Deoxyribonucleic Acid Homology Relationships Among Species of the Genus *Bifidobacterium*

V. SCARDOVI, L. D. TROVATELLI, G. ZANI, F. CROCIANI, and D. MATTEUZZI

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

Genetic relatedness among 179 strains representing 13 named species and several unnamed taxa of the genus *Bifidobacterium* from three main habitats, i.e., (i) feces of man, (ii) feces of various other animals, and (iii) the bovine rumen, was assessed by means of deoxyribonucleic acid (DNA)-DNA hybridization by using a filter-paper technique in competition experiments. Assignment of these strains to the genus *Bifidobacterium* was based on the production of lactic and acetic acids as chief products from glucose, lack of gas production, fructose-6-phosphate phosphoketolase activity, and on morphology. About 180 DNA competitors were tested with 23 reference systems. Several genetically distinct groups were recognized. Little or no DNA homology was demonstrated between some of the groups, suggesting large evolutionary divergence in this genus. *B. infantis*, *B. liberorum*, and *B. lactentis* form one of these groups; the ecological significance of this relatedness was discussed. The DNA of the following pairs are homologous: (i) *B. breve* and *B. parvulorum*, (ii) *B. thermophilum* and *B. ruminale*, and (iii) *B. pseudolongum* and *B. globosum*. Within a number of strains assigned to *B. adolescentis*, many of which were isolated from waste waters, a large genetic heterogeneity was demonstrated: in addition to *B. adolescentis*, at least three unrelated groups were recognized and are provisionally referred to as "*dentium*," "*catenulatum*," and "*angulatum*." Since these groups are not related genetically to any species of the genus and are phenotypically distinct, they may represent new species. The validity of the species *B. bifidum*, *B. longum*, and *B. suis* was confirmed at the genetic level.

The bifid bacteria are anaerobic, nonmotile, gram-positive, non-sporeforming, rodlike organisms which are often irregularly shaped, club-shaped, or rudimentarily branched in "Y" or similar forms. Glucose is fermented through the fructose-6-phosphate shunt leading to acetic and lactic acids as chief end products in the approximate ratio 1.5:1.0 (7, 39, 43). All known strains of bifidobacteria are catalase negative and do not reduce nitrate. The taxonomy of the bifid bacteria, which at present are assigned to the single genus *Bifidobacterium*, has always been a vexing problem (16, 33, 35), and the question of the relationships of the bifidobacteria with the actinomycetes, lactobacilli, and corynebacteria, particularly with respect to comparative cell wall chemistry (16) and general biochemistry (33), has further complicated the problem.

Although only a single species was originally

included by Orla-Jensen (30) in the genus *Bifidobacterium*, subsequent work (26, 27, 34, 36, 40, 41) has brought to 16 the number of named species in this genus. These species (including their subspecies and biotypes), together with their distinctive fermentation patterns and other characters, are listed in Table 1. The species have been differentiated mainly on the basis of fermentative characters, and therefore it is no wonder that there are many taxonomic uncertainties. *B. bifidum* has a very recognizable fermentation pattern. However, the distinction between the ribose-fermenting species not fermenting arabinose and xylose, i.e., *B. infantis*, *B. breve*, and *B. parvulorum*, is not easy. Also, *B. liberorum* and *B. lactentis* are not easily differentiated from each other. Their common morphologies and sources of isolation add to the difficulty. In addition, rumen and animal strains in the

TABLE 1. *Differential characters of the species of the genus Bifidobacterium*^a

Species (including subspecies and biotypes)	Growth at 46.5°C	L-Arabinose	D-Xylose	D-Ribose	D-Mannose	Cellobiose	Lactose	Trehalose	Melzitose	Starch	Inulin	Mannitol	Sorbitol	Salicin	Sodium gluconate
A Isolated from feces and vaginas of humans															
(1) <i>B. bifidum</i> (Tissier) Prévot biotype <i>a</i>	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
<i>B. bifidum</i> biotype <i>b</i>	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
(2) <i>B. infantis</i> Reuter biotype <i>a</i>	-	-	-	+	+	+	+	+	-	+	+	-	-	+	-
<i>B. infantis</i> biotype <i>b</i>	-	-	-	+	+	+	+	-	-	+	+	-	-	+	-
(3) <i>B. breve</i> Reuter biotype <i>a</i>	-	-	-	+	+	+	+	-	+	d	d	+	+	+	-
<i>B. breve</i> biotype <i>b</i>	-	-	-	+	+	+	+	+	+	d	d	+	+	+	-
<i>B. breve</i> biotype <i>c</i>	-	-	-	+	+	+	+	+	+	d	d	+	+	+	-
(4) <i>B. parvulum</i> Reuter biotype <i>a</i>	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-
<i>B. parvulum</i> biotype <i>b</i>	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-
(5) <i>B. liberorum</i> Reuter	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-
(6) <i>B. lactentis</i> Reuter	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-
(7) <i>B. adolescentis</i> Reuter biotype <i>a</i>	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-
<i>B. adolescentis</i> biotype <i>b</i>	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-
<i>B. adolescentis</i> biotype <i>c</i>	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-
<i>B. adolescentis</i> biotype <i>d</i>	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-
(8) <i>B. longum</i> Reuter subsp. <i>longum</i> Reuter biotype <i>a</i>	-	+	+	+	+	-	+	+	+	+	+	+	+	+	-
<i>B. longum</i> subsp. <i>longum</i> biotype <i>b</i>	-	+	+	+	+	-	+	+	+	+	+	+	+	+	-
B Isolated from feces of various animals and from bovine rumens															
<i>B. longum</i> Reuter subsp. <i>animalis</i> Mitsuoka biotype <i>a</i>	-	+	+	+	-	-	+	-	-	±	-	-	-	±	-
<i>B. longum</i> subsp. <i>animalis</i> biotype <i>b</i>	-	+	+	+	+	-	+	-	-	±	-	-	-	±	-
(9) <i>B. pseudolongum</i> Mitsuoka biotype <i>a</i>	-	+	+	+	+	+	+	-	-	+	+	-	-	+	-
<i>B. pseudolongum</i> biotype <i>b</i>	-	+	+	+	+	+	+	-	-	+	+	-	-	+	-
<i>B. pseudolongum</i> biotype <i>c</i>	-	+	+	+	+	+	+	-	-	+	+	-	-	+	-
<i>B. pseudolongum</i> biotype <i>d</i>	-	+	+	+	+	+	+	-	-	+	+	-	-	+	-
(10) <i>B. suis</i> Matteuzzi et al.	-	+	+	nt	+	-	+	-	-	-	-	-	-	nt	-
(11) <i>B. ruminale</i> Scardovi et al.	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
(12) <i>B. thermophilum</i> Mitsuoka biotype <i>a</i>	+	-	-	-	-	d	-	d	-	+	+	-	-	+	-
<i>B. thermophilum</i> biotype <i>b</i>	+	-	-	-	-	d	-	-	+	+	+	-	-	+	-
<i>B. thermophilum</i> biotype <i>c</i>	+	-	-	-	-	d	+	+	+	+	+	-	-	+	-
<i>B. thermophilum</i> biotype <i>d</i>	+	-	-	-	-	+	+	+	+	+	+	-	-	+	-
(13) <i>B. globosum</i> Scardovi et al.	-	+	+	+	-	d	+	-	-	+	-	+	-	nt	-
<i>B. globosum</i> (mannitol fermenting)	-	+	+	+	-	d	+	-	-	+	-	+	-	nt	-
C Isolated from the intestines of honey bees															
(14) <i>B. asteroides</i> Scardovi et al.	-	+	+	nt	-	+	-	-	-	-	nt	-	-	+	+
(15) <i>B. indicum</i> Scardovi et al.	-	+	-	nt	-	+	-	-	-	-	nt	-	-	+	+
(16) <i>B. coryneforme</i> Scardovi et al.	-	+	+	nt	-	+	-	-	-	-	nt	-	-	+	+

^a Data are taken from Reuter (36), Mitsuoka (27), Scardovi and Trovatielli (40), Scardovi et al. (41), and Matteuzzi et al. (26).

