***Bifidobacterium catenulatum*, *Bifidobacterium dentium*, and *Bifidobacterium angulatum*: Three New Species and Their Deoxyribonucleic Acid Homology Relationships**

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Previous studies by us and our colleagues suggested three distinctive deoxyribonucleic acid (DNA) homology groups among the bifidobacteria; these were provisionally named "*catenulatum*," "*dentium*," and "*angulatum*." One hundred eighty-four strains isolated from sewage, in addition to many of the strains from the previous study, were investigated and their DNA homology relationships were assessed using 23 reference systems. Strains in the *catenulatum* group were found not to differ significantly from those of *Bifidobacterium adolescentis* Reuter in their main physiological characters, such as sugars fermented and temperature, pH, and oxygen relationships; however, their DNA reciprocal homology is only some 50%, their guanine plus cytosine values were 54.7 ± 0.2 and 59.4 ± 0.4 mol %, respectively, and there were some morphological differences between them. The DNA of the *dentium* group has only about 45% homology with the DNA of *B. adolescentis* and is even less related to other members of the genus. The *dentium* strains can also be distinguished from other bifidobacteria by means of their sugar fermentations. The DNA of the *angulatum* group has little or no homology with that of any other bifidobacteria; the *angulatum* group also has a distinctive pattern of sugar fermentation and a unique morphology, resembling that of the coryneform bacteria. The three groups are named and described as new species of the genus *Bifidobacterium*: *B. catenulatum*, *B. dentium*, and *B. angulatum*. The type strains of these species are B669 (= ATCC 27539), B764 (= ATCC 27534), and B677 (= ATCC 27535), respectively. DNA-DNA homology relationships are basic to currently proposed species concepts, and data are presented confirming the reliability of critical experimental parameters influencing filter-bound DNA and thus the final relative homology values (e.g., temperature and time of incubation and annealing of DNA in the presence of homologous and heterologous competitive or nonspecific DNA, and the replicability of homology values using different homologous DNA preparations with single DNA competitor and reference DNA).

In a previous investigation (32), a large number of bifidobacteria displaying the general fermentation pattern of *Bifidobacterium adolescentis* Reuter were differentiated by means of deoxyribonucleic acid (DNA)-DNA hybridization into three groups, which were named "*catenulatum*," "*dentium*," and "*angulatum*." The *angulatum* group was well characterized by an unusual and constant "V" or angular cell disposition, suggesting the "snapping" type of cell division of the corynebacteria. The other two groups could not easily be distinguished phenotypically from *B. adolescentis*, although strains in the group named *catenulatum* commonly produced cells in chains, especially in

old cultures. However, only seven strains of *B. adolescentis* from Reuter's collection and another strain isolated by us from adult feces were available for study, and on the basis of DNA homology relatedness we could assign only 10 strains to the *catenulatum* group. The investigation of nucleic acid relatedness was later extended to over 300 strains of bifidobacteria selected from 480 isolates from sewage. Some of these strains were considered to be representative of new taxa, which will be described in a subsequent paper (30). Sixty-six strains were assigned to *B. adolescentis*, and 71 were assigned to the partially described *catenulatum* group; except for only 14 strains, all of

the others were assigned to known species of the genus *Bifidobacterium*. Furthermore, mannitol-fermenting bifidobacteria isolated from the rumens of cattle (31) and studied by Crociani et al. (5) for DNA relatedness (groups II and III of Crociani et al.) were reinvestigated thoroughly and were identified as belonging to *B. adolescentis*.

By using both DNA-DNA hybridization and physiological tests, the boundaries between the *catenulatum* and *dentium* groups and *B. adolescentis* became more easily discernible. The *catenulatum*, *dentium*, and *angulatum* groups are therefore described and named as new species of the genus *Bifidobacterium* Orla-Jensen.

TABLE 1. List of strains

Organisms	Isolated from	Culture received from
<i>Bifidobacterium adolescentis</i> Reuter		
E298b (ATCC 15705) biotype c of Reuter (27)	Feces of adult	G. Reuter
F2; F3; F5; F9; F10; F17; F21; F22; F23; F35	Sewage	Our collection
F41; F80; F108; F109; F110; F113; F119; F123; F147; F152	Sewage	Our collection
F159; F160; F161; <u>F167</u> ^a ; F200; F250; F253; F254; F268; F272	Sewage	Our collection
F275; <u>F283</u> ; F302; F305; F311; F318; F328; F332; F337; F339	Sewage	Our collection
F349; F351; F352; F353; F354; F359; F361; F363; F365; F366	Sewage	Our collection
F367; F369; F374; F377; F410; F420; F455; F470; F471	Sewage	Our collection
RU424 (reference strain of mannitol-fermenting group) (5)	Rumen of cattle	Our collection
<i>Bifidobacterium catenulatum</i> sp. nov. ^b		
B669 (Type strain) (=ATCC 27539)	Feces of adult	Our collection
F7; F11; F18; F19; F30; F44; F54; F60; F64; F67	Sewage	Our collection
F71; F72; F81; F90; <u>F94</u> ; F99; F101; F141; F142; F144	Sewage	Our collection
F163; F165; F166; F171; F187; F189; F205; F209; F216; F217	Sewage	Our collection
F223; F240; F257; F258; F260; F261; F262; <u>F276</u> ; F278; F286	Sewage	Our collection
F290; F291; F292; F293; F294; F297; <u>F320</u> ; <u>F322</u> ; F324; F331	Sewage	Our collection
F335; F336; F344; F356; F357; <u>F360</u> ; F364; F400; F412; F422	Sewage	Our collection
F428; F429; F433; F434; F435; <u>F457</u> ; F469	Sewage	Our collection
<i>Bifidobacterium dentium</i> sp. nov.		
B764 (Type strain) (=ATCC 27534)	Dental caries	Our collection
B735; B767; B781; B786; B973; B975; B976; B977; B1000; B1001	Dental caries	Our collection
B1010; B1013; B1020; B1022; B1023; B1029; B1031; B1033; B1036	Dental caries	Our collection
B1038; B1044; B1046; B1049; B1051; B1053; B1056; B1064; B1067	Dental caries	Our collection
B749; B750; B752; B754; <u>B757</u> ; B758; B760	Feces of adult	Our collection
B788; B792; B872; B932	Human vagina	Our collection
B96; B127	Oral cavity	H. Beerens
3859 <i>B. appendicitis</i> (Prévot Collection)	Human appendix	T. Mitsuoka
<i>Bifidobacterium angulatum</i> sp. nov.		
B677 (Type strain) (=ATCC 27535)	Feces of adult	Our collection
F158; F196; F406; F424; F426	Sewage	Our collection
<i>Bifidobacterium bifidum</i> (Tissier) Orla-Jensen S28a (=ATCC 15696) (26)	Feces of infant	G. Reuter
<i>Bifidobacterium breve</i> Reuter S1 (=ATCC 15700) (Type strain) (27)	Feces of infant	G. Reuter
<i>Bifidobacterium liberorum</i> Reuter S76e (=ATCC 15702) (Type strain) (27)	Feces of infant	G. Reuter
<i>Bifidobacterium longum</i> Reuter E194b (=ATCC 15707) (Type strain) (27)	Feces of adult	G. Reuter
<i>Bifidobacterium ruminale</i> Scardovi et al. RU326 (=ATCC 25866) (Type strain) (31)	Rumen of cattle	Our collection
<i>Bifidobacterium globosum</i> Scardovi et al. RU230 (=ATCC 25864) (Type strain) (31)	Rumen of cattle	Our collection
<i>Bifidobacterium suis</i> Matteuzzi et al. SU859 (=ATCC 27533) (Type strain) (20)	Feces of pig	Our collection

