Bifidobacterium animalis (Mitsuoka) comb. nov. and the "minimum" and "subtile" Groups of New Bifidobacteria Found in Sewage

V. SCARDOVI and L. D. TROVATELLI

Istituto di Microbiologia Agraria, Università di Bologna, Bologna, Italy

Twenty-two strains of bifidobacteria from the feces of chickens, rats, and rabbits, and from sewage, formed a single deoxyribonucleic acid (DNA) homology group displaying complete homology with *Bifidobacterium longum* subsp. *animalis* Mitsuoka biotype *a* strain R101-8 (=ATCC 25527). The DNA relatedness of this group of strains to the known species of the genus *Bifidobacterium* ranged from 5 to 40%. Therefore, we propose that *B. longum* Reuter subsp. *animalis* Mitsuoka biotype *a* should be elevated to species rank as *Bifidobacterium animalis* (Mitsuoka) comb. nov. The type strain is ATCC 25527. Also, a small number of strains isolated from sewage are suggested as probably being distinctive bifidobacteria and are placed in two new groups designated "minimum" and "subtile." The reference strains for these groups are F392 (=ATCC 27538) and F395 (=ATCC 27537), respectively.

Mitsuoka (2) proposed the names *Bifidobacterium thermophilum*, *Bifidobacterium pseudolongum*, and *Bifidobacterium longum* subsp. *animalis* for bifidobacteria he isolated from the feces of various animals, such as pigs, chickens, rats, and guinea pigs. *Bifidobacterium ruminale* and *Bifidobacterium globosum* were isolated in our laboratory from the rumens of cattle (8); *B. ruminale* subsequently (9) proved to be identical with *B. thermophilum* Mitsuoka, and *B. globosum* was closely related (9) to *B. pseudolongum* Mitsuoka as demonstrated by deoxyribonucleic acid (DNA)-DNA hybridizations. The question of the identities of *B. pseudolongum* and *B. globosum* was left open because of some differences in the guanine plus cytosine (G + C) contents of their DNAs: in *B. pseudolongum* the mol % G + C is 60.1 ± 0.4 , and in *B. globosum* it was 63.8 ± 0.4 (8). In the meantime, Matteuzzi et al. (1) isolated from pig feces a new species, *Bifidobacterium suis*, whose DNA homology relations and phenotypic characteristics are distinctive among the bifidobacteria.

In DNA homology experiments (9), Mitsuoka's biotypes *a* and *b* of *B. longum* subsp. *animalis* were found to be unrelated to *B. longum* Reuter. Furthermore, strain R101-8 (=ATCC 25527), representative of *B. longum* subsp. *animalis* biotype *a*, could not be assigned to any known species of *Bifidobacterium*, and

strain C10-45, representative of *B. longum* subsp. *animalis* biotype *b*, was found to be related to *B. pseudolongum*. Among a large number of bifidobacteria isolated from the feces of chickens, rats, and rabbits, and from sewage, we found 22 strains whose DNA exhibited little or no relatedness to the DNAs of known species of *Bifidobacterium*. The purpose of this study is to determine the taxonomic position of these strains and that of *B. longum* subsp. *animalis* biotype *a*.

MATERIALS AND METHODS

Bacterial strains. The strains included in this study are listed in Table 1. All of the strains were studied for their DNA relatedness to the reference strains of the various *Bifidobacterium* species listed in Table 1.

DNA-DNA hybridization. The procedures used for determining DNA relatedness were performed as previously described (5, 9).

Determination of phenotypic characters. The methods used to determine the phenotypic characters of the strains studied were also previously described (5).