Symbols: +, delayed reaction; ±, delayed and weak fermentation; d, different reaction in different strains (27); reactions concerning a few strains in some of the taxa were overlooked; nt, not tested. Additional characters: the results with other polysaccharides or glucosides (27) are similar to those with starch and salicin, respectively. *B. bifidum* biotype *a* (from adults) slowly ferments sucrose and melibiose; *B. bifidum*

biotype *b* (from infants) does not ferment sucrose but vigorously ferments melibiose (27); both biotypes do not ferment maltose or raffinose. All bifidobacteria ferment (for exceptions, see above): glucose, galactose, fructose (somewhat slow in *B. pseudolongum*), sucrose, maltose, and melibiose. Never fermented are rhamnose and glycerol. For other compounds tested, see Mitsuoka (27) and Reuter (36). Inositol is fermented only by *B. infantis* biotype *b* and *B. liberorum*; inositol is fermented slowly by *B. lactentis* (27).

species *B. ruminale* and *B. thermophilum* or *B. adolescentis* biotype *d* and *B. longum* have nearly identical fermentation patterns. The species from the intestines of bees can be distinguished mainly on morphological grounds. Further separation of the species into subspecies and biotypes would only add to the confusion.

Additional work on phenotypic characters, e.g., nutritional requirements, behavior toward oxygen (8), distribution of some enzymes like aldolase or dehydrogenases, and lipid composition (9), might be of value in the speciation of the genus *Bifidobacterium*. However, cell wall analysis (16) has already demonstrated the heterogeneity of the genus *Bifidobacterium* since the various murein types (sequence of amino acids in the interpeptide bridge) correlate well with species or groups of species. Some irregularities in the results obtained by Kandler (16), i.e., different murein types found in *B. adolescentis*, reflect the genetic heterogeneity of the strains analyzed.

Three types of electrophoretic behavior of fructose-6-phosphate phosphoketolase were found in strains of *Bifidobacterium* (38); the R_F of the enzyme is not correlated with the names of the species but rather with their main habitats: feces or body cavities of man, feces of other animals, and intestines of bees. This suggests different evolutionary trends in the various ecological niches.

Scardovi et al. (42) and Crociani et al. (3) reported experimental data concerning the genetic relatedness among some proposed species of *Bifidobacterium* from the rumen of cattle and the intestines of honey bees. Genetic relatedness was assessed by means of deoxyribonucleic acid (DNA)-DNA hybridization in competition experiments. This approach offers a direct measure of the similarity or homology of the polynucleotide sequences in DNA molecules and therefore of the genetic relatedness of many groups of bacteria. The results clearly indicated that the genus *Bifidobacterium* is not as genetically homogenous as could be argued from the narrow range of DNA base composition [60 to 65% guanine plus cytosine (GC)] and from overall phenotypic similarity.

The present study excludes strains from bees, the genetic relatedness of these strains already having been investigated by Scardovi et al. (42). This investigation is here expanded to determine the genetic relatedness of representative strains of all other named species (and certain unnamed strains) of *Bifidobacterium* to contribute to a better understanding of the taxonomy of this genus.

MATERIALS AND METHODS

Bacterial strains. The strains employed in this study, together with their sources, are listed in Table 2. All strains were repeatedly examined for adherence to species or subspecies characteristics prior to use, especially those received from foreign collections (see Table 1). Regardless of their source, strains were considered to be members of *Bifidobacterium* if glucose was fermented and yielded acetic and lactic acids in the approximate ratio of 1.5:1.0 without gas and if fructose-6-phosphate phosphoketolase activity was present in cell-free extracts. These criteria were strictly applied to strains from unusual habitats, like waste waters, where morphological forms only superficially resembling bifids could easily be overlooked.

DNA preparations. Cells for DNA extraction were grown on Trypticase-Phytone-glucose medium of the following composition: Trypticase (BBL), 0.5 g; Phytone (BBL), 0.2 g; glucose, 1.5 g; yeast extract (Difco), 0.1 g; cysteine-hydrochloride, 0.05 g; K_2HPO_4 , 0.2 g; $MgCl_2 \cdot 6H_2O$, 0.05 g; $ZnSO_4 \cdot 7H_2O$, 0.025 g; $CaCl_2$, 0.015 g and traces of $FeCl_3 \cdot 7H_2O$; distilled water, 100 ml. The substrate, distributed in the amount of 900 ml into conical flasks (1,000 ml), was sterilized at 110 C for 30 min, gassed with carbon dioxide through a porous glass disc for approximately 2 min (final pH 6.2 to 6.4), and then inoculated with about 100 ml of culture. The cells were grown to the stationary phase and then centrifuged, washed with saline, and suspended in 0.15 M NaCl and 0.1 M ethylenediaminetetraacetic acid (pH 8.0). Lysozyme followed by sodium lauryl sulfate was used to lyse the cells; this technique and the subsequent purification of DNA from cell lysates were described by Marmur (23). Labeled DNA was obtained from cells grown in the presence of 50 μ Ci each of uracil-2- ^{14}C and adenine-8- ^{14}C per liter. All DNA preparations were stored at 4 C in 0.1X SSC (0.015 M NaCl and 0.0015 M sodium citrate) with chloroform as preservative.

DNA immobilization. Native DNA (about 80 μ g/ml) was alkali-denatured (0.1 M KOH for 15 min) in 0.1X SSC at room temperature, diluted 1:10 with cold 6X SSC, and fixed by slow filtration in the cold through 15-cm membrane filters (Sartorius Membranfilter SM 11306 or Selectron filter BA 85/1; Schleicher & Schuell Co., Dassel), presoaked in 6X SSC for 12 hr. The filters were washed with 200 ml of 6X SSC, dried overnight in air, and cut with a paper punch into small filters 10.5 mm in diameter; the small filters were then dried in a vacuum oven at 80 C for 2 hr and stored at 4 C. The amount of DNA fixed on each small filter ranged from 30 to 50 μ g.

DNA hybridization. We used the single-point competition reaction and the conditions employed by Johnson and Ordal (15) for ensuring satisfactory binding and minimizing nonspecific annealing. The filters with the immobilized DNA (reference DNA) were preincubated at 69 C in a vessel containing the preincubation mixture of Denhardt (6) made up with 2X SSC. After 4 to 6 hr of preincubation, the filters were removed and excess water was blotted off. The filters were then placed singly in 4-ml screw-cap vials which contained 0.25 ml of Denhardt's mixture containing 1.0 to 2.0 μ g of denatured, labeled DNA and