^a Underlined strains were used as additional reference systems in DNA-DNA hybridization.

^b The strains of *B. catenulatum* studied previously (32) with DNA hybridization were B660, B661, B676, B684 (from feces of man), and B933 (from human vagina).

TABLE 2. Release of filter-bound DNA after incubation at different temperatures in 0.25 ml of Denhardt mixture in 2 × SSC: DNA from *B. ruminale* RU326^a

Incubation temp (C)	DNA remaining on filter (%)			
	After 6 h preincubation	After additional 12 h as for annealing	After 18 h incubation in presence of:	
			A ^b	B ^c
64.0	76.5 ± 1.2	64.3 ± 1.3	52.3 ± 0.9	55.8 ± 2.4
69.0	66.3 ± 2.7	54.9 ± 2.4		
74.0	61.9 ± 1.9	38.4 ± 0.6		
79.0	38.6 ± 2.1	29.1 ± 0.3		

^a All experiments were made in quadruplicate.^b A = 150 µg of homologous sheared denatured DNA.^c B = 150 µg of heterologous sheared denatured DNA (*B. globosum* RU230).

MATERIALS AND METHODS

Bacterial strains. The strains employed, together with their sources, are listed in Table 1. Many were cited in a previous paper (32; see Table 3) as *B. adolescentis*, but most of the strains from sewage were of recent isolation. All newly acquired strains, like those previously studied, had a functional fructose-6-phosphate phosphoketolase (F6PPK) and produced acetic and lactic acids but not gas from glucose; furthermore, they all consisted of anaerobic, non-motile, gram-positive, nonsporeforming, irregularly shaped cells.

DNA preparation, filter immobilization, and duplex formation. The general procedures for growing and harvesting cells, for purifying DNA and subsequently preparing filters, for calculating homology, etc., have been previously described (32). Some DNA preparations were unsuitable as competitors, and bad filters were obtained from others, generally whenever the DNA was collected by centrifugation during the process of purification because collection by "spooling" was meager or irregular. In these cases, new batches of cells were processed until the spooling of DNA threads on stirring rods was satisfactory, and eventually filters were prepared therefrom and used as additional reference systems (see Table 1). In a few cases, DNA was prepared by the combined methods of Marmur (17), Kirby (13), and Kirby et al. (14), as suggested by De Ley et al. (6); suitable DNA was obtained, but, probably on account of the many sophisticated steps involved, its recovery was rather low. In general, this procedure seems unsuitable for routine work when many batches of cells are processed daily. In any case, the filters obtained with DNA prepared with this procedure were not superior for their binding capacities to those prepared with DNA obtained by the carefully performed Marmur procedure alone (17).

Reliability of the method. Annealing in 1 × SSC (18) was conducted at 69 C, i.e., 25 C below the T_m of the reference DNAs (near 94 C for most bifidobacteria). It is claimed that high annealing temperatures give erroneous results in DNA homology

TABLE 3. Amounts of ¹⁴C-DNA fixed in the absence of, and in the presence of, graded amounts of homologous competitor from *B. globosum* RU230^a

Presence of competitor	¹⁴ C-DNA activity fixed (counts/min)
1. Without competitor	1,150
2. With competitor:	
50 µg	700
80 µg	540
100 µg	425
150 µg	370
200 µg	365
3. Without competitor but in 1.5 ml of 3 × SSC + DMSO 45% (dimethylsulfoxide method, [15])	330
4. As in no. 3 with threefold amount of ¹⁴ C-DNA	670

^a Input, 3,600 counts/min; specific activity of ¹⁴C-DNA, 1,125 counts per min per g. After 6 h of preincubation and 12 h of incubation for annealing at 69 C in 0.23 ml of Denhardt mixture.

determinations mainly because of an excessive spontaneous release of DNA bound to filters (2, 6, 23). Filters loaded with 14 µg of ¹⁴C-labeled DNA of *Bifidobacterium ruminale* RU326 averaged 13,260 counts/min. Sets of filters were incubated under the same conditions as in our duplexing experiments but at different temperatures. A release of filter-bound DNA occurs under the conditions used in the annealing experiments (60 C), but its magnitude, if the figures reported by De Ley and Tijtgat (7) are compared, is apparently maintained within a permissible range (Table 2). Okanishi and Gregory (23) recently observed that leaching of filter-bound DNA (from streptomycetes) can be greatly accentuated by the presence of sheared denatured DNA in solution. We tested this effect under our routine conditions using 150 µg of either homologous or heterologous

TABLE 4. Effects of the input of ^{14}C -DNA on binding in the absence of competitor and on homology values with 150 μg of DNA competitor from *B. animalis* P23 (30)^a

Amt of ^{14}C -DNA	Total activity (counts/min)	^{14}C -DNA fixed (%)	Homology values (with 150 μg of competitor DNA)
1.0 μg of ^{14}C -DNA	1,440	30.0	50
1.5 μg of ^{14}C -DNA	2,160	27.3	43
3.0 μg of ^{14}C -DNA	4,320	25.6	43
6.0 μg of ^{14}C -DNA	8,640	25.8	38

^a Reference system: *B. globosum* RU230.

competitor DNA, but there was no increased leaching (Table 2).