RESULTS

DNA homology relationships. The data in Table 2 show how closely the *animalis* group is related to reference strain P23—homology val-

TABLE 1. *Strains used*

Organisms	Isolated from	Culture received from
<i>Bifidobacterium animalis</i> (Mitsuoka) comb. nov.		
R101-8 (<i>B. longum</i> subsp. <i>animalis</i> biotype <i>a</i> , Mitsuoka (2)) (=ATCC 25527)	Feces of rat	T. Mitsuoka
P16; P17; P23; P26; P32; P39; P45	Feces of chicken	Our collection
T6; T27; T98; T160; T169	Feces of rat	Our collection
RA12; RA13; RA14; RA15; RA16; RA18; RA20; RA23	Feces of rabbit	Our collection
F437; F439	Sewage	Our collection
<i>Subtile</i> group		
F395, reference strain; F340; F394; F396; F398	Sewage	Our collection
<i>Minimum</i> group		
F392; F399	Sewage	Our collection
<i>Bifidobacterium bifidum</i> (Tissier) Orla-Jensen S28a (=ATCC 15696)	Feces of infant	G. Reuter
<i>B. breve</i> Reuter S1 (=ATCC 15700) Type strain (4)	Feces of infant	G. Reuter
<i>B. liberorum</i> Reuter S76e (=ATCC 15702) Type strain (4)	Feces of infant	G. Reuter
<i>B. adolescentis</i> Reuter E298b (=ATCC 15705) biotype <i>c</i> (3)	Feces of adult	G. Reuter
<i>B. longum</i> Reuter E194b (=ATCC 15707) Type strain (4)	Feces of adult	G. Reuter
<i>B. catenulatum</i> Scardovi and Crociani B669 (=ATCC 27539) Type strain (5)	Feces of adult	Our collection
<i>B. dentium</i> Scardovi and Crociani B764 (=ATCC 27534) Type strain (5)	Dental caries	Our collection
<i>B. angulatum</i> Scardovi and Crociani B677 (=ATCC 27535) Type strain (5)	Feces of adult	Our collection
<i>B. ruminale</i> Scardovi et al. RU326 (=ATCC 25866) Type strain (8)	Rumen of cattle	Our collection
<i>B. globosum</i> Scardovi et al. RU230 (=ATCC 25864) Type strain (8)	Rumen of cattle	Our collection
<i>B. suis</i> Matteuzzi et al. SU859 (=ATCC 27533) Type strain (1)	Feces of pig	Our collection
<i>B. asteroides</i> Scardovi and Trovatelli C51 (=ATCC 25910) Type strain (7)	Intestine of bee	Our collection
<i>B. indicum</i> Scardovi and Trovatelli C410 (=ATCC 25912) Type strain (7)	Intestine of bee	Our collection
<i>B. coryneforme</i> Scardovi and Trovatelli C215 (=ATCC 25911) Type strain (7)	Intestine of bee	Our collection

TABLE 2. *DNA homology relationships between B. animalis and other species of the genus Bifidobacterium*

Competitor strains	Reference strain <i>B. animalis</i> P23
P23 (=ATCC 27536)	100
P16; P17; P26	98-92-98
P32; P39; P45	98-98-72
T6; T27; T98; T160; T169	72-72-98-90-88
F437; F439	90-85
RA12; RA13; RA14; RA15	85-85-92-94
RA16; RA18; RA20; RA23	90-86-90-86
R101-8 (=ATCC 25527)	99
<i>Bifidobacterium bifidum</i> S28a	10
<i>B. liberorum</i> S76e	15
<i>B. breve</i> S1	22
<i>B. adolescentis</i> E298b	30
<i>B. longum</i> E194b	5
<i>B. catenulatum</i> B669	8
<i>B. dentium</i> B764	6
<i>B. angulatum</i> B677	11
<i>B. globosum</i> RU230	40
<i>B. ruminale</i> RU326	23
<i>minimum</i> group F392	5
<i>subtile</i> group F395	5

ues ranged from 72 to 99%. *B. longum* subsp. *animalis* biotype *a* strain R101-8 (=ATCC 25527) displayed 99% homology to the same reference system. Undoubtedly these strains form a very homogeneous group. The DNAs from type strains of the known species of the genus *Bifidobacterium* and of reference strains of the "*minimum*" and "*subtile*" groups competed only slightly, if at all, in the homologous reference system P23; only *B. globosum* RU230 showed some 40% homology to this reference; thus, this result and the results of the reverse reactions (see Table 4) indicate a certain similarity of some regions of the genome of *B. globosum* and the "*animalis*" reference strain.

