

## RESEARCH ARTICLE

# Molecular Analyses of the Intestinal Microbiota of Chimpanzees in the Wild and in Captivity

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Little information is available regarding the intestinal bacteria of chimpanzees in the wild, due to the technical difficulties of studying intestinal bacteria in the field. In this study, molecular-based bacterial analyses were performed to overcome this difficulty because polymerase chain reaction (PCR)-based methods, such as temperature gradient gel electrophoresis (TGGE) and amplified ribosomal DNA restriction analysis (ARDRA), of the bacterial 16S rRNA gene can be applied to ethanol-fixed fecal samples. The common presence of bacteria belonging to the *Clostridium* rRNA sub-group XIVa, such as *Ruminococcus obeum* and *Eubacterium* sp., was indicated for Bossou wild chimpanzees by ARDRA. TGGE on partial 16S rDNA followed by hierarchical clustering analysis showed a systematic difference in the composition of intestinal microbiota between wild and captive chimpanzees. However, several TGGE bands commonly shared by wild and captured chimpanzees were excised, and their sequences were obtained. They were suggested to be the *Clostridium leptum* subgroup bacteria, *Lactobacillus gasseri*-like bacterium, and *Bifidobacterium pseudocatenulatum*- or *B. catenulatum*-like bacterium. These may be considered as common intestinal bacteria for chimpanzees, and may be transmitted vertically over generations. *Am. J. Primatol.* 69:367–376, 2007. © 2006 Wiley-Liss, Inc.

**Key words:** wild chimpanzees; captured chimpanzees; fecal bacteria; 16S rDNA

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## INTRODUCTION

Little information is available regarding the intestinal microbiota of wild apes, although much is known about that of humans [Eckburg et al., 2005]. The traditional methods based on culturing bacteria require fresh material and considerable experimental equipment for anaerobic culturing, which is difficult to undertake in the field. Recent developments in molecular microbial ecology have made it possible to assess the intestinal microbiota of wild animals when fresh fecal samples are available. Bacterial DNA in feces is easily preserved by soaking the samples in ethanol or acetone [Fukatsu, 1999], which can then be safely transported (although ethanol-fixed samples are no longer suitable for bacterial culture). However, culture-independent methodologies based on the amplification of bacterial 16S rRNA genes by polymerase chain reaction (PCR) require only bacterial genomic DNA as templates. To assess the structure of a particular microbial community, the amplified ribosomal DNA can be analyzed by amplified rDNA restriction analysis (ARDRA), denaturing gradient gel electrophoresis (DGGE), or temperature gradient gel electrophoresis (TGGE) [Muyzer & Smalla, 1998].

The intestinal microbiota of chimpanzees have not been thoroughly studied. Some preliminary studies of animals in captivity have been conducted (N. Matsubayashi, unpublished), but studies in the wild have not been possible due to the technical limitations noted above. In this study we applied 16S rDNA-based analyses to the intestinal microbiota of chimpanzees in the forests of Bossou, Lola, and Republic of Guinea, and the Primate Research Institute of Kyoto University, Inuyama, Japan. We estimated the similarity and differences in the bacterial composition of intestinal microbiota of individual chimpanzees in both locations.

All of the chimpanzees at Bossou had been identified by age, sex, and lineage during a 30-year study [Sugiyama, 2004]. At Inuyama, the captive community of three generations had been developed over 40 years [Matsuzawa et al., 2006].

## MATERIALS AND METHODS

### Collection of Feces

Fresh feces were collected from 13 chimpanzees in Bossou (W1–W13) during the rainy season (July and August 2004), and from 10 chimpanzees (C1–C10) at the Primate Research Institute of Kyoto University in April 2004. In Bossou, feces of chimpanzees were collected just after defecation (within ~5 min) by the researchers and/or local guides who followed chimpanzees at a distance of ~7 m. The names of the individual chimpanzees were therefore identified. In Inuyama, feces of individual chimpanzees were collected just after defecation (within 5 min) during the morning feeding when they were individually located in separate rooms. Feces (~5 g) were transferred into sterile plastic tubes (50 ml) containing 35 ml of ethanol (>90% in Guinea and 99.9% in Japan) to maintain an ethanol concentration in excess of 70%. Caution was taken to avoid soil contamination by collecting the portion of feces that did not contact the soil or floor using sterile tweezers.

