International Journal of Systematic and Evolutionary Microbiology (2002), **52**, 1945–1951 DOI: 10.1099/ijs.0.02221-0

Unification of *Bifidobacterium infantis* and *Bifidobacterium suis* as *Bifidobacterium longum*

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The relationships between *Bifidobacterium infantis*, *Bifidobacterium longum* and *Bifidobacterium suis* were examined by means of carbohydrate fermentation, DNA–DNA hybridization, ribotyping and random amplified polymorphic DNA-PCR (RAPD-PCR). The levels of DNA–DNA hybridization among the strains of *B. infantis*, *B. longum* and *B. suis* used in this study were 67–81 % under optimal conditions (42 °C) and 63–85 % under stringent conditions (52 °C). Although the strains showed varied carbohydrate-fermentation patterns, the three species were divided into three types, namely the infantis type, the longum type and the suis type, by ribotyping and RAPD-PCR. On the basis of these results, strains of *B. infantis*, *B. longum* and *B. suis* were recognized as distinct groups within a single species. It is concluded that *B. infantis* and *B. suis* should be unified as *B. longum*, the latter species being divided into three biotypes, the infantis type, the longum type and the suis type, by molecular methods.

Keywords: *Bifidobacterium infantis*, *Bifidobacterium longum*, DNA–DNA hybridization, ribotyping, RAPD-PCR

INTRODUCTION

Bifidobacterium infantis and *Bifidobacterium longum*, isolated from children's faeces, were described on the basis of phenotypic characteristics by Reuter (1963). Two species, '*Bifidobacterium liberorum*' and '*Bifidobacterium lactentis*', initially separated from *B. infantis*, have been identified as *B. infantis* (Scardovi *et al.*, 1971) by DNA–DNA hybridization. The *B. longum* strains were grouped as biovars a and b (Reuter, 1963) on the basis of their phenotypic characteristics, and Mitsuoka (1969) subdivided *B. longum* into *B. longum* subsp. *animalis* and *B. longum* subsp. *longum*. In 1974, Scardovi & Trovatelli (1974) proposed that *B. longum* subsp. *animalis* should be elevated to the species level as *Bifidobacterium animalis* according to DNA–DNA hybridization results. Furthermore, *Bifidobacterium suis* was isolated from pig faeces and proposed by Matteuzzi *et al.* (1971).

The definition criteria for *B. infantis*, *B. longum* and *B. suis* have been based on phenotypic characteristics such as carbohydrate-fermentation patterns and the electrophoretic patterns of enzymes (Scardovi *et al.*, 1979; Scardovi, 1986). However, levels of DNA relatedness between *B. infantis* and *B. longum* were reported as 65–80 % (Scardovi, 1986) and Lauer & Kandler (1984) also showed that *B. suis* exhibited 66–78 % DNA relatedness with *B. infantis* and *B. longum*. According to the phylogenetic definition of species proposed by Wayne *et al.* (1987), approximately 70 % or greater DNA–DNA relatedness has been demonstrated among strains belonging to the same bacterial species. Therefore, it remains unclear as to whether these species represent a single species.

The present investigation was conducted to determine the taxonomic relationship of *B. infantis*, *B. longum* and *B. suis* by means of carbohydrate fermentation, DNA–DNA hybridization, ribotyping and random amplified polymorphic DNA-PCR (RAPD-PCR).

METHODS

Strains and growth conditions. The 25 strains of *B. infantis*, *B. longum* and *B. suis* examined are listed in Table 1. The

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Abbreviation: RAPD-PCR, random amplified polymorphic DNA-PCR.

