16S rRNA and 16S to 23S Internal Transcribed Spacer Sequence Analyses Reveal Inter- and Intraspecific *Bifidobacterium* Phylogeny

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In the last few years many attempts have been made to differentiate more than 20 *Bifidobacterium* species. It has been recognized that identification of bifidobacterial species is problematic because of phenetic and genetic heterogeneities. In order to contribute to our understanding of *Bifidobacterium* taxonomy, we studied *Bifidobacterium* phylogeny by performing both 16S rRNA and 16S to 23S (16S-23S) internally transcribed spacer (ITS) sequence analyses. In this study, we determined 16S rRNA sequences of five *Bifidobacterium* strains representing four species, and compared them with the sequences available in the GenBank database, and used them to construct a distance tree and for a bootstrap analysis. Moreover, we determined the ITS sequences of 29 bifidobacterial strains representing 18 species and compared these sequences with each other. We constructed a phylogenetic tree based on these sequence data and compared this tree with the tree based on 16S rRNA sequence data. We found that the two trees were similar topologically, suggesting that the two types of molecules provided the same kind of phylogenetic information. However, while 16S rRNA sequences are a good tool to infer interspecific links, the 16S-23S rDNA spacer data allowed us to determine intraspecific relationships. Each of the strains was characterized by its own ITS sequence; hence, 16S-23S rRNA sequences are a good tool for strain identification. Moreover, a comparison of the ITS sequences allowed us to estimate that the maximum level of ITS divergence between strains belonging to the same species was 13%. Our data allowed us to confirm the validity of most of the *Bifidobacterium* species which we studied and to identify some classification errors. Finally, our results showed that *Bifidobacterium* strains have no tRNA genes in the 16S-23S spacer region.

Members of the genus *Bifidobacterium* are widespread in nature, and the habitats of these organisms range from sewage (53, 54) to human and animal intestines (28, 31, 44, 52, 56). The ability to catabolize hexose by a particular pathway via fructose phosphate phosphoketolase is important for recognizing members of this genus (8, 51). In the last few years many ways to differentiate *Bifidobacterium* species have been developed. Numerical taxonomy analysis, which requires data for many characteristics, has been used to circumscribe clusters and to describe strains. Morphological traits, carbohydrate metabolism data, DNA G+C contents (3), electrophoretic patterns (22, 48, 50), serologic data (44, 58, 59), cell wall compositions (22), and rRNA gene restriction patterns (26) have been used to subdivide *Bifidobacterium* species (for a review, see reference 3). Major advances in our understanding of the taxonomy of members of the genus *Bifidobacterium* have come from the DNA-DNA hybridization studies performed by Scardovi et al. (49, 54, 55, 57). However, problems with the identification of *Bifidobacterium* species are compounded by evidence that there is phenotypic and genetic heterogeneity in these species (6). Moreover, differentiation of some species has often been based on differences in only a few traits, which has led to some controversies about species circumscription.

The use of rRNA sequences as indicators of evolutionary

divergence is now widespread, and the advantage of this method over classical taxonomic approaches has been well documented (64). 16S rRNA sequence analysis is a powerful and accurate method for determining phylogenetic relationships and is commonly used to determine inter- and intrageneric relationships. Nevertheless, as evolutionary distances decrease, there is a point at which there is insufficient base sequence diversity to differentiate strains of a given species. The inaccuracy of 16S rRNA sequence data makes defining the phylogenetic relationships of closely related species difficult (46).

In eubacterial DNA, the rRNA genetic loci include 16S, 23S, and 5S rRNA genes, which are separated by internally transcribed spacer (ITS) regions. It is known that DNA sequences in the 16S to 23S (16S-23S) intergenic spacer region exhibit a great deal of sequence and length variation (1, 33). The variations in this region have been shown to be useful for differentiating species of prokaryotes (1). Recently, it has been shown that PCR-restriction fragment length polymorphism analysis of the ribosomal intergenic spacer is a good method for differentiating strains of prokaryotes (33, 43). In this study, we investigated phylogenetic relationships among *Bifidobacterium* spp. The results of comparisons of 16S rRNA and ribosomal DNA (rDNA) sequences allowed us to determine the interspecific relationships of 18 species. In addition, to determine the intraspecific relationships, we compared the 16S-23S ITS sequences of 29 strains representing 18 species and performed phylogenetic analyses.

The aims of this study were to determine *Bifidobacterium* intrageneric relationships and to determine whether *Bifidobacterium* species are valid taxa. The results of our study of the

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TABLE 1. Bacterial strains and their 16S rRNA and 16S-23S ITS GenBank accession numbers

Species	Strain ^a	Source of isolation	GenBank accession no.	
			16S rRNA	16S-23S rDNA
<i>Gardnerella vaginalis</i>	ATCC 14018 ^T	Human vagina	M58744	
<i>Bifidobacterium</i> spp.				
<i>B. adolescentis</i>	ATCC 11146	Feces of human adult		U09511
<i>B. adolescentis</i>	ATCC 15703 ^T	Feces of human adult	M58729 ^b	
<i>B. adolescentis</i>	CIP 6459 ^T	Feces of human adult		U09512
<i>B. adolescentis</i>	CIP 6460	Feces of human adult		U09513
<i>B. adolescentis</i>	CIP 6461	Feces of human adult		U09514
<i>B. angulatum</i>	ATCC 27535 ^T	Feces of human adult		U09515
<i>B. animalis</i>	ATCC 25527 ^T	Feces of rat	X70971	U09858
<i>B. animalis</i>	ATCC 27536	Feces of chicken		L36967
<i>B. asteroides</i>	ATCC 25910 ^T	Intestine of <i>Apis mellifera</i>	M58730 ^b	U09516
<i>B. bifidum</i>	ATCC 11147	Unknown		U09526
<i>B. bifidum</i>	ATCC 29521 ^T	Feces of human infant	M38018 ^b	
<i>B. bifidum</i>	CIP 56-7 ^T	Feces of human infant		U09517
<i>B. bifidum</i>	CIP 6465	Feces of human infant		U09831
<i>B. breve</i>	ATCC 15700 ^T	Feces of human infant	M58731 ^b	
<i>B. breve</i>	CIP 6469 ^T	Feces of human infant	X70973	U09520
<i>B. breve</i>	CIP 6468	Feces of human infant		U09519
<i>B. breve</i>	CIP 6470	Feces of human infant		U09521
<i>B. breve</i>	ATCC 15698	Feces of human infant	X70972	U09518
<i>B. catenulatum</i>	ATCC 27539 ^T	Feces of human adult	M58732 ^b	U09522
<i>B. choerinum</i>	CIP 103378 ^T	Feces of pig		L36968
<i>B. coryneforme</i>	ATCC 25911 ^T	Intestine of <i>Apis mellifera</i>	M58733 ^b	U09523
<i>B. cuniculi</i>	ATCC 27916 ^T	Feces of rabbit	M58734 ^b	
<i>B. cuniculi</i>	CIP 103379 ^T	Feces of rabbit		U09790
<i>B. dentium</i>	ATCC 27534 ^T	Oral cavity		U10434
<i>B. dentium</i>	ATCC 15423	Lung abscess in human	M58735 ^b	
<i>B. globosum</i>	ATCC 25865 ^T	Rumen cattle	M58736 ^b	U09524
<i>B. indicum</i>	ATCC 25912 ^T	Intestine of <i>Apis cerana</i>	M58737 ^b	U09791
<i>B. infantis</i>	ATCC 15697 ^T	Feces of human infant	M58738 ^b	U09792
<i>B. infantis</i>	ATCC 25962	Feces of human infant		U09525
<i>B. infantis</i>	CIP 6378	Unknown		U09527
<i>B. longum</i>	ATCC 15707 ^T	Feces of human adult	M58739 ^b	
<i>B. longum</i>	ATCC 15708	Feces of human infant	U10152	U09832
<i>B. magnum</i>	ATCC 27540 ^T	Feces of rabbit	M58740 ^b	U09878
<i>B. minimum</i>	ATCC 27538 ^T	Sewage	M58741 ^b	
<i>B. pseudolongum</i>	ATCC 25526 ^T	Feces of pig	M58742 ^b	U09879
<i>B. suis</i>	ATCC 27533 ^T	Feces of pig	M58743 ^b	
<i>B. suis</i>	CIP 103377 ^T	Feces of pig		
<i>B. thermophilum</i>	ATCC 25525 ^T	Feces of pig	U10151	U09528