Samples of feces were kept at ambient temperature in Bossou and at 4 °C in Japan until the analyses were performed. After homogenization by vigorous shaking by hand, a portion of the suspension was centrifuged (10,000 × *g*, for 10 min 4 °C) to collect pellets. The pellets were subjected to the DNA extraction procedure proposed by Godon et al. [1997].

### PCR Amplification for TGGE

A forward primer, U968-GC (5'-CGCCCGGGGCGCGCCCC GGGCGGGGCG GGGGCACGGGGGGAACGCGAAGAACCTCAC-3'), and a reverse primer, L1401 (5'-GCGTGTGTACAAGACCC-3') [Nübel et al., 1996], were used to amplify regions V6–V8 of the bacterial 16S rRNA genes. All individual samples (13 from Bossou chimpanzees and 10 from Inuyama chimpanzees) were subjected to TGGE analysis. The GC clamp in the forward primer creates PCR products suitable for separation by TGGE [Muyzer et al., 1993]. PCRs were performed with recombinant (*r*-) *Taq* DNA polymerase (Toyobo, Osaka, Japan) and its antibody for hot-start PCR (Anti-*taq* high; Toyobo). Each 50  $\mu$ l PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each of deoxynucleoside triphosphate (dNTP), 2U *r-Taq* DNA polymerase, 2U Anti-*taq* high, 0.4  $\mu$ M of each primer, and 1  $\mu$ l extracted bacterial DNA. The samples were amplified in a Gene Amp System 2400 (Perkin-Elmer, Branchburg, NJ) using the following program: initial denaturation at 95 °C for 1 min; 40 thermal cycles of 95 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 50 sec; and final elongation at 72 °C for 7 min. The size and amount of the PCR products were confirmed by analyzing 5  $\mu$ l samples by 1% agarose gel (wt/vol) electrophoresis and ethidium bromide staining.

### TGGE Analysis on PCR Products

The D-code system (Bio-Rad, Tokyo, Japan) was used for the sequence-specific separation of PCR products [Inoue & Ushida, 2003a]. Electrophoresis was performed in 1 mm polyacrylamide gel (8% [wt/vol] acrylamide, 0.28% [wt/vol] methylene-bis-acrylamide, a 1.25  $\times$  Tris-(hydroxymethyl)aminomethane-acetate-EDTA [pH 8.0](TAE) buffer, and 8 M urea) with 1  $\times$  TAE as the electrophoresis buffer at a fixed voltage of 60 V for 20 hr. A gradient from 67 °C to 70 °C was applied (ramping rate = 0.2 °C/hr). Gels were developed by silver staining using a Wako silver staining kit (Wako, Osaka, Japan). The gel image was taken using a digital camera (Nikon Cool Pix 990; Nikon, Tokyo, Japan) and transferred to a personal computer. The profiles were analyzed using Gel-Pro Analyzer 3.1 (Media Cybernetics, Silver Spring, MD), and cluster analysis of the TGGE band profiles was performed using hierarchical clustering analysis with Euclidean square distances using the Excel (Microsoft, Seattle, WA) macro program developed by S. Aoki (Gunma University, Utsunomiya, Japan; program available at <http://aoki2.si.gunma-u.ac.jp/lecture/stats-by-excel/vba/html/clustan.html>). A corresponding dendrogram was constructed with Ward linkages. In this study, very weakly stained bands with an optical density (OD) of <0.1 in the analyzing software were not taken into account, because on occasion such bands cannot be identified automatically by the software and this could result in artifacts in the analyses. The density of the bands, which was measured by the software, was not used in this analysis. If a lane had a band at a certain position, an assignment of "1" was made; if there was no band at the same position, an assignment of "0" was made. Hierarchical clustering analysis is a statistical method for finding relatively homogenous clusters of cases based on measured characteristics. It is widely used in various factor analyses, such as the analysis of DNA microarrays [Eisen et al., 1998].

