Description of *Bacillus carboniphilus* sp. nov.

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We observed activation of growth with six aerobic spore-forming isolates on an otherwise nonpermissive medium when a carbon material, such as graphite or activated charcoal, was added to the medium (Bacto Antibiotic Medium 3). On the basis of the phenotypic characteristics and physiological and biochemical properties of these organisms and DNA homology data we concluded that they belong to a new *Bacillus* species, which is designated *Bacillus carboniphilus*. Strain Kasumi 6 (= JCM 9731) is the type strain of this new species.

Several workers have reported previously that activated charcoal enhances the growth of cultures of some bacteria, including *Neisseria gonorrhoeae* and *Neisseria meningitidis* (5), *Haemophilus pertussis* (11), and *Legionella pneumophila* (4). In recent studies Matsuhashi et al. isolated *Bacillus* strains which require a carbon material, such as graphite or activated charcoal, for growth on otherwise nonpermissive nutrient agar plates containing 1% KCl at 44°C (9) and described important tools that can be used to investigate the mechanism of the growth-promoting effects of carbon materials on bacteria (9, 10). In this study these *Bacillus* strains were characterized taxonomically, and in this paper they are described as members of a new species.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains which we used are listed in Table 1. The new isolates (strains Kasumi 1, Kasumi 2A, Kasumi 3, Kasumi 4, Kasumi 6^T [T = type strain], and Kasumi 7) were grown on slants of Trypto-Soya Agar (Nissui Co., Tokyo, Japan) at 30°C for 48 to 72 h, and the other *Bacillus* strains were grown on slants of Bacto Nutrient Agar (Difco) at 30°C for 48 to 72 h. Working stocks were prepared on agar slants or agar plates and were stored at 4°C.

Isolation of bacteria. Strains Kasumi 1, Kasumi 2A, Kasumi 3, Kasumi 4, Kasumi 6^T, and Kasumi 7 were isolated from the air of Kasumi district in Numazu, Japan. These strains were isolated on agar plates containing 17.5 g of Bacto Antibiotic Medium 3 per liter and 15 g of agar (Wako Pure Chemical Industries, Osaka, Japan) per liter. Bacto Antibiotic Medium 3 contains 0.05 M potassium and 0.06 M sodium, which ordinarily did not permit the new isolates to survive. However, about 200 µl of a thick suspension (in 1% Triton X-100) of Graphite C (Toyo Carbon Co., Tokyo, Japan) that had been thoroughly sterilized by heating for 1 min at 700°C (9) was spotted on the surface of each agar plate,

and the plates were incubated for 48 to 96 h at 44°C. Bacterial colonies appeared around the spots of graphite and were collected, and single-cell cultures were obtained by streaking the bacteria on Trypto-Soya Agar plates.

Phenotypic characterization. Unless indicated otherwise, the methods described by Gordon et al. (6) and Claus and Berkeley (1) were used to characterize bacteria. The media and growth temperature used to determine physiological characteristics were the media and temperature described above unless indicated otherwise. Tests for hydrolysis of urea and utilization of nitrate were performed by using the methods described by Komagata (8). Indophenol oxidase was detected with commercial cytochrome oxidase test paper (Eiken, Tokyo, Japan). Anaerobic growth tests were performed with a commercial anaerobic growth test system (Anaero Pack; Mitsubishi Gas Co., Tokyo, Japan). Tests for production of indole and hydrogen sulfate were performed by using SIM medium (Nissui Co., Tokyo, Japan) containing (per liter of distilled water) 3 g of beef extract, 28 g of peptone, 0.025 g of sodium thiosulfate, 1 g of ammonium iron citrate, and 3 g of agar; the pH of this medium was 7.2. DNase production was determined by using DNase test agar (Nissui) containing (per liter of distilled water) 15 g of peptone, 5 g of soybean peptone, 5 g of sodium chloride, 2 g of DNA, 0.1 g of toluidine blue O, and 15 g of agar; the pH of this medium was also 7.2. Tolerance to salinity was determined by using Trypto-Soya Broth (Nissui) supplemented with 2 to 10% (wt/vol) NaCl. Hydrolysis of Tween 80 was determined by the method described by Cowan (2), and hydrolysis of esculin was determined on bile esculin agar (Difco). Decomposition of arginine, decomposition of lysine, and decomposition of ornithine were determined in Moeller decarboxylase broth base (BBL Microbiology Systems, Cockeysville, Md.). The *o*-nitrophenyl-β-D-galactopyranoside test for β-galactosidase was performed with commercial *o*-nitrophenyl-β-D-galactopyranoside disks (Nissui).

