New approach to phylogenetic analysis of the genus *Bifidobacterium* based on partial HSP60 gene sequences

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The partial 60 kDa heat-shock protein (HSP60) genes of 36 *Bifidobacterium* strains representing 30 different *Bifidobacterium* species and subspecies and of the type strain of *Gardnerella vaginalis* were cloned and sequenced using a pair of universal degenerate HSP60 PCR primers. The HSP60 DNA sequence similarities were determined for the taxa at various ranks as follows: 99.4–100% within the same species, 96% at the subspecies level, and 73–96% (mean 85%) at the interspecies level (and 98% in the case of two groups of closely related species, *Bifidobacterium animalis* and *Bifidobacterium lactis*, *Bifidobacterium infantis*, *Bifidobacterium longum* and *Bifidobacterium suis*, whose 16S rRNA sequence similarities are all above 99%). The HSP60 DNA sequence similarities between different *Bifidobacterium* species and *G. vaginalis*, a closely related bacterium according to 16S rRNA analysis, ranged from 71 to 79% (mean 75%). Although the topology of the phylogenetic tree constructed using the HSP60 sequences determined was basically similar to that for 16S rRNA, it seemed to be more clear-cut for species delineation, and the clustering was better correlated with the DNA base composition (mol% G+C) than that of the 16S rRNA tree. In the HSP60 phylogenetic tree, all of the high-G+C (55–67 mol%) bifidobacteria were grouped into one cluster, whereas the low-G+C species *Bifidobacterium inopinatum* (45 mol%) formed a separate cluster with *G. vaginalis* (42 mol%) and *Bifidobacterium denticolens* (55 mol%); a *Bifidobacterium* species of intermediate G+C content formed another cluster between the two. This study demonstrates that the highly conserved and ubiquitous HSP60 gene is an accurate and convenient tool for phylogenetic analysis of the genus *Bifidobacterium*.

Keywords: *Bifidobacterium*, HSP60 gene sequences, 16S rRNA sequences, phylogenetic analysis

INTRODUCTION

Members of the genus *Bifidobacterium* are Gram-positive, anaerobic, non-motile bacteria. The cells are pleomorphic, irregular, non-spore-forming rods. Uniquely, they possess fructose-6-phosphate phosphoketolase and produce acetic and lactic acid as the end-products of glucose metabolism. The habitats of bifidobacteria range from sewage to the intestines of humans, animals and honey bees (Scardovi, 1986), so they are believed to be predominantly a constituent of the intestinal flora. In recent years, new species of bifidobacteria have also been isolated from human dental caries (Crociani *et al.*, 1996), fermented milk

(Meile *et al.*, 1997) and an anaerobic digester (Dong *et al.*, 2000). So far, 32 species, including two subspecies, have been assigned to this genus.

Miyake *et al.* (1998) analysed the 16S rRNA sequences of type strains of 31 *Bifidobacterium* species and of *Gardnerella vaginalis*. The result indicated that all of the species formed a compact cluster involving similarity levels ranging from 93 to 99%, except for the two species *Bifidobacterium inopinatum* and *Bifidobacterium denticolens*, which have relatively low G + C contents (45 and 55 mol%, respectively). For some species, the 16S rRNA sequence similarities were even above 99%. So, although 16S rRNA sequence-hom-

ology analysis is demonstrated to be a useful tool for bacterial phylogenetic study, it cannot provide sufficient resolution to discriminate between closely related bacterial strains (Stackebrandt & Goebel, 1994).

Lauer & Kandler (1983) determined that DNA–DNA hybridization was an effective standard for species designation within the genus *Bifidobacterium*, and that DNA–DNA homology would be 70% or higher for the members within the same species. However, DNA–DNA hybridization is a laborious and inconvenient process. To determine the taxonomic position of a bacterial strain, one has to perform hybridizations between this strain and all the existing members of the genus. To study the relationship between different strains, even more pairwise hybridizations must be performed. In addition, the results of DNA–DNA hybridizations might not be as stable and comparable as those involving nucleotide sequences, because of the different experimental methods and conditions involved.