^a The strains were obtained from the American Type Culture Collection (ATCC) or the Collection de l'Institut Pasteur (CIP). The type strains were designated by Reuter (45). The following pairs of strains are synonymous: *B. adolescentis* ATCC 15703^T and *B. adolescentis* CIP 6459^T; *B. bifidum* ATCC 29521^T and *B. bifidum* CIP 56-7^T; *B. breve* ATCC 15700^T and *B. breve* CIP 6469^T; *B. cuniculi* ATCC 27916^T and *B. cuniculi* CIP 103379^T; and *B. suis* ATCC 27533^T and *B. suis* CIP 103377^T.

^b 16S rRNA sequence obtained by Larsen et al. (21).

ITS polymorphisms allowed us to identify each *Bifidobacterium* strain accurately.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains were provided directly by either the American Type Culture Collection or the Collection de l'Institut Pasteur. The strains whose sequences were determined in this study and the reference strains used for comparison when phylogenetic trees were constructed are shown in Table 1. Table 1 includes the strain designations, the sources of the strains, and the GenBank accession numbers for the 16S rRNA and 16S-23S ITS sequences.

All *Bifidobacterium* strains were cultivated in the same medium, as described previously (4).

Isolation and purification of RNA. Undegraded RNA was isolated by using a single guanidinium thiocyanate step and phenol-chloroform extraction (7).

PCR amplification and purification of product. Genomic DNAs from all of the strains were prepared as described previously by Marmur (27). The bacterial cells were lysed as described previously by using lysosyme (27); none of the species was lysis resistant when the lysosyme was used at the end of the exponential growth phase. PCR amplification of the ITS was performed by using a GeneAmp kit (Perkin-Elmer) (30). The PCR primers used to amplify the spacer region were selected from the sequences of 16S and 23S rRNA genes conserved in various bacteria (5, 20, 32–34, 36, 61, 62). Primer L was immediately adjacent

to the 16S-23S spacer; the sequence of this oligonucleotide was 5'-GCTGGAT CACCTCCTTTCT-3', which corresponded to a conserved sequence at the 3' end of the 16S rRNA (from position 1525 to position 1543 on the *Escherichia coli* 16S rRNA). The sequence of primer R was deduced from an alignment of 23S rRNA 5' sequences (from position 23 to position 40 on the *E. coli* 23S rRNA); the sequence of primer R was 5'-CTGGTGCCAAGGCATCCA-3'. These primers were supplied by Eurogentec, Seraing, Belgium. The PCR conditions which we used were as follows: 30 cycles consisting of 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min, followed by one 5-min chain elongation step at 72°C. All amplification reactions were performed in a PREM temperature controller (LEP Scientific). The total volume of each amplification reaction mixture was 100 µl, and 100 ng of total DNA was used as the template. The amplification products were purified on cDNA spun columns (Pharmacia) as recommended by the manufacturer.

Sequencing methods. (i) **16S rRNA sequencing.** rRNA sequences were determined as described previously (20, 42) by using α-³⁵S-labeled dATP and a reverse transcriptase RNA sequencing kit (United States Biochemicals). DNA primers complementary to 10 universally conserved regions of 16S rRNA were either kindly provided R. Christen or obtained from Eurogentec. The 10 primers complemented positions 98 to 119 (primer S2; TTACTCACCCGTCCGC CACTC), 342 to 356 (primer S4; CTGCTGCCTCCCGTA), 518 to 534 (primer S6; TATTACCGCGGCTGCTG), 684 to 702 (primer S8; CTACGCATTTTAC CGCTAC), 786 to 802 (primer S9; CTACAGGGTATCTAAT), 906 to 925 (primer S10; CCGTCAATTCATTTGAGTTT), 1099 to 1115 (primer S12;

AAGGGTTGCGCTCGTTG), 1223 to 1240 (primer S14; CATTGTAG CACGTGTGTA), 1384 to 1400 (primer S15; CGGTGTGTACAAGGCC), and 1491 to 1509 (primer S17; GGTACCTTGTACGACTT) of the 16S rRNA (*E. coli* nucleotide numbering).

For sequencing we used the RT RNA sequencing kit and the standard procedures recommended by the manufacturer.

(ii) 16S-23S spacer sequencing. For amplified DNA sequencing we used Vent (exo-)DNA polymerase as recommended by the manufacturer and a Circum Vent thermal cycle dideoxy DNA sequencing kit (Biolabs). In each reaction either primer L or primer R was annealed to a complementary single-stranded stretch of DNA. Repetitive cycles of denaturation, annealing, and chain extension from small amounts of template molecules resulted in linear amplification of reaction products and a strong sequencing signal. In each cycle, the temperature was increased to 95°C to denature the double-stranded DNA, decreased to 55°C to anneal the primer to the template, and then increased to 72°C for the elongation step involving enzymatic synthesis. A total of 25 cycles were completed. The nucleotide fragments produced in the chain elongation reactions were separated on 55-cm wedge-shaped 5% (wt/vol) polyacrilamide-42% urea gels by using a Sequi-Gen nucleic acid sequencing cell (Bio-Rad).

Analysis of sequence data. The 16S rRNA gene sequences which we determined and the sequences of the other bifidobacteria and reference strains obtained from the GenBank database and the Ribosomal Database Project (21) were aligned by using the MUST package (37). The 16S rRNA sequences were first aligned by using the Clustal V program (17), and then the alignments were corrected by hand and analyzed by the neighbor-joining method of Saitou and Nei (47) by using the neighbor-joining program of MUST.