TABLE 2. *List of strains*

Organism	Isolated from	Culture received from
<i>Bifidobacterium bifidum</i> (Tissier) Orla-Jensen		
<i>B. bifidum</i> biotype <i>a</i> (Reuter) E319f	Feces of adult	G. Reuter
<i>B. bifidum</i> biotype <i>b</i> (Reuter) S28a (ATCC 15696)	Feces of infant	G. Reuter
<i>Lactobacillus bifidus</i> subsp. <i>pennsylvanicus</i> Gyorgy (ATCC 11683)	Feces of infant	
<i>B. bifidum</i> biotype? B884; B888; B953	Human vagina	Our collection
<i>Bifidobacterium infantis</i> Reuter S12 (ATCC 15697) [type strain (37)]	Feces of infant	G. Reuter
<i>Bifidobacterium parvulorum</i> Reuter		
<i>B. parvulorum</i> biotype <i>a</i> S50 (ATCC 15698) [type strain (37)]	Feces of infant	G. Reuter
<i>B. parvulorum</i> biotype <i>b</i> S17c (ATCC 15699)	Feces of infant	G. Reuter
<i>Bifidobacterium liberorum</i> Reuter S76e (ATCC 15702) [type strain (37)]	Feces of infant	G. Reuter
<i>Bifidobacterium lactentis</i> Reuter 659 (ATCC 25962) [type strain (37)]	Feces of infant	G. Reuter (isolated by Dehnert)
<i>Bifidobacterium breve</i> Reuter		
<i>B. breve</i> biotype <i>a</i>		
S1 (ATCC 15700) [type strain (37)]	Feces of infant	G. Reuter
B609; B624; B637; B648	Feces of infant	Our collection
B860; B912; B943	Human vagina	Our collection
<i>B. breve</i> biotype <i>b</i>		
S46 (ATCC 15701)	Feces of infant	G. Reuter
B622; B626; B628; B644	Feces of infant	Our collection
B828; B862; B941	Human vagina	Our collection
<i>B. breve</i> biotype? Cellobiose negative		
B627; B632; B633; B641; B644	Feces of infant	Our collection
B799; B898	Human vagina	Our collection
Unassigned strains: B721; B723	Human vagina	Our collection
<i>Bifidobacterium longum</i> Reuter		
<i>B. longum</i> biotype <i>a</i> E194b (ATCC 15707) [type strain (37)]	Feces of adult	G. Reuter
<i>B. longum</i> biotype <i>b</i> S3 (ATCC 15708)	Feces of infant	G. Reuter
B612; B647; B686; B688	Feces of infant	Our collection
B800; B821; B923; B929	Human vagina	Our collection
<i>B. longum</i> Reuter subsp. <i>animalis</i> Mitsuoka		
biotype <i>a</i> R101-8 (ATCC 25527) [type strain (27)]	Feces of rat	T. Mitsuoka
biotype <i>b</i> C10-45	Feces of calf	T. Mitsuoka
<i>Bifidobacterium globosum</i> Scardovi et al.		
RU224 (ATCC 25865) [type strain (41)]	Rumen of cattle	Our collection
RU230 (ATCC 25864); RU240; RU255; RU256	Rumen of cattle	Our collection
RU257; RU323; RU324; RU457	Rumen of cattle	Our collection
<i>Bifidobacterium pseudolongum</i> Mitsuoka		
<i>B. pseudolongum</i> biotype <i>a</i> PNC-2-9G (ATCC 25526) [type strain (27)]	Feces of pig	T. Mitsuoka
<i>B. pseudolongum</i> biotype <i>b</i> 28T	Feces of chicken	T. Mitsuoka
<i>B. pseudolongum</i> biotype <i>c</i> 29SRT	Feces of chicken	T. Mitsuoka
<i>B. pseudolongum</i> biotype <i>d</i> Mo2-10	Feces of mouse	T. Mitsuoka
<i>Bifidobacterium thermophilum</i> Mitsuoka		
<i>B. thermophilum</i> biotype <i>a</i> P2-91 (ATCC 25525) [type strain (27)]	Feces of pig	T. Mitsuoka
<i>B. thermophilum</i> biotype <i>b</i> 14-44	Feces of pig	T. Mitsuoka
<i>B. thermophilum</i> biotype <i>c</i> P16-6	Feces of pig	T. Mitsuoka
<i>B. thermophilum</i> biotype <i>d</i> Nissin	Feces of pig	T. Mitsuoka
<i>Bifidobacterium ruminale</i> Scardovi et al.		
RU326 (ATCC 25866); RU263; RU268; RU279	Rumen of cattle	Our collection
RU290; RU356; RU381; RU417; RU431; RU445 (ATCC 25867)	Rumen of cattle	Our collection
<i>R variants</i> RU276; RU348; RU354	Rumen of cattle	Our collection

TABLE 2.—(Continued)

Organism	Isolated from	Culture received from
<i>Bifidobacterium adolescentis</i> Reuter		
<i>B. adolescentis</i> biotype <i>a</i> E194a (ATCC 15703) [type strain (37)]	Feces of adult	G. Reuter
A205-14	Feces of adult	T. Mitsuoka
3859 (<i>B. appendicitis</i> Prévot)	Appendix	T. Mitsuoka
B624; B627	Feces of infant	Our collection
B735; B764; B767	Dental caries	Our collection
H28	Feces of adult	G. Reuter
<i>B. adolescentis</i> biotype <i>b</i>		
E305 (ATCC 15704)	Feces of adult	G. Reuter
A202-16; A216-21	Feces of adult	T. Mitsuoka
H39a	Feces of adult	G. Reuter
B654; B749; B750; B751; B752; B754	Feces of adult	Our collection
B757; B758; B760	Feces of adult	Our collection
B872; B876; B932	Human vagina	Our collection
Melezitose negative: B781; B786	Dental caries	Our collection
Melezitose positive: B973; B975; B976	Dental caries	Our collection
B977; B1000; B1001; B1013; B1010; B1020	Dental caries	Our collection
B1022; B1023; B1029; B1031; B1033; B1036	Dental caries	Our collection
B1038; B1044; B1046; B1049; B1051; B1053	Dental caries	Our collection
B1056; B1064; B1067	Dental caries	Our collection
<i>B. adolescentis</i> biotype <i>c</i> E298b (ATCC 15705)		
A201-3; A204-4	Feces of adult	G. Reuter
B617; B618	Feces of infant	T. Mitsuoka
B660; B661; B665; B669; B676; B682; B684	Feces of adult	Our collection
B839; B933	Human vagina	Our collection
<i>B. adolescentis</i> biotype <i>d</i>		
E319a (ATCC 15706); H33	Feces of adult	G. Reuter
B608	Feces of infant	Our collection
B677	Feces of adult	Our collection
B788; B792; B841; B947	Human vagina	Our collection
Miscellaneous strains		
Unassigned Group " <i>angulatum</i> ": F92; F102	Waste waters	Our collection
F158; F196; F251; F313; F380; F399; F406	Waste waters	(Morphology similar to that of strain B677; see under <i>B. adolescentis</i> biotype <i>d</i>)
Unassigned group " <i>catenulatum</i> ": F55; F71; F81; F85; F86; F94; F98; F99	Waste waters	(Morphology similar to that of strain B669; see under <i>B. adolescentis</i> biotype <i>c</i>)
<i>Bifidobacterium suis</i> Matteuzzi et al.		
SU859 [type strain (26)]	Feces of pig	Our collection

150 μ g of denatured competitor DNA in 2X SSC. These DNA preparations, in 0.1X SSC, had been sheared by passage through a French pressure cell at about 10,000 psi (21) and prior to use were denatured by heating in a boiling-water bath for 10 min. The amount of competitor DNA was sufficient to reach the necessary saturation plateau for single-point competition test. The vials were gently shaken by means of a wrist-action shaker for 12 hr at the temperature of 69 C, 25 C below the melting temperature (T_m) as determined in 1X SSC. After incubation, the filters were washed on both sides with 2X SSC at 69 C, dried under an infrared lamp, and counted with a liquid-scintillation counter. Homology or similarity values were calculated in per cent of binding with respect to the depression measured with the homologous system, i.e., with the equation (counts without competitor DNA - counts with heterologous DNA)/(counts without competitor DNA - counts with homologous DNA) $\times$ 100.

DNA base composition. The per cent GC (moles of guanine plus cytosine) was determined by using the thermal denaturation method of Marmur and Doty (24). Curves of optical density variations and temperature were recorded with a T_m analyzer (Beckman Instruments Inc.).

RESULTS

Homology relationships among type strains of the species of *Bifidobacterium*. Species from human feces. Outlines of the DNA homology among the type strains of the species isolated from human feces and proposed by Reuter (36, 37) are reported in Table 3.

B. breve and *B. parvulorum* are undoubtedly more strongly related to each other than to any other species studied in this work. The two

strains of *B. parvulorum*, S50 and S17c, have 88 and 94% homology to strain S1 (*B. breve*) as reference organism, and in reciprocal reaction the strains S1 and S46 (*B. breve*) display 98 and 106% homology with strain S50 (*B. parvulorum*). Strain S12 of *B. infantis* displayed lower, but still considerable, levels of similarity to the references *B. breve* (strain S1) and *B. parvulorum* strain S50: 50 and 45%, respectively. Similar levels of similarity to *B. breve* and *B. parvulorum* were found with DNA competitors from *B. liberorum* and *B. lactentis*. The DNA from other species has little or no homology with that of *B. breve*.