Thus, both approaches have limitations with respect to the phylogenetic analysis of bifidobacteria. In recent years, some housekeeping proteins such as the 60 kDa heat-shock protein (HSP60) (Viale *et al.*, 1994) and elongation factor (Kamla *et al.*, 1996), because of their highly conserved primary structures and ubiquity, have been considered as useful phylogenetic markers. HSP60 belongs to the chaperonin family, members of which are present in all bacteria and eukaryotic cell organelles such as mitochondria and chloroplasts. These chaperonins facilitate the intracellular folding and assembly (into oligomeric complexes) of various polypeptide chains. Viale *et al.* (1994) and Gupta (1995) have previously observed that evolutionary trees constructed from the protein sequences of these molecules in eubacteria demonstrated remarkable similarity to those derived from 16S rRNA genes. It is also known that almost all bacterial species have a single copy of the HSP60 gene in their genomes (Segal & Ron, 1996). Thus, it is hypothesized that the HSP60 gene is not easily transferred from one bacterium to another, and is a suitable macromolecule for the phylogenetic study of closely related bacterial species and strains. Kwok *et al.* (1999) cloned and sequenced the partial HSP60 protein genes of 36 validly described strains representing 28 different staphylococcal species or subspecies within this genus. The results showed that the partial HSP60 gene provided a convenient and accurate tool for species-specific identification and phylogenetic analysis of staphylococci.

In this study, a fragment (of approximately 0.6 kb) of the HSP60 gene of 36 *Bifidobacterium* strains, including 31 type strains of different *Bifidobacterium* species or subspecies, five bifidobacterial isolates, and a type strain of the closely related micro-organism *G. vaginalis* were amplified, cloned and sequenced. The phylogenetic analysis based on these sequences showed that the partial HSP60 DNA sequence was a powerful

tool for phylogenetic analysis of the genus *Bifidobacterium*.

METHODS

Bacterial strains. Thirty-seven bacterial strains, including 31 type strains of *Bifidobacterium* species, five bifidobacterial strains isolated from commercial dairy products, and one type strain of *G. vaginalis* were used in this study (Table 1). The strains were kindly provided by the Japan Culture Collection of Microorganism (JCM), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), the American Type Culture Collection (ATCC), the China General Micro-organism Culture Collection Center, or were previously isolated and identified in our laboratory.

The strains were routinely grown in tryptone peptone yeast-extract glucose medium (Scardovi, 1986). The media were prepared in glass tubes sealed with butyl rubber stoppers and screw caps. The gas phase was N₂. After inoculation, tubes were incubated anaerobically at 37 °C.

DNA extraction. The chromosomal DNA was extracted using a modified version of the method of Marmur (1961). As the cell walls of bifidobacteria were hard to lyse with lysozyme and SDS, 0.5 mg penicillin G ml⁻¹ culture was added to the cultures at the late exponential phase to assist cell breakage. The cultures were incubated for another 16–20 h. Proteinase K (100 µg ml⁻¹, 37 °C for 1.5–2 h.) was used instead of phenol to remove the protein contamination.

PCR amplification. The HSP60 degenerate PCR primers, designed on the basis of the conserved protein sequences GDGTTATV and AVKAPGFGD in HSP60 (Rusanganwa *et al.*, 1992), were H60F and H60R. The nucleotide sequences of H60F and H60R were 5'-GG (ATGC) GA (CT) GG (ATGC) AC (ATGC) AC (ATGC) AC (ATGC) GC (ATGC) AC (ATGC) GT-3' and 5'-TC (ATGC) CC (AG) AA (ATGC) CC (ATGC) GG (ATGC) GC (CT) TT (ATGC) AC (ATGC) GC-3', respectively. The 25 µl PCR mixture consisted of approximately 300 ng DNA template, 2.5 nmol each dNTP, 2.5 µl *Taq* DNA polymerase, 10 × buffer, 50 pmol each of H60F and H60R (Sangon) and 2.5 U recombinant *Taq* DNA polymerase (TaKaRa). The PCR thermal cycling conditions were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. Finally, the reaction was maintained at 72 °C for 10 min. The PCR amplification was performed with a Thermolyne Amplitron I (Barnstead Thermolyne). After amplification, the reaction mixtures were electrophoresed through a 1% (w/v) agarose gel (Promega) in 1 × TAB buffer (50 mM Tris acetate, 1 mM EDTA, pH 8.0) and visualized with ethidium bromide under UV light.