DNA base composition and DNA homology. The chromosomal DNAs used to determine base composition and levels of hybridization were isolated from bacterial cells by the method of Saito and Miura (13). DNA base compositions were determined by the method of Tamaoka and Komagata (14). DNA-DNA hybridization was performed with photobiotin-labeled probes in microplate wells as described by Ezaki et al. (3). The DNA probes used for hybridization were prepared from strain Kasumi 1, Kasumi 3, Kasumi 4, and Kasumi 7 chromosomal

TABLE 1. Bacterial strains used in this study^a

Strain(s)	Source	History
Kasumi 1, Kasumi 2A, Kasumi 3, Kasumi 4, Kasumi 6 ^T , Kasumi 7	Tokai University, Numazu, Japan	Isolated from air on plates containing Bacto Antibiotic Medium 3 agar and carbon graphite
<i>B. megaterium</i> JCM 2506 ^T	JCM	CCM 2007 from R. E. Gordon
<i>B. flexus</i> DSM 1320 ^T	DSM	NRS 665 from R. E. Gordon
<i>B. simplex</i> DSM 1321 ^T	DSM	NRS 960 from R. E. Gordon
<i>B. lentus</i> JCM 2511 ^T	JCM	CCM 2214 from NCIB 8773

^a JCM, Japan Collection of Microorganisms, Saitama, Japan; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; CCM, Czech Collection of Microorganisms, Brno, Czech Republic; NRS, Collection of Nathan R. Smith, U.S. Department of Agriculture, Peoria, Ill.; NCIB, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland.

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TABLE 2. Phenotypic characteristics of the new isolates

Characteristic	New isolates
Cell width (μm)	0.5–0.9
Cell length (μm)	3.0–5.0
Sporangia swollen	— ^a
Gram reaction	+
Anaerobic growth	—
Catalase activity	+
Oxidase activity	+
Nitrate reduction	—
Voges-Proskauer reaction	—
pH of Voges-Proskauer broth of >7.0	—
Utilization of:	
Citrate	—
Acetate	—
Fumarate	—
L-Malate	—
DL-Lactate	—
Succinate	—
L-Glutamate	—
Hydrolysis of:	
Casein	+
Gelatin	+
DNA	V
Starch	+
Tween 80	+
Urea	—
Hippurate	+
Deamination of phenylalanine	—
Decomposition of tyrosine	—
Growth at:	
pH 5.6 (nutrient broth)	+
17–47°C	+
Growth in the presence of 7.0% NaCl	+
ONPG reaction ^b	—
Resistance to 0.02% sodium azide	+
Acid produced from:	
D-Glucose	—
D-Fructose	—
D-Galactose	—
Lactose	—
Sucrose	—
Trehalose	—
D-Mannitol	—
Salicin	—
Gas produced from:	
D-Glucose	—
L-Arabinose	—
D-Xylose	—
D-Mannitol	—

^a —, negative; +, positive; V, variable.^b ONPG, *o*-nitrophenyl- β -D-galactopyranoside.

DNAs, and these probes were used to estimate the levels of hybridization with total chromosomal DNAs from all six new isolates. The DNAs were hybridized at 37°C for 12 h.

RESULTS

Phenotypic properties. Some of the morphological and biochemical characteristics of the six new isolates are shown in Table 2. These six strains were rod shaped, gram positive, oxidase positive, catalase positive, and motile by means of peritrichous flagella. Ellipsoidal spores were formed. The spores were central or terminal in sporangia which were not swollen.

The data described above indicate that all of the new isolates are members of the genus *Bacillus*, and these organisms probably belong to a new species, since the most closely related previously described *Bacillus* species (aerobic species that form

TABLE 3. Characteristics that differentiate the new isolates from *B. megaterium*, *B. flexus*, *B. simplex*, and *B. lentus*

Characteristic	New isolates	<i>B. lentus</i> ^a	<i>B. megaterium</i> ^b	<i>B. simplex</i> ^b	<i>B. flexus</i> ^b
Nitrate reduction	— ^c	—	—	+	—
Voges-Proskauer reaction	—	—	—	—	—
pH in Voges-Proskauer broth	6.0–7.0	6.0–7.0	<6.0	V	V
Growth at pH 5.6	+	NT	+	+	+
Deamination of phenylalanine	—	—	+	—	—
Decomposition of tyrosine	—	—	V	V	V
Hydrolysis of:					
Casein	+	—	+	+	+
Gelatin	+	—	+	+	+
Urea	—	+	+	—	+
Utilization of citrate	—	—	V	+	+
Acid produced from:					
D-Glucose	—	+	+	+	+
L-Arabinose	—	—	+	V	—
D-Xylose	—	V	V	V	—
D-Mannitol	—	V	+	V	+

^a Data from reference 12.^b Data from reference 7.^c —, negative; +, positive; V, variable; NT, not tested.

ellipsoidal spores in unswollen sporangia and have G+C contents of 36 to 40 mol%; *Bacillus megaterium* [7], *Bacillus flexus* [7], *Bacillus simplex* [7], and *Bacillus lentus* [12]) could be distinguished from the new isolates by many phenotypic characteristics, as shown in Table 3.

Activation of growth of the six isolates was observed when carbon material, such as graphite or activated charcoal, was added to Bacto Antibiotic Medium 3.

