Bacillus ehimensis sp. nov. and *Bacillus chitinolyticus* sp. nov., New Chitinolytic Members of the Genus *Bacillus*

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Five chitin-degrading bacteria were isolated from soil. These organisms were strictly aerobic and rod shaped, formed spores, contained menaquinone 7 as the major isoprenoid quinone and 12-methyltetradecanoic acid as the major cellular fatty acid, and had guanine-plus-cytosine contents of 51.3 to 54.9 mol%, characteristics which indicate that they belong to the genus *Bacillus*. These five strains were divided into two groups on the basis of physiological characteristics and the results of a DNA-DNA hybridization study. As low levels of DNA relatedness were found between our isolates and previously described *Bacillus* strains, we propose that our isolates should be classified in two new *Bacillus* species, *Bacillus ehimensis* and *Bacillus chitinolyticus*. The type strains of *B. ehimensis* and *B. chitinolyticus* are strains IFO 15659 and IFO 15660, respectively.

Chitin is a polymer of β -1,4-linked *N*-acetylglucosamine and is widely distributed in nature as a structural component of crustacea, fungi, protozoa, and insects. The polymer is degraded enzymatically by the sequential action of two hydrolytic enzymes chitinase (EC 3.2.1.14) and chitobiase (EC 3.2.1.29) (12). Two types of chitinase activity, exochitinase activity and endochitinase activity, have been distinguished. Exochitinase activity is defined as a progressive activity that starts at the nonreducing ends of chitin chains and successively releases diacetylchitobiose units, while endochitinase activity involves random cleavage at internal points in chitin chains (25). Microbial degradation of chitin has been observed with various bacteria, especially bacteria isolated from marine environments. Well-known chitinolytic bacteria include members of the genera *Alteromonas* (2), *Bacillus* (6), *Chitinophaga* (27), *Cytophaga* (22), *Flexibacter* (22), *Herpetosiphon* (24), *Lysobacter* (23), *Micromonospora* (10), *Paenibacillus* (6), *Photobacterium* (3), *Planomonospora* (10), *Serratia* (15, 16), *Streptomyces* (4), and *Vibrio* (3). In the genus *Bacillus*, chitin-degrading activity has been found in the type strains of seven species (*Bacillus brevis*, *Bacillus laterosporus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus thiaminolyticus*, and *Bacillus thuringiensis*) and in some other strains of *Bacillus* species (6, 21, 31). Three chitinolytic *Bacillus* species, *Bacillus alvei*, *Bacillus macerans*, and *Bacillus pulvificiens*, have been transferred to the genus *Paenibacillus* as *Paenibacillus alvei*, *Paenibacillus macerans*, and *Paenibacillus pulvificiens*, respectively (1, 9).

We searched soil samples for chitinolytic bacteria and obtained five isolates. These bacteria were considered members of the genus *Bacillus* but could not be assigned to any previously described species of the genus. The isolates were divided into two groups on the basis of physiological characteristics and DNA relatedness data, and two new species, *Bacillus chitinolyticus* and *Bacillus ehimensis*, are described below.

MATERIALS AND METHODS

Isolation. Soil samples were obtained from five garden sites in Matsuyama, Ehime Prefecture, Japan, and three forest sites in Kaya, Kagoshima Prefecture, Japan. To isolate organisms, 1 g of a soil sample was suspended in 2 ml of sterile distilled water, and aliquots of the resulting suspension were inoculated onto M9 agar plates (14) supplemented with 0.1% yeast extract and 0.3% colloidal chitin (Funakoshi, Tokyo, Japan). After incubation for 3 days at 30°C, about 2,000 colonies per sample were examined for chitin-degrading activity, and the colonies that had clear halos were subcultured onto Luria-Bertani agar, which contained 1% peptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar (pH 7.0).

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. The biomass used for isoprenoid quinone and cellular fatty acid analyses was prepared by growing the strains for 1 day at 28°C with shaking in Luria-Bertani medium. The cells were harvested by centrifugation, washed with distilled water, and lyophilized.