The strains of the species *B. infantis*, *B. liberorum*, and *B. lactentis* form what appears to be a well-defined homology group: their relative homology values, measured with each of the strains used as reference, fall within a range of 70 to 100% (see the third and the last two columns of Table 3).

The two strains of *B. bifidum* do not share any appreciable polynucleotide sequences with other species but are completely related to each other; the same is true for the strains of the biotypes of *B. adolescentis*, although some discrepancies were observed in the reciprocal competition experiments. The two proposed biotypes of *B. longum* can be sharply distinguished from the other species by virtue of their similarity values but they can also be distinguished from each other. They seem to be somewhat less related to each other than the biotypes within other species of *Bifidobacterium*. The other species which seem to be

TABLE 3. Deoxyribonucleic acid homology values among strains (including type strains) of *Bifidobacterium* species isolated from man^a

Competitor strains		Reference strains												
		S1	S46	S12	S50	S17c	E319f	E194a	E298b	E319a	E194b	S3	S76e	659
S1	<i>B. breve</i> biotype a	100	84	45	106		27		4			43	34	25
S46	<i>B. breve</i> biotype b	86	100	39	98		25		5					
S12	<i>B. infantis</i>	50		100	45		42					60	77	92
S50	<i>B. parvulorum</i> biotype a	88		45	100	84			0				31	57
S17c	<i>B. parvulorum</i> biotype b	94		45	86	100			0					
E319f	<i>B. bifidum</i> biotype a	10		20	13		100					2		
S28a	<i>B. bifidum</i> biotype b	20		28			100		3					
E194a	<i>B. adolescentis</i> biotype a	10		10				100	63	60				
E305	<i>B. adolescentis</i> biotype b	5		5				95	70	80		20		
E298b	<i>B. adolescentis</i> biotype c	6		10	30		13	96	100	82	30			
E319a	<i>B. adolescentis</i> biotype d	5		5				87	90	100				
E194b	<i>B. longum</i> biotype a	12		15	34		15			0	100	70	13	43
S3	<i>B. longum</i> biotype b									0	75	100		
S76e	<i>B. liberorum</i>	42		77	45	24				0		52	100	92
659	<i>B. lactentis</i>	17		70	40	22				0		46	77	100

^a Homology or similarity value is calculated in percentage of binding depression in respect to depression of the homologous system (=100).

related to *B. longum* with little more than 50% homology are *B. liberorum*, *B. lactentis*, and *B. infantis*.

Species from animals. The DNA homology values of the type strains of the species found in the feces of various animals and in the rumen of cattle relative to six reference organisms are reported in Table 4. Three of the four biotypes of *B. thermophilum* (i.e., *a*, *b*, *c*) behave as expected and are 100% homologous with the type strain of *B. thermophilum*, P2-91, as reference, whereas biotype *d* strain Nissin is 83% homologous. The other reference organism to which the biotypes of *B. thermophilum* appear to be highly related is the neotype strain of *B. ruminale*, RU326: the similarity values were found to range from 60 to 72%. Furthermore, the DNA from *B. ruminale* is 100% homologous to that of strain P2-91 (*B. thermophilum* biotype *a*).

B. pseudolongum biotypes *c* and *d* are genetically not distinct and are very closely related to the type strain of *B. globosum*, RU230 (70 and 65%). Strain C10-45, *B. longum* subsp. *animalis* biotype *b*, is 100% homologous with *B. pseudolongum* but not with strain R101-8, thought to belong to biotype *a* of the same subspecies; strain R101-8 was not related to any of the species from animals.

Strain SU859 (*B. suis*) is not related genetically to any other strain in this study.

Relationships between "human" and "animal" species. Some competition tests were performed to substantiate at the genetic level

the phenotypic unrelatedness between the species of *Bifidobacterium* found primarily in humans and those found in animal feces and rumens. On the basis of the results reported in Tables 3 and 4, four strains were selected as reference organisms: S1 (*B. breve* biotype *a*), E298-b (*B. adolescentis* biotype *c*), P2-91 (*B. thermophilum* biotype *a*), and S3 (*B. longum* biotype *b*). The relative similarity values of the DNA from some competitor strains and the DNA of these reference strains are given in Table 5.

There was very insignificant DNA homology between strains from human and other animal sources even when phenotypic resemblances like those between *B. breve* and *B. thermophilum*, *B. longum* and *B. pseudolongum*, or *B. longum* subsp. *animalis*, existed. A good example of such unrelatedness was given by *B. longum* subsp. *animalis* and *B. longum* subsp. *longum*. *B. longum* subsp. *animalis* was proposed by Mitsuoka (27) as a subspecies (variety) of *B. longum*, a species so far found exclusively in the feces of man, on the basis of similar fermentative characters. However, neither biotype *a* nor *b* of *B. longum* subsp. *animalis* displays any homology with *B. longum* subsp. *longum*. Biotype *b* is actually *B. pseudolongum*, and the relationships of biotype *a* remain obscure.

These results (see Table 5) correlate well with data obtained from the migration rate of fructose-6-phosphate phosphoketolase in starch-gel electrophoresis (38).

Homology relationships among strains assigned to species of *Bifidobacterium* on a

TABLE 4. Deoxyribonucleic acid homology values among strains (including type strains) of *Bifidobacterium* species isolated from feces of various animals and bovine rumen^a

Competitor strains		Reference strains					
		P2-91	Mo2-10	C10-45	RU230	RU326	SU859
P2-91	<i>B. thermophilum</i> biotype <i>a</i>	100	23	0	8	60	12
14-44	<i>B. thermophilum</i> biotype <i>b</i>	100	9	14		72	10
P16-6	<i>B. thermophilum</i> biotype <i>c</i>	100	6	16	0	70	5
Nissin	<i>B. thermophilum</i> biotype <i>d</i>	83	0	0		62	5
PNC-2-9G	<i>B. pseudolongum</i> biotype <i>a</i>	13	70	65	70	20	5
29SrT	<i>B. pseudolongum</i> biotype <i>c</i>	14	100	100	70		0
Mo2-10	<i>B. pseudolongum</i> biotype <i>d</i>	23	100	90	65	13	0
R101-8	<i>B. longum</i> subsp. <i>animalis</i> biotype <i>a</i>	9	34	30		16	27
C10-45	<i>B. longum</i> subsp. <i>animalis</i> biotype <i>b</i>	10	100	100	70	23	46
RU230	<i>B. globosum</i>	10	75		100	35	10
RU326	<i>B. ruminale</i>	100	17		30	100	5
SU859	<i>B. suis</i>		0		25	15	100

^a See footnote to Table 3.

TABLE 5. Deoxyribonucleic acid homology relationships between strains of some "human" and "animal" species of *Bifidobacterium*^a

Competitor strains		Reference strains			
		S1	E298-b	P2-91	S3
S1	<i>B. breve</i> biotype <i>a</i>	100		21	
P2-91	<i>B. thermophilum</i> biotype <i>a</i>	22		100	
Nissin	<i>B. thermophilum</i> biotype <i>d</i>	10	3		6
E298-b	<i>B. adolescentis</i> biotype <i>c</i>		100		
S12	<i>B. infantis</i>			12	
S50	<i>B. parvulorum</i> biotype <i>a</i>			22	
659	<i>B. lactentis</i>			12	
S76e	<i>B. liberorum</i>			0	
PNC-2-9G	<i>B. pseudolongum</i> biotype <i>a</i>		5		13
29SrT	<i>B. pseudolongum</i> biotype <i>c</i>		10		30
C10-45	<i>B. longum</i> subsp. <i>animalis</i> biotype <i>b</i>	5	23	5	9
R101-8	<i>B. longum</i> subsp. <i>animalis</i> biotype <i>a</i>	5	10		0
S3	<i>B. longum</i> biotype <i>b</i>			10	100

^a See footnote to Table 3.

phenotypic basis. DNA hybridization experiments can often lead to taxonomic misinterpretations when too few strains are employed, even if these are the type strains of the presumed species, especially when the phenotypic characters of these strains, like those of the bifidobacteria, are not too well known. Therefore, additional competition experiments were performed with the strains of our collection obtained from different habitats and identified as known species on the basis of morphology and fermentation patterns. This survey had two objectives: to assess the genetic homogeneity of the named species of *Bifidobacterium* and to determine whether there are groups of bifidobacteria with DNA-homology relationships not fitting the patterns which emerge from the study of the type strains.

