Bifidobacterium gallinarum sp. nov.: a New Species Isolated from the Ceca of Chickens

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Bifidobacterium gallinarum sp. nov. is described. Ten strains of this species were isolated from chicken ceca. These strains are obligately anaerobic, gram-positive, nonsporeforming, nonmotile, slightly curved, short rod-shaped organisms with pointed ends; the cells occur singly, in pairs, and sometimes in short chains. The fermentation products from glucose or fructose are lactic acid and acetic acid in a molar ratio of $1:4.0 \pm 0.2$ in broth. These isolates differ from other species in the genus *Bifidobacterium* in morphology, carbohydrate fermentation pattern, guanine-plus-cytosine content of deoxyribonucleic acid, and deoxyribonucleic acid homologies. The type strain of *B. gallinarum* is Ch206-5 (= ATCC 33777).

In 1969, Mitsuoka (10) described strains of bifidobacteria isolated from human feces and from the intestines of chickens and other animals. In this study, Mitsuoka differentiated chicken strains from human strains by carbohydrate fermentation patterns and the ability of the chicken strains to grow at 46.5°C, and he proposed two new species, *Bifidobacterium pseudolongum* and *Bifidobacterium thermophilum*. Trovatelli et al. (18) isolated a new *Bifidobacterium* species from the feces of chickens and named it *Bifidobacterium pullorum*. Strains of *Bifidobacterium animalis* were also isolated from the feces of chickens (16).

Some strains of bifidobacteria isolated by us from the ceca of chickens have not been identified. Barnes and Impey (1) also isolated unidentified *Bifidobacterium* strains from the ceca of chickens. In this study, the phenotypic characteristics and genetic relationships of the fowl strains are compared with those of the known *Bifidobacterium* species to clarify the taxonomic status of these strains.

MATERIALS AND METHODS

Bacterial strains. The designations and origins of the strains examined are listed in Table 1.

Cultivation of cultures. All of the organisms were grown on BL agar and in EG broth (12) at 37°C in anaerobic steel wool jars in an atmosphere of 100% CO₂. Strains were maintained by monthly transfers on prerduced EG liver agar slants supplemented with H₂CO₃-CO₂ buffer (12) and storage at 4°C.

Cultural and biochemical tests. The methods used for carbohydrate fermentation tests (10) and other biochemical characterization tests (7) have been described previously. For fermentation tests, 24-h-old cells were harvested by centrifugation at $2,500 \times g$ for 10 min, suspended to one-half the original volume in

the basal medium (10) without fermentable carbohydrate, and inoculated into the test medium. Fermentation products were analyzed by the method of Holdeman et al. (5). Fructose-6-phosphate phosphoketolase was demonstrated photometrically (3).

Preparation of DNA. To prepare deoxyribonucleic acid (DNA), cells were grown in EG broth supplemented with 0.5% sucrose. Cells were harvested in the late logarithmic phase and washed twice with 0.15 M NaCl-0.1 M ethylenediaminetetraacetic acid (pH 8.0). DNA was isolated by a modification of the procedures of Marmur (8). The purity and the amount of DNA were estimated by measuring the hyperchromic shift during thermal denaturation (4). Tritium-labeled DNA was prepared by using the nick-translation system (New England Nuclear Corp., Boston, Mass.) adapted from the procedure of Rigby et al. (14).

DNA base composition. The guanine-plus-cytosine (G+C) contents of the DNAs were determined by the thermal melting point method (9), using an automatic recording spectrophotometer (Komatsumi Electronics, Tokyo, Japan). Calf thymus DNA was included in each set as a standard.

DNA homology experiments. DNA homology experiments were performed by the S1 nuclease procedure, as described by Johnson et al. (6). S1 nuclease digestions were conducted with 0.5 U of S1 nuclease (Tokyo Kasei Ltd., Tokyo, Japan). After incubation for 15 min at 37°C, the remaining double-stranded DNA segments were precipitated with cold 10% trichloroacetic acid and collected on a membrane filter (type HA; Millipore Corp., Bedford, Mass.). The membranes were dried, and the radioactivity was measured in a toluene-based scintillation fluid with a liquid scintillation counter (model 3330; Packard Instrument Co., Inc., Rockville, Md.).

