Phenotypic and Genomic Analyses of Human Strains Belonging or Related to *Bifidobacterium longum*, *Bifidobacterium infantis*, and *Bifidobacterium breve*

DRISS BAHAKA,¹ CHRISTEL NEUT,² ABDELKRIM KHATTABI,¹ DANIEL MONGET,³
AND FRANÇOISE GAVINI^{1,4*}

Institut National de la Recherche Agronomique, Laboratoire d'Ecologie et de Physiologie du Système Digestif, Domaine du C.E.R.T.I.A., 59650 Villeneuve d'Ascq Cedex,¹ Service de Bactériologie, Faculté de Pharmacie, 59045 Lille Cedex,² Biomerieux-Unité de Bactériologie, La Balme les Grottes, 38390 Montalieu Vercieu,³ and Institut National de la Santé et de la Recherche Médicale,⁴ France

A numerical analysis based on phenotypic characteristics (89 enzymatic tests and 49 carbohydrate acidification tests), in which experimental strips from Biomerieux-API, La Balme les Grottes, France, were used, was performed to characterize 82 new isolates belonging or related to *Bifidobacterium longum*, *Bifidobacterium infantis*, and *Bifidobacterium breve*. A total of 72 strains were isolated from child or adult feces, and the other strains were obtained from human vaginas and bronchi. In this study we also included 38 type and reference strains that were representative of all species of the genus *Bifidobacterium* and 6 strains belonging to the genus *Lactobacillus*. DNA-DNA relationships between *B. longum* and *B. infantis* were determined by using 19 strains related to these species, as determined by the numerical analysis. The degree of DNA binding was determined by the S1 nuclease method. The phenotypic study revealed that there were six main clusters, which were subdivided into nine subclusters. Subcluster Va contained the type strains of *B. longum* and *B. infantis*. The DNA-DNA relatedness values of some of the new isolates were very similar to the DNA-DNA relatedness values of the type strain of *B. longum*. On the basis of these data, it was difficult to isolate *B. infantis* strains and then to define *B. infantis* as a single species separated from *B. longum*. Subclusters IVb to IVf comprised reference strains of *B. breve*. Cluster III and subcluster Ia were not identified.

At the present time, the genus *Bifidobacterium* includes 29 species, 10 of which are of human origin. Four of these species, *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Bifidobacterium infantis*, and *Bifidobacterium breve*, have been studied with increasing interest because of their role in physiological relationships in human (especially infant and child) gastrointestinal tracts (2, 3, 29). Furthermore, today, the use of these bacteria in food microbiology (dairy products) makes accurate differentiation of these organisms necessary (19).

The type species of the genus, *B. bifidum*, is well separated from the other species on the basis of its phenotypic characteristics, as well as its DNA-DNA hybridization values. Identification of the three other species studied in this work is much more uncertain; in particular, distinction between *B. longum* and *B. infantis* is difficult.

On the basis of the results of phenotypic studies, Reuter (18) defined and subdivided *B. longum* into two biovars, biovars a and b, and Mitsuoka (17) divided *B. longum* into *B. longum* subsp. *longum*, and *B. longum* subsp. *animalis* variants a and b. Variant a was elevated to species rank as *Bifidobacterium animalis* on the basis of the results of DNA-DNA hybridization experiments (22); however, phenotypic differentiation of this taxon from *B. longum* was still being discussed by Yaeshima et al. in 1991 (28).

The definitions of *B. infantis* and *B. breve*, as described by Reuter (18), were revised in 1971 by Scardovi et al. (24); *B. infantis* included *Bifidobacterium liberorum* and *Bifidobacterium lactentis*, species that were initially separated from *B. infantis*, while *B. breve* included *Bifidobacterium parvulum* (18).

The purpose of this study was to determine by numerical analysis and DNA-DNA hybridization the phenotypic and genomic characteristics of *B. longum* and *B. infantis* and the relationships of these species to *B. breve*.

MATERIALS AND METHODS

Bacterial strains. The strains which we used are listed in Table 1. A total of 126 strains were used in this study, including 82 new isolates (72 isolates from child and adult feces or intestines, 2 isolates from human vaginas, 2 isolates from bronchi, and 6 isolates of unknown origin) and 44 type and reference strains (6 of which belonged to the genus *Lactobacillus*). Every new isolate except strain R12H2 from an ileal reservoir came from the Service de Bactériologie, Faculté de Pharmacie de Lille, Lille, France.

Phenotypic characterization. All of the strains were considered members of the genus *Bifidobacterium* because they exhibited the characteristics described previously for this genus (10), especially the presence of fructose-6-phosphate phosphoketolase. The same tests performed previously (10), including 89 experimental enzymatic strip tests and 49 carbohydrate acidification tests (Biomerieux-API, La Balme les Grottes, France), were used for numerical analysis.

Numerical analysis. A total of 24 tests which were either positive or negative for all of the strains were not included in the numerical analysis. The levels of similarity between bacterial strains were calculated by using the Dice index (9). Aggregation was based on the unweighted pair group average-linkage method (7, 26).

G+C contents and DNA-DNA hybridization. DNA was extracted by using lysozyme (15) in addition to achromopeptidase treatment (1). After the lytic phase, purified DNA was obtained by the method of Marmur (15). The thermal dena-

* Corresponding author.