Table 1. Strains of *B. infantis*, *B. longum* and *B. suis* used in this study

Strain	Source	Isolation date
B. infantis		
JCM 1210 (= ATCC 25962 = DSM 20223)	Faeces of human infant	1963
JCM 1222^T (= ATCC 15697^T = DSM 20088^T)	Faeces of human infant	1963
JCM 1260 (= ATCC 17930 = DSM 20218)	Faeces of human infant	1956
JCM 1272 (= ATCC 15702 = DSM 20090)	Faeces of human infant	1963
JCM 7007	Faeces of human infant	1969
JCM 7009	Faeces of human infant	1969
JCM 7010	Faeces of human infant	1969
JCM 7011	Faeces of human infant	1969
JCM 11344	Faeces of human infant	1997
JCM 11345	Faeces of human infant	1997
JCM 11346	Faeces of human infant	1997
JCM 11347	Faeces of human infant	1997
B. longum		
JCM 1217^T (= ATCC 15707^T = DSM 20219^T)	Faeces of adult human	1963
JCM 7050	Faeces of adult human	1969
JCM 7052	Faeces of adult human	1969
JCM 7053	Faeces of human infant	1969
JCM 7054 (= ATCC 15708)	Faeces of human infant	1963
JCM 7055	Faeces of human infant	1969
JCM 7056	Faeces of human infant	1969
JCM 11340	Faeces of human infant	1980
JCM 11341	Faeces of human infant	1980
JCM 11342	Faeces of human infant	1980
JCM 11343	Faeces of human infant	1980
B. suis		
JCM 1269^T (= ATCC 27533^T = DSM 20211^T)	Faeces of pig	1971
JCM 7139 (= ATCC 27532)	Faeces of pig	1971

strains of *B. infantis* and *B. longum* were isolated from the faeces of infant and/or adult humans, while the strains of *B. suis* were isolated from pig faeces. All strains were incubated for 48 h at 37 °C on BL agar (Nissui Seiyaku) with 5 % horse blood in an anaerobic jar with 100 % CO_2.

Carbohydrate-fermentation test. Twenty-five carbohydrate-fermentation tests were conducted in PYF broth (Benno *et al.*, 1984) containing 0·5 % (w/v) of various carbohydrates (L-arabinose, D-xylose, ribose, glucose, mannose, fructose, sucrose, maltose, cellobiose, lactose, trehalose, melibiose, raffinose, melezitose, dextrin, starch, glycerol, inulin, mannitol, sorbitol, inositol or salicin) or 0·25 % (w/v) aesculin or amygdalin. Carbohydrate fermentation was determined using a semi-automatic system for bacterial identification (Benno, 1996).

DNA–DNA hybridization. DNAs were extracted from the cells harvested from MRS broth (Difco) containing 0·05 % (w/v) L-cysteine hydrochloride monohydrate after growth for 12 h at 37 °C in an anaerobic jar and were purified by the method of Saito & Miura (1963), with modifications (i.e. the cell suspensions were lysed with *N*-acetylmuramidase at a final concentration of 0·1 mg ml^{-1}). Levels of DNA–DNA hybridization were determined by the method of Ezaki *et al.* (1989), using photobiotin and a microplate. Hybridization was carried out under optimal conditions (42 °C) and under stringent conditions (52 °C) for 16 h. The hybridization conditions were decided on the basis of the G+C content (58 mol%) of the type strains of *B. infantis* and *B. longum* (Ezaki *et al.*, 1989).

Ribotyping. The automated ribotyping device, the RiboPrinter microbial characterization system (Qualicon), was used for ribotyping. Standard reagents were used in all stages of the analysis. The method involves the release of DNA from cells, *Eco*RI digestion of chromosomal DNA and separation of the resulting fragments by agarose gel electrophoresis, followed by Southern hybridization probing using a sulfonated probe comprising the *rrnB* rRNA operon from *Escherichia coli* (Brosius *et al.*, 1981) and a chemiluminescent substrate. Images were obtained with a charge-coupled-device camera. In addition to the RiboPrinter-system automated analysis, numerical analysis of ribopatterns was performed using the GelCompar system (version 4.0; Applied Maths), which normalizes fragment-pattern data for band intensity and relative band position compared with the molecular mass marker. The similarity between all pairs was expressed using Pearson's coefficient correlation, and the unweighted pair-group method with arithmetic averages (UPGMA) was used for construction of the dendrograms.

RAPD-PCR. DNAs were extracted from cells harvested from BL agar with 5 % horse blood after growth for 48 h at 37 °C in an anaerobic jar with 100 % CO_2 and then purified by the method of Saito & Miura (1963). PCR amplification was performed using the random primers 103 (5′ GTGACGCCGC 3′), 127 (5′ ATCTGGCAGC 3′) and 173 (5′ CAGGCGGCGT 3′) (University of British Columbia). PCR amplifications were performed in a total volume of 25 µl containing 0·1 µg DNA, 1·25 U TaKaRa Ex *Taq* (Takara Shuzo), 2·5 µl 10× buffer (Mg^{2+}-free), 3 mM $MgCl_2$, 2 µl dNTP mixture (2·5 mM each), 0·2 µM primer and 15·92 µl water. PCR amplification was performed using a TaKaRa PCR Thermal Cycler MP (Takara Shuzo) with 45 cycles consisting of denaturation at 94 °C for 60 s, annealing at 30 °C for 90 s and extension at 72 °C for 120 s (according to Vincent *et al.*, 1998). After analysis of the RAPD-PCR patterns using NIH Image (National Institutes of Health, USA), the levels of similarity between the RAPD-PCR patterns were calculated using BioCLUST version 1.0 (Hiraishi *et al.*, 2000). Dendrograms were prepared by the program TreeEdit version 1.0 (Evolutionary Biology Group, University of Oxford, UK) using the neighbour-joining method (Saitou & Nei, 1987).