The stability of relationships was assessed by using the bootstrap method (10). A total of 1,000 bootstrap trees were generated for each data set by using the NJboot program of MUST. A similar analysis was performed by using the ITS sequences.

Nucleotide sequence accession numbers. The 16S rRNA gene and ITS sequences which we determined have been deposited in the GenBank Data Library under the accession numbers shown in Table 1.

RESULTS

Construction of a phylogenetic tree based on 16S rRNA sequence comparison results. The 16S rRNA sequences of the following *Bifidobacterium* strains belonging to four species were determined: *Bifidobacterium breve* ATCC 15698 and CIP 6469^T (T = type strain), *Bifidobacterium longum* ATCC 15708, *Bifidobacterium animalis* ATCC 25527^T, and *Bifidobacterium thermophilum* ATCC 25525^T. The 16S rDNA sequences of 16 other *Bifidobacterium* species were described by Larsen et al. for the Ribosomal Database Project (21). The five *Bifidobacterium* 16S rRNA sequences which we determined were compared with the sequences available in the GenBank database. The levels of 16S rRNA sequence similarity are shown in Table 2. The levels of similarity for the 18 *Bifidobacterium* species examined ranged from 92 to 99%. Despite considerable phenotypic diversity, the members of the genus *Bifidobacterium* exhibit rather high levels of relatedness, which are reflected by their similar DNA G+C contents (for a review, see reference 3).

A phylogenetic tree was constructed by using distance matrix data obtained from the 16S rRNA sequences and the neighbor-joining method (47). This tree was based on 1,437 unambiguously aligned positions, and 181 of these positions were informative under the parsimony criterion. The tree was rooted by using the genus *Gardnerella*, which was the genus that was most closely related to the genus *Bifidobacterium* (35). Figure 1 is a dendrogram which shows the phylogeny of the genus *Bifidobacterium*. All of the bifidobacteria fell into six major clusters of species which were characterized by high bootstrap values (>84%). The phylogenetic relationships determined in our analysis were in general consistent with a previous dendrogram based on DNA-DNA homology data (22), but there were some differences, which are discussed below.

Clusters 1 and 4. The species *Bifidobacterium minimum* and *Bifidobacterium bifidum* were included in clusters 1 and 4, respectively. These species can be easily recognized by their

murein types, Lys-Ser and Orn (Lys)-D-Ser-D-Asp, respectively (for a review, see reference 3). Although *B. bifidum* is the type species of the genus, it exhibited no significant affinity with any other species. These data are consistent with those of Lauer and Kandler (22), who showed that *B. bifidum* exhibited only a low level of relatedness (about 25%) with all of the other species used in this study and that there was no indication of a distinct affinity with any of the other clusters.

Cluster 2. Cluster 2 included *B. thermophilum*, *Bifidobacterium coryneforme*, and *Bifidobacterium asteroides*. The last two species are bacteria that inhabit the intestines of honeybees (52). Since *B. coryneforme* and *B. asteroides* have numerous fermentative characteristics in common (3), produce the same fructose-6-phosphate phosphoketolase electrophoretic pattern (3), and are considered phenotypically synonymous (29), we expected that they would be found to be closely related, and this did occur (Fig. 1). We also expected that *Bifidobacterium indicum*, which exhibited a level of DNA-DNA similarity with *B. coryneforme* of 60%, would also be included in cluster 2 (2). However, our results showed that *B. indicum* ATCC 25912^T is not a member of cluster 2 but belongs in cluster 6 and revealed a convincing relationship between this organism and *Bifidobacterium infantis* ATCC 15697^T.

Cluster 3. Cluster 3 was composed of *Bifidobacterium catenulatum*, *Bifidobacterium dentium*, and *Bifidobacterium adolescentis* strains. The close phylogenetic relationship of the last two species was expected since Biavati et al. (3) found previously that these organisms cannot be distinguished phenotypically (they have the same murein type and produce the same fructose-6-phosphate phosphoketolase electrophoretic pattern).

Cluster 5. Included in cluster 5 were *Bifidobacterium magnum*, *Bifidobacterium globosum*, *Bifidobacterium cuniculi*, and *B. animalis*. The last two species are characterized by having the same murein type (Orn [Lys]-Ser [Ala]-Ala₂) (for a review, see reference 3). This group is consistent with the group described by Lauer and Kandler (22) but is not consistent with the group described by Biavati et al. (3), suggesting that *B. magnum* could be related to *B. infantis* and *B. longum*.

Cluster 6. Cluster 6 was composed of *B. breve*, *B. indicum*, *B. infantis*, *B. longum*, *Bifidobacterium pseudolongum*, and *Bifidobacterium suis*. The close relationship of *B. breve*, *B. infantis*, and *B. longum* was revealed previously by the results of DNA-DNA hybridization experiments (22). In contrast, the close relationship of *B. infantis* ATCC 15698^T and *B. indicum* ATCC 25912^T was not expected because these two organisms did not exhibit high levels of DNA-DNA hybridization and did not have the same phenotypic characteristics (22). Identical restriction patterns for the genes coding for rRNA (rDNA) were observed only when a Southern blot analysis was performed (data not shown). On the basis of the results of previous studies (for a review, see reference 55) we expected that *B. pseudolongum* would be in cluster 5 (since it is similar to *B. globosum*) rather than cluster 6.

The significance of the branching order of the trees was estimated by performing a bootstrap analysis. First, the bootstrap analysis results provided rather strong support for the monophyly of the clusters (bootstrap values, 86, 86, 97, and 99). They also showed that it would be impossible to determine the relationships of the six clusters (bootstrap values, 21, 24, 48, and 67) by using the complete 16S rRNA sequences and to determine the relationships of closely related species. Indeed, the variations in the 16S rRNA sequences of closely related strains are not sufficient to allow clear determinations of evolutionary distances. The evolutionary rate of the 16S rRNA molecule is too low to provide useful information concerning

TABLE 2. Levels of similarity for portions of the 16S rRNA sequences of 21 representatives of the genus *Bifidobacterium* and the type strain of *G. vaginalis*