DNA base composition and DNA homology. The G+C contents and levels of DNA homology of the new strains are shown in Table 4. The DNA base compositions of the six new strains ranged from 37.8 to 38.1 mol% G+C. The DNAs of these six strains were hybridized with the DNAs of strains Kasumi 1, Kasumi 3, Kasumi 4, and Kasumi 7 and were found to be closely related; therefore, the six new strains belong to the same species. In contrast, when strain Kasumi 1 and Kasumi 3 DNA probes were hybridized with DNAs from phenotypically related *Bacillus* strains having similar G+C contents (*B. megaterium*, *B. flexus*, *B. simplex*, and *B. lentus* strains), the levels of hybridization were less than 10%, indicating that the new isolates are not closely related to the other *Bacillus* species studied (Table 5).

TABLE 4. DNA compositions and levels of DNA homology for the new isolates

Strain	G+C content (mol%)	% DNA-DNA reassociation with ^a :			
		Kasumi 1	Kasumi 3	Kasumi 4	Kasumi 7
Kasumi 1	38.0	100	80	100	88
Kasumi 2A	38.0	84	100	100	100
Kasumi 3	38.0	100	100	100	100
Kasumi 4	37.8	63	76	100	75
Kasumi 6 ^T	37.9	89	59	100	76
Kasumi 7	38.1	99	72	100	100

^a The values are the averages of the values from two determinations; the maximum variation observed was 9%.

TABLE 5. DNA base compositions and levels of homology for *B. megaterium*, *B. flexus*, *B. simplex*, *B. lentus*, and the new isolates

Strain	G+C content (mol%)	% DNA-DNA reassociation with ^a :	
		Kasumi 1	Kasumi 3
Kasumi 1	38.0	100	80
Kasumi 3	38.0	100	100
<i>B. megaterium</i> JCM 2506 ^T	39.8	6	10
<i>B. flexus</i> DSM 1320 ^T	37.8	8	10
<i>B. simplex</i> DSM 1321 ^T	38.1	2	7
<i>B. lentus</i> JCM 2511 ^T	36.9	NT ^b	0

^a The values are the averages of the values from two determinations; the maximum variation observed was 9%.

^b NT, not tested.

DISCUSSION

Carbonaceous materials, such as charcoal, have been used in the isolation of fastidious organisms. For example, charcoal-yeast extract agar facilitated the primary isolation of *Legionella pneumophila* (4). By incorporating charcoal or graphite in isolation media, we isolated a number of *Bacillus* strains under stressed conditions, and Matsuhashi et al. demonstrated that these isolates require graphite to grow in the presence of 1 to 2% KCl at 44°C (9). High levels of intragroup DNA relatedness indicated that the new isolates were homogeneous genetically. Low levels of DNA relatedness with strictly aerobic species having G+C contents of about 38 mol% indicated that the new isolates were genetically distinct from previously described species. Phenotypically, the new isolates were homogeneous but distinctly different from previously described species. These observations suggest that the new isolates are members of new species, for which the name *Bacillus carboniphilus* is proposed.

Description of *Bacillus carboniphilus* sp. nov. *Bacillus carboniphilus* (car.bo.ni'phi.lus. L. n. *carbo*, coal, carbon; Gr. adj. *philos*, loving; M. L. adj. *carboniphilus*, carbon loving). Cells are rod shaped (0.5 to 0.9 by 3.0 to 5.0 µm) and gram positive. Motile by means of peritrichous flagella. Ellipsoidal spores are formed. The spores are central or terminal in sporangia which are not swollen. Colonies on nutrient agar are flat, smooth, grayish yellow, and circular with entire edges. A brown-red pigment is produced on Tryptone-Soya Agar. Growth occurs at 17 to 47°C. Positive for catalase activity; oxidase activity; hydrolysis of casein, gelatin, Tween 80, starch, and hippurate; and growth in the presence of 7.0% NaCl. Negative for the Voges-Proskauer reaction; anaerobic growth; reduction of nitrate; the *o*-nitrophenyl-β-D-galactopyranoside reaction; hydrolysis of esculin and urea; decomposition of arginine, lysine, and ornithine; utilization of citrate, propionate, acetate, fumarate, L-malate, D- and L-lactate, succinate, L-glutamate, L-aspartate, alginate, D-gluconate, 2-oxoglutarate, malonate, pyruvate, and tartrate; acid production from D-glucose, L-arabinose, D-fructose, D-galactose, maltose, lactose, sucrose, D-xylose, trehalose, glycerol, D-mannitol, cellobiose, D-ribose, salicin, D-sorbose, D-mannose, melibiose, L-rhamnose, raffinose, *meso*-inositol, erythritol, adonitol, and starch; and gas production from D-glucose, L-arabinose, D-xylose, and D-mannitol.

The DNA base composition ranges from 37.8 to 38.1 mol% G+C. Strain Kasumi 6 is the type strain. The G+C content of the type strain is 37.9 mol%. The type strain has been deposited in the Japan Collection of Microorganisms as strain JCM 9731.

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