Preparation of cell-free extracts. Cells were grown anaerobically in EG broth for 24 h at 37°C. Cells were harvested by centrifugation at $8,000 \times g$ for 10 min and washed twice with 0.05 M phosphate buffer (pH 7.4) containing 0.04% (vol/vol) 2-mercaptoethanol. Cells resuspended in the same buffer (150 mg [wet weight]

TABLE 1. Strains used in this study

Strain	Source ^a	Origin
<i>B. gallinarum</i>		
Ch206-5 ^T (= ATCC 33777)	Our collection	Chicken cecum
Barnes EBF 77/12B	Barnes	Chicken cecum
Barnes EBF 77/14B	Barnes	Chicken cecum
Barnes EBF 90/38 (= ATCC 33778)	Barnes	Chicken cecum
Barnes EBF 90/40A	Barnes	Chicken cecum
Barnes EBF 90/51A	Barnes	Chicken cecum
Barnes EBF 90/82	Barnes	Chicken cecum
Barnes EBF 90/90	Barnes	Chicken cecum
Barnes EBF 92/91	Barnes	Chicken cecum
Barnes 92/68	Barnes	Chicken cecum
<i>B. pseudolongum</i>		
PNC-2-9G ^T	Our collection	Pig feces
T-26	Our collection	Chicken feces
H5	Our collection	Pig feces
<i>B. thermophilum</i>		
P2-91 ^T	Our collection	Pig feces
14-44	Our collection	Pig feces
T-111	Our collection	Chicken feces
<i>B. animalis</i>		
R101-S ^T	Our collection	Rat feces
G11-3	Our collection	Guinea pig feces
<i>B. longum</i>		
Reuter E194b ^T	Reuter	Adult feces
M101-2	Our collection	Adult feces
<i>B. infantis</i>		
Reuter S12 ^T	Reuter	Infant feces
21143	Our collection	Infant feces
<i>B. breve</i> Reuter S1 ^T	Reuter	Infant feces
<i>B. parvulorum</i> Reuter S50 ^T	Reuter	Infant feces
<i>B. bifidum</i>		
Beerens Ti ^T (Tissier strain)		Infant feces
Reuter S28a	Reuter	Infant feces
<i>B. adolescentis</i> Reuter E194a ^T	Reuter	Adult feces
<i>B. angulatum</i> Reuter B677 ^T	Reuter	Adult feces
<i>B. catenulatum</i> Scardovi B669 ^T	Scardovi	Adult feces
<i>B. dentium</i> Scardovi B764 ^T	Scardovi	Dental caries
<i>B. pseudocatenulatum</i> Scardovi B1279 ^T	Scardovi	Infant feces
<i>B. minimum</i> Scardovi F392 ^T	Scardovi	Sewage
<i>B. subtile</i>		
Scardovi F394 ^T	Scardovi	Sewage
Scardovi F395	Scardovi	Sewage
<i>B. pullorum</i> Scardovi P145 ^T	Scardovi	Chicken feces
<i>B. cuniculi</i> Scardovi RA93 ^T	Scardovi	Rabbit feces
<i>B. boum</i> Scardovi RU917 ^T	Scardovi	Bovine rumen
<i>B. choerinum</i> Scardovi SU806 ^T	Scardovi	Piglet feces
<i>B. magnum</i> DSM 20222 ^T	DSM	Rabbit feces
<i>B. indicum</i>		
Scardovi C410 ^T	Scardovi	Bee intestine
DSM 20216	DSM	Bee intestine
<i>B. suis</i> ATCC 27533 ^T	ATCC	Pig feces

^a Cultures were received from the following sources: Barnes, E. M. Barnes, Agricultural Research Service Food Research Institute, Norwich, NR4 7UA, England; Reuter, G. Reuter, Institut für Lebensmittelhygiene, Freie Universität Berlin, Berlin, Federal Republic of Germany; Scardovi, V. Scardovi, Instituto di Microbiologia Agraria, Università di Bologna, Bologna, Italy; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany; ATCC, American Type Culture Collection, Rockville, Md.

per ml) were sonicated for 1 to 3 min at 0°C with a Sonic 300 Dismembrator (Artek, Farmingdale, N.Y.) fitted with a 0.7-cm-diameter tip, at an energy setting of 40%. After centrifugation at 20,000 × g for 30 min,

the supernatants were used for electrophoretic enzyme assays; 1 ml of fluid was mixed with 0.15 ml of 0.1% bromophenol blue in 40% sucrose and stored at -20°C until use.