Strain ^a	% Sequence similarity																			
<i>B. adolescentis</i> ATCC 15703 ^T	92.87	94.32	95.71	95.31	98.85	98.63	94.59	95.50	94.22	93.67	94.10	94.40	99.21	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. animalis</i> ATCC 25527 ^T	94.65	92.85	95.71	95.31	98.85	98.63	94.59	95.50	94.22	93.67	94.10	94.40	99.21	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. asteroides</i> ATCC 25910 ^T	94.53	92.85	95.71	95.31	98.85	98.63	94.59	95.50	94.22	93.67	94.10	94.40	99.21	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. bifidum</i> ATCC 29521 ^T	94.95	94.42	94.38	95.32	98.85	98.63	94.59	95.50	94.22	93.67	94.10	94.40	99.21	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. breve</i> ATCC 15700	94.76	94.68	94.13	95.32	98.85	98.63	94.59	95.50	94.22	93.67	94.10	94.40	99.21	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. breve</i> CIP 6469 ^T	94.19	94.63	94.81	95.32	98.85	98.63	94.59	95.50	94.22	93.67	94.10	94.40	99.21	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. breve</i> ATCC 15698	95.56	95.59	95.79	95.15	97.99	98.63	94.59	95.50	94.22	93.67	94.10	94.40	99.21	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. catenulatum</i> ATCC 27539 ^T	94.22	94.18	98.10	95.42	94.62	94.42	94.73	95.50	94.22	93.67	94.10	94.40	99.21	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. coryneforme</i> ATCC 25911 ^T	93.36	95.99	94.72	94.23	94.08	93.88	94.34	93.33	94.22	93.67	94.10	94.40	99.21	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. cuniculi</i> ATCC 27916 ^T	96.61	93.30	95.81	95.39	95.08	94.59	94.38	96.63	94.71	93.67	94.10	94.40	99.21	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. dentium</i> ATCC 15423	93.29	96.42	94.70	94.36	94.78	94.37	94.82	93.67	94.92	96.21	95.47	94.36	99.21	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. globosum</i> ATCC 25865 ^T	95.36	93.49	94.07	95.71	97.08	97.93	96.34	94.65	94.82	93.54	95.17	94.40	99.21	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. indicum</i> ATCC 25912 ^T	95.44	93.55	93.88	95.55	97.08	97.05	96.32	94.67	94.51	93.28	95.35	94.09	99.21	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. infantis</i> ATCC 15697 ^T	95.03	93.30	94.01	95.37	96.50	96.62	96.26	95.16	94.70	93.71	95.47	94.36	99.21	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. longum</i> ATCC 15707 ^T	94.46	93.14	93.67	94.61	96.33	96.43	95.93	94.59	94.13	93.07	95.04	93.86	98.75	98.82	99.07	92.76	93.42	95.22	99.86	93.88
<i>B. longum</i> ATCC 15708	92.88	93.64	93.53	93.86	94.07	94.09	93.89	93.01	92.46	94.57	93.37	94.86	93.47	93.06	93.34	92.76	93.42	95.22	99.86	93.88
<i>B. magnum</i> ATCC 27540 ^T	94.89	93.90	95.55	95.30	94.79	94.95	94.82	95.01	96.21	93.94	94.87	94.43	95.20	94.96	95.08	94.44	93.42	95.22	99.86	93.88
<i>B. minimum</i> ATCC 27538 ^T	95.18	93.44	94.30	95.45	96.74	96.86	96.57	95.32	94.76	93.69	95.56	94.35	99.34	99.19	99.86	99.05	93.33	95.05	99.86	93.88
<i>B. pseudolongum</i> ATCC 25526 ^T	95.24	93.36	94.29	95.57	96.63	96.74	96.45	95.37	94.82	94.98	95.60	94.49	99.35	99.20	99.72	98.92	93.47	95.05	99.86	93.88
<i>B. suis</i> ATCC 27533 ^T	93.55	93.73	95.85	94.94	93.83	93.99	93.93	95.00	95.56	93.41	94.36	93.77	93.70	93.83	93.83	94.08	92.30	94.05	93.98	93.88
<i>B. thermophilum</i> ATCC 25525 ^T	92.88	91.80	93.44	93.54	93.52	93.44	93.23	93.30	94.06	92.21	92.89	92.35	93.08	93.06	93.02	92.58	91.39	94.09	93.13	92.55
<i>G. vaginalis</i> ATCC 14018 ^T																				

^a The sequences of *B. animalis* ATCC 25527^T, *B. breve* CIP 6469^T, *B. breve* ATCC 15698, *B. longum* ATCC 15708, and *B. thermophilum* ATCC 25525^T were determined in this study; the other sequences were obtained from reference 21.

FIG. 1. Dendrogram based on 16S rRNA sequences, showing the relationships of members of the genus *Bifidobacterium*. The tree was rooted with *G. vaginalis* and was constructed by using the neighbor-joining method and bootstrap values calculated from 1,000 trees. Each number on a branch indicates the number of times (out of 100) that the node was supported by the bootstrap analysis results. Bar = 0.43% sequence divergence. The six major clusters (clusters 1 through 6) are indicated.

strains belonging to the same species. Thus, the evolutionary distances between strains belonging to *B. breve* or *B. longum* could not be determined from the results of 16S rRNA sequence comparisons.

16S rRNA sequence analysis is not an appropriate method for delineating species and measuring intraspecies relationships (60). To assess intraspecific phylogenetic relationships, we decided to study a more variable sequence, the 16S-23S ITS sequence.

Construction of a phylogenetic tree based on the 16S-23S ITS sequence comparisons (i) Length polymorphism of the PCR-amplified ITS sequences. A total of 30 16S-23S rDNA spacer regions, obtained from 18 *Bifidobacterium* species, were amplified by PCR. Using data from previous studies, we determined the minimal number of *rrn* loci in *Bifidobacterium* strains; this number varied between 2 and 5 for the species which we studied (4, 24). Although it is known that *Bifidobacterium* species have more than one *rrn* locus, in each case only one PCR product was obtained, suggesting that the different ITS copies in each chromosome did not differ in size.

However, the sizes of the PCR products of the strains varied from 263 to 552 bp (Table 3). In most cases only small variations in ITS sizes (less than 2%) were observed with strains belonging to the same species. The one exception was the *B. infantis* strains, whose ITS sizes differed dramatically; the ITS sizes for the three *B. infantis* strains studied were 263 bp (*B. infantis* ATCC 25962), 443 bp (*B. infantis* CIP 6378), and 477 bp (*B. infantis* ATCC 15697^T). In addition, the ITS sizes of *B. adolescentis* ATCC 11146 and *B. bifidum* ATCC 11147 were one-half those observed in other strains of these two species (Table 3). The variations in ITS length observed could have been due in part to variations in the number and type of tRNA sequences found in the spacers (5, 23).

Secondary-structure models were constructed for the ITS rDNA sequences of *B. breve* CIP 6469^T, *B. magnum* ATCC 27540^T, and *B. pseudolongum* ATCC 25526^T by using the Program Manual for the Wisconsin Package Computer Group software (13). However, tRNA structures were never found, indicating that no tRNA genes were located between the 16S and 23S rRNA genes in *Bifidobacterium* strains. Consistent with these results, a comparison of the 29 ITS sequences which we studied with 12 tRNA sequences published previously by