Morphological and physiological characteristics. Motility was determined with an optical microscope by the hanging drop method. Catalase activity was determined by bubble formation in a 3% hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1% tetramethyl-*p*-phenylenediamine on filter paper. Hydrolysis of casein, hydrolysis of gelatin, and hydrolysis starch were determined as described by Cowan and Steel (7). Hydrolysis of chitin was determined by a plate method. An actively growing culture of each strain was inoculated onto CT-6 agar containing colloidal chitin (23). After incubation for 3, 7, 10, and 14 days, the presence of clear halos surrounding the colonies was evidence of chitin degradation. Other phenotypic characteristics were determined by the methods described by Claus and Berkeley (5). All tests were performed at 28°C.

Analysis of isoprenoid quinones. Menaquinones were extracted from 200 mg of freeze-dried cells with chloroform-methanol (2:1, vol/vol), separated by thin-layer chromatography with hexane-dimethyl ether (85:15) as the solvent, extracted with diethyl ether, dried under a nitrogen stream, and then analyzed by high-performance liquid chromatography (HPLC) with a model LC-5A apparatus (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a Zorbax octyldecyl silane column (4.6 by 150 mm). Methanol-isopropyl ether (7:1) was used as the mobile phase.

Analysis of cellular fatty acids. Fatty acids were extracted from 50 mg of freeze-dried cells by acid methanolysis and were analyzed with a model GC-9A gas-liquid chromatograph (Shimadzu Co., Ltd.) equipped with a glass column (2 mm by 5 m) containing 10% diethylene glycol succinate on Chromosorb W at 180°C (29).

DNA base composition. DNA was isolated from bacterial cells grown overnight in Luria-Bertani medium containing 0.5% glucose at 28°C by the method of Saito and Miura (26). The G+C content of the DNA was determined by the method of Mesbah et al. (13); after the DNA was treated with P₁ nuclease and alkaline phosphatase, the G+C content was determined by HPLC with a model LC-6AD apparatus (Shimadzu Co., Ltd.) equipped with a Cosmosil 5C₁₈-AR column (4.6 by 150 mm; Nacalai Tesque, Inc., Kyoto, Japan). The mobile phase used was 0.2 M ammonium phosphate-acetonitrile (40:1, vol/vol).

DNA-DNA hybridization. Levels of DNA relatedness were determined fluorometrically by the method of Ezaki et al. (8) by using biotinylated DNA.

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TABLE 1. Bacterial strains used

Strain ^a	Source ^a	History ^a
Group 1 strains		
EAG-1 (= IFO 15657)	Ehime University	R. Takata, isolated from garden soil in Matsuyama, Ehime Prefecture, Japan
EAG-2 (= IFO 15658)	Ehime University	R. Takata, isolated from garden soil in Matsuyama, Ehime Prefecture, Japan
EAG-5 ^T (= IFO 15659 ^T)	Ehime University	R. Takata, isolated from garden soil in Matsuyama, Ehime Prefecture, Japan
Group 2 strains		
EAG-3 ^T (= IFO 15660 ^T)	Ehime University	R. Takata, isolated from forest soil in Kaya, Kagoshima Prefecture, Japan
EAG-4 (= IFO 15661)	Ehime University	R. Takata, isolated from forest soil in Kaya, Kagoshima Prefecture, Japan
<i>Bacillus agri</i> IFO 15538 ^T	NRRL NRS-1219 ^T	N. R. Smith strain 1219 from C. Lamanna strain 13
<i>Bacillus borstelensis</i> IFO 15714 ^T	NRRL NRS-818 ^T	Through O. Shida from NRRL from J. R. Poter (= JCM 9022 ^T)
<i>Bacillus centrosporus</i> IFO 15540 ^T	NRRL NRS-664 ^T	N. R. Smith strain 664 from B. S. Henry strain 120
<i>Bacillus choshinensis</i> IFO 15518 ^T	O. Shida strain HPD52 ^T	H. Takagi et al., isolated from soil (= JCM 8505 ^T)
<i>Bacillus lautus</i> IFO 15380 ^T	NRRL NRS-666 ^T	N. R. Smith strain 666 from University of Washington
<i>Bacillus parabrevis</i> IFO 12334 ^T	ATCC 10027 ^T	N. R. Smith strain 605 from University of Washington
<i>Bacillus subtilis</i> IFO 13719 ^T	ATCC 6051 ^T	H. J. Cohn strain Marburg
<i>Bacillus thiaminolyticus</i> IFO 15656 ^T	JCM 8360 ^T	Hokkaido University AHU 1393 (= NRRL B-4156 ^T)
<i>Bacillus</i> sp. strain IFO 15377	NRRL NRS-381	N. R. Smith strain 381, isolated from soil