Cloning and sequencing of PCR products. The desired PCR products, which approximately matched nucleotide positions 250–840 in the HSP60 gene, were purified using the UNIQ-5 column DNA gel extraction kit (Sangon and NSBC) as recommended by the manufacturer. The purified fragments were ligated to pUCm-T (Sangon) with T₄ DNA ligase (MBI). The ligation products were then transformed into competent *Escherichia coli* DH5α, which was prepared by the CaCl₂ method. Ampicillin (100 µg ml⁻¹) and a blue-white selection (Sambrook *et al.*, 1989) were used to choose the required transformants, and the plasmids were extracted using the alkaline lysis method. To verify the presence of a correct insert of the partial HSP60 gene, PCR protocols, as

Table 1. List of strains and GenBank accession numbers of partial HSP60 gene sequences of bifidobacteria and related bacterial strains

Species	Strain	GenBank no.
<i>Bifidobacterium adolescentis</i>	JCM 1275 ^T	AF210319
<i>Bifidobacterium angulatum</i>	JCM 7096 ^T	AF240568
<i>Bifidobacterium animalis</i>	JCM 1190 ^T	AY004273
<i>Bifidobacterium asteroides</i>	JCM 8230 ^T	AF240570
<i>Bifidobacterium bifidum</i>	JCM 1255 ^T	AY004280
<i>Bifidobacterium boum</i>	JCM 1211 ^T	AY004285
<i>Bifidobacterium breve</i>	JCM 1192 ^T	AF240566
<i>Bifidobacterium catenulatum</i>	JCM 1194 ^T	AY004272
<i>Bifidobacterium choerinum</i>	JCM 1212 ^T	AY013247
<i>Bifidobacterium coryneforme</i>	JCM 5819 ^T	AY004275
<i>Bifidobacterium cuniculi</i>	JCM 1213 ^T	AY004283
<i>Bifidobacterium denticolens</i>	DSM 10105 ^T	AF240565
<i>Bifidobacterium dentium</i>	JCM 1195 ^T	AF240572
<i>Bifidobacterium gallicum</i>	JCM 8224 ^T	AF240575
<i>Bifidobacterium gallinarum</i>	JCM 6291 ^T	AY004279
<i>Bifidobacterium indicum</i>	JCM 1302 ^T	AF240574
<i>Bifidobacterium infantis</i>	JCM 1222 ^{T†}	AF240577
<i>Bifidobacterium infantis</i>	JCM 1210	AF240576
<i>Bifidobacterium infantis</i>	Isolate BB52	AY004288
<i>Bifidobacterium infantis</i>	Isolate 9901	AY004286
<i>Bifidobacterium inopinatum</i>	DSM 10107 ^T	AY004281
<i>Bifidobacterium lactis</i>	DSM 10140 ^{T†}	AY004282
<i>Bifidobacterium lactis</i>	Isolate 9950	AY004287
<i>Bifidobacterium lactis</i>	Isolate 9952	AF286735
<i>Bifidobacterium longum</i>	JCM 1217 ^T	AF240578
<i>Bifidobacterium magnum</i>	JCM 1218 ^T	AF240569
<i>Bifidobacterium merycicum</i>	JCM 8219 ^T	AY004277
<i>Bifidobacterium minimum</i>	JCM 5821 ^T	AY004284
<i>Bifidobacterium pseudocatenulatum</i>	JCM 1200 ^T	AY004274
<i>Bifidobacterium pseudolongum</i> subsp. <i>globosum</i>	JCM 5820 ^T	AF286736
<i>Bifidobacterium pseudolongum</i> subsp. <i>pseudolongum</i>	JCM 1205 ^T	AF240573
<i>Bifidobacterium pullorum</i>	JCM 1214 ^T	AY004278
<i>Bifidobacterium ruminantium</i>	JCM 8222 ^T	AF240571
<i>Bifidobacterium suis</i>	JCM 1269 ^T	AY013248
<i>Bifidobacterium thermacidophilum</i>	AS 1.2282 ^T	AY004276
<i>Bifidobacterium thermophilum</i>	JCM 1207 ^T	AF240567
<i>Gardnerella vaginalis</i>	ATCC 14018 ^T	AF240579
<i>Escherichia coli</i>	K-12	AE000487*