TABLE 1. Strains used in the numerical analysis

Cluster	Subcluster	Strain ^a	Name as received	Isolated from:
I (5 strains)	Ia	CUETM 89-129	?	Child feces
		CUETM 89-218	?	Child feces
		CUETM 89-244	?	Child feces
		CUETM 89-271	?	Child feces
	Unclustered	DSM 20097 (= C10-45)	<i>B. longum</i>	Calf feces
II (11 strains)		DSM 20089 ^T	<i>B. asteroides</i>	Honeybee
		(= ATCC 25910 ^T = C51 ^T)		
		DSM 20214 ^T	<i>B. indicum</i>	Honeybee
		(= ATCC 25912 ^T = C410 ^T)		
		DSM 20434 ^T	<i>B. choerinum</i>	Pig feces
		(= ATCC 27686 ^T = SU806 ^T)		
		ATCC 25865 ^T	<i>B. globosum</i>	Bovine rumen
		(= DSM 20092 ^T = RU224 ^T)		
		DSM 20099 ^T	<i>B. pseudolongum</i>	Pig feces
		(= ATCC 25526 ^T = PNC-2-9G ^T)		
		DSM 20211 ^T	<i>B. suis</i>	Pig feces
		(= ATCC 27533 ^T = SU859 ^T)		
		NCFB 2242 ^T	<i>B. animalis</i>	Rat feces
		(= ATCC 25527 ^T = R101-8 ^T)		
		DSM 20222 ^T	<i>B. magnum</i>	Rabbit feces
III (3 strains)	IIIa	(= ATCC 27540 ^T = RA3 ^T)		
		DSM 20011	<i>L. casei</i> subsp. <i>casei</i>	?
		DSM 20079	<i>L. acidophilus</i>	?
IV (34 strains)	IVa (2 strains)	DSM 20082	<i>B. bifidum</i>	Adult feces
		(= E319f)		
	Unclustered	CUETM 89-158	?	Adult intestine
		CUETM 89-164	?	Child feces
	Unclustered	DSM 20096 ^T	<i>B. subtilis</i>	Sewage
		(= ATCC 27537 ^T = F395 ^T)		
	IVb (11 strains)	DSM 20210 ^T	<i>B. thermophilum</i>	Pig feces
		(= ATCC 25525 ^T = P2-91 ^T)		
	IVb (11 strains)	DSM 20432 ^T	<i>B. boum</i>	Bovine rumen
		(= ATCC 27917 ^T = RU917 ^T)		
	IVb (11 strains)	CUETM 89-188	?	Child feces
		CUETM 89-205	?	Child feces
		CUETM 89-197	?	Child feces
		CUETM 89-185	?	Child feces
		CUETM 89-248	?	Child feces
		CUETM 89-156	?	?
		CUETM 89-161	?	Child feces
		CUETM 89-155	?	?
		CUETM 89-211	?	Child feces
		ATCC 15701	<i>B. breve</i>	Child feces
		(= S46)	variant b	
	IVc (6 strains)	DSM 20091	<i>B. breve</i>	Child feces
		(= ATCC 15698 = S50)		
		CUETM 89-220	?	Child feces
		CUETM 89-154	?	Human vagina
		CUETM 89-243	?	Child feces
		CUETM 89-175	?	Human vagina
	IVd (2 strains)	CUETM 89-145	?	Child feces
		CUETM 89-149	?	Child feces
		CUETM 89-160	?	Adult feces
		CUETM 89-183	?	Child feces
	IVe (4 strains)	CUETM 89-182	?	Child feces
		CUETM 89-192	?	?
		CUETM 89-190	?	?
	IVf (4 strains)	CUETM 89-159	?	Child feces
		CUETM 89-246	?	Child feces
		CUETM 89-272	?	Child feces
		CUETM 89-181	?	Child feces
	Unclustered	CUETM 89-163	?	Child feces
		CUETM 89-184	?	Child feces
		CUETM 89-203	?	Child feces
		NCFB 2257 ^T	<i>B. breve</i>	Infant intestine
		(= DSM 20213 ^T = CCUG 18365 ^T = S1 ^T)		
		CUETM 89-199	?	?