The results of similar competition experiments conducted with reference strains F392 (*minimum* group) and F395 (*subtile* group) are reported in Table 3. Only low levels of homology were demonstrated between the *minimum* and *subtile* groups, and, similarly, low or even zero levels of homology were found with DNA competitors covering all the specific taxa of the genus *Bifidobacterium*. Because of some morphological resemblance of the *mini-*

TABLE 3. DNA homology relationships between the minimum and subtile groups and species of the genus *Bifidobacterium*

Competitor strains	Reference strains	
	F392 (Group mini- mum)	F395 (Group subtile)
<i>minimum</i> group		
F392; F399	100-103	16-20
<i>subtile</i> group		
F340; F394; F395	10-20-31	70-94-100
F396; F398	20-23	70-80
<i>Bifidobacterium bifidum</i> S28a	4	4
<i>B. liberorum</i> S76e	4	5
<i>B. breve</i> S1	10	17
<i>B. adolescentis</i> E298b	5	13
<i>B. longum</i> E194b	7	23
<i>B. catenulatum</i> B669	30	12
<i>B. dentium</i> B764	5	00
<i>B. angulatum</i> B677	23	8
<i>B. globosum</i> RU230	35	10
<i>B. ruminale</i> RU326	4	15
<i>B. suis</i> SU859	10	8
<i>B. asteroides</i> C51	00	2
<i>B. indicum</i> C410	00	00
<i>B. coryneforme</i> C215	4	00
<i>B. animalis</i> P23	5	10

mum strains with *B. indicum* from the intestine of the bee (7), preparations of DNA from the type strain of *B. indicum*, ATCC 25912, were included; they proved absolutely unrelated to both reference systems (*minimum* and *subtile*). In the experiments reported in Table 4, DNA-DNA hybridizations were performed in reverse, i.e., by using the DNAs of *B. animalis*, *minimum*, and *subtile* as competitors in 14 reference systems. The data correlate well with those reported in Tables 2 and 3.

Morphology. The cellular morphology of the *animalis* group exhibits some recognizable traits: the central portions, probably where division into two daughter cells did not go to completion, are often enlarged; branchings can occur at this point to form cross-like elements with branches of nearly the same length (see Fig. 6 of Plate 1); cell extremities are generally enlarged to clavate or spatula-like forms, with occasional rudimentary branchings. Cells of this group display a morphology similar to those of *B. globosum* and *B. pseudolongum*, where short, enlarged, and tapered cells are frequent; however, branching tendencies are not found with the latter two species.

Cells of the shape and size of the two *minimum* strains (see Plate 2) have not been found in any other bifidobacteria, with the sole exception of *B. asteroides* when grown in deficient media (see Fig. 5 and 6 of Plate 2 of reference 7); they are very minute, short and thin, with tapered ends, and are sometimes irregularly branched. Star-like aggregates of cells, like those characteristic of *B. asteroides*, are absent here. Had it not been for their biochemical characters, the *minimum* strains could easily be overlooked or mistaken for corynebacteria or anaerobic actinomycetes.

Subtile cells, as the name indicates, are characteristically thinner than those of the usual bifidobacteria; their general shape is reminiscent of *B. breve* Reuter (see Fig. 4 and 5 of Plate 3), but the cells of the latter are often shorter and thicker, swollen and branched (4) (our unpublished observations). Branchings or swellings were not observed in the *subtile* strains. Because of the few *minimum* and *subtile* strains observed, the present morphological descriptions, although generally unusual for bifidobacteria, should be considered as tentative subject to the study of more strains.