TABLE 3. Sizes of 16S-23S ITS sequences of *Bifidobacterium* strains

Strain	Size of 16S-23S ITS sequence (bp)
<i>B. adolescentis</i> ATCC 11146	274
<i>B. adolescentis</i> CIP 6459 ^T	487
<i>B. adolescentis</i> CIP 6460	496
<i>B. adolescentis</i> CIP 6461	487
<i>B. angulatum</i> ATCC 27535 ^T	472
<i>B. animalis</i> ATCC 25527 ^T	509
<i>B. animalis</i> ATCC 27536	506
<i>B. asteroides</i> ATCC 25910	452
<i>B. bifidum</i> CIP 56-7 ^T	499
<i>B. bifidum</i> CIP 6465	491
<i>B. bifidum</i> ATCC 11147	275
<i>B. breve</i> ATCC 15698	488
<i>B. breve</i> CIP 6468	489
<i>B. breve</i> CIP 6469 ^T	489
<i>B. breve</i> CIP 6470	496
<i>B. catenulatum</i> ATCC 27539 ^T	492
<i>B. choerinum</i> CIP 103378 ^T	552
<i>B. coryneforme</i> ATCC 25911 ^T	459
<i>B. cuniculi</i> CIP 103379 ^T	490
<i>B. dentium</i> ATCC 27534 ^T	494
<i>B. globosum</i> ATCC 25865 ^T	548
<i>B. indicum</i> ATCC 25912 ^T	471
<i>B. infantis</i> ATCC 15697 ^T	477
<i>B. infantis</i> CIP 6378	443
<i>B. infantis</i> ATCC 25962	263
<i>B. longum</i> ATCC 15708	476
<i>B. magnum</i> ATCC 27540 ^T	391
<i>B. pseudolongum</i> ATCC 25526 ^T	541
<i>B. thermophilum</i> ATCC 25525 ^T	478

Wawrousek et al. (63) revealed no similarity. This finding is consistent with previous data (34, 41, 61) which clearly showed that no tRNA genes occur in the 16S-23S ITS sequences of *Frankia* or *Streptomyces* strains or any other member of the order *Actinomycetales*.

(ii) ITS sequence comparison. The complete ITS sequences of 29 *Bifidobacterium* strains were determined by directly sequencing PCR-amplified 16S-23S rDNA spacer products. We found that 2 of the 29 16S-23S ITS sequences examined, (the *B. adolescentis* ATCC 11146 and *B. bifidum* ATCC 11147 sequences) were very similar (they differed at less than five positions) but were too heterogeneous to be compared with the sequences of the other *Bifidobacterium* strains used (levels of divergence, more than 75%), even the *B. adolescentis* and *B. bifidum* sequences. Moreover, the restriction patterns and the results of ribotyping and hybridization experiments revealed no relationship between *B. adolescentis* ATCC 11146 and the other *B. adolescentis* strains (24, 26). These data are consistent with the results of Scardovi and Trovatielli (51), who concluded that strains ATCC 11146 and ATCC 11147 were erroneously identified as members of the genus *Bifidobacterium*. A comparison of the *B. adolescentis* ATCC 11146 and *B. bifidum* ATCC 11147 16S-23S ITS sequences with the sequences available in the GenBank database revealed striking similarities with 16S-23S ITS sequences of *Lactobacillus* strains, especially the sequences of *Lactobacillus brevis* and *Lactobacillus heteriochii* (32). This finding is consistent with the results of Kagermeier-Callaway and Lauer (18), who showed by using DNA-DNA hybridization experiments (levels of similarity, 89 to 98%) and G+C content analysis that there is a close phylogenetic relationship between members of the genus *Lactobacillus* and the two strains *B. adolescentis* ATCC 11146 and *B. bifidum* ATCC 11147.

A comparison of the remaining 27 ITS sequences led to the construction of a similarity matrix. The 16S-23S ITS sequence similarity values are shown in Table 4. We found that each of the strains had a unique ITS sequence, which allowed rapid identification of the organisms. Moreover, the levels of ITS sequence similarity between members of the same species were often greater than the levels of similarity between members of different species; the minimum level of sequence similarity for members of the same species was about 87%. A comparison of *B. animalis*, *B. bifidum*, and *B. breve* strains revealed levels of sequence similarity of more than 93%. In contrast, we estimated that the average level of sequence similarity for *B. infantis* strains was only 39%. *B. infantis* ATCC 25962 exhibited a level of ITS sequence similarity with *B. infantis* ATCC 15697^T or CIP 6378 of only 30%, whereas the level of ITS sequence similarity between strains ATCC 15697^T and CIP 6378 was 52%. This result, which indicates that the *B. infantis* strains are not closely related, is consistent with the results of restriction fragment length polymorphism and ribotyping analyses, which revealed no similarity in the patterns of the three strains used in this study (24, 25).

Nevertheless, low levels of ITS sequence similarity were commonly obtained when we compared strains belonging to distinct species; these values could be as low as 26%. A low level of ITS similarity was found when we compared *B. asteroides* ATCC 25910^T and *B. infantis* ATCC 25962. Surprisingly, the following four pairs of strains belonging to distinct species exhibited high levels of sequence similarity: *B. pseudolongum* ATCC 25526^T and *B. globosum* ATCC 25865^T (level of similarity, 88.9%); *B. cuniculi* CIP 103379^T and *B. dentium* ATCC 27534^T (level of similarity, 96.3%); *B. infantis* ATCC 15697^T and *B. longum* ATCC 15708 (level of similarity, 87.3%); and *B. infantis* ATCC 15697^T and *B. indicum* ATCC 25912^T (level of similarity, 97.7%). These data raised questions about the previously described taxonomy of these strains.

(iii) Limitation of ITS-based phylogeny because of high evolutionary rates. Figure 2 is an ITS tree which shows *Bifidobacterium* phylogeny. The significance of the branching order of our trees was estimated by performing a bootstrap analysis. First, it should be noted that the high levels of 16S-23S ITS sequence variation did not allow us to determine the relationships among distantly related organisms. The lack of ITS sequence similarity and the high frequency of insertion-deletion events could prevent accurate sequence alignment and therefore make phylogenetic relationships difficult to assess. Moreover, because of the high evolutionary rate of the ITS, multiple substitutions often occur at the same nucleotide position. This implies that there has been mutational saturation of ITS sequences, which is known to confuse phylogenetic inferences (for example, see reference 38).

However, importantly, we did determine whether ITS sequences could be a reliable tool for inferring phylogenetic relationships between closely related organisms. This was accomplished by comparing the ITS tree with the tree obtained in the 16S rRNA sequence analysis.

(iv) ITS tree and comparison with the 16S tree. Overall, the phylogenetic relationships observed in our two trees were similar, which confirmed the hypothesis that ITS sequences could be used in phylogenetic analyses. The ITS tree clearly contained 10 strongly supported groups. The majority of the strains fell into five major groups, which were designated clusters 2 to 6 because of the numbers used on the 16S rRNA sequence tree, and 5 of the 10 groups consisted of single strains (*B. infantis* ATCC 25962, *B. magnum* ATCC 27540^T, *B. catenulatum* ATCC 27539^T, *B. infantis* CIP 6378, and *B. angulatum* ATCC 27535^T) (Fig. 2). However, the two trees did differ in

some respects. The phylogenetic dendrogram based on the ITS sequences (Fig. 2) differed the dendrogram based on the 16S rRNA sequences (Fig. 1) by the positions of three species. These differences are discussed below.