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TABLE 1—Continued

Cluster	Subcluster	Strain ^a	Name as received	Isolated from:	
V (52 strains)	Va (38 strains)	CUETM 89-327	?	Child feces	
		CUETM 89-247	?	Child feces	
		CUETM 89-287	?	Child feces	
		CCUG 24071	?	Child feces	
		CCUG 18157	?	?	
		CUETM 89-174	?	Child feces	
		CUETM 89-186	?	Child feces	
		CUETM 89-193	?	Child feces	
		CUETM 89-263	?	Child feces	
		CUETM 89-216	?	Adult intestine	
		CUETM 89-215	?	Child feces	
		CUETM 89-238	?	Child feces	
		CUETM 89-281	?	Child feces	
		CUETM 89-260	?	Child feces	
		CUETM 89-257	?	Child feces	
		CUETM 89-259	?	Child feces	
		CUETM 89-267	?	Child feces	
		CUETM 89-239	?	Child feces	
		CUETM 89-276	?	Child feces	
		CUETM 89-245	?	Child feces	
		R 12 H2	?	Pouchite	
		C 76	?	Child feces	
		CUETM 89-177	?	Child feces	
		NCTC 11818 ^T	<i>B. longum</i>	Adult feces	
		(= ATCC 15707 ^T = DSM 20219 ^T = E194b ^T)			
		CUETM 89-290	?	Child feces	
		CCUG 15137	?	Adult intestine	
		CUETM 89-172	?	?	
		CUETM 89-171	?	Child feces	
		CUETM 89-268	?	Child feces	
		CUETM 89-265	?	Child feces	
		CUETM 89-251	?	Child feces	
		CUETM 89-256	?	Child feces	
		CUETM 89-157	?	Child feces	
		CUETM 89-284	?	Child feces	
		CUETM 89-179	?	Child feces	
		DSM 20088 ^T	<i>B. infantis</i>	Child feces	
		(= ATCC 15697 ^T = S12 ^T)			
		CUETM 89-170	?	Adult feces	
		ATCC 15708	<i>B. longum</i>	Child feces	
		(= S3)			
		DSM 20218	<i>B. infantis</i>	?	
		(= ATCC 17930)			
	Unclustered	ATCC 33777 ^T	<i>B. gallinarum</i>	Chicken feces	
		(= CH206-5 ^T)			
		ATCC 15704	<i>B. adolescentis</i>	Adult feces	
		(= E305)			
		CUETM 89-213	?	?	
		Vb (11 strains)	CUETM 89-280	?	Child feces
			CUETM 89-250	?	Child feces
			CUETM 89-262	?	Child feces
			CUETM 89-204	?	Child feces
CUETM 89-128			?	Child feces	
CUETM 89-252			?	Child feces	
CUETM 89-167			?	Child feces	
CUETM 89-240			?	Child feces	
CUETM 89-253			?	Child feces	
CUETM 89-217			?	Child feces	
CUETM 89-269			?	Child feces	
VI (7 strains)		CUETM 89-236	?	Bronchus	
		CUETM 89-285	?	Child feces	
		CCUG 18363 ^T	<i>B. adolescentis</i>	Adult feces	
	(= DSM 20083 ^T)				
	= ATCC 15703 ^T = E194a ^T)				
	CUETM 89-196	?	Child feces		
	DSM 20438 ^T	<i>B. pseudocatenulatum</i>	Child feces		
	(= ATCC 27919 ^T = B1279 ^T)				

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TABLE 1—Continued

Cluster	Subcluster	Strain ^a	Name as received	Isolated from:
Unclustered		CCUG 18366 ^T (= ATCC 27539 ^T = DSM 20103 ^T = B669 ^T)	<i>B. catenulatum</i>	Human feces
		CCUG 18367 ^T (= ATCC 27534 ^T = DSM 20436 ^T = B764 ^T)	<i>B. dentium</i>	Dental caries
		DSM 20216 ^T (= ATCC 25911 ^T = C215 ^T)	<i>Bifidobacterium coryneforme</i>	Honeybee
		DSM 20102 ^T (= ATCC 27538 ^T = F392 ^T)	<i>Bifidobacterium minimum</i>	Sewage
		DSM 20098 ^T (= ATCC 27535 ^T = B677 ^T)	<i>Bifidobacterium angulatum</i>	Adult feces
		CCUG 23084	?	?
		DSM 20433 ^T (= ATCC 27685 ^T = B145 ^T)	<i>Bifidobacterium pullorum</i>	Chicken feces
		CUETM 89-209	?	Bronchus
		DSM 20016 ^T (= ATCC 23272 ^T = F275 ^T)	<i>Lactobacillus reuteri</i> (= <i>Lactobacillus fermentum</i>)	Adult intestine
		DSM 20074 ^T (= ATCC 9649 ^T = NCIB 8130 ^T)	<i>Lactobacillus delbrueckii</i> subsp. <i>delbrueckii</i>	?
		CUETM 89-166	?	Child feces
		CCUG 18650	?	?
		DSM 20093 ^T (= P6 ^T)	<i>Bifidobacterium gallicum</i>	Human feces
		DSM 20435 ^T (= ATCC 27916 ^T = RA93 ^T)	<i>Bifidobacterium cuniculi</i>	Rabbit feces
		DSM 20505 ^T (= NCIB 11720 ^T)	<i>Lactobacillus sharpae</i>	Sewage
		CIP 3179	<i>Lactobacillus plantarum</i>	?

^a ATCC, American Type Culture Collection, Rockville, Md.; C. Faculté de Pharmacie, Lille, France; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; CIP, Collection Institut Pasteur, Paris, France; CUETM, Collection Unité Ecotoxicologie, Villeneuve d'Ascq, France; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, Germany; NCFB, National Collection of Food Bacteria, Shinfield, Reading, England; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom; R. P. Raibaud, Laboratoire d'Ecologie et de Physiologie du Système Digestif, Institut National de la Recherche Agronomique, Jouy-en-Josas, France. upa ATCC, American Type Culture Collection, Rockville, Md.; C. Faculté de Pharmacie, Lille, France; CCUG, Culture Collection, University of Göteborg, ermentum)

turation and guanine-plus-cytosine (G+C) values were determined as described previously (8, 16). The degrees of DNA binding were determined by the S1 nuclease-trichloroacetic acid method (5, 6, 12), and the ΔT_m values (ΔT_m is the difference between the temperature at which one-half of the reassociated DNA in the homologous reaction is denatured and the temperature at which one-half of the reassociated DNA in the heterologous reaction is denatured) were calculated (5, 12). The strains used in the DNA-DNA binding

experiments and the results of these experiments are shown in Table 2.