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

RESULTS AND DISCUSSION

Chitinolytic strains EAG-1, EAG-2, and EAG-5^T (T = type strains) were isolated from three garden soil samples collected in Matsuyama, and strains EAG-3^T and EAG-4 were isolated from two forest soil samples collected in Kaya. Tables 2 and 3 show the morphological, biochemical, and chemotaxonomic characteristics of these organisms. These strains belong to the genus *Bacillus* because they are strictly aerobic, produce rod-shaped cells and oval spores in swollen sporangia (Fig. 1), are motile by means of peritrichous flagella, and contain menaquinone 7 as the major quinone and 12-methyltetradecanoic acid (anteiso-C_{15:0}) as the major cellular fatty acid.

The G+C contents of the five new isolates ranged from 51.3 to 54.9 mol%. The previously described chitinolytic *Bacillus* strains (6) include *B. brevis* ATCC 8246^T, *B. laterosporus* ATCC 64^T, *B. lentus* NRRL B-369^T, *B. licheniformis* ATCC 14580^T, *B. megaterium* ATCC 14581^T, and *B. thuringiensis* ATCC 10792^T, and the G+C contents of these strains range from 34 to 47 mol%

(5). Therefore, our isolates can be clearly differentiated from these six previously described chitinolytic species on the basis of G+C contents. Although some *Bacillus* strains, including "*Bacillus apirius*" NRRL B-1438, *Bacillus circulans* WL-12A, *Bacillus* sp. strain ATCC 4513 (previously named "*Bacillus chitinosporus*") (11), and *B. thiaminolyticus* S327 to S330 (21), have been referred to as chitinolytic (6, 29), they are not type strains, and their G+C contents have not been determined.

Table 4 shows the levels of DNA relatedness between the new isolates and the type strains of previously described *Bacillus* species which are mesophilic and have G+C contents of more than 49 mol%. *Bacillus* sp. strain IFO 15377 was used as the *B. circulans* group G reference strain (20). The low levels of DNA relatedness with the reference strains (range, 0 to 18%) strongly suggested that the five new isolates did not belong to the previously described species.

The five new chitinolytic isolates were divided into two groups on the basis of physiological and biochemical charac-

TABLE 2. Differential phenotypic characteristics of the new chitinolytic isolates

Characteristic	Strain EAG-1	Strain EAG-2	Strain EAG-5 ^T	Strain EAG-3 ^T	Strain EAG-4
Colony morphology					
Color	Creamy	Pale brown	Creamy	Pale brown	Pale yellow
Form	Circular	Circular	Circular	Irregular	Irregular
Elevation	Convex	Convex	Convex	Raised	Raised
Margin	Entire	Entire	Entire	Undulate	Undulate
Gram reaction	V ^a	—	V	V	V
Arrangement of flagella	ND	Peritrichous	Peritrichous	Peritrichous	Peritrichous
Oxidase activity	+	+	+	+	—
Voges-Proskauer test	—	—	—	W	W
Acid produced from:					
L-Arabinose	+	+	+	—	—
D-Xylose	+	+	+	—	—
D-Mannitol	+	+	+	—	—
Hydrolysis of:					
Casein	—	+	+	—	+
Gelatin	+	+	+	—	+
Starch	+	+	+	—	—
Growth at:					
45°C	+	+	+	—	—
50°C	+	+	—	—	—
55°C	—	—	—	—	—

^a V, variable; —, negative; +, positive; ND, not determined; W, weakly positive.

TABLE 3. Major menaquinone and cellular fatty acid compositions of the new chitinolytic isolates

Strain	Major quinone ^a	Fatty acid composition (% of total cellular fatty acids)									Unknown ^b
		Saturated acids									
		C ₁₄	C ₁₅	C ₁₆	iso-C ₁₄	iso-C ₁₅	iso-C ₁₆	iso-C ₁₇	anteiso-C ₁₅	anteiso-C ₁₇	
Group 1 strains											
EAG-1	MK-7	1.2	0.8	12.6	1.8	5.5	8.4	5.6	53.0	10.0	2.3
EAG-2	MK-7	1.8	0	10.7	3.0	5.2	12.3	2.4	52.3	7.8	4.5
EAG-5 ^T	MK-7	1.0	0	16.5	1.2	5.7	5.9	8.0	49.6	10.6	1.5
Group 2 strains											
EAG-3 ^T	MK-7	1.7	0	21.3	1.7	0	4.2	3.4	58.0	7.8	1.4
EAG-4	MK-7	3.2	0	16.6	3.2	0	7.4	2.5	59.0	5.1	3.7