The amount of competitor DNA (150 μg) used in the single-point competition procedure adopted is sufficient to reach the saturation plateau (Table 3). The method of Legault-Démare et al. (15), in which dimethylsulfoxide (DMSO) is added to the hybridization mixture, has been advocated as giving the most reliable results (7). We observed that, probably on account of the large volume of the annealing mixture used (1.5 ml), much larger amounts of labeled DNA are necessary for obtaining the levels of DNA fixed in the Denhardt-Johnson procedure (see last figures of Table 3). Furthermore, from the data reported by De Ley and Tijtgat (see Fig. 6 and 8 of reference 7), 400 to 700 μg of competitor DNA should be used per competition experiment. The use of such large amounts of DNA is impractical whenever, as in the case in point, the same DNA preparation is used in an unpredictable number of competition experiments with more than one reference system.

In general, we recommend the use of low amounts of labeled DNA both in direct and competition annealing reactions (some 1 to 2 μg) with respect to the amount of the reference DNA fixed to the filter. Because, however, of sizeable differences in DNA labeling from one batch of cells to another (variations from 700 to 2,000 counts/min per μg of ^{14}C -DNA are common under our conditions), we preferred to adjust the amount of labeled DNA to initial inputs of 3,000 to 4,500 counts/min, producing confidence on fixation levels of nearly 30%. Variations in the amounts of ^{14}C -DNA, such as those reported in Table 4, do not seem to affect the extent of the fixation (in the absence of competitor) nor the homology levels measured in the presence of 150 μg of heterologous competitor; some deviation can be expected at low initial inputs of ^{14}C -DNA (see Table 4). In certain cases, DNA competed slightly or not at all, irrespective of the reference systems used. These bad preparations were generally obtained by centrifugation instead of by spooling with a glass rod. Undue variability in the competitive effectiveness of different preparations from the same strain could not be excluded, however, since 40 to 50% of filter-fixed DNA is lost during incubation at 69 C. We assessed, therefore, the overall reproducibility of the competition method of determining homology by testing the relatedness to the same reference system of homologous and heterol-

TABLE 5. Homology values obtained with different preparations of DNA of *B. globosum* (RU230) and *B. animalis* (P23) (see 30) used as competitor in the same reference system (RU230)

DNA competitor	Percent homology to reference DNA
Strain RU230 prepn:	
no. 1	100 ^a
no. 2	92.2 $\pm$ 1.5
no. 3	93.5 $\pm$ 0.7
no. 4	95.8 $\pm$ 0.6
no. 5	102.1 $\pm$ 2.1
no. 6	92.0 $\pm$ 1.3
no. 7	93.2 $\pm$ 0.7
no. 8	98.2 $\pm$ 1.1
Strain P23 prepn:	
no. 1	40.7 $\pm$ 1.5
no. 2	43.7 $\pm$ 3.6
no. 3	33.2 $\pm$ 4.5
no. 4	38.7 $\pm$ 1.7
no. 5	39.7 $\pm$ 1.2
no. 6	37.2 $\pm$ 2.5
no. 7	32.7 $\pm$ 2.2

^a The value of 100% homology was calculated from the figures obtained with the DNA used for the preparation of filters. All experiments were made in quadruplicate.

ogous competitor DNAs obtained from different batches of cells of the same strains (Table 5). Provided that single filters were derived from the same mother filter, the percentage of DNA relatedness was not seriously influenced by the competitor DNA coming from different batches of cells.

DNA base composition. The thermal melting point (T_m) of DNA dissolved in 1 $\times$ SSC was determined using a T_m Analyzer (Beckman Instruments Inc.) and the mol % of guanine plus cytosine (G + C) was calculated by the formula of Marmur and Doty (19). Ethanol-dehydrated DNA fibers were routinely spooled onto glass rods and preserved in vacuum over CaCl_2 at 4 C.

Physiological characters. Trypticase (BBL)-phytone (BBL)-yeast extract (Difco)-glucose (TPY) medium

of the composition already reported (32) was used throughout this study. Bromocresol purple (30 mg/liter) was used as indicator. Substrates (0.5% wt/vol) in the fermentation tests were autoclaved in the basal medium except for pentoses, which were sterilized by filtration and added aseptically. Growth and color changes were recorded after 8 to 10 days of incubation in anaerobic jars at 39 C.

Oxygen tolerance and CO₂ requirements were determined from the growth characteristics of agar stabs and slant cultures incubated in air in closed jars to prevent evaporation, in 10% CO₂ plus 90% N₂, and in 10% CO₂ plus 90% air. For comparative purposes only, organisms were considered to be "normally" or "highly" anaerobic if the oxygen inhibition zones were less than or greater than 5 mm in depth, respectively, from the surfaces of agar stabs.

Temperature relations were ascertained using the temperature gradient incubator of Oppenheimer and Drost-Hansen (24).

Acidic products from the fermentation of glucose in 7-day-old cultures were determined. Titrations of the original broth and steam distillate were calculated as total acidity and acetic acid, respectively. Proteins were precipitated with ZnSO₄-NaOH, and the cleared supernatant was then steam-distilled. Lactic acid production and the configuration of the lactic acid were determined from analyses of ethyl ether extracts using the enzymatic procedure and products of Boehringer (Biochemia, Milano). The presence of propionic and butyric acids in steam-distilled fractions was tested for by means of gas chromatography using a Perkin-Elmer model F11 instrument equipped with hydrogen flame detector. A stainless steel column (2 m by 0.3 cm) was packed with Trimer acid (Company source) and dinonylnaphthalenedisulfonic acid (10/0.4, wt/wt) coated on chromosorb W (60/80 mesh). The temperature of the injection block was 280 C, and the column temperature was programmed at 5 C per min from 80 to 160 C. Volatile acids present in the steam distillate were dissolved in 5% formic acid before injection (4). Nitrogen was the carrier gas at a flow rate of 25 ml/min.