RESULTS

Phenotypic characterization. Our dendrogram (Fig. 1) contained six main clusters (clusters I to VI), which were subdivided into nine subclusters (subclusters Ia, IVa to IVf, Va, and Vb). Fourteen strains did not fall into any cluster,

TABLE 2. G+C contents of 19 strains belonging to subclusters Va and Vb and levels of DNA-DNA relatedness between these strains and *B. longum* and *B. infantis* type strains

Subcluster	No. of strains	Name as received	Strain	G+C content (mol%)	% DNA-DNA relatedness to:	
					<i>B. longum</i> NCTC 11818 ^T	<i>B. infantis</i> DSM 20088 ^T
Va	15	<i>B. longum</i>	NCTC 11818 ^T	57	100	66
		<i>Bifidobacterium</i> sp.	CCUG 15137	57	96	65
		<i>Bifidobacterium</i> sp.	CUETM 89-239	59	89	68
		<i>Bifidobacterium</i> sp.	CCUG 24071	57	89 (2.0) ^a	60 (5.5)
		<i>Bifidobacterium</i> sp.	CCUG 18157	57	86 (3.0)	64 (5.6)
		<i>Bifidobacterium</i> sp.	CUETM 89-260	57	86	62
		<i>Bifidobacterium</i> sp.	CUETM 89-259	56	84	67
		<i>B. longum</i>	ATCC 15708	57	83	67
		<i>Bifidobacterium</i> sp.	CUETM 89-193	57	82 (2.7)	71 (4.2)
		<i>Bifidobacterium</i> sp.	CUETM 89-284	58	79 (3.2)	64 (5.4)
		<i>Bifidobacterium</i> sp.	R12H2	57	79 (3.8)	64 (5.6)
		<i>Bifidobacterium</i> sp.	CUETM 89-290	57	78	65
		<i>Bifidobacterium</i> sp.	CUETM 89-170	57	76 (2.5)	61 (5.9)
		<i>B. infantis</i>	DSM 20218	56	70 (4.8)	80 (3.8)
		<i>B. infantis</i>	DSM 20088 ^T	56	67 (5.2)	100
Vb	4	<i>Bifidobacterium</i> sp.	CUETM 89-250	59	98 (3.2)	62 (5.7)
		<i>Bifidobacterium</i> sp.	CUETM 89-252	57	95 (2.7)	62 (5.4)
		<i>Bifidobacterium</i> sp.	CUETM 89-262	58	94 (3.0)	63 (5.3)
		<i>Bifidobacterium</i> sp.	CUETM 89-240	57	77 (2.2)	70 (4.9)

^a The numbers in parentheses are ΔT_m values (in degrees Celsius).

FIG. 1. Phenotypic dendrogram based on unweighted pair group average linkage.

and 10 strains were not included in any subcluster. The phenotypic characteristics of the clusters and subclusters are shown in Tables 3 and 4.

Cluster I. Cluster I comprised five strains, four of which were in subcluster Ia and were isolated from child feces. No type or reference strains were present in this cluster. Strain DSM 20097 (= C10-45), isolated from calf feces, was unclustered; its taxonomic position is discussed below.

Cluster II. Among the 11 strains in cluster II, 8 were type strains of *Bifidobacterium* species of animal origin, 2 were reference strains belonging to *Lactobacillus casei* and *Lactobacillus acidophilus*, and 1 was *B. bifidum* DSM 20082 (= E319f). Most of the type strains of animal origin and the

Lactobacillus strains were in one cluster. This artificial grouping could be easily explained by the phenotypic differentiation of these organisms from strains belonging to the other clusters.

Cluster III. The two strains in cluster III were isolated from an adult intestine and child feces. One strain, *Bifidobacterium subtilis* DSM 20096^T (= F395^T) (T = type strain), which was isolated from sewage, was unclustered.

Cluster IV. The 34 strains in cluster IV were subdivided into six subclusters, and 5 strains were unclustered. Subcluster IVa contained the type strains of *Bifidobacterium thermophilum* (strain DSM 20210 [= P2-91]) and *Bifidobacterium boum* (strain DSM 20432 [= RU 917]), which were isolated