RESULTS

Carbohydrate-fermentation test

The carbohydrate-fermentation patterns of the strains are shown in Table 2. All of the *B. longum* strains and *B. suis* fermented L-arabinose, unlike the *B. infantis* strains. Melezitose fermentation was positive in four *B. infantis* strains, eight *B. longum* strains and two *B. suis* strains, whereas eight *B. infantis* strains and three *B. longum* strains were melezitose-negative. Inulin fermentation was positive in four *B. longum* strains. On the other hand, four *B. infantis* strains isolated in 1997 fermented melezitose and inositol, but the *B. longum* strains isolated in 1980, except for *B. longum* JCM 11343, were melezitose-negative. For some strains of *B. infantis* and *B. longum*, the carbohydrate-fermentation patterns differed from those of the type strains.

DNA–DNA hybridization

The results of DNA–DNA hybridization experiments performed under optimal conditions (42 °C) and under stringent conditions (52 °C) using three *B. infantis* strains, two strains of *B. longum* and one *B. suis* strain are shown in Table 3. *Bifidobacterium bifidum* JCM 1209^T, the type strain of the type species of the genus *Bifidobacterium*, was used as a negative control. At 42 °C, the level of DNA–DNA hybridization between *B. infantis* JCM 1222^T and *B. longum* JCM 1217^T was 80–81 %, while that for the strains of *B. infantis* and *B. longum* was 67–81 %. Under stringent conditions (52 °C), as well as under optimal conditions, *B. infantis* JCM 1222^T showed high levels of DNA relatedness (75–76 %) to *B. longum* JCM 1217^T and showed 66–67 % relatedness to *B. longum* JCM 7054. Two reference strains of *B. infantis* demonstrated 63–85 % relatedness to *B. longum* JCM 1217^T and 60–70 % relatedness to *B. longum* JCM 7054. *B. suis* also exhibited 75–78 % relatedness to the type strain of *B. infantis* and 75–78 % relatedness to the type strain of *B. longum* under optimal conditions. Furthermore, the

Table 2. Carbohydrate-fermentation characteristics of strains used in this study

Strains: 1–12, *B. infantis* strains JCM 1222^T (1), JCM 1210 (2), JCM 1260 (3), JCM 1272 (4), JCM 7007 (5), JCM 7009 (6), JCM 7010 (7), JCM 7011 (8), JCM 11346 (9), JCM 11344 (10), JCM 11345 (11) and JCM 11347 (12); 13–23, *B. longum* strains JCM 1217^T (13), JCM 7052 (14), JCM 7050 (15), JCM 11343 (16), JCM 7053 (17), JCM 7054 (18), JCM 7055 (19), JCM 7056 (20), JCM 11340 (21) JCM 11341 (22) and JCM 11342 (23); 24–25, *B. suis* strains JCM 1269^T (24) and JCM 7139 (25). All strains were positive for the fermentation of fructose, glucose, lactose, maltose, melibiose, ribose, raffinose and sucrose; all strains were negative for the fermentation of amygdalin, cellobiose and glycerol.

Substrate	*B. infantis*												*B. longum*											*B. suis*	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
L-Arabinose	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	−	+	−	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	+
Trehalose	−	−	+	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Melezitose	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−
Dextrin	−	−	−	+	+	+	−	−	+	+	−	−	+	+	−	−	−	−	−	+	−	−	+	−	−
Starch	−	−	−	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Inulin	+	−	+	−	+	+	−	+	+	−	−	+	−	+	+	+	−	−	+	−	−	−	−	−	−
Mannitol	−	+	−	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sorbitol	−	−	−	+	+	−	−	+	+	+	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−
Inositol	−	−	+	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Aesculin	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Salicin	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Table 3. Levels of DNA–DNA hybridization

Values are percentages of DNA–DNA hybridization at 42/52 °C. NT, Not tested.