The reference organisms used were selected with the results given in Tables 3 and 4 as guidelines. The strains used for the extraction of DNA competitors are listed in Table 2.

Homology among strains identified as *B. breve*. The strains used were isolated from infants' feces and human vaginas and were assigned to *B. breve* because they fermented mannitol and sorbitol and not arabinose and xylose; biotypes *a* and *b* were distinguished on the basis of differences in trehalose and melezitose fermentations (Table 1). Contrary to the reports of Mitsuoka (27), many of our strains had the fermentation pattern of biotype *b* but did not ferment cellobiose. The DNA-homology values of this group of strains with type strain

S1 (*B. breve* biotype *a*) as reference are reported in Table 6.

Of 24 strains investigated, only two (B629

TABLE 6. Homology values of strains phenotypically identified as *Bifidobacterium breve* and measured with the type strain of *B. breve* as the reference organism^a

Competitor strains	Reference
	S1 (type strain)
<i>B. breve</i> biotype <i>a</i> strain S1 (type strain)	100
B609; B624, from human feces	84-100
B637; B648, from human feces	82-89
B912; B943; B860, from human vagina	62-89-82
<i>B. breve</i> biotype <i>b</i> S46	86
Cellobiose-positive strains	
B622; B626, from human feces	62-86
B628; B629, from human feces	86-16
B828; B862; B941, from human vagina	83-87-85
Cellobiose-negative strains	
B627; B632; B633, from human feces	100-100-82
B641; B644, from human feces	4-86
B799; B898, from human vagina	82-75
Unidentified strains	
B721; B723, from human vagina	90-91

^a See footnote to Table 3.

and B641 from feces) failed to demonstrate homology to *B. breve* S1 and remained unassigned at the end of multiple trials with all reference organisms. The remaining 22 strains, with the exception of B912 from the vagina and B622 from feces, gave values in the range of 75 to 100%, thus demonstrating the high genetic homogeneity of this group of isolates as referred to the type strain of *B. breve*, regardless of the behavior toward cellobiose.

Also homologous to *B. breve* were two strains from the human vagina, i.e., B721 and B723; these strains are not phenotypically related to *B. breve*. They do not ferment mannitol and sorbitol and ferment arabinose but not xylose. This uncommon fermentation pattern, reminiscent of that reported by Malyoth and Bauer (22), is accompanied by some peculiarities in morphology that will be described in a later paper. No doubt these two strains are *B. breve* from a genetic standpoint.

Homology among strains identified as *B. adolescentis*. The phenotypic distinction between biotype *d* of *B. adolescentis* and *B. longum* is based only upon the fermentation of cellobiose and glucosides (see Table 1); the biotypes *a*, *b*, and *c* of *B. adolescentis* are better characterized by their behavior toward mannitol and sorbitol. We tested the fermentation of gluconate by strains of this species received from Reuter, viz. E194a, E305, E298b, H28, H39a, and H33. All of these strains fermented gluconate, and gas formation was easily observed by using the technique of Gibson and Abd-el-Malek (10). However, less acidity was produced than from glucose (45). From routine work on our own isolates from various sources, we concluded (40) that the fermentation of gluconate was a property shared by the species from honey bees and by the strains having the fermentation patterns of the biotypes of *B. adolescentis*; the mannitol-fermenting bifidobacteria from the bovine rumen, closely related genetically to *B. adolescentis* (3), ferment gluconate regularly. Therefore, the fermentation of gluconate was taken as an additional valuable tool in the selective work preliminary to the present study.

From isolates of our collection, obtained from human feces, vaginas, and dental caries, we selected for study 60 strains identified on fermentation patterns as *B. adolescentis*: 5 from infant feces, 17 from adult feces, 9 from vaginas, and 29 from dental caries. Six strains received from Mitsuoka and three from Reuter (besides the type and the reference strains from the same authors) were added to the list (see Table 2). The majority of our strains were

assigned to biotype *b* of *B. adolescentis*. Most strains were assayed for their DNA homology with Reuter's strain E298b (biotype *c*) as reference organism. The pertinent data are reported in Table 7.

It is immediately apparent that only a very few strains displayed high genetic relatedness to *B. adolescentis*, viz. H28, H39a, and H33 from Reuter; A205-14 and A216-21 from Mitsuoka; and B618 from our collection. It is striking that the vast majority of our isolates, mostly from dental caries but from human feces and from vaginas as well, displayed homologies never exceeding 50% to the reference strain used. Therefore, they are not *B. adolescentis* on a genetic basis. Our isolates from dental caries were extremely uniform from a phenotypic standpoint and fermented mannitol like the oral strains of Beerens, Gérard, and Guillaume (1).

To assess the genetic homogeneity of our large group of isolates, a second reference organism was used, strain B757 isolated from human feces and only 30% homologous with *B. adolescentis* strain E298b. The results reported in Table 7 show that the DNA from 41 strains displayed homologies with the new reference organism ranging from 69 to 105%. Such strains form, therefore, a rather highly homogeneous group. This group, consisting of mannitol-fermenting strains (with only a very few exceptions), will be referred to hereafter as Unassigned Group "*dentium*" to indicate what seems to be one of the preferred habitats of these organisms. (We prefer to refer to our Unassigned Groups by latinized words instead of by numerals or letters to avoid confusion with the designations used for other groups or types reported in the literature).

The data in Table 7 fail however to indicate any genetic relatedness between 14 strains and the reference organism B757 (group "*dentium*"), i.e., the homology values rarely exceed 40%. All these strains, some from human feces and some from vaginas, have the fermentation pattern of biotype *c* of *B. adolescentis*. They characteristically ferment sorbitol. These strains may form a new genetically distinct group of bifidobacteria.

The cells of this second group of unassigned strains characteristically are arranged in irregular, sometimes long, chains and vigorously ferment sorbitol but not mannitol; they ferment gluconate as well. Several additional strains with these characters were found among a large number of bifidobacteria isolated from waste waters and muds and are now under study in our laboratory. These strains are

TABLE 7. *Deoxyribonucleic acid homologies among strains with the phenotype of Bifidobacterium adolescentis measured with B. adolescentis biotype c strain E298b and strain B757 as reference organisms^a*

Competitor strains	Reference strains	
	E298b	B757
<i>B. adolescentis</i> biotype <i>a</i>		
E194a (type strain)	65	
A205-14	60	21
3859	24	55
H28	82	
B735; B764; B767	27-31-21	100-100-100
<i>B. adolescentis</i> biotype <i>b</i>		
E305	70	
A202-16; A216-21	32-87	56-45
H39a	70	7
B654; B749; B750; B751	11-21-nt ^b -24	23-69-78-96
B752; B754; B758; B760	nt-nt-14-40	86-84-69-85
B757	30	100
B872; B876; B932	nt-55-15	95-40-105
Melezitose-negative strains		
B781; B786	3-21	100-100
Melezitose-positive strains		
B973; B975; B976; B977	20-25-20-30	89-100-92-100
B1000; B1001; B1010; B1013		100-83-100-100
B1020; B1022; B1023; B1029		100-85-75-90
B1031; B1033; B1036; B1038		100-111-88-105
B1044; B1046; B1049; B1051		95-102-100-85
B1053; B1056; B1064; B1067		96-95-92-100
<i>B. adolescentis</i> biotype <i>c</i>		
E298b	100	33
A201-3; A204-4	44-41	43-27
B617; B618	33-71	30-38
B660; B661; B665; B669	46-42-14-10	27-14-17-15
B676; B682; B684	40-40-40	36-35-50
B839; B933	34-36	20-26
<i>B. adolescentis</i> biotype <i>d</i>		
E319a	82	
H33	70	20
B608	22	
B677		3
B788; B792; B841; B947	20-10-7-27	81-77-45-37

^a See footnote to Table 3.