Cluster 2. The three species belonging to cluster 2 exhibited high levels of ITS sequence similarity. *B. coryneforme* and *B. asteroides* were more closely related to each other (level of similarity, 83%) than to *B. thermophilum* (level of similarity, 61%). This result is consistent with the results of DNA-DNA hybridization experiments, which showed that the level of DNA-DNA similarity between *B. coryneforme* and *B. asteroides* was 35% (22). In contrast, *B. thermophilum* exhibited lower levels of DNA-DNA similarity with *B. coryneforme* and *B. asteroides*. Nevertheless, cluster 2 is equivalent to the 16S rRNA group shown in Fig. 1.

Cluster 3. Cluster 3 was divided into two subgroups, subclusters 3A and 3B. Whereas subcluster 3B contained only *B. catenulatum* ATCC 27539^T, subcluster 3A contained *B. adolescentis*, *B. dentium*, and *B. cuniculi* strains. Consistent with the results of the 16S rRNA investigations, we found that *B. adolescentis* and *B. dentium* were closely related (average level of similarity, approximately 77%; bootstrap value, 100). Three of the four *B. adolescentis* strains were closely related, whereas ATCC 11146 was clearly misclassified as a *Bifidobacterium* strain (see above). Although three of the *B. adolescentis* strains examined exhibited relatively high levels of sequence divergence (maximum level of divergence within the species, 13%), the bootstrap value (100) supported the hypothesis that *B. adolescentis* is a phylogenetically significant group. A *B. adolescentis* intraspecific relationship was supported by a high bootstrap value (100), which showed that the ITS sequence comparison technique is a good tool for determining intraspecific phylogenetic relationships.

The presence of *B. cuniculi* CIP 103379^T in subcluster 3A was completely unexpected. Even more remarkable was the highly significant association of this strain (level of similarity, 96.3%) with *B. dentium* ATCC 27534^T. In our previous 16S rRNA sequence analysis we included *B. cuniculi* ATCC 27916^T in cluster 5. Thus, because the *B. cuniculi* strains used in the two analyses were obtained from two different collections, we wondered whether the currently available strains *B. dentium* ATCC 27534^T and *B. cuniculi* CIP 103379^T are the same organism. Our suspicion that *B. cuniculi* CIP 103379^T is not a member of *B. cuniculi* was confirmed by the results of a restriction fragment length polymorphism analysis. Indeed, restriction fragment length polymorphism experiments performed with *B. dentium* ATCC 27534^T and *B. cuniculi* CIP 103379^T revealed that these organisms differed by only one high-molecular-weight fragment in a *Pvu*II digest (unpublished data). This finding demonstrated that the two strains are related. Thus, it seems that *B. cuniculi* CIP 103379^T was misclassified as a member of the species *B. cuniculi* and instead is a *B. dentium* strain. 16S rRNA nucleotide sequence data will be required to shed further light on the taxonomy of these strains.

Another surprising result was the absence of *B. catenulatum* in subcluster 3A, as found in the 16S rRNA analysis. One way to explain the differences between the two trees is to involve multiple changes that accumulated in the ITS sequence, simulated false identities, and masked the actual number of evolutionary events. According to Philippe et al. (38, 39), multiple changes can be checked by comparing inferred substitutions with observed differences in the ITS sequences. Such a comparison clearly indicated that none of the ITS sequences except that of *B. catenulatum* (data not shown), was mutationally saturated, indicating that the position of *B. catenulatum* on the ITS sequence tree should not be taken into consideration.

TABLE 4. Levels of ITS sequence similarity

Strain	% Sequence similarity																									
	<i>B. adolescentis</i> CIP 6459 ^T	<i>B. adolescentis</i> CIP 6460	<i>B. adolescentis</i> CIP 6461	<i>B. angulatum</i> ATCC 27535 ^T	<i>B. animalis</i> ATCC 25527 ^T	<i>B. animalis</i> ATCC 27536	<i>B. asteroides</i> ATCC 25910 ^T	<i>B. bifidum</i> CIP 56-7 ^T	<i>B. bifidum</i> CIP 6465	<i>B. breve</i> CIP 6469 ^T	<i>B. breve</i> CIP 6468	<i>B. breve</i> CIP 6470	<i>B. breve</i> ATCC 15698	<i>B. catenulatum</i> ATCC 27539 ^T	<i>B. choerthum</i> CIP 103378 ^T	<i>B. coryneforme</i> ATCC 25911 ^T	<i>B. cuniculi</i> CIP 103379 ^T	<i>B. dentium</i> ATCC 27534 ^T	<i>B. globosum</i> ATCC 25865 ^T	<i>B. indicum</i> ATCC 25912 ^T	<i>B. infantis</i> ATCC 15697 ^T	<i>B. infantis</i> ATCC 25962	<i>B. infantis</i> CIP 6378	<i>B. longum</i> ATCC 15708	<i>B. magnum</i> ATCC 27540 ^T	<i>B. pseudolongum</i> ATCC 25526 ^T
<i>B. adolescentis</i> CIP 6460																										
<i>B. adolescentis</i> CIP 6461	82.47																									
<i>B. angulatum</i> ATCC 27535 ^T	87.05	94.53																								
<i>B. animalis</i> ATCC 25527 ^T	65.43	64.46	64.26																							
<i>B. animalis</i> ATCC 27536	41.18	39.83	40.75	41.30																						
<i>B. asteroides</i> ATCC 25910 ^T	40.89	39.90	40.71	40.90	94.04																					
<i>B. bifidum</i> CIP 56-7 ^T	42.85	43.21	44.32	42.78	38.55	37.82																				
<i>B. bifidum</i> CIP 6465	52.57	52.38	52.48	53.35	43.37	42.63	43.86																			
<i>B. breve</i> CIP 6469 ^T	50.76	50.48	50.57	52.00	41.77	40.96	43.78	95.95																		
<i>B. breve</i> CIP 6468	62.65	62.22	63.27	57.99	41.49	41.60	47.51	57.39	55.75																	
<i>B. breve</i> CIP 6470	61.88	61.84	62.70	57.88	40.82	41.82	47.51	56.82	55.18	95.51																
<i>B. breve</i> ATCC 15698	63.29	62.79	63.34	59.21	41.88	41.91	47.34	58.32	56.49	95.75	93.77															
<i>B. catenulatum</i> ATCC 27539 ^T	62.70	62.46	63.32	58.97	40.36	40.36	47.79	57.73	56.46	95.91	95.28	93.77														
<i>B. choerthum</i> CIP 103378 ^T	55.79	56.09	56.47	50.29	40.78	40.04	40.50	50.10	48.69	53.85	53.10	54.50	54.72													
<i>B. coryneforme</i> ATCC 25911 ^T	36.76	36.64	37.61	39.79	51.86	51.77	36.02	40.88	40.49	39.36	39.48	39.69	39.73	39.48												
<i>B. cuniculi</i> CIP 103379 ^T	45.39	45.85	46.78	44.53	40.08	39.72	83.34	44.85	44.57	48.39	48.00	48.59	48.47	42.25	37.68											
<i>B. dentium</i> ATCC 27534 ^T	77.25	79.26	80.64	62.78	40.61	40.57	46.29	54.13	52.39	61.16	59.70	61.57	61.39	56.90	36.87	46.11										
<i>B. globosum</i> ATCC 25865 ^T	75.74	77.61	78.94	62.04	39.58	39.62	44.93	52.95	59.42	59.70	58.26	59.93	60.31	55.88	36.67	45.22	96.32									
<i>B. indicum</i> ATCC 25912 ^T	39.53	39.63	40.24	39.83	56.92	56.85	36.49	42.96	41.88	41.30	40.11	41.26	40.93	39.63	60.90	38.06	39.42	39.02								
<i>B. infantis</i> ATCC 15697 ^T	58.47	58.78	58.74	55.24	38.79	38.66	44.34	55.88	54.53	79.20	78.63	78.25	79.88	51.42	37.98	45.13	56.13	54.93	38.12							
<i>B. infantis</i> ATCC 25962	60.35	61.03	61.00	56.93	40.08	39.76	45.79	57.01	55.67	81.09	80.33	80.12	82.19	55.64	39.24	45.91	57.83	56.80	39.36	97.68						
<i>B. infantis</i> CIP 6378	32.12	32.96	53.66	30.06	27.53	28.22	26.67	31.60	30.51	31.56	30.79	30.34	31.95	29.58	27.49	28.66	32.24	32.25	29.19	30.34	30.88					
<i>B. longum</i> ATCC 15708	55.80	56.23	57.68	56.83	37.55	37.55	44.83	50.39	50.20	56.75	56.24	56.25	57.49	48.26	36.10	45.57	56.95	55.19	38.35	52.08	53.85	30.36				
<i>B. magnum</i> ATCC 27540 ^T	59.39	59.24	59.58	56.11	41.46	40.43	47.23	57.50	56.54	76.80	76.05	76.64	77.83	50.94	39.41	46.96	57.75	56.90	40.96	85.54	87.30	31.13	54.64			
<i>B. pseudolongum</i> ATCC 25526 ^T	41.78	41.21	40.92	39.97	40.47	40.47	34.89	38.98	37.94	42.36	41.81	41.75	42.07	38.54	36.48	36.79	39.71	38.96	38.73	38.37	39.30	33.26	42.78	38.61		
<i>B. thermophilum</i> ATCC 25525 ^T	38.61	39.29	39.56	38.39	54.47	54.69	36.13	43.06	42.15	39.22	38.71	39.77	38.85	38.04	58.72	38.10	38.95	38.81	88.87	37.08	38.31	29.11	36.26	39.39	38.00	
	45.19	45.83	46.67	44.45	41.61	40.39	61.09	46.06	45.24	47.60	46.86	47.53	47.49	41.12	41.90	61.08	46.26	45.66	41.70	45.09	45.76	29.89	46.32	45.31	39.27	40.44