Strain	Source of labelled DNA						
	1	2	3	4	5	6	7
1. *B. infantis* JCM 1222^T	100/100	89/82	83/85	81/76	71/58	78/75	35/20
2. *B. infantis* JCM 1260	91/92	100/100	87/95	73/85	73/67	NT/NT	44/32
3. *B. infantis* JCM 1272	89/75	83/77	100/100	71/63	68/60	NT/NT	32/10
4. *B. longum* JCM 1217^T	80/75	77/69	70/73	100/100	84/70	75/73	39/25
5. *B. longum* JCM 7054	68/66	67/61	72/70	79/77	100/100	NT/NT	36/16
6. *B. suis* JCM 1269^T	75/65	NT/NT	NT/NT	78/75	NT/NT	100/100	28/19
7. *B. bifidum* JCM 1255^T	43/23	33/26	37/28	34/31	41/25	46/14	100/100

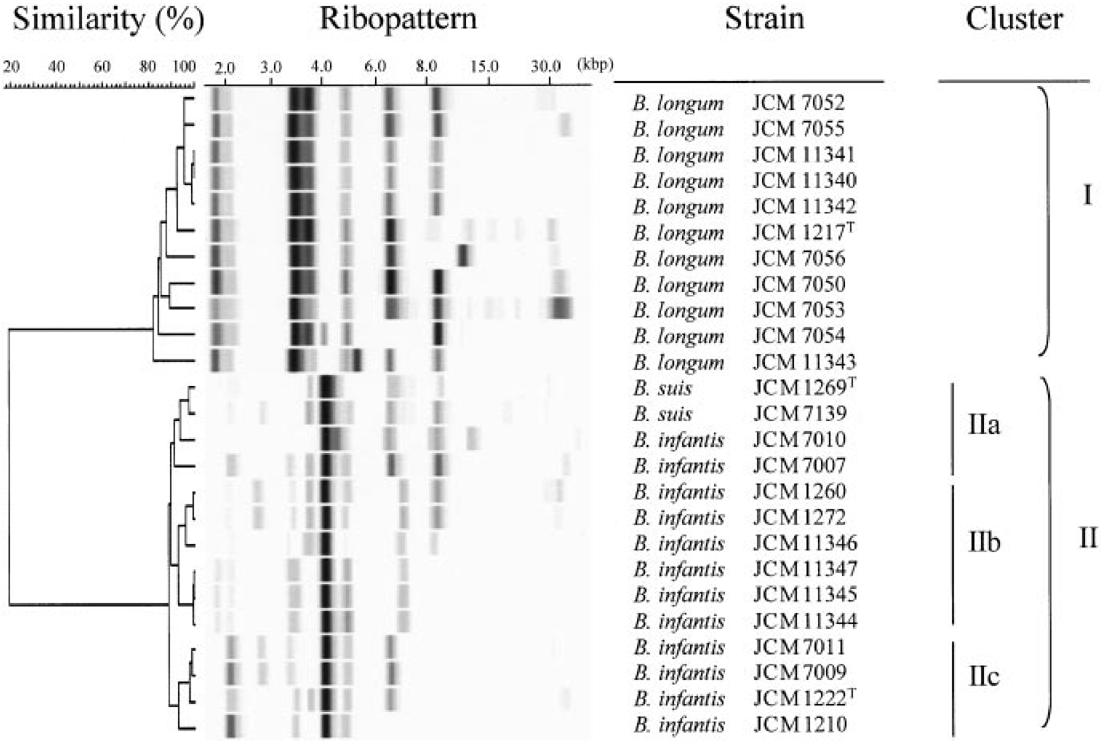

Fig. 1. Dendrogram of *B. infantis*, *B. longum* and *B. suis* strains based on *Eco*RI ribotyping.

levels of DNA–DNA hybridization among the three species were high (65–75%) under stringent conditions.