^b Not tested.

tentatively referred to as Unassigned Group "catenulatum" because of the characteristic arrangement of their cells in chains.

B677, a strain isolated from feces from an adult, showed an unusual "V" cell disposition under all cultural conditions; this disposition of the cells, strongly suggesting the "snapping" type of cell division in the genus *Corynebacterium* (17), has so far never been observed by us in any bifidobacterium except for some

isolates from waste waters. These isolates and strain B677, all fermenting pentoses but not mannitol and sorbitol (they do not ferment gluconate), are placed in a third Unassigned Group, "angulatum," so-called because of the unique disposition of their cells.

The DNA-homology patterns of the aforementioned Unassigned Groups and the genetic relatedness between their reference strains and the selected competitor DNA from all known

species of *Bifidobacterium* are reported in Table 8. It appears that the three Unassigned Groups, "dentium," "catenulatum," and "angulatum," are quite distinct genetically and do not share appreciable DNA polynucleotide sequence similarity with any of the named species of the genus *Bifidobacterium*. The general morphology and arrangement of the cells of organisms in the Unassigned Groups "dentium," "catenulatum," and "angulatum" are given in Fig. 1 to 6.

Homology among strains identified as *B. longum*. Biotypes *a* and *b* of *B. longum* are reported (see Table 1) characteristically not to ferment cellobiose, starch, and salicin. On the other hand, according to Mitsuoka (27), cellobiose is fermented very slowly by all biotypes

of *B. adolescentis*. Starch fermentation is in general erratic among bifidobacteria. Mitsuoka (27) reported that melezitose is characteristically fermented by *B. longum* but only slowly fermented by *B. adolescentis*; this statement is not generally valid because our strains, genetically found to be *B. longum* (Table 9), do not ferment this carbohydrate. The behavior toward salicin and gluconate would probably be of more differential value. The bifidobacteria which ferment pentoses but not gluconate and salicin could be assigned to *B. longum*. Among our isolates only a few could be found to test for DNA homology with the type strain E194b and a reference strain S3 of *B. longum*; only one strain, B654, not assigned in the experiments with *B. adolescentis* (Table 7) was

TABLE 8. Deoxyribonucleic acid homology patterns of strains of the Unassigned Groups "dentium," "catenulatum," and "angulatum"^a

Competitor strains	Reference strains of groups		
	"dentium" B764	"catenulatum" B669	"angulatum" B677
Unassigned group "dentium"			
B764	100	20	5
B757	93	27	3
Unassigned group "catenulatum"			
B669 reference strain	27	100	23
B660; B661	25-15	97-97	26-37
B676; B684	35-50	95-100	
F71; F81	28-34	73-68	33-33
F94; F99 ^b	33-21	78-65	31-34
B933		70	35
Unassigned group "angulatum"			
B677 reference strain	5	28	100
F92; F158; F196	27-8-10	60-35-35	77-90-80
F424; F426 ^b	15-20	25-20	76-83
S1 <i>B. breve</i> biotype <i>a</i>	10	14	16
S12 <i>B. infantis</i>	25	28	13
S50 <i>B. parvulorum</i> biotype <i>a</i>	4	15	32
S76e <i>B. liberorum</i>	26	12	20
659 <i>B. lactentis</i>	13	30	30
S28a <i>B. bifidum</i> biotype <i>b</i>	4	17	5
E298b <i>B. adolescentis</i> biotype <i>c</i>	10	40	0
RU326 <i>B. ruminale</i>	20	26	23
SU859 <i>B. suis</i>	12	38	28
RU230 <i>B. globosum</i>	15	0	24
P2-91 <i>B. thermophilum</i> biotype <i>a</i>	17	23	27
Mo2-10 <i>B. pseudolongum</i> biotype <i>d</i>	28		10
E194b <i>B. longum</i> biotype <i>a</i>	12	15	7
S3 <i>B. longum</i> biotype <i>b</i>	28	27	38

^a See footnote to Table 3.

^b Not all the data obtained with the strains from waste waters (see Table 2) are reported in this table.

FIG. 1-6. Morphology of unassigned groups. Fig. 1-3. Group "angulatum" strains F424, B677 (reference strain), and F196. Fig. 4. Group "catenulatum" strain B669 (reference strain). Fig. 5 and 6. Group "dentium" strains B757 and B764. Cells from anaerobic stabs in Trypticase-Phytone-yeast extract-glucose medium. Phase contrast. X 1,500.

included in this group of isolates. The results are given in Table 9. Although the homology values were not so high as in preceding groups, it should be noted that, except for strain B686 which remained unassigned in this work, the strains tested as competitors displayed an homology level to *B. longum* biotype *a* very close to that of strain S3 of *B. longum* biotype *b*. All our strains, however, do not ferment mannose. Therefore, they should be considered closer phenotypically to *B. longum* biotype *a* than to *B. longum* biotype *b*; the data reported in Table 9 indicate that the homology values of the group are higher when they are measured in the homologous system of strain S3 (*B. longum* biotype *b*).

DISCUSSION

McCarthy and Bolton (21) and Hoyer et al. (14) showed that competition reactions, wherein the interference of nonlabeled DNA fragments in binding of labeled DNA to homologous DNA is measured, provides a means for assessing DNA interactions with a minimal number of experimental variables. Annealing of single-strand DNA with fixed reference DNA must, however, be carried out under "stringent" conditions, i.e., those permitting only specific pairing of complementary polynucleotide sequences.

The temperature of duplex formation is one of the critical parameters in the formation of specific complementary double strands (15, 20). Theoretically, with increasing "stringent"

conditions (i.e., at temperatures at least 25 C below T_m) as recognized first by Marmur and Doty (24), one selects against imperfections of annealing. Precoating the cellulose-nitrate membranes with the albumin-polymer solution of Denhardt (6) lowers the amount of unspecific attachments. In the present study, these requirements were satisfied, and the true degree of relatedness was therefore evaluated. The fact that relatedness in the range from 0 to 100% was assessed among organisms like those studied here, all falling in a very narrow range of per cent GC (60 to 65), is a confirmation of this assumption. The reasonably good agreement of relative relatedness in all reciprocal reactions seems to rule out gross differences in genome size. Recently, however, De Ley and Tijtgat (5) drew attention to the spontaneous release of DNA from filters at high temperatures; the high temperatures required, even at 25 C below T_m , could not be suitable for organisms with over 65% GC. In the range of 50 to 65% GC, the reliability of the results obtained with the procedure of Denhardt (6) should depend on the quality of the filter. We experienced some difficulties in obtaining good filters with some DNA, irrespective of the amount of DNA fixed; spontaneous release of DNA during incubation cannot be excluded. In many of these cases "Selectron" filters were used preferably (see above).

The results obtained by DNA-binding experiments are generally trusted when they correlate reasonably with the taxonomic groupings deter-

TABLE 9. Homology values of strains phenotypically identified as *Bifidobacterium longum* measured with the type strain of *B. longum* and strain S3 as reference organisms^a

Competitor strains	Reference strains	
	E194b (type strain)	S3
<i>B. longum</i> biotype <i>a</i> E194b (type strain)	100	70
<i>B. longum</i> biotype <i>b</i> S3	75	100
Strains isolated from feces of infants:		
B612; B647	70-68	71-80
B686; B688	25-60	20-73
Strains isolated from human vaginas:		
B800; B821	70-87	85-92
B923; B929	67-67	85-86
Strain isolated from feces of adult:		
B654 ^b	65	71

^a See footnote to Table 3.