FIG. 2. Dendrogram based on 16S-23S ITS sequences, showing the inter- and intraspecific relationships of members of the genus *Bifidobacterium*. The tree was constructed by using the neighbor-joining method and bootstrap values calculated from 1,000 trees. Each number on a branch indicates the number of times (out of 100) that the node was supported by the bootstrap analysis results. The nodes to the left of the vertical line were not solved by the ITS phylogeny analysis, whereas the nodes to the right side were solved accurately. The root was placed arbitrarily. Bar = 3.6% sequence divergence. The designations on the right are cluster and subcluster designations.

Cluster 4. Cluster 4 was phenotypically coherent. This cluster contained all of the *B. bifidum* strains except *B. bifidum* ATCC 11147, which is clearly misclassified as a *Bifidobacterium* strain (see above). As observed in the 16S rRNA analysis, bootstrap data indicated that *B. bifidum* exhibited no significant affinity with any other organism and clearly belonged to a distinct group. The same observation has been reported previously (22).

Cluster 5. Cluster 5 was divided into two subgroups, subclusters 5A and 5B. Consistent with the results of the 16S rRNA analysis and the results of Lauer and Kandler (22), *B. animalis* and *B. globosum* were found to be related and were included in subcluster 5A. The presence of both *B. pseudolongum* and *B. globosum* in subcluster 5A was expected since workers have reported previously that these two species are related, as determined by DNA-DNA hybridization data (levels of similarity, 69 to 73%) (2), phenotypic characteristics (22, 29), and serologic characteristics (58). Therefore, the high level of ITS sequence similarity (>88%) between *B. pseudolongum* and *B. globosum* provided additional evidence that these two taxa should be combined. Thus, our ITS sequence data are consistent with the data of Yaeshima et al. (65), who proposed that these two species should be designated subspecies of the same species. However, these data are not consistent with the data obtained in the 16S rRNA sequence analysis, which showed convincingly that *B. pseudolongum* ATCC 25526^T and *B. longum* ATCC 15707^T are related to each other but not to *B. globosum* ATCC 25865^T. Taking into account the high level of 16S rRNA sequence similarity between *B. pseudolongum* ATCC 25526^T and *B. longum* ATCC 15707^T (the sequences of these organisms differed at only six positions), it seems likely that the strain used by Larsen et al. (21) in their 16S sequence analysis was misclassified as *B. pseudolongum* ATCC 25526^T and probably is a *B. longum* strain.

Subcluster 5B contained only *B. magnum* ATCC 27540^T. Our ITS sequence analysis failed to reveal which strain is the strain that is most closely related to ATCC 27540^T (Fig. 2).

Cluster 6. As shown above, cluster 6 included *B. breve*, *B.*

longum, *B. infantis* ATCC 15697^T, and *B. indicum* ATCC 25912^T. We were not able to confirm that these species are closely related to *B. suis* because no PCR product was generated when *B. suis* CIP 103377^T was used.

Both the 16S rRNA analysis and the 16S-23S ITS sequence analysis revealed that *B. infantis* ATCC 15697^T and *B. indicum* ATCC 25912^T are closely related (level of 16S rRNA sequence similarity, 99.8%; level of ITS sequence similarity, 97.7%), suggesting that these organisms belong to the same species. The level of evolutionary divergence between *B. indicum* ATCC 25912^T and *B. infantis* ATCC 15697^T seems to be more consistent with classification of these strains as members of a single species than with classification as members of separate species in the same genus. These data contrast with results obtained previously in DNA-DNA hybridization experiments (level of DNA-DNA similarity between *B. indicum* ATCC 25912^T and *B. coryneforme* ATCC 25911^T, 100%) (52), with the fermentative characteristics of the organisms, and with the murein type (22), which suggested that *B. indicum* is related to *B. coryneforme*. Our data suggest that the *B. indicum* strain used in our two analyses is a *B. infantis* strain and differs from *B. indicum* ATCC 25912^T as described by Scardovi and Trovati (52).