Ribotyping

The ribopatterns of digestion with *Eco*RI are shown in Fig. 1. Twenty-five strains were divided into two clusters at a similarity of 82%. Cluster I contained 11 *B. longum* strains and cluster II contained 12 *B. infantis* strains and two *B. suis* strains. Cluster II was subdivided into three subclusters (IIa, IIb and IIc) at a similarity of 93%. Two strains of *B. infantis* and *B. suis* fell into subcluster IIa. Subclusters IIb and IIc contained respectively six and four *B. infantis* strains. Cluster I yielded three specific bands, of approximately 0·7, 2·7 and 4·2 kbp, and cluster II yielded one specific band, of approximately 4·2 kbp. Subclusters IIa, IIb and IIc each yielded specific bands (IIa, 6·2 and 9 kbp; IIb, 7·5 kbp; IIc, 2·0 kbp).

RAPD-PCR

The RAPD-PCR patterns obtained using random primers 103, 127 and 173 are shown in Fig. 2. Twenty-five strains were divided into eight clusters at a

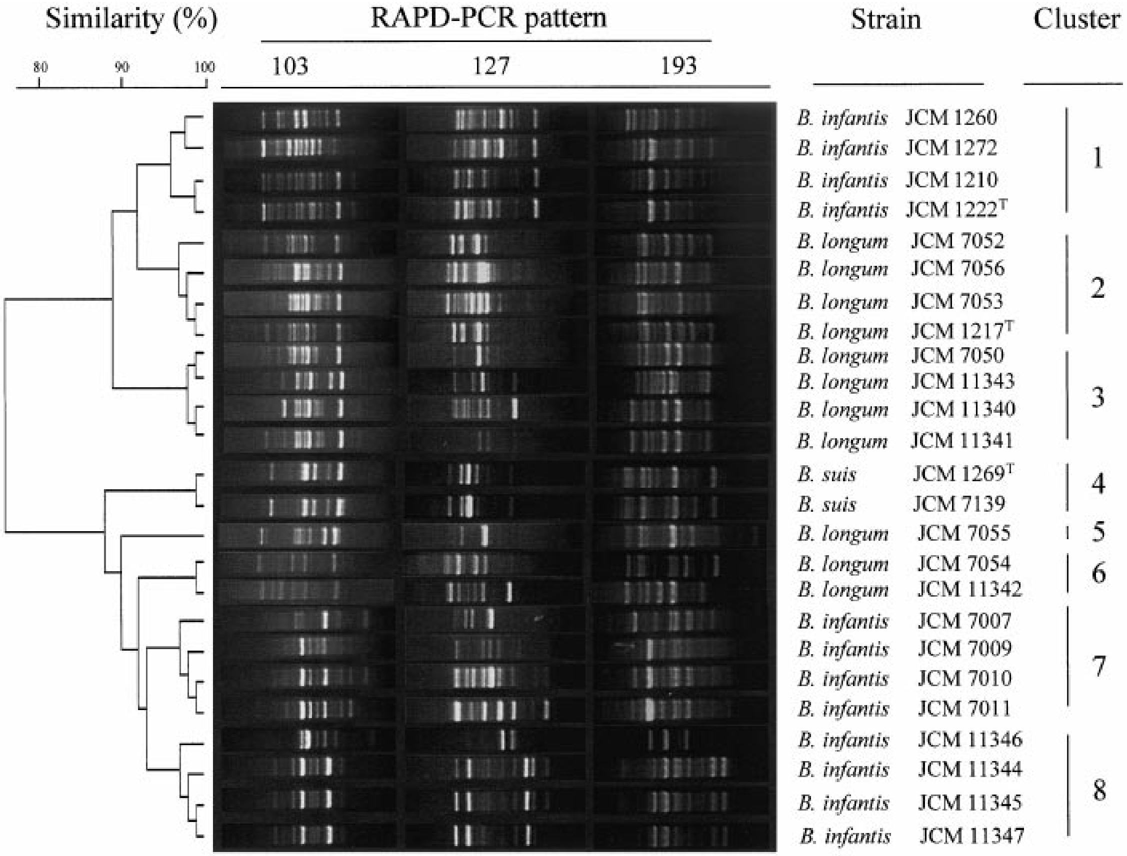

Fig. 2. Dendrogram of *B. infantis*, *B. longum* and *B. suis* strains based on RAPD-PCR.

similarity of 93 %. The *B. infantis* strains were divided into three clusters (1, 7 and 8) and the *B. longum* strains were divided into four clusters (2, 3, 5 and 6). The *B. suis* strains fell into cluster 4.