^b Not assigned in the preceding experiments (see Table 7).

mined by classical methods or better by taxometric analysis (2, 13). It must be remembered, however, that the DNA-DNA hybridization technique takes into account the entire genome, whereas taxometric analysis involves only those parts of it whose phenotypic expression can be measured (13); furthermore, similar enzymatic expressions may result from enzymes containing quite different amino-acid sequences. In the case of the bifidobacteria, there are probably insufficient characters to permit extensive taxometric analyses. At present, the DNA homology approach in the study of the taxonomy of the bifidobacteria, as well as in other newly discovered and incompletely examined microorganisms, seems to be the method of choice; it serves not only to confirm the validity of species at the genetic level but also to pave the way for fruitful comparative physiological and biochemical investigations with genetically homogeneous bacterial populations.

B. bifidum. *B. bifidum* is the most easily recognized species of *Bifidobacterium* because of its characteristic fermentation pattern (see Table 1). Kandler (16) found in five isolates of this species and in *Lactobacillus bifidus* subsp. *pennsylvanicus* ATCC 11863 the same characteristic murein. Although few strains of this species were investigated here, the results were striking. Strains E319f and S28a, almost identical phenotypically but isolated from feces from an adult and an infant, respectively, are 100% homologous; three other isolates from the human vagina and *L. bifidus* subsp. *pennsylvanicus* are 100% homologous and are also homologous to either of two reference organisms. Little if any similarity was found relative to other species of the genus *Bifidobacterium*. The validity of this species is therefore undeniable. However, its homogeneity should be better assessed with the study of more strains.

B. infantis, B. liberorum, and B. lactentis. Although differing reports have been given (44), most pertinent literature data indicate that *B. infantis*, *B. liberorum*, and *B. lactentis* are the predominant bifidobacteria in the feces of breast-fed infants. Dehnert (thesis, Universitäts-Kinderklinik, Heidelberg, 1961; reference 4), Petuely and Lindner (32), Liebscher (19), Gyllenberg and Carlberg (11), Müller and Pech (29), and Haenel et al. (12) generally recognize as "Brustkind-Bifidus" the bifidobacteria which ferment xylose and mannitol [nutritional type A of Gyllenberg and Carlberg (11), group IV Dehnert (4)]. Reuter (36) named this type *B. lactentis* (Table 1). Reuter further distinguished as *B. liberorum* a novel xylose-positive type

similar to *B. lactentis*; however, *B. liberorum* does not ferment mannitol; the strong phenotypic relatedness of *B. liberorum* and biotype c of Dehnert's group IV (J. Dehnert, thesis, Universitäts-Kinderklinik, Heidelberg, 1961) was recognized by Reuter (36). He also found a biotype he named *B. infantis* which did not ferment pentoses and polyols (Table 1) and which was very similar in morphology to *B. liberorum*. Although they were distinct serologically, Reuter stated that between *B. infantis* and *B. lactentis* "... gewisse Beziehungen hinsichtlich von Teilantigenen bestehen" (36, p. 494). A biotype of *B. infantis* was found by Mitsuoka (27) (*B. infantis* type b) which ferments inositol like *B. liberorum* and *B. lactentis* (Table 1). Some fermentation characters are thus apparently common to these three species: slow fermentation of ribose and mannose, no reaction with cellobiose or melezitose, fermentation of inulin and inositol, and similar behavior toward glucosides.

Our results, obtained with the type strains of these species, clearly indicate the close genetic relatedness existing among these strains. *B. infantis* strain S12 is 77 and 92% homologous with *B. liberorum* and *B. lactentis* (see last two columns of Table 3), and these species are 77 and 70% similar to the type strain S12 of *B. infantis*. Although this group will be circumscribed with more confidence when larger numbers of strains are examined, the proposal is made here to merge *B. infantis*, *B. liberorum*, and *B. lactentis* into a single species, for which the name *B. infantis* should be used because it has priority (page priority) over the other two names.

Bifidobacterium parvulorum and B. breve. *B. parvulorum* biotype a and *B. breve* were the designations given by Reuter (36) to bifidobacteria corresponding to biotypes b and a, respectively, of the group III established by Dehnert (thesis, Universitäts-Kinderklinik, Heidelberg, 1961). Reuter named as *B. parvulorum* biotype (variety) b an organism isolated from feces from an infant and subsequently referred to as a new type (type 6) by Werner and Seeliger (46); these are also found in the human vagina. No doubt these "types," separated on the basis of fermentation characters, have much in common in their morphology, serology, and probably ecology. They are apparently abundant in the feces of infants where, especially *B. parvulorum* biotype a "... schien in mehreren Fällen der Ersatz für den zurückgehenden Typ IV (*B. infantis*) zu sein" (36, p. 492). Their serological relatedness has been recognized by Reuter (36). Our results indicate that these

types are strongly related genetically (more than 86% homology, see Table 3) and that many strains behaving phenotypically like *B. breve* share most polynucleotide sequences of their DNA (Table 6). Of 21 strains tested (each isolated from a different specimen), only 2 (strains B629 and B641) were not identifiable with any named species.

Many of the strains tested were cellobiose-negative; Reuter (36), Mitsuoka (27), and Dehnert (4) reported on the contrary that cellobiose was characteristically fermented by the strains of this type. Once again this strengthens the notion that some fermentation characters may be of little value in the speciation of the genus *Bifidobacterium*. A clear example was offered by the strains isolated from the bovine rumen which, although fermenting pentoses and mannitol, are 85 to 90% homologous with *B. globosum*, a species which does not ferment these compounds (3). Although we did not test mannitol- and sorbitol-negative isolates which could be assigned to *B. parvulorum* (Table 1), the most logical conclusion to be drawn from our DNA homology experiments is to merge *B. breve* and *B. parvulorum* into a single species, *B. breve*, this name having priority over *B. parvulorum*. Other phenotypic types described by Malyoth and Bauer (22), such as those fermenting arabinose but not xylose, probably belong to this species (see our strains B721 and B723 in Table 6). Phenotypic characters that might be used for identifying this species require further consideration.

B. longum. *B. longum*, sometimes found in the feces of infants (Dehnert's group V), predominates together with *B. adolescentis* in the feces of adults. Here again Reuter (36) distinguished a biotype (variety) *a*, mostly found in adults, and a biotype *b*, predominating in the feces of infants. The level of genetic similarity between these two biotypes is apparently lower than that between the biotypes of any other bifidobacteria.

Our isolates, phenotypically closer to biotype *a* but isolated from feces of infants and vaginas, seem to be more related genetically to biotype *b* (Table 9); this could be perhaps more in accord with their ecology. *B. longum* is clearly distinct genetically from the other bifidobacteria, and hence its validity as a species is confirmed. A certain degree of relatedness seems to exist between *B. longum* and the group *B. infantis-liberorum-lactentis*. The similarity values of these last three species tested as competitors in the reactions with *B. longum* biotype *b* are 60, 50, and 46%, respec-

tively. This interesting point merits further experimental work. *B. bifidum* and *B. adolescentis* (see below) are far less related to *B. longum*. The distinction of a subspecies (variety) (*B. longum* subsp. *animalis*) in the species *B. longum* as suggested by Mitsuoka (27) did not find any experimental support. Strain C10-45 (type *b* of Mitsuoka's subspecies) is evidently *B. pseudolongum*, and strain R101-8 (type *b* of the same subspecies) remained unassigned to any species. The electrophoretic behavior of fructose-6-phosphate phosphoketolase of strain C10-45 was found to fit its DNA-homology pattern. The migration rate of this enzyme in starch electrophoresis was found to be identical to that of *B. pseudolongum* and other species from animals and very different from that of *B. longum* and other species from human sources (38).

B. adolescentis and unassigned groups. Reuter (36) named as *B. adolescentis* those pentose-fermenting bifidobacteria he first found to predominate, along with *B. longum*, in the feces of adults; he distinguished four biotypes (varieties), *a*, *b*, *c*, and *d*, differing in their fermentation patterns and serological behavior. Mitsuoka (27) also distinguished four biotypes and confirmed the ecology of this species. The genetic validity of the species *B. adolescentis* was confirmed by the DNA sequence similarity among the reference strains of the biotypes proposed by Reuter and a few other strains (Table 7). From a practical point of view, however, the phenotype of this species must be carefully assessed and compared with that of the Unassigned Groups discussed below.