Other physiological tests were as follows: ammonia from arginine or asparagine (0.3% wt/vol) added to TPY medium, as described by Gasser (11); urease production by incubation of a 1-ml cell suspension in phosphate buffer (0.1 N, pH 7) containing 25 µmol of urea and subsequent nesslerization; nitrate reduction in a modified TPY medium containing KNO₃ and glucose (each 0.1% wt/vol), as detected by the sulfanilic-naphthylamine reagent; and indole and acetylmethylcarbinol production by the usual tests (3).

Enzymology. Cells were mechanically disrupted by shaking with glass beads in a Nossal apparatus. The supernatant cell extracts, containing 6 to 12 mg of protein per ml, were assayed for aldolase by the procedure of Sibley and Lehninger (35) as modified by Dounce et al. (10), and for F6PPK as described by Schramm et al. (34). The glucose-6-phosphate dehydrogenase (G6PD) assay system contained the following: 2.6 ml of glycyl glycine buffer, 0.1 M, pH 7.5; 0.20 ml of MgCl₂, 0.1 M; 0.1 ml of G6P sodium salt,

0.3 M; 0.10 ml of nicotinamide adenine dinucleotide phosphate (NADP) sodium salt, 0.03 M; and 0.01 ml of cell extract; the final volume was 3 ml. The 6-phosphogluconate dehydrogenase (6PGD) assay system contained the following: 2.65 ml of glycyl glycine buffer, 0.1 M, pH 7.6; 0.10 ml of gluconate-6-P, cyclohexammonium salt, 0.3 M; 0.10 ml of MgCl₂, 0.1 M; 0.10 ml of NADP sodium salt, 0.03 M; and 0.05 ml of cell extract; final volume, 3 ml. The G6PD and 6PGD activities were detected spectrophotometrically by change of absorbance at 340 nm. Starch-gel electrophoresis of F6PPK was performed as previously described (29); crude cell-free extracts of *B. bifidum* S28a served as the "human" and *B. globosum* RU230 as the "animal" reference type of bifidobacterial F6PPK. Proteins were determined by the method of Lowry et al. (16). Assay compounds were obtained from Boehringer, Mannheim, Germany.

RESULTS

B. adolescentis homology group. Strains of the "catenulatum," "dentium," and "angulatum" groups exhibited fermentation patterns similar to those of biotypes *b* and *c* of *B. adolescentis* Reuter (26). The "angulatum" group was recognizable by its characteristic coryneform-like morphology, whereas the other two groups were, on a phenotypic basis, hardly distinguishable from *B. adolescentis*.

The study of a large number of strains forming a homogenous DNA homology group with the type strain of *B. adolescentis* was essential to an understanding of the relationships of the "catenulatum," "dentium," and "angulatum" groups to the previously described species of *Bifidobacterium*. Such strains were numerous among the isolates from sewage. Table 6 contains data on the DNA relatedness of 59 of these strains to five reference systems: (i) *B. adolescentis* Reuter E298b; (ii) B669, our reference strain for the "catenulatum" group; (iii) B764, our reference strain for the "dentium" group; (iv) B677, our reference strain for the "angulatum" group; and (v) strain RU424 (now known to be indistinct from strains of *B. adolescentis*) as representative of the mannitol-fermenting bifidobacteria isolated from the rumens of cattle (31).

In a previous study (5), the DNAs from many mannitol-fermenting bifidobacteria from rumens displayed 80 to 100% homology with the DNA from strain RU424, although there were some differences in the sugars fermented (see group II and group III, p. 102, reference 5). In further DNA-DNA homology experiments, strain RU424 was highly related to some bifidobacteria isolated from human feces and having the fermentation pattern of *B. adoles-*

centis. The present data confirm previous results and establish the DNA relatedness of strain RU424 to *B. adolescentis* Reuter. Furthermore, the cell wall peptidoglycan of strain RU424 contains an ornithine-(lysine)-aspartate peptide typical of that of *B. adolescentis* and quite different from those of rumen strains of *B. globosum* and *B. ruminale* (33). The DNA homogeneity of the *B. adolescentis* group was further demonstrated with many infragroup DNA-DNA hybridizations made with the additional reference systems F167, F283, F354, and F420, the data on which are not reported here. The relatedness levels of the DNAs of strains of *B. adolescentis* to the DNA reference from *B. adolescentis* strain E298b (Table 6) conclusively show the correctness of using this reference DNA to define the boundaries between our new groups and *B. adolescentis* Reuter. Among the homology values of the DNAs of the *adolescentis* group to the reference strain RU424, there are high values, such as 110 to 112%; other similar results are reported in Table 7. Homology values for each group were obtained from many hybridization experiments in which different DNA preparations were often used as competitors in the reference system; variability in the quality of

the reference DNAs may account for such apparently atypical results.

Catenulatum homology group. Among 320 bifidobacteria from sewage, 64 strains from 36 specimens displayed high DNA relatedness to strain B669, earlier selected as the reference strain of the *catenulatum* group. Strains F71, F81, F94, and F99 had been studied previously (30). The data in Table 8 indicate that this important group of strains is a homogeneous assemblage whose DNA relatedness relative to other DNA reference systems distinguishes it quite sharply from *B. adolescentis*, *B. longum*, and the *dentium* and *angulatum* groups. Some strains, like F99, F240, and F257, were included in the *catenulatum* group because their DNAs were clearly unrelated to the DNAs from the other groups. In some runs there was only some 65% homology to the reference strain B669 (see Table 8); however, if additional DNA preparations of presumed better quality were used as competitors or references (filters), these low DNA homologies were often increased to values approaching 100%. For practical reasons, however, this was done only in a limited number of cases (see underlined strains of Table 1).