TABLE 3. Characteristics for differentiating clusters I through VI^a

Characteristic	% of strains positive					
	Cluster I (n = 5) ^b	Cluster II (n = 11)	Cluster III (n = 3)	Cluster IV (n = 34)	Cluster V (n = 52)	Cluster VI (n = 7)
Enzymatic tests						
L-Tyrosine arylamidase	0	100	100	41	41	9
L-Phenylalanine arylamidase	0	100	100	97	90	18
L-Hydroxyproline arylamidase	0	100	100	100	100	100
Glycine arylamidase	0	82	0	68	83	9
L-Asparagine arylamidase	89	100	0	85	100	18
S-benzyl-cysteine arylamidase	0	100	67	62	65	0
Leucyl-glycine arylamidase	0	91	0	62	71	0
L-Seryl-tyrosine arylamidase	0	91	0	62	71	0
L-Proline arylamidase	0	100	67	88	96	100
L-Histidyl-L-leucyl-L-histidine arylamidase	40	100	0	88	96	27
L-Leucyl-L-alanine arylamidase	0	100	0	18	56	0
L-Prolyl-L-arginine arylamidase	0	45	0	3	85	18
L-Histidyl-L-phenylalanine arylamidase	0	73	0	0	0	0
α -D-Galactosidase	0	100	100	97	85	55
α -L-Arabinosidase	100	64	0	0	96	100
α -D-Glucosidase	0	100	100	74	62	36
β -D-Glucosidase	0	91	67	76	12	55
β -D-Fucosidase	0	64	67	82	81	36
Esterase C4	0	100	100	59	79	36
Esterase C5	0	91	100	62	83	36
Carbohydrate acidification tests						
L-Arabinose	100	27	0	3	90	55
D-Xylose	100	9	0	6	90	45
Melezitose	80	0	0	6	65	0
Starch	0	27	33	47	100	100
Amygdalin	0	36	0	79	0	27
Mannitol	0	18	67	82	2	0
Sorbitol	0	9	100	82	4	27

^a The type strains of the following species were included in the clusters: cluster III, *B. subtilis*; cluster IV, *B. boum*, *B. thermophilum*, and *B. breve*; cluster V, *B. longum* and *B. infantis*; cluster VI, *B. adolescentis*, *B. dentium*, and *B. catenulatum*.

^b n is the number of strains tested.

from pig feces and a bovine rumen, respectively. The other subclusters, subclusters IVb to IVf, contained only human strains. Subcluster IVb included two reference strains, *B. breve* ATCC 15701 (= S46) (variant b) and *B. breve* DSM 20091 (= ATCC 15698 = S50) (variant a). The four other subclusters contained only new isolates. *B. breve* NCFB 2257^T (= S1^T) was unclustered, but not far from subcluster IVc.

Cluster V. Cluster V is the most important cluster (52 strains) and is divided into subcluster Va (38 strains) and subcluster Vb (11 strains). The following strains were not members of either of these subclusters: *Bifidobacterium gallinarum* ATCC 33777^T (= CH206-5^T), *Bifidobacterium adolescentis* ATCC 15704 (= E305), (variant b), and one strain whose origin was unknown.

The type strains of *B. longum* (strain NCTC 11818 [= E194b]) and *B. infantis* (strain DSM 20088 [= S12]) were in the same subcluster, subcluster Va, along with two reference strains, *B. longum* ATCC 15708 (= S3) (variant b) and *B. infantis* DSM 20218 (= ATCC 17930). Subcluster Vb did not contain any type or reference strains. All of these organisms were isolated from child feces.

Cluster VI. Cluster VI contained the type strains of species belonging to the *B. adolescentis* complex (21, 23), including *B. adolescentis* CCUG 18363 (= E194a), *Bifidobacterium pseudocatenulatum* DSM 20438 (= B1279), *Bifidobacterium catenulatum* CCUG 18366 (= B669), and *Bifidobacterium dentium* CCUG 18367 (= B764), and three wild strains, one isolated from a bronchus and two isolated

from child feces. Thirteen strains were unclustered, including six type strains, four strains belonging to the genus *Lactobacillus*, and three new isolates (Table 1).

G+C contents and DNA-DNA hybridization. The G+C contents of 15 strains belonging to subcluster Va and 4 strains belonging to subcluster Vb ranged from 56 to 59 mol% (Table 2).

The DNA relatedness values for these 19 strains, including the type strains of *B. longum* and *B. infantis*, are shown in Table 2. The levels of DNA-DNA base sequence similarity between these two type strains were 66 and 67%. A higher value (70%), with a ΔT_m of 4.8°C, was obtained between *B. infantis* DSM 20218 and *B. longum* NCTC 11818^T (= E194b^T), while a level of relatedness of 80% and a ΔT_m of 3.8°C were observed between *B. infantis* DSM 20218 and *B. infantis* DSM 20088^T (= S12^T).

The 11 new isolates belonging to subcluster Va and the 4 isolates belonging to subcluster Vb studied by DNA-DNA hybridization yielded levels of DNA relatedness with *B. longum* NCTC 11818^T (= E194b^T) of more than 76%. The reciprocal results obtained by using *B. infantis* DSM 20088^T (= S12^T) as the reference strain ranged from 60 to 71%, with ΔT_m values of less than 6.0°C.