TABLE 4. DNA homology relationships between *B. animalis*, the minimum and subtile groups (used as competitors) and species of the genus *Bifidobacterium* (used as references)

Reference strains	Percent homology of DNA from competitor strains		
	P23 (<i>B. ani- malis</i>)	F392 (group mini- mum)	F395 (group subtile)
<i>Bifidobacterium animalis</i>			
P23 (ATCC 27536) ..	100	5	8
<i>minimum</i> group F392 ..	8	100	15
<i>subtile</i> group F395	10	10	100
<i>B. bifidum</i> S28a	10	5	5
<i>B. liberorum</i> S76e	10	6	5
<i>B. breve</i> S1	20	10	14
<i>B. adolescentis</i> E298b ..	25	4	15
<i>B. longum</i> E194b	8	10	20
<i>B. catenulatum</i> B669 ..	10	25	8
<i>B. dentium</i> B764	4	00	00
<i>B. angulatum</i> B677	10	20	11
<i>B. globosum</i> RU230 ...	30	5	8
<i>B. ruminale</i> RU326 ...	25	10	18
<i>B. suis</i> SU859	20	7	11
<i>B. asteroides</i> C51	5	3	5
<i>B. indicum</i> C410	00	00	4
<i>B. coryneforme</i> C215 ..	00	4	00

PLATE 1. Morphology of *Bifidobacterium animalis* (Mitsuoka) *comb. nov.* Fig. 1 and 2. Strain P23: cells grown anaerobically and under low oxygen tension, respectively, in Trypticase-phytone-yeast extract-glucose-agar medium. Fig. 3 and 4. Strain P16: cells grown in an anaerobic stab and in a broth medium with glucose, respectively. Fig. 5. Cells of strain P17 from an anaerobic stab. Fig. 6. Cells of strain P39 from a 6-day-old broth culture. Fig. 7 to 14. Cells from anaerobic stabs of strains T6, T27, T98, T169, RA13, RA14, F439, and RA20, respectively. Fig. 15. Cells of Mitsuoka strain R101-8 from an anaerobic stab. Phase contrast. $\times 1,500$. Fig. 16 and 17. Side and top views, respectively, of surface colonies of the strain P23 on anaerobic slants. $\times 10$.

PLATE 2. Morphology of the group *minimum*. Fig. 1 to 3. Strain F392: cells from an anaerobic stab (the first transfer after isolation); cells from a 24-h-old broth culture (DNA extraction); and cells from a 6-day-old slant incubated anaerobically, respectively. Fig. 4 to 6. Strain F399: the same as above. Phase contrast. $\times 1,500$.

Physiology: fermentation patterns. Strains of the *animalis* group fermented arabinose and xylose (Table 5). Strains P16, P17, P23, and P32 were also investigated by W. E. C. Moore (personal communication), who obtained a contradictory negative result with arabinose, a behavior reported for the *B. infantis*-*B. liberorum*-*B. lactentis* group (9). We cannot explain this discrepancy between our results and Moore's. Pending resolution of this discrepancy, our results suggest that the *animalis* group can be distinguished from other pentose-fermenting species of *Bifidobacterium* by the fact that 83% and 100% of the *animalis* strains ferment cellobiose and salicin, respectively, and 100% of the strains do not ferment gluconate.

The fermentation pattern of the *minimum* strains is unusual among bifidobacteria. The two strains of the *minimum* group, like the *B. thermophilum*-*B. ruminale* group and the species from bees, do not ferment lactose; they ferment only glucose, fructose, maltose, dextrin, and, albeit slowly, sucrose and starch. This pattern is evidently quite different from that of *B. bifidum*, the species of the genus with the most restricted fermentation spectrum, i.e., the latter ferments glucose, fructose, lactose, and galactose and is variable in saccharose and melibiose fermentation (2, 3).

The *subtile* strains also did not ferment lactose, arabinose, or xylose, but they differed from lactose-negative species such as *B. indicum* and *B. ruminale* in that the *subtile* strains fermented sorbitol and gluconate.

Other physiological and biochemical characteristics. The physiological and biochemical characteristics of *B. animalis* and of the *minimum* group are in line with those of most bifidobacteria (see below). The *subtile* strains have an optimum growth temperature near 35 C and a maximum not higher than 40 C; these temperature relationships are not shared by any known species of *Bifidobacterium*. Furthermore, these strains (*subtile* group) are unique bifidobacteria in that their cell-free extracts possess both "animal" and "human" electrophoretic types of fructose-6-phosphate phosphoketolase (F6PPK) (6).