Dentium homology group. Forty strains in

TABLE 6. DNA relatedness of the *adolescentis* group to reference DNAs from other groups or types

Competitor strains	Percent homology to reference DNA from:				
	<i>B. adolescentis</i> E298b	Mannitol-fermenting from rumen <i>B. adolescentis</i> RU424	<i>B. catenulatum</i> B669 (Type strain)	<i>B. dentium</i> B764 (Type strain)	<i>B. angulatum</i> B677 (Type strain)
E298b <i>B. adolescentis</i>	100	95	40	32	30
F2; F3; F5; F9	90-88-83-83	*-107-105-100 ^a	25-30-34-*	30-36-34-*	30-38-35-32
F10; F17; F21; F22	92-85-94-88	*-100-111-112	20-26-38-38	46-23-31-32	44-33-38-36
F23; F35; F41; F80	83-100-85-98	100-86-92-*	34-40-32-33	30-25-35-30	32-25-35
F108; F109; F110; F113	92-102-92-90	87-97-100-87	44-48-38	30-31-35	25-25-28
F119; F123; F147; F152	85-82-90-98	81-76-95-100	24-40-39-41	24-32-35-*	26-28-30-*
F159; F160; F161; F167	80-102-98-78	75-94-*	32-36-56	*-40	*-32
F200; F250; F253; F254	78-81-80-77	71-103-92-85	42-34-34-41	*-49-44-32	*-30-37-*
F268; F272; F275; F283	74-75-82-95	*-80-100	45-40-30-25	*-*	*-*
F302; F305; F311; F318	85-95-86-75	108-87-109-76	40-50-57-52	42-40-*	36-40-*
F328; F332; F337; F339	82-80-75-75	73-70-87-73	40-30-47-27	*-*	*-*
F349; F351; F352; F353	95-72-75-102	100-86-100	52-27-45-28	*-*	*-*
F354; F359; F361; F363	90-80-70-80	94-94-87-75	30-50-40-40	24-30-25	26-25-32
F365; F366; F367; F369	75-77-78-70	88-96-96	54-40-41-40	*-32	*-25
F374; F377; F410; F420	80-100-85-95	83-80-98	37-30-35-30	30-34-23	31-25
F455; F470; F471	83-70-95	92-80-98	30-40-30	25-25	26-20
Range	70-102	71-112	20-57	24-49	20-44

^a Asterisk indicates not tested.

TABLE 7. DNA relatedness of the *dentium* group to reference DNAs from other groups and *B. longum*

Competitor strains	Percent homology to reference DNA from:				
	<i>B. dentium</i> B764 (Type strain)	<i>B. adolescentis</i> E298b	<i>B. catenulatum</i> B669 (Type strain)	<i>B. angulatum</i> B677 (Type strain)	<i>B. longum</i> E194b (Type strain)
B764 (Type of strain) <i>B. dentium</i>	100	25	20	5	7
From feces of adult: B749; B750; B751; B752 B754; B757; B758; B760	69-78-96-86 84-92-69-85	*- *- *- * ^a *-35- *- *	*- *- *- * *-27- *- *	*- *- *- * *-10- *- *	6- 6-00- * 00- 5- *- *
From human vagina B788; B792; B872; B932	81-77-83-105	30-24- *-28	25-37- *-18	*- *- *- *	13- *- *- *
From dental caries B735; B767; B781; B786 B973; B975; B976; B977 B1000; B1001; B1010; B1013 B1020; B1022; B1023; B1029 B1031; B1033; B1036; B1038 B1044; B1046; B1049; B1051 B1053; B1056; B1064; B1067 B96; B127 (Beerens strains) 3859 (<i>B. appendicitis</i>)	104-101-88-110 89-95-92-99 102-83-97-103 99-85-75-90 99-108-88-106 95-101-100-99 83-96-92-98 92-81 83	*- *- *- * 35-33-35- * 45-31-41-20 30-28-28-17 17-26-28-57 25-42-44-30 37-23- *- * 30-28 25	*- *- *- * 30-28-32- * 20-25-21-19 32-24-30-20 16-25-31-45 21-35-32-24 32-20- *- * 30-25 19	*- *- *- * 15-12-14- * 8-13-15-10 20-15-15-12 9-12-16-26 14-25-18-12 15-8- *- * 10-12 *	*- *- *- * 10- 5- 8- * 2-10- 6-00 12-12- 8- 5 00- 4- 9-12 8-13-10- 4 6-00- *- * 10-10 7
Range	69-110	15-57	16-45	5-26	0-13

^a Asterisk indicates not tested.TABLE 8. DNA relatedness of the *catenulatum* group to references from other groups and *B. longum*

Competitor strains	Percent homology to reference DNA from:				
	<i>B. catenulatum</i> B669 (Type strain)	<i>B. adolescentis</i> E298b	<i>B. dentium</i> B764 (Type strain)	<i>B. angulatum</i> B677 (Type strain)	<i>B. longum</i> E194b (Type strain)
B669 (Type strain of <i>B. catenulatum</i>)	100	40	32	22	20
F7; F11; F18; F19 F30; F44; F54; F60 F64; F67; F71; F72 F81; F86; F90; F94 F99; F101; F141; F142 F144; F163; F165; F166 F171; F187; F189; F205 F209; F216; F217; F223 F240; F257; F258; F260 F261; F262; F276; F278 F286; F290; F291; F292 F293; F294; F297; F320 F322; F324; F331; F335 F336; F344; F356; F357 F360; F364; F400; F412 F422; F428; F429; F433 F434; F435; F457; F469	84-93-83-88 93-82-74-95 85-92-73-70 68-83-81-78 65-83-92-71 100-100-85-93 82-81-98-83 83-70-75-104 98-66-83-71 78-75-104-82 78-83-82-90 87-90-86-84 87-78-74-92 74-81-72-100 92-67-90-67 68-84-93-75 76-81-71-72	25-56-39-57 50-30-44-42 40-22- *-40 *40-48-42 *56-32-40 57-56-57-57 30-47-50-40 53-30-30-54 35-40-27-38 37-48-45- * 30-35-25- * *36-45-55 *51-40-50 *- *-38-36 28-50-45-43 40- *- *-44 *44-50-39	20-30-36-37 37-41-37-30 32-25-28-14 34-42-35-33 21-36-29- * 42-48-30- * *- *-40-35 24-20-14-34 3- *- *-25 *- *-30- * *- *- *-20 18- *- *-33 *- *-25- * 15- *- *-20- * 20- *-25- * *- *-30-20 20-30-30-25	15- *-2-28 ^a 18-23- *- * *- *-33- * 33-20-20-20 *- *-36- * 32-27-32-37 *- *- *-20 *- *- *-38 17- *- *-10 *- *-20- * 12- *- *-12 15- *- *-14 *- *-12- * 10- *- *-13 10- *-20- * *- *-12-8 *- *-10-18	*- *-16-17 13- 2- *-15 *- *-15- * *- *-10-15 *- *-10- * 00-20- 3-4 *- *- *- * *- *- *-00 5- 5- *- * 14-00- 4- * 13-13- *-6 8- *-26 *- *-00- * 4- *- *-36 8-00-10- * 3- *- *-10 *- *-14-20
Range	65-104	22-57	3-48	2-37	0-26