* Previously reported GenBank sequence.

† Strains in the same species used for the HSP60 sequence similarity analysis.

described above, were performed using approximately 50 ng plasmid DNA as the template. Nucleotide sequencing was performed by TaKaRa using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) and an ABI PRISM 377XL DNA sequencer. The T7 promoter (5'-TAA TAC GAC TCA CTA TAG GG-3') was used as the sequencing primer.

Analysis of sequence data, and phylogenetic tree construction. The partial HSP60 sequences determined in this study, and that of *E. coli* from the database, were aligned by the CLUSTAL W software (version 1.5). Continuous fragments of 538 bp, omitting the primer sequences, were used for

analysis. The similarities were calculated and converted into a distance matrix with the Jukes–Cantor coefficient by the DNADIST program (PHYLIP, version 3.5; Felsenstein, 1993). A phylogenetic tree rooted with *E. coli* was constructed by the Fitch method (PHYLIP, version 3.5) and the topology of the tree was evaluated by 1000 trials of bootstrap analysis with the SEQUENCEBOOTS program (PHYLIP, version 3.5). Partial HSP60 DNA sequences were also translated into protein sequences by using the PRIMER PREMIER program, version 5.00 (Biosoft International). The protein sequence similarities were calculated and converted into a distance matrix with the DNAMAN program, version 4.0 (Lynnon Biosoft).

RESULTS

Cloned 600 bp partial HSP60 gene sequences of bifidobacteria

A library of 37 cloned 600 bp HSP60 PCR products, representing 30 validly described *Bifidobacterium* species and subspecies and *G. vaginalis*, was generated. These PCR products were cloned and sequenced, and their GenBank accession numbers are listed in Table 1.

Sequence similarity in partial HSP60 genes and proteins among different *Bifidobacterium* species and subspecies

HSP60 DNA and protein sequence similarities among the four *Bifidobacterium infantis* and three *Bifidobacterium lactis* strains listed in Table 1 were determined. For the strains within the same species of *B. infantis* or *B. lactis*, the HSP60 DNA sequences were

highly conserved, the similarity ranging from 99.4 to 100% (mean 99.5%); the HSP60 protein sequence similarities were consistently 100%.

At the interspecies level, HSP60 DNA sequence similarity among 30 different *Bifidobacterium* species ranged generally from 73 to 96% (mean 85%), but was 98% for the two groups of closely related species, *Bifidobacterium animalis* and *B. lactis*, as well as *B. infantis*, *Bifidobacterium longum* and *Bifidobacterium suis*, whose 16SrRNA sequence similarities are all above 99%. The HSP60 DNA sequence similarity between the two subspecies of *B. pseudolongum*, *B. pseudolongum* subsp. *pseudolongum* and *B. pseudolongum* subsp. *globosum*, was 96%. The lowest similarity levels (73%) were determined between *B. inopinatum* and *Bifidobacterium cuniculi*, *B. inopinatum* and *B. pseudolongum* subsp. *pseudolongum*, and *B. inopinatum* and *B. pseudolongum* subsp. *globosum*. It is noteworthy that both of *B. denticolens* and *B.*