Polyacrylamide gel electrophoresis. The gels used to detect enzymes were prepared by a modification of the method of Davis (2, 13). The separation gels contained 7.5% acrylamide, and the pH of each gel was adjusted to 8.9. Electrode buffer [tris(hydroxymethyl)aminomethane, 6.0 g; glycine, 28.8 g; water to 1,000 ml] was diluted 1:10 before use. The lower electrode served as the anode, and samples were electrophoresed at 15 mA for 30 min. The current was then increased to 35 mA and held constant at this level until the bromophenol blue front arrived at the bottom of the gel. The gel was stained specifically to reveal lactate dehydrogenase with a mixture containing following: 0.1 M tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 7.4), 20 ml; lithium L-lactate, 200 mg; nicotinamide adenine dinucleotide, 40 mg; Nitro Blue Tetrazolium chloride, 30 mg; phenazine methosulfate, 6 mg; fructose 1,6-diphosphate, 6 mg.

RESULTS

Morphology. The colonial appearance and cellular appearance of strains of the new species *Bifidobacterium gallinarum* are shown in Fig. 1. All strains were gram-positive, nonsporeforming, rod-shaped organisms. The cells of strains Ch206-5^T (type strain), Barnes EBF 92/91, and Barnes EBF 92/68 were predominantly short rods (0.5 to 1.0 μm wide by 1.0 to 1.5 μm long) and occurred in pairs or, occasionally, in short chains. The cells of strains Barnes EBF 77/12B and Barnes EBF 77/14A were short rods (1.0 μm wide by 1.5 μm long) with two or three globular elements arranged in chains. The cells of the other strains were slender, irregularly shaped

rods (0.5 to 1.0 μm wide by 1.5 to 2.5 μm long); chains of globular elements were absent. Only cells in aged liquid cultures had short branches.

Colonies of strains Ch206-5^T, Barnes EBF 77/14B, Barnes EBF 92/91, and Barnes EBF 92/68 on BL agar were 0.3 to 0.7 mm in diameter, circular, smooth, flat, transparent to translucent, glistening, entire, and soft. Colonies of the other strains on BL agar were 0.5 to 1.0 mm in diameter, circular, smooth, convex, translucent, grayish, glistening, entire, and soft.

Biochemical properties. All strains fermented xylose, ribose, fructose, sucrose, maltose, melibiose, raffinose, inulin, dextrin, esculin, salicin, and amygdalin. Rhamnose, starch, glycogen, mannitol, sorbitol, and inositol were not fermented. Strain Barnes EBF 92/68 failed to ferment glucose. No strain reduced nitrate or produced indole or hydrogen sulfide.

All strains produced acetic acid and lactic acid in ratios of 3.8:1 to 4.2:1 from the fermentation of glucose or fructose.

The fructose-6-phosphate phosphoketolase tests were positive, in accordance with the results obtained for bifidobacteria by De Vries et al. (3). The biochemical properties that were variable among the strains are shown in Table 2.

G+C contents and DNA homology. Table 3 shows the G+C contents of the DNA preparations and the DNA homologies among strains examined by the S1 nuclease method. All isolates gave high levels of DNA homology with

FIG. 1. Morphology of *B. gallinarum* sp. nov. (A) Cells of type strain Ch206-5^T from a 2-day-old surface colony on BL agar. Bar = 10 μm . (B) Cells of strain Barnes EBF 77/12B from a 2-day-old surface colony on BL agar. (C) Cells of strain Barnes EBF 90/38 from a 2-day-old surface colony on BL agar. (D) Two-day-old surface colonies of type strain Ch206-5^T on BL agar. Bar = 5 mm. (E) Surface colonies of strain Barnes EBF 90/38 on BL agar.

TABLE 2. Biochemical characteristics of 10 *B. gallinarum* strains

Strain	Final pH in glucose or fructose broth	Ratio of end products of glucose fermentation ^a	Acid from: ^b									Litmus milk ^b	
			Arabinose	Glucose	Mannose	Galactose	Cellobiose	Lactose	Trehalose	Melezitose	α -Methylglucoside	Acid	Curd
Ch206-5 ^T	4.6	1:3.8	(+)	+	—	+	—	(+)	+	+	±	—	—
Barnes EBF 77/12B	4.7	1:3.9	+	+	+	+	—	+	+	—	+	+	+
Barnes EBF 77/14A	5.1	1:4.1	+	+	+	+	+	+	+	—	(+)	+	+
Barnes EBF 90/38	4.9	1:4.2	+	+	+	+	+	+	+	—	+	+	+
Barnes EBF 90/40A	5.0	1:4.2	+	+	+	+	+	+	+	—	+	+	+
Barnes EBF 90/51A	4.7	1:4.0	+	+	+	+	+	±	±	—	+	+	(+)
Barnes EBF 90/82	4.9	1:3.9	+	+	+	+	+	(+)	(+)	—	+	—	—
Barnes EBF 90/90B	4.8	1:3.8	+	+	+	+	+	+	+	—	+	+	—
Barnes EBF 92/91	4.5	1:4.0	±	+	+	(+)	+	(+)	+	+	—	—	—
Barnes EBF 92/68	5.0	1:4.1	—	—	—	—	—	—	—	—	+	—	—