^a Asterisk indicates not tested.

the *dentium* group were previously studied (32). Additional strains studied here are B96 and B127, isolated by Beerens from the oral cavity, and 3859, labeled *Bifidobacterium appendicitis* Prévot, received from T. Mitsuoka. The results of the study confirm previous findings (32) that this group is a separate entity. Strains from the human oral cavity previously studied by Beerens et al. (1) are homogeneous with our *dentium* group of strains. It is of interest to note that the strain labeled *B. appendicitis* 3859 from Prévot's collection also belongs to this group. However, another strain of Prévot's collection, *B. appendicitis* 2727B, has the fermentation pattern of *B. adolescentis* Reuter biotype *c*, and its DNA is fully related to the DNA of *B. adolescentis*. The general characters of these two strains of Prévot's collection bearing the label *B. appendicitis* are not those reported by Prévot (25) (Scardovi and co-workers, unpublished data). Strains belonging to the *dentium* group were never isolated from sewage.

Angulatum homology group. The DNA homology relationships of the strains in this group

are reported in Table 9. This study confirmed previously reported results on these strains (32) and was extended to include *B. adolescentis* and *B. longum*. Unfortunately, we were unable to find new strains of the *angulatum* group among the recent isolates from sewage or other habitats, but the six strains studied had an unusual coryneform-like morphology, and their DNA behaved distinctively in having little or no homology with the DNAs of other bifidobacteria.

Relatedness of the catenulatum, dentium, and angulatum groups to the named species of Bifidobacterium. DNA homologies between type strains of the novel groups and type strains of the known species of the genus *Bifidobacterium* (except those isolated from the intestine of bees) were investigated. The reference DNAs were from: *B. bifidum* strain S28a; *B. liberorum* S76e for the group *liberorum-infantis-lactentis*; *B. breve* S1 for the group *breve-parvulorum*; *B. globosum* RU230 for the group *globosum-pseudolongum*, and *B. ruminale* RU326 as representative of the *ruminale-thermophilum* group (32); *B. longum* E194b; and

TABLE 9. DNA relatedness of the angulatum group to reference DNAs from other groups and *B. longum*

Competitors strains	Percent homology to reference DNA from:				
	<i>B. angulatum</i> B677 (Type strain)	<i>B. catenulatum</i> B669 (Type strain)	<i>B. dentium</i> B764 (Type strain)	<i>B. adolescentis</i> E298b	<i>B. longum</i> E194b (Type strain)
B677 (Type strain of <i>B. angulatum</i>)	100	20	15	25	8
F158; F196; F406 F424; F426	95-90-80 76-83	30-35-35 25-20	15-8-10 15-20	25-30-28 22-20	13-6-8 12-10
Range	76-95	20-35	8-20	20-30	6-13

TABLE 10. DNA relatedness of type strains of the new species to type strains of the known species of the genus *Bifidobacterium* used as references

Competitor strains	Percent homology to reference DNA from:						
	<i>B. bifidum</i> S28a	<i>B. liberorum</i> S76e (Type strain)	<i>B. breve</i> S1 (Type strain)	<i>B. longum</i> E194b (Type strain)	<i>B. globosum</i> RU230 (Type strain)	<i>B. ruminale</i> RU326 (Type strain)	<i>B. suis</i> SU859 (Type strain)
B669 (Type strain of <i>B. catenulatum</i>)	20	15	10	12	5	20	25
B764 (Type strain of <i>B. dentium</i>)	5	20	12	7	12	20	20
B677 (Type strain of <i>B. angulatum</i>)	7	20	10	8	20	18	20

B. suis SU859. The type strains of the proposed three new species share only a small number of polynucleotide sequences with DNA of the reference strains used (Table 10).

Distinctive phenotypic characters of the groups. (i) Morphology. The general morphology of the cells of the three groups and their arrangement are given in Plates 1, 2, and 3.

PLATE 1. Morphology of *Bifidobacterium catenulatum* sp. n. Fig. 1 and 2. Cells of the type strain (B669) from fresh anaerobic stabs. Fig. 3. Cells of strain B669 from a broth culture (DNA extraction). Fig. 4. Cells of strain B660 from an old anaerobic stab. Fig. 5 to 15. Cells from 2-day-old anaerobic stabs of strains B684, F60, F71, F81, F99, F141, F141, F335, F428, and F429, respectively. Phase contrast. $\times 1,500$. Fig. 16. Surface colonies of strain B669 in a slant culture incubated anaerobically under carbon dioxide. Fig. 17. Same as Fig. 16, side view.

PLATE 2. Morphology of *Bifidobacterium dentium* sp. n. Fig. 1 and 2. Cells of the type strain (B764) from a fresh anaerobic stab and from a broth culture (DNA extraction). Fig. 3. Cells of strain B975 from a broth culture (DNA extraction). Fig. 4 to 7. Cells of strains B1000, B1001, B1022, and B1029, respectively, from fresh anaerobic stabs. Fig. 8 to 13. Cells of strains B1031, B1033, B1036, B1049, B1051, and B1038, respectively, from 4-day-old anaerobic stabs. Fig. 14 and 15. Cells of strain B96 (Beerens) and B127 (Beerens), respectively, from fresh stabs. Phase contrast. $\times 1,500$. Fig. 16 and 17. Surface colonies in slants incubated anaerobically under carbon dioxide.

Arrangement in irregular chains, especially in the first transfers or in aged cultures (4 to 5 days) is characteristic of the cells of the *catenulatum* group. The cells of *angulatum*

strains can be promptly recognized from their "V-" or "palisade-" like disposition, which has never been observed by us among some thousands of other bifidobacteria. The mor-

PLATE 3. Morphology of *Bifidobacterium angulatum* sp. n. Fig. 1 and 2. Cells of the type strain (B677) from fresh anaerobic stabs. Fig. 3. Cells of strain B677 from an 8-day-old broth culture. Fig. 4 and 5. Cells of strain F158 from broth cultures with glucose and gluconate, respectively. Fig. 6. Cells of strain F196 from a 24-h-old broth culture used for DNA extraction. Fig. 7 and 8. Cells of strain F424 from fresh anaerobic stabs. Fig. 9. Cells of strain F426 from an old anaerobic stab. Phase contrast. $\times 1,500$. Fig. 10 and 11. Surface colonies in slants incubated anaerobically under carbon dioxide.

phology of *dentium* cells is not characteristic; it is in general very similar to that of *B. longum* (our unpublished data).