^a MK-7, menaquinone with seven isoprene units.^b Nonpolar acid.

teristics. Group 1 consisted of strains EAG-1, EAG-2, and EAG-5^T, which produced acid from L-arabinose, D-xylose, and D-mannitol, hydrolyzed starch, grew at 45°C, and were negative in the Voges-Proskauer test. Group 2 consisted of strains EAG-3^T and EAG-4, did not produce acid from L-arabinose, D-xylose, and D-mannitol, did not hydrolyze starch, did not grow at 45°C, and were positive in the Voges-Proskauer test. These groups were confirmed by the results of a DNA-DNA hybridization study. The members of each group exhibited high

levels of DNA relatedness with each other (89 to 105%) but low levels of DNA relatedness with members of the other group (7 to 18%) (Table 4). These results indicated that the two groups correspond to two independent species. It should be noted that the group 1 strains were isolated from garden soil samples obtained in Matsuyama, Ehime Prefecture, and the group 2 strains were isolated from forest soil samples obtained in Kaya, Kagoshima Prefecture. The physiological and biochemical criteria used to distinguish these two groups from

FIG. 1. Phase-contrast micrographs of sporulating cells of *B. ehimensis* IFO 15659^T (A) and *B. chitinolyticus* IFO 15660^T (B) grown on soil extract agar containing 0.5% peptone, 0.3% meat extract, 0.2% yeast extract, 1.5% agar, and 50% soil extract (pH 7.0). The cultures were incubated for 3 days at 30°C. The soil extract was prepared as follows: 400 g of farm soil in 1 liter of tap water was autoclaved for 30 min, the solids were removed by filtration, and then the filtrate was autoclaved for 20 min on each of 2 successive days.

TABLE 4. Levels of DNA relatedness between the new chitinolytic isolates and *Bacillus* reference strains

Strain	G+C content (mol%) ^a	% Similarity with labeled DNA from:				
		Strain EAG-1	Strain EAG-2	Strain EAG-5 ^T	Strain EAG-3 ^T	Strain EAG-4
<i>Bacillus subtilis</i> IFO 13719 ^T	44.1	4	1	5	9	18
<i>Bacillus agri</i> IFO 15538 ^T	52.6	1	12	1	13	5
<i>Bacillus borstelensis</i> IFO 15714 ^T	51.3	2	6	8	8	5
<i>Bacillus centrosporus</i> IFO 15540 ^T	50.3	4	0	0	6	0
<i>Bacillus choshinensis</i> IFO 15518 ^T	49.1	7	0	1	13	0
<i>Bacillus lautus</i> IFO 15380 ^T	50.2	9	4	8	2	7
<i>Bacillus parabrevis</i> IFO 12334 ^T	50.0	12	0	9	14	10
<i>Bacillus thiaminolyticus</i> IFO 15656 ^T	53.5	9	12	18	15	6
<i>Bacillus</i> sp. strain IFO 15377	50.2	5	17	3	13	6
EAG-1	54.3	100	91	104	13	7
EAG-2	52.9	91	100	98	17	13
EAG-5 ^T	54.9	89	97	100	18	11
EAG-3 ^T	51.3	9	14	9	100	105
EAG-4	52.8	7	15	7	104	100

^a Determined by HPLC.

seven other species that are mesophilic and have G+C contents of more than 49 mol% are shown in Table 5. Chitin-degrading activity was found in *B. thiaminolyticus* IFO 15656^T but not in the type strains of the other six species studied.

The results of this study demonstrated that the five chitinolytic isolates could be distinguished from all previously described *Bacillus* species and warrant new taxa. Therefore, below we describe two new species for the group 1 and 2 strains, *Bacillus ehimensis* and *Bacillus chitinolyticus*, respectively.