DISCUSSION

The carbohydrate-fermentation pattern is an important characteristic for distinguishing strains of *B. infantis*, *B. longum* and *B. suis*. The production of acid from L-arabinose and melezitose was proposed as a criterion to define *B. infantis* and *B. longum*/*B. suis* (Lauer & Kandler, 1983; Scardovi, 1986). In this study, the carbohydrate patterns could not distinguish *B. infantis* from *B. longum*/*B. suis* according to Lauer & Kandler (1983), because the strains showed varied melezitose-fermentation patterns. In previous studies, strains have been reported that showed carbohydrate-fermentation patterns that were different from those of the type strains (Bahaka *et al.*, 1993; Yaeshima *et al.*, 1991). Although the time of isolation does not necessarily affect the carbohydrate-fermentation pattern, it can be considered at least as a factor making identification difficult. Hence, identification by using carbohydrate-fermentation patterns is difficult, and a molecular method is needed to facilitate identification of the strains. Further work is need to clarify why the carbohydrate-fermentation patterns of the strains change according to the time of isolation.

Previous studies (Bahaka *et al.*, 1993; Lauer & Kandler, 1983; Yaeshima *et al.*, 1996) reported that the levels of DNA–DNA relatedness between the type strains of *B. infantis* and *B. longum* were 59–67 %. Bahaka *et al.* (1993) also reported a higher value (70 %, ΔT_m of 4·8 °C) for *B. infantis* DSM 20218 (= JCM 1260) and *B. longum* NCTC 11818^T (= JCM 1217^T). Strains isolated from the faeces of children showed high levels of DNA–DNA relatedness (70–82 %, ΔT_m of 2·2–2·7 °C) in comparison with the type strains of *B. infantis* and *B. longum*. Thus, there are strains that could be considered as part of a ‘continuum’ between *B. infantis* and *B. longum* (Bahaka *et al.*, 1993; Scardovi *et al.*, 1979). Furthermore, Lauer & Kandler (1983) reported that *B. infantis*, *B. longum* and *B. suis* showed a high degree of relatedness (62–78 %). In the present study, our results clearly indicated the close genetic relatedness existing between the three species under optimal and stringent conditions (Table 2). A species is defined as a group of strains that shares approximately 70 % or more DNA–DNA relatedness with a ΔT_m of 5 °C or less

(Wayne *et al.*, 1987). Phylogenetic analysis of the genus *Bifidobacterium* based on 16S rDNA (Miyake *et al.*, 1998) and a heat-shock protein (Jian *et al.*, 2001) also showed close relatedness between *B. infantis*, *B. longum* and *B. suis*. These results support the unification of these three species into a single species.

Using ribotyping, we demonstrated that the 25 strains used in this study were divided into four clusters containing three subclusters. Mangin *et al.* (1994) applied ribotyping to discriminate species of *Bifidobacterium*, and found that similar-sized molecular bands were generally observed in closely related strains. Hence, each cluster that yielded specific bands is considered as a distinct group within a single species. RAPD-PCR divided the strains into many more groups than did ribotyping and allows differentiation not only between species of the same genus and between strains of a given species but also between mutants belonging to the same clone (Martin *et al.*, 2000). Therefore, RAPD-PCR is a powerful tool for identifying bacteria and is highly sensitive and specific (Lin *et al.*, 1996). These results indicated that the strains of *B. infantis*, *B. longum* and *B. suis* should be considered as biotypes within a species.

The patterns for enzymes such as L-lactate dehydrogenase (*ldh*) are classification criteria for *Bifidobacterium* (Lauer & Kandler, 1983) and protein-coding sequence data are an effective tool for describing the diversity of bacteria (Palys *et al.*, 1997; Roy & Sirois, 2000). However, the populations of bacteria categorized using protein-coding sequences are ecological units, and are not necessarily in agreement with the units obtained by DNA–DNA hybridization. Hence, *B. infantis*, *B. longum* and *B. suis* should be unified into a single species on the basis of DNA–DNA hybridization.

In conclusion, *B. infantis*, *B. longum* and *B. suis* would be unified into a single species on the basis of DNA–DNA hybridization values. Meanwhile, in this study, it was possible to distinguish *B. infantis*, *B. longum* and *B. suis* by means of ribotyping and RAPD-PCR. These groups should be recognized as distinct biotypes within the same species. We conclude that *B. infantis* and *B. suis* should be unified as *B. longum* and that the latter species can be divided into three biotypes, namely the infantis type, the longum type and the suis type, by molecular methods.

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