(ii) **Fermentation patterns.** The sugars which

are fermented by the groups are reported in Table 11. Group *catenulatum* cannot be distinguished on this basis from *B. adolescentis* biotype *c* of Reuter (26): most strains ferment

TABLE 11. Fermentation patterns of *B. catenulatum*, *B. dentium*, and *B. angulatum* and of their type strains^a

Compounds	<i>B. catenulatum</i>		<i>B. dentium</i>		<i>B. angulatum</i>	
	68 strains	Type strain (ATCC 27539)	44 strains	Type strain (ATCC 27534)	6 strains	Type strain (ATCC 27535)
D-Xylose	+ ^b (66/68) ^c	+	+	+	+	+
D-Ribose	+ ^d	+	+	—	+	+
D-Mannose	— (44/68) ^e	—	+	+	— (6/6)	—
D-Cellobiose	+ (66/68) ^f	+	+	—	— (5/6) ^f	—
D-Trehalose	+ (43/68) ^g	—	+	+	— (6/6)	—
Melezitose	— (65/68) ^h	—	+	+	— (6/6)	—
Dextrin	+ (36/68)	+	+	+	+	+
Starch	+ (35/68) ⁱ	+	+	+	+	+
Inulin	+ (35/68)	—	—	+	+	+
Mannitol	— (57/68) ^j	—	+	+	— (6/6)	—
Sorbitol	+ (66/68) ^k	+	—	+	— (5/6) ^q	—
Gluconate	+ (52/68) ^l	+	+	+	— (4/6) ^r	—

^a All strains fermented arabinose, glucose, fructose, galactose, sucrose, maltose, lactose, melibiose, raffinose, and salicin. None fermented glycerol, rhamnose, or lactate.

^b +, fermented; —, not fermented.

^c F99 and F94 were negative. The type strain (ATCC 27539) occasionally fermented arabinose and xylose slowly; this behavior was observed in *B. catenulatum* strains isolated from human feces and vagina (see Table 1).

^d Only the strains from human feces and vagina were tested (see Table 1).

^e F19, F30, F54, F72, F86, F90, F101, F142, F163, F166, F209, F217, F223, F240, F257, F258, F260, F322, F331, F356, F400, F434, and F435 were negative.

^f F262 and F508 were negative.

^g F30, F141, F163, F187, F189, F205, F216, F257, F262, F286, F291, F292, F293, F297, F320, F324, F344, F364, F422, F428, F433, F434, F457, F469, and B669 (type strain) were negative.

^h F322, F331, and F435 were positive.

ⁱ Twenty strains fermented these three carbohydrates; 15 strains did not ferment any; 17 strains fermented starch and dextrin but not inulin; 16 strains fermented only inulin.

^j F19, F71, F90, F99, F189, F209, F262, F293, F336, F364, and F469 were positive.

^k F336 and F457 were negative. ^l F18, F54, F72, F86, F101, F187, F257, F262, F322, F331, F336, F400, F434, F435, F457, and F469 were negative.

^m B781 and B1000 were negative.

ⁿ B788 and B1000 were negative.

^o B767 was positive.

^p F406 was positive.

^q F406 was positive.

^r F158 and F196 were positive.

sorbitol and not mannitol. The data obtained by W.E.C. Moore (personal communication) with 20 strains of the group and with the following additional compounds—adonitol, amygdalin, dulcitol, erythritol, esculin, glycogen, inositol, and sorbose—were of no help in distinguishing this group from *B. adolescentis*. The *dentium* strains, including strains B96 and B127 from H. Beerens, had a more uniform pattern, corresponding to that of *B. adolescentis* biotype *b* (26): they fermented mannitol but not sorbitol. *B. appendicitis* strain B859 fermented sorbitol slowly. However, in contrast with the strains of *B. adolescentis* biotype *b*, the majority of the *dentium* strains fermented melezitose. The fermentation pattern of the *angulatum* strains is like that of strains of biotype *b* of *B. adolescentis* (26).

Physiological and biochemical characters.

Very few differences in the physiological and biochemical characters of the groups, reported in the detailed descriptions below, are apparent: *dentium* strains can develop at somewhat lower temperatures (24 C instead of 28 to 29 C); *angulatum* strains are more sensitive to free oxygen, and their anaerobic growth is highly stimulated by the presence of CO₂. However, a difference of more weight was revealed in the DNA base composition: the G + C values of strains of the *catenulatum* group, which range from 54.2 to 55.7 mol %, are significantly lower than those found in most bifidobacteria (59 to 61 mol %).

Because DNA homology was taken as a guideline in our studies on the speciation of the genus *Bifidobacterium*, the data presented here

concerning the DNA homology relationships between our three groups of strains and the previously described species of the genus constitute the principal argument on which our proposal of recognizing these groups as new species is based. Morphology and DNA base composition provide additional distinctive characters of importance for the *catenulatum* and *angulatum* groups; the strains of the *dentium* group did not display any distinctive phenotypic character. However, the phenotypic expression of the large divergencies in the DNA base sequences shown to exist among the species of the genus *Bifidobacterium* (see reference 32) remains to be discovered.

Detailed descriptions of the three new species of the genus *Bifidobacterium* follow.

1. *Bifidobacterium catenulatum* sp. n. (M.L. adj. *catenulatus* having small chains).

Cell characteristics. Gram-positive, non-motile, nonsporeforming cells, generally with three to four globular elements arranged in chains 1.3 to 1.7 μ m in diameter; when not fragmented, the chains are 0.6 to 0.8 by 2.0 to 4.0 μ m. The distal ends of the chains are usually tapered. Distinct branchings, club-swellings, or spatula-like extremities are generally absent. (Plate 1, Fig. 1-15)

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

Relationships to oxygen and CO₂. Anaerobic; CO₂ is without affect upon oxygen sensitivity or anaerobic growth.

Temperature relationships. Optimum, 39 to 41 C; minimum, 28 to 28.5 C; maximum, 42.5 to 43 C; no growth at 26 C or 44 C.

pH relationships. Initial optimum, 6.5 to 6.9; delayed growth at 6.3 or 7.2; no growth at 4.5 or 8.0.