DISCUSSION

***B. infantis* and *B. longum* (subclusters Va and Vb).** *B. infantis* and *B. longum* strains, including the type strains, were not differentiated phenotypically, even on the basis of

TABLE 4. Characteristics for differentiating subclusters IVa through IVf and subclusters Va and Vb^a

Characteristic	% of strains positive							
	Subcluster IVa (n = 2) ^b	Subcluster IVb (n = 11)	Subcluster IVc (n = 6)	Subcluster IVd (n = 2)	Subcluster IVe (n = 4)	Subcluster IVf (n = 4)	Subcluster Va (n = 38)	Subcluster Vb (n = 11)
Enzymatic tests								
L-Phenylalanine arylamidase	100	100	100	100	75	100	95	64
L-Alanine arylamidase	0	100	100	50	50	100	95	91
S-benzyl-cysteine arylamidase	100	91	83	0	0	25	79	27
Leucyl-glycine arylamidase	50	82	100	50	0	25	92	0
L-Isoleucine arylamidase	0	55	100	100	0	75	84	45
L-Threonine arylamidase	0	91	100	100	0	100	63	45
L-Alanyl-L-phenylalanyl-L-proline arylamidase	100	100	67	0	50	25	66	0
L-Histidyl-L-serine arylamidase	0	9	17	100	0	25	71	9
L-Lysyl-L-alanine arylamidase	0	45	83	100	0	75	58	9
α -D-Galactosidase	100	100	100	100	100	75	97	18
α -D-Glucosidase	100	91	67	50	100	0	74	9
β -D-Fucosidase	50	100	100	100	100	0	92	27
β -D-Lactosidase	0	64	100	50	0	0	5	9
β -D-Xylosidase	0	18	100	50	0	0	0	0
Esterase C4	100	75	67	100	25	0	97	0
Esterase C5	100	75	33	100	75	0	100	0
Esterase C10	100	82	33	50	25	0	18	0
Carbohydrate acidification tests								
L-Arabinose	0	0	0	0	0	0	92	100
Ribose	0	82	67	100	100	100	61	64
D-Xylose	0	0	0	0	0	0	89	100
Cellobiose	0	9	50	50	100	0	3	0
Trehalose	0	50	0	0	0	0	0	0
Melezitose	0	0	17	0	0	0	68	55
Mannitol	0	91	100	0	100	100	3	0
Sorbitol	0	91	100	0	100	100	5	0
Starch	50	9	83	100	50	25	3	0

^a The type strains and reference strains of the following species were included in the subclusters: subcluster IVa, *B. thermophilum* and *B. boum*; subcluster IVb, *B. breve* variants a and b; subcluster Va, *B. longum* and *B. infantis*.

^b n is the number of strains tested.

a large number of tests (carbohydrate acidification and enzymatic tests). Scardovi et al. (20) referred to the sugar fermentation patterns extensively shared by these two species. L-Arabinose and D-xylose, which are not fermented by strains identified as *B. infantis* according to Reuter (18), were acidified by most of the *B. infantis* strains studied by Scardovi et al. (20). All strains were melezitose negative according to both studies. Melezitose and D-ribose reactions could be positive or negative with members of the *B. longum* group according to Scardovi et al. (20), while melezitose and L-arabinose reactions were nearly always positive for *B. longum* strains according to Biavati et al. (4). These authors observed overlapping of zymograms (transaldolases and 6-phosphogluconate dehydrogenases) for the two species. In 1991, Roy et al. (19) described, on the basis of a few reference strains, specific biochemical tests for the differentiation of *Bifidobacterium* species. L-Arabinose acidification and melezitose acidification were used by these authors to separate *B. infantis* and *B. longum* after a numerical analysis based on carbohydrate acidification tests in which API Rapid CH galleries obtained from Analytab Products were used. The absence of new isolates made it impossible to conclude that these tests were sufficient to separate the two species.

Strains in subclusters Va and Vb were L-arabinose and D-xylose positive, as reported by Scardovi et al. (20) for their *B. infantis* strains. However, 61 and 64% of the strains in subclusters Va and Vb were ribose positive, respectively,

and 68 and 55% of the strains were melezitose positive, respectively. These results are consistent with the results described by Scardovi et al. (20) for *B. longum* strains, but not with the results of Biavati et al. (4), who found that 92% of their *B. longum* strains were melezitose positive. Therefore, these characteristics could not be used either to differentiate the two species or to identify subclusters Va and Vb as members of one of these species (Table 4). Differentiation between the two subclusters was based on enzymatic tests, and the data indicated that there are two different phenotypes for the *B. longum*-*B. infantis* group.

The levels of DNA relatedness between the two species were found to vary from 65 to 80% (20, 23, 25). Later, in 1983, Lauer and Kandler (14) performed DNA-DNA hybridization experiments with the type strains and obtained values of 62 to 63%. Furthermore, DNAs from strains isolated from calves were as closely related (75 to 80%) to *B. infantis* strains as to *B. longum* strains and thus could represent part of a "continuum" between the two species (20).

Our results for 19 human strains (Table 2) showed that their levels of DNA-DNA relatedness were 67 to 98 and 61 to 80% when *B. longum* and *B. infantis*, respectively, were used as the reference strains. Of the 12 ΔT_m values calculated (Table 2), 11 were less than 5°C in the experiments performed with *B. longum* as the reference strain; the ΔT_m for the type strain of *B. infantis* was 5.2°C. In contrast, eight

ΔT_m values were more than 5°C in the experiments performed with *B. infantis* as the reference strain, but three values were less than 5°C. According to the definition of a species, as cited by Wayne et al. (27), and the standard errors calculated by Grimont et al. (13) (about 4% in the S1 nuclease method and about 1.7°C in ΔT_m determinations), only one strain (DSM 20218) could be considered a *B. infantis* strain. Two new isolates (CUETM 89-193 and CUETM 89-240) could represent part of the continuum described by Scardovi et al. (20). All of the other strains are *B. longum* strains.