DISCUSSION

16S rRNA analysis. Comparisons of 16S rRNAs have proven to be extremely useful for determining phylogenetic relationships among organisms from the level of domains to the level of moderately closely related species (60). The results which we obtained in this study led to the conclusion that the evolutionary distances exhibited by most bifidobacteria are such that these organisms belong to the same genus. Despite considerable phenotypic diversity, all of the bifidobacteria whose sequences have been determined are closely related (levels of 16S rRNA similarity, >93%). However, the relationships among the different lineages within the genus *Bifidobacterium* are largely uncertain (Fig. 1). Judging from the short branch lengths between some nodes, the six clusters appear to have diverged over a relatively short period.

It has been assumed that *Gardnerella* and *Bifidobacterium* strains belong to the same genus (9) for the following reasons. The length of the branch from the root of the *Gardnerella-Bifidobacterium* cluster to where the branch joins the other actinomycetes was similar to the lengths of the branches that separate the most distantly related mycelium- and spore-forming actinomycete taxa. The genera *Gardnerella* and *Bifidobacterium* exhibit close phylogenetic proximity (21). The levels of 16S rRNA similarity between *Gardnerella vaginalis* and representatives of the genus *Bifidobacterium* (Table 2) are consistent with placing *G. vaginalis* and *Bifidobacterium* strains in a single genus. However, the opposite result was obtained previously by Greenwood and Pickett (15) and Piot et al. (40), who concluded on the basis of DNA-DNA hybridization data that *G. vaginalis* is not a member of the genus *Bifidobacterium*. Another difference is the G+C content of *G. vaginalis* (42 to 44 mol%) (16), which is significantly lower than the G+C contents of *Bifidobacterium* spp. (55 to 67 mol%) (3). According to Goodfellow and O'Donnel (14), "if the chromosomal base composition of species in a genus differs by more than 10-12% G+C, they must be so distantly related that classification into separate genera is warranted." This is another piece of evidence which supports the view that the genera *Gardnerella* and *Bifidobacterium* should be considered two separate taxa.

ITS analysis is complementary to 16S rRNA analysis. The evolutionary rate of the 16S-23S rDNA spacer region is 10 times greater than the evolutionary rate of the 16S rDNA.

Hence, these two molecules provide different complementary phylogenetic information. While the 16S rDNA sequence is a good tool for inferring inter- and intrageneric relationships (11), 16S-23S rDNA spacer sequence comparisons provide information concerning intraspecific links (this study) and allow workers to detect recently diverged species.

ITS tree topology allowed species validity to be confirmed. Our data provide the first demonstration that 16S-23S ITS sequences allow workers to determine relationships among closely related species. In most cases, the ITS tree topology (Fig. 2) showed that the strains belonging to a species are more closely related to the strains of that species than to members of another species. This was true for all strains belonging to *B. animalis*, *B. bifidum* (except ATCC 11147), and *B. breve*, and thus the identities of these strains were confirmed.

In contrast, the *B. infantis* strains fell into three different areas on the phylogenetic tree. Because of the lack of phylogenetic coherence in this species, these strains should be placed in separate species. Only the characteristics of *B. infantis* ATCC 15697^T are exactly consistent with previously determined *B. infantis* characteristics (which are similar to *B. longum* characteristics). Additional cooperative work, however, needs to be performed to determine the taxonomic status of the other two *B. infantis* strains (CIP 6378 and ATCC 25962).

ITS sequence data versus bacterial species. Levels of ITS sequence divergence between strains belonging to the same species may help clarify species definitions that have up to now been controversial. However, it is not possible to determine precise boundary limits on the basis of levels of ITS rDNA sequence relatedness to delineate species. As an approximation, most species groups exhibit levels of 16S-23S sequence divergence of <13%. In this study the maximum level of intraspecific ITS sequence divergence was observed with *B. adolescentis* (12.7%), and for most species the levels of intraspecific divergence were much lower (<6%). We have reported previously that the 16S rRNA sequences of any two species differ by at least 1.5%; hence, it could be argued that two strains belong to the same species if their ITS sequences do not differ by more than 13%. Nevertheless, the exact 16S-23S rDNA similarity limits for defining a taxon will have to be determined for each bacterial family.

Most of the strains belonging to distinct species exhibit levels of ITS sequence divergence of more than 13%. Only the following four pairs of strains did not: *B. pseudolongum* ATCC 25526^T and *B. globosum* ATCC 25865^T; *B. cuniculi* CIP 103379^T and *B. dentium* ATCC 27534^T; *B. infantis* ATCC 15697^T and *B. longum* ATCC 15708; and *B. infantis* ATCC 15697^T and *B. indicum* ATCC 25912^T. An examination of these pair of strains indicated that these taxa should not be viewed as distinct species but should be considered single species (see below). Despite the very good agreement between the two sets of data (the 16S rRNA and ITS sequence data), there were significant differences between the two tree topologies. These differences revealed culture collection classification errors. For instance, we found that *B. cuniculi* CIP 103379^T should be reclassified as a *B. dentium* strain.

The most controversial problem concerns the species *B. infantis* and *B. longum*, which are difficult to distinguish by traditional methods. Phylogenetic data obtained from both the 16S rRNA sequence analysis (level of divergence, <0.8%) and the 16S-23S ITS sequence analysis (level of divergence, 12.7%) clearly demonstrated that *B. longum* ATCC 15707^T and *B. infantis* ATCC 15697^T are closely related. When the guideline value of 13% is used in conjunction with tree topology information (including confidence values), it seems likely that *B. longum* ATCC 15708 and *B. infantis* ATCC 15697^T should be

considered subspecies of a single species. Our doubt concerning the advisability of recognizing *B. infantis* and *B. longum* as separate species is also supported by DNA-DNA hybridization data which showed that the levels of DNA similarity between the two species range from 65 to 80% (48).

With the exception of *B. breve*, the same taxonomic question could be asked concerning each of the species belonging to cluster 6. The position of *B. suis* is questionable. Lauer and Kandler (22) previously supported the idea that *B. infantis*, *B. longum*, and *B. suis* should be considered members of a single species on the basis of 16S rRNA similarity data (level of similarity, 99.2%), DNA-DNA similarity data, fermentative characteristics, and murein types (for a review, see reference 22). On the basis of DNA-DNA similarity and 16S rRNA sequence analysis data, we agree with Lauer and Kandler (22), who combined *B. longum*, *B. suis*, and *B. infantis* in a single species, *B. longum*.

In conclusion, ITS sequence analysis is a useful technique for identifying bifidobacteria. There is a good correlation between strain relationships determined by ITS sequence studies and corresponding data derived from DNA-DNA hybridization studies.

Previous studies have demonstrated that it is feasible to distinguish bifidobacterial species by using either probes directed at 16S rRNA (12, 66) or random cloning probes (26). As we have shown previously, rDNA polymorphisms are useful characterizing bifidobacteria found in the human colonic flora (25). Despite ribotyping experiments which allowed us to characterize all strains, it has not been possible to assign each strain to a particular species. In this study, we found that the data obtained by sequencing the 16S-23S ITS region are useful for rapid identification or intraspecific phylogenetic studies of strains. Further experiments to identify strain-specific oligonucleotides are in progress.

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