### Identification of Several TGGE Bands

Six TGGE bands were excised and put into 20  $\mu$ l of sterile water. The excised gel in water was left overnight at 4 °C to elute the DNA [Ohashi et al., 2004].

These bands (indicated in Fig. 1) were selected because they appeared to be common for all individuals tested. After the extraction step was performed, a portion of the eluted DNA solution (3  $\mu$ l) was amplified with primers L1401 and 968F, which does not contain the 40 bases GC crump. The PCR conditions were principally the same as indicated above except for the cycle number and the length of each thermal step: 25 cycles (30 sec each for three thermal steps) and 3 min of final elongation. Amplified products were sequenced after TA-cloning, as described elsewhere [Inoue & Ushida, 2003b]. Briefly, PCR amplicons were ligated into pGEM-T vector (Biorad, Tokyo, Japan) and *Escherichia coli* JM109 was transformed. Clones harboring amplified DNA were selected by blue-white screening, and 32 white clones were randomly selected from each plate (one plate for each TGGE band). Colony PCR using SP6 and T7 primers was performed to check the size of inserted DNA with *r-Taq* polymerase. The following thermal cycle was applied: 94 °C for 3 min of initial denaturation followed by 25 cycles of 94 °C for 30 sec, 48 °C for 30 sec, and 72 °C for 50 sec, with a final elongation at 72 °C for 7 min. RFLP analyses on PCR products were performed with *Hae* III (Toyobo, Tokyo), *Hha* I (Toyobo), or *Rsa* I (Toyobo) according to the manufacturer's instructions to group the obtained *E. coli* clones. Although several clone groups (up to four) were recognized for each excised TGGE band, the most abundant clone group was selected and the insert of one randomly selected clone from the clone group was sequenced. Sequencing was performed at Shimadzu Genomic Research, Co. Ltd. (Kyoto, Japan). Sequences (up to ~450 bp) were compared via the BLAST program at NCBI (<http://www.ncbi.nlm.nih.gov/blast>) with those registered in databases (such as DDBJ/EMBL/GenBank) to suggest possible taxonomic names.

### Amplified Ribosomal DNA Restriction Analyses (ARDRA)

DNA extracted as above from fecal samples of 13 Bossou chimpanzees was subjected to ARDRA. The V3–V8 region of the 16S rRNA gene was amplified using primer 338f (5'-CTCCTACGGGAGGCAG-3') and L1401. After an initial denaturation at 95 °C for 4 min, 12 thermal cycles of 95 °C for 30 sec, 52 °C for 30 sec, and 72 °C for 50 sec were performed. The reaction was completed with a final elongation at 72 °C for 7 min.

Amplified products were ligated into a pGEM-T vector. *E. coli* JM109 was transformed, and positive clones were obtained by blue-white selection as described above.

Ninety-six positive clones were randomly picked from a plate raised from the feces of each individual chimpanzee (one plate for each individual). A total of 1,248 clones (96  $\times$  13) were obtained and maintained in 96-well plates. Restriction analyses were performed using *Hae* III, *Hha* I, and *Rsa* I to group these 1,248 *E. coli* clones. Of the 596 clone groups, the groups commonly shared by at least two individuals were subjected to further sequence analyses. One *E. coli* clone was selected randomly from each group, and its plasmid insert was then reamplified using T7 and SP6 primers. Amplicons were sequenced at Shimadzu Genomic Research (Kyoto, Japan) to compare registered DNA sequence in public databases via the BLAST program as described above.