The results of this study show conclusively that the 22 strains of bifidobacteria we isolated from the feces of chickens, rats, and rabbits, and from sewage, are identical to *B. longum* subsp. *animalis* biotype *a* strain R101-8 isolated by Mistuoka from the feces of rat because they displayed complete DNA relatedness to this strain and had the same phenotypic characteristics. However, these bifidobacteria cannot be included in a subspecies of *B. longum* Reuter

PLATE 3. Morphology of the group *subtile*. Fig. 1. Cells of strain F395 from a 24-h-old anaerobic stab. Fig. 2 and 3. Cells of strains F394 and F396, respectively, as above. Fig. 4 and 5. Cells of strain F395 from 6-day-old broth cultures containing glucose and gluconate, respectively. Fig. 6. Cells of strain F396 from a 24-h-old broth culture (DNA extraction). Phase contrast. $\times 1,500$.

because they do not share with the type strain of this species any common DNA base sequences, as revealed by levels of DNA homology of $<10\%$. Therefore, we propose the creation of the new species *Bifidobacterium animalis* (Mitsuoka) comb. nov. The *minimum* and *subtile* groups are not proposed as new species because of the small number of strains studied.

The main characters of this new species are summarized as follows.

Bifidobacterium animalis (Mitsuoka) comb. nov. (*B. longum* Reuter subsp. *animalis* Mitsuoka biotype *a*).

Cell characteristics. Gram-positive, non-motile, nonsporeforming short rods (2 to 4 μm) with the central area generally slightly enlarged; in longer cells, distal portions may be clavate or spatula-like. Branchings can occur to form cross-like aggregates of four cells distally inflated. (Plate 1, Fig. 1-15)

Surface colonies. Smooth, convex, entire edges, cream to white, glistening and soft. (Plate 1, Fig. 16 and 17)

Relationships to oxygen and CO_2 . Anaerobic; CO_2 has no effect upon oxygen sensitivity and upon anaerobic growth. No growth in

slants incubated in air or in air enriched with carbon dioxide.

Temperature relationships. Optimum, 39 to 41 $^{\circ}\text{C}$; minimum, about 25 $^{\circ}\text{C}$; maximum, 44.5 to 45 $^{\circ}\text{C}$; no growth at 24 $^{\circ}\text{C}$ or 46 $^{\circ}\text{C}$.

pH relationships. Initial optimum, 6.4 to 7.0; retarded growth at 6.0 or 7.4; no growth at 5.0 or 8.0.

End products from glucose. Lactic and acetic acids are produced in a molar ratio of $1:3.6 \pm 0.3$ in Trypticase-phytone-yeast extract medium. Isomeric type of lactic acid produced: L(+). Propionic and butyric acids are not produced.

Fermentation pattern. The fermentation pattern is given in Table 5. The fermentation of cellobiose and salicin is not observed in the other species of *Bifidobacterium* which ferment pentoses but not gluconate.

Enzymology. Fructose-1,6-diphosphate aldolase: approximately 3 to 4 mU of specific activity; glucose-6-phosphate (G6P) dehydrogenase (oxidized nicotinamide adenine dinucleotide phosphate [NADP^+] dependent): demonstrable (nearly 2 mU of specific activity); 6-phosphogluconate (6PG) dehydrogenase (NADP^+ dependent): some 40 to 50 mU of

TABLE 5. Fermentation patterns of *Bifidobacterium animalis* and the minimum and subtile groups^a