***B. breve* (subclusters IVb to IVf).** Although the type strain of *B. breve*, strain NCFB 2257, was unclustered in subclusters IVb to IVf, the five subclusters could be assigned to the species *B. breve* on the basis of acidification of carbohydrates according to Scardovi et al. (24) and Biavati et al. (4). The acidification of mannitol and the acidification of sorbitol (except for the two strains in subcluster IVd) are particularly important. All of the strains were L-arabinose or D-xylose negative. In subclusters IVb to IVf 33% of the strains were cellobiose negative. Subcluster IVb included two reference strains, *B. breve* ATCC 15701 (= S46) and DSM 20091 (= ATCC 15698 = S50). Strain ATCC 15698 was not grouped, according to Roy et al. (19), with the other two *B. breve* strains tested by these authors (strains ATCC 15700 [= S1] and ATCC 15701 [= S46]). It is difficult to differentiate a species on the basis of only three strains, particularly when one strain has atypical characteristics. Enzymatic tests were useful in this study in view of the separation of the five subclusters (Table 4).

The DNA-DNA relatedness values for most of the strains phenotypically identified as *B. breve* when type strain S1 was used as the reference strain were very similar (between 75 and 91%) (24).

Cluster VI. Cluster VI contained four type strains, *B. adolescentis* CCUG 18363 (= E194a), *B. pseudocatenulatum* DSM 20438 (= B1279), *B. catenulatum* CCUG 18366 (= B669), and *B. dentium* CCUG 18367 (= B764), and three new isolates. These genospecies were defined by Scardovi et al. (21, 23) on the basis of the phenospecies described by Reuter (18). These species were previously placed in the *B. adolescentis* homology group (21) and the *B. pseudocatenulatum* group (23).

Unidentified clusters. (i) **Cluster I.** It was interesting that one of the strains (CUETM 89-271) isolated from child feces was also studied by Gavini et al. (10) and clustered in their group VIIa but was not assigned to any known species. In this study, strain DSM 20097 (= C10-45) was unclustered in cluster I. It was received from the Deutsche Sammlung von Mikroorganismen as *B. longum* because of its high DNA-DNA relatedness to the *B. longum* type strain (89%) according to Lauer and Kandler (14). In 1971, Scardovi et al. (24) defined this strain as *B. longum* subsp. *animalis* biotype b, as Mitsuoka did (17), but established that it was 100% homologous with *Bifidobacterium pseudolongum* biotype c strain 29SrT and 65% homologous with *B. pseudolongum* biotype a strain PNC-2-9G, which is the type strain of the species at the present time. Its taxonomic position in our study did not permit us to determine to which species it belonged. The discrepancies between the results of Scardovi et al. (24) and those of Lauer and Kandler (14) might be explained by differences in the strains used since they were obtained from different collections and were studied by different authors.

(ii) **Cluster III.** Cluster III comprised subcluster IIIa, with only two strains, and the type strain of *B. subtile*, strain DSM 20096. One of the two strains, CUETM 89-158, was

clustered by Gavini et al. (10) with the *B. catenulatum* type strain (CCUG 18366). In our analysis, this type strain was included in cluster VI, along with other type strains belonging to the *B. adolescentis* complex. This difference in taxonomic position could be explained by the small number of strains belonging to this complex which were studied.

In conclusion, we make the following observations. *B. longum* and *B. infantis* could not be differentiated on the basis of a large number of phenotypic characteristics. The genomic data obtained in this study lead us to ask whether the existence of strains named *B. infantis* is a reality, since no new strains have been isolated.

Type strain *B. infantis* DSM 20088 and reference strain *B. infantis* DSM 20218 could be considered marginal strains belonging to the species *B. longum*. *B. breve* has been divided into five phenospecies; however, this finding probably is not supported by genomic data if we consider previous DNA-DNA hybridization results for different phenotypes. The strains of human origin in cluster I and subcluster IIIa, which phenotypically are related to *B. longum*, *B. infantis*, and *B. breve*, could not be identified as members of any known species. Further DNA-DNA hybridization studies must be carried out to define the taxonomic position of these organisms.