Fig. 1. Phylogenetic tree based on partial HSP60 DNA sequences. The tree was rooted with *Escherichia coli* and constructed using the Fitch method with bootstrap values calculated from 1000 trees. The number at each branch point represents the percentage bootstrap support. Bar, 5% sequence divergence.

inopinatum showed exceptionally low similarities with respect to all of the other members of the genus *Bifidobacterium* (76–80 %, mean 78 %, and 73–78 %, mean 75 %, respectively). The HSP60 DNA sequence similarities between *G. vaginalis* and different *Bifidobacterium* species ranged from 71 to 79 % (mean 74 %).

HSP60 protein sequence similarities among the 30 *Bifidobacterium* species were also examined. Because of the degeneracy of genetic codes, it is not surprising that the amino acid sequence similarity between any given pair was always higher than that of the corresponding DNA sequence. The amino acid sequence similarity at the interspecies level ranged from 83 to 100 % (mean 92 %). The highest similarity was between *B. animalis* and *B. lactis* (100 %), *B. longum* and *B. suis* (100 %), and among *Bifidobacterium boum*, *Bifidobacterium thermacidophilum* and *Bifidobacterium thermophilum* (all 100 %), and the lowest similarity was between *Bifidobacterium merycicum* and *B. inopinatum* (83 %). As expected, *B. denticolens* and *B. inopinatum* showed quite low amino acid sequence similarities with other *Bifidobacterium* species (84–92 %, mean 86 %; 83–92 %, mean 87 %, respectively). The amino acid sequence similarity between *G. vaginalis* and all the *Bifidobacterium* species studied ranged from 86 to 93 % (mean 91 %).

Phylogenetic analysis

A phylogenetic tree rooted with *E. coli* was constructed using the Fitch method with bootstrap values calculated from 1000 trees (Fig. 1). On the basis of the HSP60 DNA sequences, the *Bifidobacterium* species were grouped into three clusters: most of the species (28 out of 30) were grouped into cluster 1, *B. denticolens* formed cluster 2, and *B. inopinatum* and *G. vaginalis* formed cluster 3. The species in cluster 1 were further grouped into subclusters: *Bifidobacterium indicum*, *Bifidobacterium coryneforme*, *Bifidobacterium asteroides* (the three species isolated from the intestines of bees) and *Bifidobacterium minimum* formed one subcluster, while the three thermophilic species, *B. thermophilum*, *B. thermacidophilum* and *B. boum*, formed another one; the remaining 21 species were grouped into the third subcluster, having a profile quite similar to that determined on the basis of 16S rRNA (Miyake *et al.*, 1998).

DISCUSSION

Inter- and intra-species sequence similarities of partial HSP60 versus the 16S rRNA gene of *Bifidobacterium*

Because of the limitations of 16S rRNA analysis in the phylogenetic study of closely related bacterial taxa and the inconvenience of DNA–DNA hybridization, partial HSP60 DNA sequence analysis was conducted for most of the *Bifidobacterium* species described in this study. The result has shown that the partial HSP60 DNA sequence similarity among different *Bifido-*