^a Ratio of lactic acid to acetic acid.^b +, Positive reaction; (+), weak positive reaction; ±, negative or delayed weak reaction; —, negative reaction.

strains Ch206-5^T, Barnes EBF 90/38, and Barnes EBF 92/91. The DNAs of the other bifidobacteria tested had little or no homology with strains Ch206-5^T, Barnes EBF 90/38, and Barnes EBF 92/91. The mean G+C content of the DNAs from the *B. gallinarum* isolates tested was 65.7 ± 1.5 mol%.

Electrophoretic analysis. The ranges of the electrophoretic mobilities of the lactate dehydrogenases of some strains of *B. gallinarum*, *B. longum*, and *B. thermophilum* are shown in Fig. 2. The mobilities of the *B. gallinarum* lactate dehydrogenases were similar.

DISCUSSION

Our DNA homology results demonstrate that there is very low or no genomic relatedness between the strains isolated from chicken ceca and the previously described species of the genus *Bifidobacterium*. Although there were some differences in their carbohydrate fermentation patterns and in their morphological characteristics, the strains isolated from chicken ceca were closely related genetically. Therefore, we propose the name *Bifidobacterium gallinarum* sp. nov. for these strains and designate strain Ch205-6 (= ATCC 33777) as the type strain.

Although strain Barnes EBF 92/68 did not ferment glucose, it grew well in the medium containing sucrose and had fructose-6-phosphate phosphoketolase, the key enzyme for hexose degradation in bifidobacteria (3). Because of high DNA homology with the type strain, strain Barnes EBF 92/68 is a member of the species *B. gallinarum*.

At present, the DNA homology approach is basic to the study of the taxonomy of the bifidobacteria. However, it is especially important to

compare physiological and biochemical characteristics to differentiate genetically distinct species (11, 17). The G+C content of the DNA of *B. gallinarum* is 65.7 mol% and thus is similar to the value for *B. pullorum* DNA (66 to 68 mol% [18]), but there is a low level of DNA homology between these two species. The biochemical and morphological properties of these two species also differ. *B. gallinarum* strains can be differentiated from other *Bifidobacterium* species by the following characteristics: small cell size; transparent to translucent small colonies on BL agar; and specific fermentation pattern of polysaccharides. The ability of *B. gallinarum* to ferment inulin and extrin distinguish this species from the other starch-negative bifidobacteria.

Bifidobacterium gallinarum sp. nov. (gal.li.na'-rum L. n. *gallina* hen; L. gen. pl. n. *gallinarum* of hens). Gram-positive, nonmotile, nonspore-forming, slightly curved, short rods (0.5 to 1.0 μ m wide by 1.0 to 2.5 μ m long) with tapered ends, usually arranged singly or in short chains. Swellings or club-shaped cells are frequent. In liquid aged cultures some cells have short branches.

Surface colonies on BL agar after 2 days of anaerobic incubation are punctiform to 1 mm in diameter, circular, entire, flat to convex, gray to grayish white, transparent to translucent, and glistening.

Strictly anaerobic.

Final pH of glucose or fructose broth: 4.5 to 5.1.

Acid from xylose, ribose, fructose, sucrose, maltose, melibiose, raffinose, inulin, dextrin, esculin, salicin, and amygdalin. Arabinose, glucose, mannose, galactose, cellobiose, lactose, trehalose, and α -methylglucoside usually fer-

TABLE 3. DNA homologies among strains of *B. gallinarum* and other *Bifidobacterium* species