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REFERENCES

1. Barsotti, O., J. J. Morrier, J. Freney, F. Renaud, G. Benay, D. Decorot, and J. Dumont. 1988. Achromopeptidase for rapid lysis of oral anaerobic Gram-positive rods. *Oral Microbiol. Immunol.* 3:86-88.
2. Bezkorovainy, A. 1989. Ecology of bifidobacteria. In A. Bezkorovainy and R. Miller-Catchpole (ed.), *Biochemistry and physiology of bifidobacteria*. CRC Press, Boca Raton, Fla.
3. Biavati, B., P. Castagnoli, F. Crociani, and L. D. Trovatielli. 1984. Species of *Bifidobacterium* in the feces of infants. *Microbiologica (Bologna)* 7:341-345.
4. Biavati, B., V. Scardovi, and W. E. C. Moore. 1982. Electrophoretic patterns of proteins in the genus *Bifidobacterium* and proposal of four new species. *Int. J. Syst. Bacteriol.* 32:358-373.
5. Brenner, D. J. 1978. Characterization and clinical identification of *Enterobacteriaceae* by DNA hybridization. *Prog. Clin. Pathol.* 7:71-117.
6. Crosa, J. M., D. J. Brenner, and S. Falkow. 1973. Use of a single-strand-specific nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and heteroduplexes. *J. Bacteriol.* 115:904-911.
7. Delabre, M., A. Bianchi, and M. Veron. 1973. Etude critique des méthodes de taxonomie numérique. Application à une classification des bactéries aquicoles. *Ann. Inst. Pasteur Microbiol.* 124A:489-506.
8. De Ley, J. 1970. Reexamination of the association between melting point, buoyant density, and chemical base composition of deoxyribonucleic acid. *J. Bacteriol.* 101:738-754.
9. Gavini, F., B. Lefebvre, and H. Leclerc. 1976. Positions taxonomiques d'entérobactéries H2S- par rapport au genre *Citrobacter*. *Ann. Inst. Pasteur Microbiol.* 127A:275-295.
10. Gavini, F., A. M. Pourcher, C. Neut, D. Monget, C. Romond, C. Oger, and D. Izard. 1991. Phenotypic differentiation of bifidobacteria of human and animal origins. *Int. J. Syst. Bacteriol.* 41:548-557.
11. Gavini, F., M. B. Romond, A. Beji, C. Bernard, and D. Izard. 1990. Genomic and phenotypic characterizations of *Pseudomonas alcaligenes* and *Comamonas* species with a special reference to *C. testosteroni*. *Curr. Microbiol.* 21:279-282.
12. Grimont, P. A. D. 1988. Use of DNA reassociation in bacterial classification. *Can. J. Microbiol.* 34:541-546.

13. Grimont, P. A. D., M. Y. Popoff, F. Grimont, C. Coynault, and M. Lemelin. 1980. Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. *Curr. Microbiol.* 4:325-330.
14. Lauer, E., and O. Kandler. 1983. DNA-DNA homology, murein types, and enzyme patterns in the type strains of the genus *Bifidobacterium*. *Syst. Appl. Microbiol.* 4:42-64.
15. Marmur, J. 1961. A procedure for isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* 3:208-218.
16. Marmur, J., and P. Doty. 1962. Determination of base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. Mol. Biol.* 5:109-118.
17. Mitsuoka, T. 1969. Vergleichende Untersuchungen über die Bifidobakterien aus dem Verdauungstrakt von Menschen und Tieren. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig.* 210:52-64.
18. Reuter, G. 1963. Vergleichende Untersuchungen über die Bifidus-Flora im Säuglings- und Erwachsenenstuhl. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig.* 191:486-507.
19. Roy, D., P. Ward, C. J. Toupin, and P. Chevalier. 1991. Phenotypic characterization of *Bifidobacterium* spp. of human origin using rapid method. *Microbiol. Aliments Nutr.* 9:133-138.
20. Scardovi, V., F. Casalicchio, and N. Vincenzi. 1979. Multiple electrophoretic forms of transaldolase and 6-phosphogluconic dehydrogenase and their relationships to the taxonomy and ecology of the bifidobacteria. *Int. J. Syst. Bacteriol.* 29:312-327.
21. Scardovi, V., and F. Crociani. 1974. *Bifidobacterium catenulatum*, *Bifidobacterium dentium*, and *Bifidobacterium angulatum*: three new species and their deoxyribonucleic acid homology relationships. *Int. J. Syst. Bacteriol.* 24:6-20.
22. Scardovi, V., and L. D. Trovatielli. 1974. *Bifidobacterium animalis* (Mitsuoka) comb. nov. and the "minimum" and "subtile" groups of new bifidobacteria found in sewage. *Int. J. Syst. Bacteriol.* 24:21-28.
23. Scardovi, V., L. D. Trovatielli, B. Biavati, and G. Zani. 1979. *Bifidobacterium cuniculi*, *Bifidobacterium choerinum*, *Bifidobacterium boum*, and *Bifidobacterium pseudocatenulatum*: four new species and their deoxyribonucleic acid homology relationships. *Int. J. Syst. Bacteriol.* 29:291-311.
24. Scardovi, V., L. D. Trovatielli, G. Zani, F. Crociani, and D. Mateuzzi. 1971. Deoxyribonucleic acid homology relationships among species of the genus *Bifidobacterium*. *Int. J. Syst. Bacteriol.* 21:276-294.
25. Scardovi, V., G. Zani, and D. Trovatielli. 1970. Deoxyribonucleic acid homology among the species of the genus *Bifidobacterium* isolated from animals. *Arch. Microbiol.* 72:318-325.
26. Sneath, P. H. A., and R. R. Sokal. 1973. Numerical taxonomy. W. H. Freeman, San Francisco.
27. Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper. 1987. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. *Int. J. Syst. Bacteriol.* 37:463-464.
28. Yaeshima, T., T. Fujisawa, and T. Mitsuoka. 1991. Differential characteristics of *Bifidobacterium longum* and *Bifidobacterium animalis*. *Syst. Appl. Microbiol.* 14:169-172.
29. Yamazaki, S., K. Machii, S. Tsuyuki, H. Momose, T. Kawashima, and K. Ueda. 1985. Immunological responses to monoassociated *Bifidobacterium longum* and their relation to prevention of bacterial invasion. *Immunology* 56:43-50.