka University, Yamada-ka, Suita-shi, Osaka 565, Japan.

ADDENDUM IN PROOF

We were recently informed by T. Kaneko, R. Nozaki, and K. Aizawa that they observed close similarities in the G+C contents and DNA-DNA hybridizations of *Bacillus anthracis*, *B. cereus*, and *B. thuringiensis* (Microbiol. Immunol. [Tokyo], in press; for offprints contact: T. Kaneko, The Institute of Physical

and Chemical Research, Wako-shi, Saitama 351, Japan).

LITERATURE CITED

1. Brenner, D. J., G. R. Fanning, K. E. Johnson, R. V. Citarella, and S. Falkow. 1969. Polynucleotide sequence relationships among members of *Enterobacteriaceae*. *J. Bacteriol.* **98**:637-650.
2. Brenner, D. J., G. R. Fanning, F. J. Skerman, and S. Falkow. 1972. Polynucleotide sequence divergence among strains of *Escherichia coli* and closely related organisms. *J. Bacteriol.* **109**:953-965.
3. Crosa, J. H., D. J. Brenner, W. H. Ewing, and S. Falkow. 1973. Molecular relationships among the salmonellae. *J. Bacteriol.* **115**:307-315.
4. Gibson, T. 1935. The urea-decomposing microflora of soils. I. Description and classification of the organisms. *Zentralbl. Bakteriologie. Parasitenkunde. Infektionskrankheiten. Hygiene*. **2**:364-380.
5. 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 & Wilkins Co., Baltimore.
6. Gordon, R. E., W. C. Haynes, and C. H.-N. Pang. 1973. The genus *Bacillus*. *Agricultural handbook no. 427*. U.S. Department of Agriculture, Washington, D.C.
7. Kaneda, T. 1967. Fatty acids in the genus *Bacillus*. I. Iso- and anteiso-fatty acids as characteristic constituents of lipids in 10 species. *J. Bacteriol.* **93**:894-903.
8. Kaneda, T. 1968. Fatty acids in the genus *Bacillus*. II. Similarity in the fatty acid compositions of *Bacillus thuringiensis*, *Bacillus anthracis*, and *Bacillus cereus*. *J. Bacteriol.* **95**:2210-2216.
9. Krieg, A. 1969. *In vitro* determination of *Bacillus thuringiensis*, *Bacillus cereus*, and related bacilli. *J. Invertebrate Pathology*. **15**:313-320.
10. Lovett, P. S., and F. E. Young. 1969. Identification of *Bacillus subtilis* NRRL B-3275 as a strain of *Bacillus pumilus*. *J. Bacteriol.* **100**:658-661.
11. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* **3**:208-218.
12. Seki, T., T. Oshima, and Y. Oshima. 1975. Taxonomic study of *Bacillus* by deoxyribonucleic acid-deoxyribonucleic acid hybridization and interspecific transformation. *Int. J. Syst. Bacteriol.* **25**:258-270.
13. Smith, N. R., R. E. Gordon, and F. E. Clark. 1952. *Aerobic sporeforming bacteria*. *Agricultural monograph no. 16*. U.S. Department of Agriculture, Washington D.C.
14. Somerville, H. J., and M. L. Jones. 1972. DNA competition studies within the *Bacillus cereus* group of bacilli. *J. Gen. Microbiol.* **73**:257-265.
15. Spizizen, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxynucleate. *Proc. Natl. Acad. Sci. U.S.A.* **44**:1072-1078.