bacterium species ranged from 73 to 96 % (mean 85 %). Exceptionally high similarities (98 %) were determined among the group comprising *B. infantis*, *B. longum* and *B. suis*, as well as between *B. animalis* and *B. lactis*. However, the 16S rRNA similarities of the two groups all reached levels above 99 %. By comparison, the 16S rRNA sequence similarities among all the *Bifidobacterium* species ranged from 90.2 to 99.9 % (mean 95 %) and were exceptionally high for five groups: (1) *B. indicum* and *B. coryneforme* (similarity 99.1 %), (2) *Bifidobacterium catenulatum* and *Bifidobacterium pseudocatenulatum* (similarity 99.5 %), (3) *Bifidobacterium saeculare*, *Bifidobacterium gallinarum* and *Bifidobacterium pullorum* group (similarity 99.3–99.9 %), (4) *B. longum*, *B. infantis* and *B. suis* group (similarity 99.1–99.2 %), (5) *B. animalis* and *B. lactis* (similarity 99 %) (Miyake *et al.*, 1998). Although the HSP60 DNA sequence similarities of these groups were higher than the mean, the discriminatory power was still stronger than that of 16S rRNA. For example, the HSP60 DNA sequence similarity was 91 % between *B. indicum* and *B. coryneforme*, 93 % between *B. catenulatum* and *B. pseudocatenulatum*, 96 % between *B. gallinarum* and *B. pullorum*, 98 % among *B. longum*, *B. infantis* and *B. suis*, and 98 % between *B. animalis* and *B. lactis*. For the strains within the same species of *B. infantis* or *B. lactis*, the HSP60 DNA sequence similarities were 99.4–100 %. Thus, analysis of partial HSP60 gene similarity is a powerful tool for the phylogenetic study of *Bifidobacterium* species, has a resolution power higher than that of 16S rRNA homology analysis, and is much more convenient than DNA–DNA hybridization.

Phylogenetic analysis of bifidobacteria and *gardnerella* by using partial HSP60 gene similarities

In the phylogenetic tree based on 16S rRNA (Miyake *et al.*, 1998), bifidobacteria were grouped into two clusters. The two species isolated from human dental caries, *B. denticolens* and *B. inopinatum*, formed cluster 1; all of the other members, including *G. vaginalis*, formed cluster 2. In the present study, based on partial HSP60 gene similarities, a phylogenetic tree including the *Bifidobacterium* species and *G. vaginalis* was constructed (Fig. 1). The topology was basically similar to that of 16S rRNA but with the following differences: (1) instead of clustering with most of the *Bifidobacterium* species in the 16S rRNA phylogenetic tree, *G. vaginalis* formed a relatively independent branch with *B. inopinatum*, a similar low-G + C *Bifidobacterium* species, which confirmed the genus status of genus *Gardnerella*; (2) *B. denticolens*, which formed one cluster with *B. inopinatum* in the 16S rRNA tree, was in an independent branch of the HSP60 tree, indicating that the phylogenetic relationship between *B. denticolens* and *B. inopinatum* may be more distant than was estimated from 16S rRNA sequence similarity. Furthermore, the clustering of strains in the tree of the HSP60 gene was better correlated with the DNA base composition than that of 16S rRNA. In the tree,

all of the high-G+C (55–67 mol%) bifidobacteria were grouped into one compact cluster, whereas the only two low-G+C members, *B. inopinatum* (45 mol%) and *G. vaginalis* (42 mol%), formed another cluster; *B. denticolens* (55 mol%) formed an intermediate cluster between the other two.

HSP60 protein sequence similarities of bifidobacteria

It is not surprising that the HSP60 protein sequence similarities should be higher than those of the corresponding DNA sequences. However, an interesting observation is shown for the group comprising *B. thermophilum*, *B. thermacidophilum* and *B. boum*. Although their HSP60 DNA did not show very high similarities (94–95%), the HSP60 protein sequence similarities were all 100%, the highest among all the *Bifidobacterium* species. It is suggested that there is some association between their thermophilic characteristics and the shared primary structures of their heat-shock proteins.

Removal of *B. denticolens* and *B. inopinatum* from the genus *Bifidobacterium*

It is noteworthy that, on the basis of HSP60 DNA sequences, *B. denticolens* and *B. inopinatum* were quite distant from other *Bifidobacterium* species. This result was in accordance with that of 16S rRNA sequence homology analysis, and led to the view that these two organisms should be removed from the genus *Bifidobacterium* and proposed as new genera in the family *Bifidobacteriaceae* (Stackebrandt *et al.*, 1997). Further studies, such as the analysis of other conserved molecules, are needed to confirm this view.

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