End products from glucose. Lactic and acetic acids in a molar ratio of $1:2.15 \pm 0.15$ are produced in TPY medium. Isomeric type of lactic acid produced: L(+). CO₂ is formed only in the fermentation of gluconate.

Fermentation pattern. The fermentation pattern is given in Table 11. Most strains (approximately 85%) ferment sorbitol but not mannitol, as do strains of biotype *c* of *B. adolescentis* Reuter.

Enzymology. Fructose-1,6-diphosphate aldolase: <2 mU of specific activity; G6P dehydrogenase (NAD⁺ or NADP⁺ dependent) not demonstrable; 6PG dehydrogenase (NADP⁺ dependent): some 40 mU of specific activity; electrophoretic type of F6PPK: "human."

Biochemical reactions. Catalase or pseudo-

catalase (hemin) not produced; nitrate not reduced; indole not produced; acetylmethylcarbinol produced; gelatin not liquefied; skim milk acidified and coagulated; ammonia produced from asparagine but not from urea or arginine.

G + C content of DNA. 54.7 ± 0.2 mol % (T_m).

DNA homology relationships. More related to *B. adolescentis* Reuter (some 45% homology) than to any other species of the genus.

Source. Feces of adult man, human vagina, and sewage.

Type strain. B669 of the Collection of the Istituto di Microbiologia Agraria, Universita di Bologna, Italia; deposited in the American Type Culture Collection (ATCC), Rockville, Md. under the number 27539.

2. *Bifidobacterium dentium* sp. n. (L. mas. n. *dens* tooth; L. plural gen. n. *dentium* of teeth).

Cell characteristics. Gram-positive, non-motile, nonsporeforming, slender rods 2 to 8 μ m long, with the extremities often swollen, dumb-bell shaped or enlarged to give a spatula-like appearance; branchings occur at the distal ends of the cells; chains of globular elements are absent. The general morphology is similar to that of *B. longum* Reuter. (Plate 2, Fig. 1-15)

Surface colonies. Flat or umbonate when aged, with entire or undulate margins, often raised, glistening, cream to white, soft. (Plate 2, Fig. 16 and 17)

Relationships to oxygen and CO₂. Anaerobic; CO₂ does not affect sensitivity to oxygen or anaerobic growth.

Temperature relationships. Optimum, 39 to 41 C; minimum, 24 to 25 C; maximum, 42 to 42 C; no growth at 23 or 44 C.

pH relationships. Initial optimum, 6.5 to 6.9; delayed growth at 6.3 or 7.2; no growth after two days at 4.5 or 8.0.

End products from glucose. Lactic and acetic acids in a molar ratio of $1:2.5 \pm 0.2$ are produced in TPY medium. Isomeric type of lactic acid produced: L(+). CO₂ is formed only in the fermentation of gluconate.

Fermentation pattern. The fermentation pattern is given in Table 11. Most strains (approximately 90%) ferment mannitol but not sorbitol and can be distinguished from strains of biotype *b* of *B. adolescentis* Reuter because they ferment melezitose.

Enzymology. Fructose-1,6-diphosphate aldolase: <2 mU of specific activity; G6P dehydrogenase (NAD⁺ or NADP⁺ dependent) not demonstrable; 6PG dehydrogenase (NADP⁺ dependent): approximately 2 mU of specific

activity; electrophoretic type of F6PPK: "human."

Biochemical reactions. Catalase or pseudocatalase (hemin) not produced; nitrate not reduced; indole not produced; acetylmethylcarbinol produced; ammonia produced from asparagine but not from urea or arginine.

G + C content of DNA. 61.2 ± 0.4 mol % (T_m).

DNA homology relationships. Insignificantly related (some 30%) to *B. adolescentis*, *B. catenulatum*, *B. infantis*, *B. liberorum*, *B. lactentis*, *B. ruminale*, and *B. suis*; still less related or not at all to the other species of the genus *Bifidobacterium*.

Source. The only bifidobacterium found in human dental caries; present in human feces (adults) and the human vagina.

Type strain. B764 of the Collection of the Istituto di Microbiologia Agraria, Università di Bologna, Italia; deposited in the ATCC under the number 27534.

3. *Bifidobacterium angulatum* sp. n. (L part. adj. *angulatus* with angles, angular).

Cell characteristics. Gram-positive, nonmotile, nonsporeforming cells, 0.6 to 0.7 by 1.5 to 3.0 μ m, generally disposed in "V" or "palisade" arrangements like the corynebacteria; rarely enlarged at the extremities; branchings are absent. This morphological type is unique among the known species of the genus *Bifidobacterium*. (Plate 3, Fig. 1-9)

Surface colonies. Circular, pulvinate, smooth with entire margins, porcelain white, glistening, and of soft consistency. (Plate 3, Fig. 10 and 11)

Relationships to oxygen and CO₂. Anaerobic, but more sensitive to oxygen than most bifidobacteria (from the depth of growth in stab); CO₂ does not affect the sensitivity to oxygen, but it strongly enhances anaerobic growth.

Temperature relationships. Optimum, 39 to 40 C; maximum, 42 to 42 C; minimum, 28 to 29 C; no growth at 27 or 44 C.

pH relationships. Initial optimum, 6.5 to 6.9; delayed growth at 6.3 or 7.2; no growth after two days at 4.5 or 8.0.

End products from glucose. Lactic and acetic acids in a molar ratio of $1:2.2 \pm 0.06$ are produced in TPY medium. Isomeric type of lactic acid produced: L(+). Propionic and butyric acids not produced. CO₂ is formed only in the fermentation of gluconate.

Fermentation pattern. The fermentation pattern is given in Table 11.

Enzymology. Fructose-1,6-diphosphate aldo-

lase: <2 mU of specific activity; G6P dehydrogenase (NAD⁺ or NADP⁺ dependent) not demonstrable; 6PG dehydrogenase (NADP⁺ dependent): approximately 10 mU of specific activity; electrophoretic type of F6PPK: "human."

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

G + C content of DNA. 59.0 ± 0.1 mol % (T_m).

DNA homology relationships. Very little or no relationship to the other known species of the genus *Bifidobacterium*.

Source. First isolated from human feces and then found in sewage.

Type strain. B677 of the Collection of the Istituto di Microbiologia Agraria, Università di Bologna, Italia; deposited in the ATCC under the number 27535.

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REPRINT REQUESTS

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