The results we obtained with a rather large number of strains isolated from human feces, vaginas, dental caries, and waste waters demonstrated that the fermentation patterns of *B. adolescentis* and its biotypes are actually displayed by at least three additional bacterial groups distantly related genetically from each other and from other genetic species of the genus *Bifidobacterium* (Tables 7 and 8).

The heterogeneity of *B. adolescentis* had previously been indicated. Kandler (16) found different types of murein and varying ornithine-lysine ratios in different strains of this species, and some differences in DNA homology relationships were noted by Crociani et al. (3). The amplitude of the divergencies, however, was quite unexpected. The recognition of the existence of sharply distinct genetic groups within the phenotype of *B. adolescentis*, referred to here as the "*dentium*," "*catenulatum*," and "*angulatum*" groups, may help in resolving

some of the problems of bifidobacteria taxonomy.

The mannitol-fermenting strains isolated by Beerens, Gérard, and Guillaume (1) from the human mouth might be related to our Unassigned Group "*dentium*." We found these unique organisms in dental caries; this type is present, however, also in the feces of adults and in the vagina. The sorbitol-fermenting bifidobacteria isolated by Orla-Jensen et al. (31) from human feces may well be related to our Unassigned Group "*catenulatum*." Our preliminary records indicate that strains of this group are abundant in waste waters or muds; whether these organisms can withstand extrabody conditions of proliferate in these habitats is a question that merits further investigation. Our strains display the biochemical characters of true bifidobacteria; hence, they probably do not have anything in common with the pleomorphic, chain-forming, anaerobic bacteria found by Lerche and Reuter (18) or with the "Kettenformige Bifidobakterien" referred to by Mitsuoka, Sega, and Yamamoto (28), bacterial forms which are being assigned to the genus *Catenabacterium* (Kandler and Mitsuoka, *personal communication*).

Our Unassigned Group "*angulatum*" merits comment. Its morphology (Fig. 1–3) has never been observed before in bifidobacteria, and the cell disposition suggests a type of division apparently unknown in this group of bacteria (see above). The strains we studied were identified as bifidobacteria on the basis of their overall metabolism and biochemistry; the reference strain B677 possesses a "human" electrophoretic type of fructose-6-phosphate phosphoketolase (38; Scardovi, *unpublished data*) and a per cent GC of 59.0 ± 0.12 . Only one strain (strain B677) was isolated from human feces; others, although not numerous, were found in waste waters. A particular ecology is thus indicated.

A small number of strains phenotypically assigned to *B. adolescentis* were not related genetically to any of the reference organisms used in this work. Among these were strains B617, B665, and B682, all assigned phenotypically to biotype *c* of *B. adolescentis*. In a previous study by Crociani et al. (3), a group of mannitol-fermenting bifidobacteria isolated from rumens (reference strain: RU424) was found closely related genetically to some bifidobacteria from feces of humans and having the fermentation pattern of *B. adolescentis* biotype *c*. These "human" strains were B617, B665 and B682 (3 and Table 3).

The electrophoretic pattern of fructose-6-

phosphate phosphoketolase of strain RU424 (38), as well as its uniform murein type, Lys-Asp (Kandler, *personal communication*), confirmed this relatedness. All of this could be taken as evidence for the existence of an additional group of bifidobacteria genetically distinct from but having a fermentation pattern similar to that of *B. adolescentis*; additional work is needed to elucidate this point.

***B. ruminale* and *B. thermophilum*.** *B. ruminale* and *B. thermophilum* cannot be distinguished on the basis of DNA homology (Tables 4 and 10). *B. ruminale* strains exhibit the same range of homology when tested as competitors in the systems RU326 (*B. ruminale* neotype strain) and P16-6 (*B. thermophilum* biotype *c*). There is also great phenotypic similarity. The fermentation patterns are identical (Table 1); both *B. ruminale* and *B. thermophilum* ferment ribose, a sugar not tested in previous work on bifidobacteria from the rumen (41). Lactose-fermenting strains were found in *B. ruminale* (see *B. ruminale* R in Table 10). Kandler (*personal communication*) found that *B. thermophilum* and *B. ruminale* have the same murein type, with D-glutamic acid as the only constituent of the interpeptide bridge. The only distinguishing character is that *B. thermophilum* can grow at 46.5°C, whereas *B. ruminale* cannot. It is doubtful whether these two species are separate specific taxa, and they should be merged into a single species. The correct name of this species remains to be determined, both *B. thermophilum* and *B. ruminale* having been published in 1969.

***B. pseudolongum* and *B. globosum*.** As in the preceding case, high levels of genetic relatedness were detected between *B. pseudolongum* and *B. globosum*. There was 78 to 106% homology between our strains of *B. globosum* and strain RU230 as reference, and 69 to 73% with *B. pseudolongum* biotype *c*, strain Mo2-10 as reference. However, these data (Table 10) indicate that the genetic homology between these two species is not so great as that between *B. ruminale* and *B. thermophilum*. Nevertheless, *B. pseudolongum* and *B. globosum* have many common phenotypic traits. The characteristic shape (short to coccoid rods) of the cells of *B. globosum*, aptly described by the specific epithet [see photographs in Scardovi et al. (41)], appears also to be a trait of both *B. pseudolongum* and *B. longum* subsp. *animalis*.

B. globosum does not ferment pentoses. However, Crociani et al. (3) found that, among mannitol-fermenting bifidobacteria from the rumen, many isolates were genetically like *B. globosum* but fermented pentoses like *B.*

TABLE 10. Deoxyribonucleic acid homology relationships of *Bifidobacterium ruminale*, *B. globosum*, *B. thermophilum*, and *B. pseudolongum*^a

Competitor strains	Reference strains			
	RU326	RU230	P16-6	Mo2-10
<i>B. ruminale</i> strains				
RU326	100	30	71	27
RU263	86	15	74	23
RU268	88	14	74	8
RU279	62	0	69	2
RU290	70	50	72	6
RU356	85	0	77	15
RU381	83	19	84	12
RU417	42	30		46
RU431	98	15	79	10
RU445	96	17	84	23
(range)	62-98 ^b	0-50 ^b	69-84	2-27 ^b
<i>B. globosum</i> strains				
RU224 (type strain)	30	78	50	72
RU230	35	100	36	71
RU240	39	94	38	73
RU255	30	106	38	69
RU256	18	92	20	69
RU257	25	92	26	72
RU323	54	95	42	73
RU457	34	82	30	73
(range)	18-54	78-106	20-50	69-73
<i>B. ruminale</i> R strains				
RU276	64	5	70	0
RU348	64	10	65	5
RU354	70	5	70	5
<i>B. thermophilum</i> biotype c P16-6 ..	70	5	100	6
<i>B. pseudolongum</i> biotype d Mo2-10 .	13	70	20	100

^a See footnote to Table 3.

^b Value of strain R417, previously not identified, was omitted.

pseudolongum. Mitsuoka (27) did not record any pentose-negative strains of *B. pseudolongum*. Some strains of *B. pseudolongum* were isolated from sheep and bovine rumens (27), habitats where *B. globosum* proliferates. Kandler (*personal communication*) found the same murein type, L-Orn (or L-Lys)- (L-Ala)₃, in both species. The two species diverge, however, in their DNA base composition. In Mitsuoka's strains C10-45 (*B. longum* subsp. *animalis* biotype b), PNC-2-9G, 29SrT, and Mo2-10 (*B. pseudolongum* biotypes a, c and d, respectively) we found the values 59.7 ± 0.28 , 59.5 ± 0.36 , 59.9 ± 0.69 , and 61.1 ± 0.47 , respectively. In *B. globosum*, previously reported (41) as having a higher per cent GC, we confirmed the value of 63.80 ± 0.40 .

We feel, therefore, some reluctance to con-

clude that these two species are identical; it seems more advisable to investigate further their similarity at the macromolecular level before drawing definite conclusions.

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