Strain	% Homology with reference DNA from:						G+C content (mol%)
	<i>B. gal- linarum</i> Ch206-5 ^T	<i>B. gal- linarum</i> Barnes EBF 77/12B	<i>B. gal- linarum</i> Barnes EBF 90/38	<i>B. gal- linarum</i> Barnes EBF 92/91	<i>B. pseu- dolongum</i> T-26	<i>B. ther- mophilum</i> 14-44	
<i>B. gallinarum</i>							
Ch206-5 ^T	100	93	84	82	4	2	66.4
Barnes EBF 77/12B	66	100	65	79	4	3	64.7
Barnes EBF 77/14A	80	84	74	70	4	5	64.8
Barnes EBF 90/38	68	71	100	80	NT ^a	7	64.2
Barnes EBF 90/40A	64	105	102	65	NT	0	66.4
Barnes EBF 90/51A	74	87	104	74	7	1	64.1
Barnes EBF 90/82	65	76	72	77	0	2	66.3
Barnes EBF 90/90B	77	77	92	93	2	0	66.4
Barnes EBF 92/91	64	76	83	100	2	0	66.8
Barnes EBF 92/68	71	75	94	102	1	7	67.5
<i>B. pseudolongum</i>							
NPC-2-9G ^T	7	NT	4	NT	NT	NT	NT
T-26	4	7	2	3	100	6	61.8
H5	5	5	4	5	62	10	NT
<i>B. thermophilum</i>							
P2-91 ^T	3	NT	5	NT	NT	NT	NT
14-44	4	3	3	6	0	100	61.8
T-111	4	6	4	0	NT	74	NT
<i>B. animalis</i>							
R101-S ^T	7	NT	9	NT	NT	NT	NT
G11-3	6	2	3	2	4	7	NT
<i>B. longum</i>							
Reuter E194b ^T	7	NT	6	NT	NT	NT	NT
M101-2	10	8	8	9	1	3	NT
<i>B. infantis</i>							
Reuter S12 ^T	3	NT	4	NT	NT	NT	NT
21143	2	9	2	0	NT	2	NT
<i>B. breve</i> Reuter S1 ^T	4	8	2	1	0	3	NT
<i>B. parvulorum</i> Reuter S50 ^T	2	1	2	4	0	0	NT
<i>B. bifidum</i>							
Beerens Ti ^T	3	NT	7	NT	NT	NT	NT
Reuter S28a	7	9	4	5	1	3	NT
<i>B. adolescentis</i> Reuter E194a ^T	6	4	1	0	0	0	NT
<i>B. angulatum</i> Scardovi B677 ^T	2	1	4	0	3	5	NT
<i>B. catenulatum</i> Scardovi B669 ^T	1	7	5	8	0	7	NT
<i>B. dentium</i> Scardovi B764 ^T	6	5	3	0	1	5	NT
<i>B. pseudocatenulatum</i> Scar- dovi B1279 ^T	1	1	0	0	0	3	NT
<i>B. minimum</i> Scardovi F392 ^T	5	8	1	1	2	1	NT
<i>B. subtile</i>							
Scardovi F394 ^T	4	NT	3	NT	NT	NT	NT
Scardovi F395	5	9	2	0	16	2	NT
<i>B. pullorum</i> Scardovi P145 ^T	4	5	1	8	2	2	NT
<i>B. cuniculi</i> Scardovi RA93 ^T	3	3	2	6	12	1	NT
<i>B. boum</i> Scardovi RU917 ^T	3	6	3	0	1	0	NT
<i>B. choerinum</i> Scardovi SU806 ^T	3	5	0	1	1	4	NT
<i>B. magnum</i> DSM 20222 ^T	7	5	3	3	1	5	NT
<i>B. indicum</i>							
Scardovi C410 ^T	4	NT	2	NT	NT	NT	NT
DSM 20216	4	1	1	0	0	2	NT
<i>S. suis</i> ATCC 27533 ^T	3	8	4	0	0	1	NT

^a NT, Not tested.

mented. Rhamnose, starch, glycogen, mannitol, sorbitol, and inositol not fermented. Melezitose usually not fermented.

No gas formation from glucose or fructose. Fermentation products from glucose or fructose are lactic acid and acetic acid in a molar

FIG. 2. Electrophoretic mobilities of the lactate dehydrogenases of some *Bifidobacterium* strains. Lane 1, *B. gallinarum* type strain Ch206-5^T; lane 2, *B. gallinarum* Barnes EBF 77/12B; lane 3, *B. gallinarum* Barnes EBF 90/38; lane 4, *B. gallinarum* Barnes EBF 92/68; lane 5, *B. longum* M101-2; lane 6, *B. pseudolongum* T-26; lane 7, *B. thermophilum* 14-44; lane 8, *B. thermophilum* T-111.

ratio of $1:4.0 \pm 0.2$ in EG broth containing these carbohydrates. Propionic acid and butyric acid are never formed.

Nitrate not reduced.

Indole not produced.

Hydrogen sulfide not produced.

Ammonia not produced from arginine.

Litmus milk usually acidified.

Gelatin not liquefied.

G+C content: 65.7 ± 1.5 mol%.

Source: chicken caeca.

Type strain: Ch206-5 (= ATCC 33777).

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