Description of *Bacillus ehimensis* sp. nov. *Bacillus ehimensis* (e.hi.men'sis. M. L. adj. *ehimensis*, referring to Ehime Prefecture, Japan, the source of the soil samples from which the organisms were isolated). Cells are rod shaped (0.4 to 0.6 by 1.7 to 5 µm). The gram reaction is usually positive, although a number of cells are gram negative. Motile by means of peritrichous flagella. Ellipsoidal spores are formed in swollen sporangia. Catalase and oxidase are produced. Aerobic. Acethyl-methylcarbinol is not produced. The pH in Voges-Proskauer

broth ranges from 5.8 to 6.3. Nitrate is reduced to nitrite. Acid, but no gas, is produced from D-glucose, L-arabinose, D-xylose, and D-mannitol. Gelatin and starch are hydrolyzed. Hydrolysis of casein is variable. Chitin is decomposed. Citrate and propionate are not utilized. The optimum growth temperature ranges from 28 to 40°C; the maximum growth temperature ranges from 50 to 53°C; and the minimum growth temperature ranges from 18 to 20°C. The G+C content of the DNA ranges from 52.9 to 54.9 mol%. Strains were isolated from garden soil samples obtained in Matsuyama, Ehime Prefecture, Japan.

The type strain is strain IFO 15659 (= EAG-5).

Description of *Bacillus chitinolyticus* sp. nov. *Bacillus chitinolyticus* (chi.ti.no.ly'ti.cus. M. L. n. *chitinum*, chitin; M. L. adj. *lyticus*, dissolving; M. L. adj. *chitinolyticus*, decomposing chitin). Cells are rod shaped (0.4 to 0.6 by 1.7 to 3 µm). The gram reaction is usually positive, although a number of cells are gram negative. Motile by means of peritrichous flagella. Ellipsoidal spores are formed in swollen sporangia. Catalase is

TABLE 5. Characteristics that differentiate *B. chitinolyticus*, *B. ehimensis*, and related *Bacillus* species

Characteristic	<i>Bacillus ehimensis</i> ^a	<i>Bacillus chitinolyticus</i> ^a	<i>Bacillus thiaminolyticus</i> ^b	<i>Bacillus agri</i> ^c	<i>Bacillus lautus</i> ^d	<i>Bacillus centrosporus</i> ^e	<i>Bacillus parabrevis</i> ^e	<i>Bacillus choshinensis</i> ^e	<i>Bacillus borstelensis</i> ^f
Anaerobic growth	— ^g	—	+	—	+	—	—	—	—
Voges-Proskauer test	+	+	+	—	+	—	—	—	—
pH of Voges-Proskauer broth									
<6.0	+	+	+	—	+	—	—	—	—
>7.2	—	—	—	+	—	+	+	+	+
Reduction of nitrate	+	+	+	—	+	d	+	—	+
Acid produced from:									
Arabinose	+	—	d	d	+	—	—	—	—
Glucose	+	+	+	+	+	+	+	+	d
Mannitol	+	—	d	+	+	+	+	d	—
Xylose	+	—	—	—	+	—	—	—	—
Hydrolysis of:									
Chitin ^a	+	+	+	—	—	—	—	—	—
Casein	+	d	+	+	—	—	+	—	+
Gelatin	+	d	ND	+	ND	ND	+	—	+
Starch	+	—	+	—	+	—	—	—	—
G+C content (mol%)	52–55	51–53	52–54	52–55	50–52	49–51	50–53	49–50	50–52

^a Data from this study.^b Data from reference 18.^c Data from reference 19.^d Data from reference 17.^e Data from reference 30.^f Data from reference 28.

produced. Aerobic. Acetylmethylcarbinol is produced. The pH in Voges-Proskauer broth ranges from 5.4 to 5.6. Nitrate is reduced to nitrite. Acid, but no gas, is produced from D-glucose. No acid is produced from L-arabinose, D-xylose, and D-mannitol. Hydrolysis of casein and hydrolysis of gelatin are variable. Starch is not hydrolyzed. Chitin is decomposed. Citrate and propionate are not utilized. The optimum growth temperature ranges from 25 to 37°C; the maximum growth temperature ranges from 42 to 45°C; and the minimum growth temperature ranges from 18 to 20°C. The G+C content of the DNA ranges from 51.3 to 52.8 mol%. Strains were isolated from forest soil samples obtained in Kaya, Kagoshima Prefecture, Japan.

The type strain is strain IFO 15660 (= EAG-3).

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