Compound	<i>B. animalis</i>		minimum group (2 strains)	subtile group (5 strains)
	23 strains	Type strain (ATCC 25527) ^b		
L-Arabinose	+ ^c (23/23)	+	—	— (5/5)
D-Xylose	+ (23/23)	+	—	— (5/5)
D-Ribose	+ (23/23)	+	—	+ (4/5) ^j
D-Mannose	— (18/23) ^d	—	—	— (5/5)
Galactose	+ (23/23)	+	—	+ (5/5)
Sucrose	+ (23/23)	+	+	+ (5/5)
D-Cellobiose	+ (19/23) ^e	—	—	— (4/5) ^k
Lactose	+ (23/23)	+	—	— (5/5)
D-Trehalose	— (17/23) ^f	—	—	— (4/5) ^k
Melibiose	+ (22/23) ^g	+	—	+ (5/5)
Raffinose	+ (23/23)	+	—	+ (5/5)
Melezitose	— (14/23) ^h	—	—	+ (5/5)
Dextrin	+ (22/23) ⁱ	±	+	+ (5/5)
Starch	+ (22/23) ⁱ	±	+	+ (5/5)
Inulin	— (23/23)	—	—	— (4/5) ^k
Sorbitol	— (23/23)	—	—	+ (5/5)
Salicin	+ (23/23)	+	—	— (3/5) ^l
Gluconate	— (23/23)	—	—	+ (5/5)

^a All strains ferment glucose, fructose, and maltose; none ferments mannitol, glycerol, rhamnose, or lactate.

^b The fermentation pattern of the type strain corresponds to that given by Mitsuoka (2) for *B. longum* subsp. *animalis*.

^c Symbols: +, fermented; —, not fermented; ±, very delayed reaction.

^d P26, T6, T27, T160, and RA23 were positive.

^e P45, T98, RA23, and R101-8 were negative.

^f P17, P26, P32, P39, T27, and T160 were positive.

^g RA12 was negative.

^h P26, P32, P39, T6, T27, T160, RA14, RA16, and RA23 were positive.

ⁱ Only strain R101-8 ferments these sugars very slowly.

^j F395 was negative.

^k Only strain F396 ferments these sugars.

^l F395 and F396 were positive.

specific activity; electrophoretic type of F6PPK: "animal."

Biochemical reactions. Catalase or pseudo-catalase (hemin) not produced; nitrate not reduced; indole not produced; acetylmethylcarbinol produced; gelatin not liquefied; skim milk acidified and coagulated; ammonia not produced from urea or arginine.

G + C content of DNA. 60.1 ± 0.3 mol % (T_m).

DNA homology relationships. Little or no relationship to all other known species of the genus.

Sources. The feces of chickens, rats, and rabbits, and sewage.

Type strain. R101-8 (=ATCC 25527); designated as the type strain by Mitsuoka (2), who isolated it from the feces of a rat.

Distinctive characters of the *minimum* and *subtile* strains are summarized as follows.

1. *Minimum* group.

Cell characteristics. Very small cells, 0.3 by 1.3 to 1.5 μ m, with tapered ends and sometimes irregularly branched. Star-like aggregates, like those in *B. asteroides* Scardovi and Trovati, are absent.

End products from glucose. Lactic and acetic acids are produced in a molar ratio of 1:3.3. L(+) lactic acid is formed. Propionic and butyric acids are not produced.

Fermentation pattern. The fermentation pattern is given in Table 5.

Enzymology. Only 6PG dehydrogenase is measurable; the "animal" electrophoretic type of F6PPK is produced.

DNA homology relationships. Unrelated to

any other species of *Bifidobacterium* from man or other animals, including bees.

Source. Sewage.

Reference strain. F392 (=ATCC 27538).

2. *Subtile* group.

Cell characteristics. Slender rods, 0.5 by 2 to 3 μ m, with rounded or tapered ends, sometimes curves; branchings are not evident.

Temperature relationships. Optimum, 34 to 35.5 C; maximum, near 40 C; minimum, 22 C.

Fermentation pattern. The fermentation pattern is given in Table 5.

Enzymology. G6P dehydrogenase activity (both oxidized nicotinamide adenine dinucleotide and NADP⁺ dependent) is rather evident (some 10 to 15 mU of specific activity in strain F395); both "animal" and "human" electrophoretic types of F6PPK are present.

DNA homology relationships. Unrelated to any known species of the genus *Bifidobacterium*.

Source. Sewage.

Reference strain. F395 (=ATCC 27537).

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REPRINT REQUESTS

Address reprint requests to: Dr. V. Scardovi, Istituto di Microbiologia Agraria, Università di Bologna, Bologna, Italy.

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