Throughout this experiment, sterile water was used as the negative control for PCR. In the case of TGGE, marker strains were used as positive controls for PCR.

Chemicals were obtained from Nacalai Tesque (Kyoto, Japan) and Wako Purechemical (Osaka, Japan) unless otherwise stated.

## RESULTS

As shown by the TGGE images (Fig. 1), the band profiles apparently differed between the Bossou and Inuyama chimpanzees, although there were still several commonly shared bands. Sequence analyses on these bands suggested that they belong to the genera *Clostridium*, *Lactobacillus*, and *Bifidobacterium* (Table I). There were bacteria belonging to the *Clostridium leptum*-subgroup (bands A and B), *L. gasseri*-like bacteria (bands C and D), and *B. pseudocatenulatum*- (band E) or *B. catenulatum*-like (band F) bacteria. The dendrogram clearly indicates the difference in the bacterial composition of these two groups of chimpanzees (Fig. 2). The difference in bacterial composition within a group appeared to be small (Euclid square distance <20). In this dendrogram, individual chimpanzees harboring a similar bacterial composition belonged to one particular cluster. For

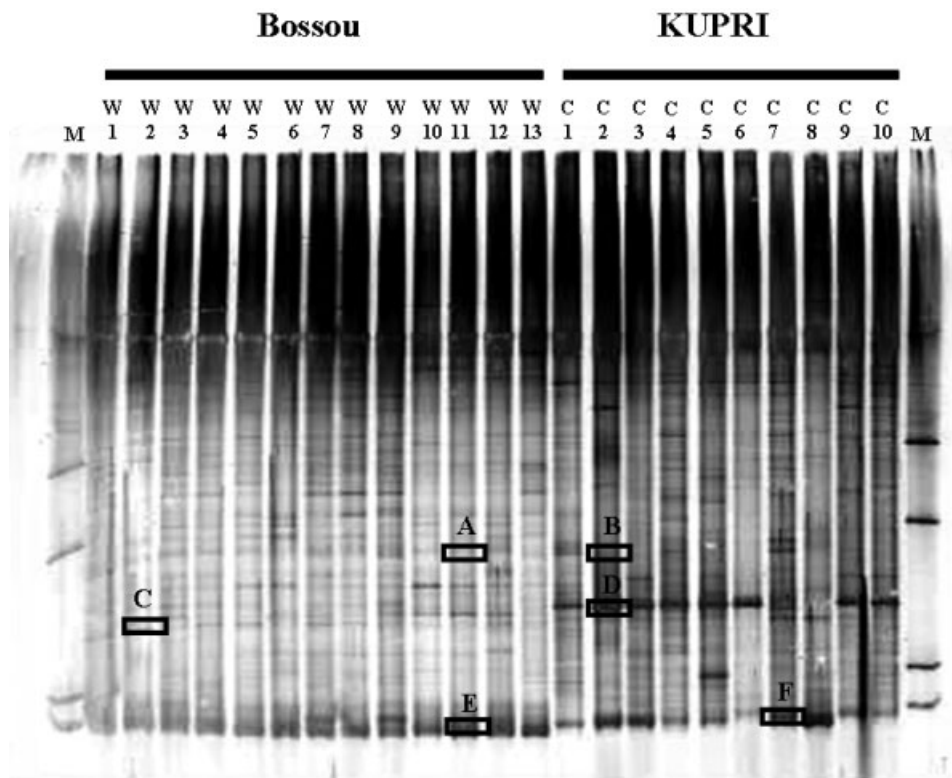


Fig. 1. Gel image of TGGE of bacterial 16S rDNA retrieved from the feces of chimpanzees. W1–W13: Bossou wild chimpanzees. The individual names are given in parentheses: W1 (Jire), W2 (Foaf), W3 (Pama), W4 (Peley), W5 (Fotayu), W6 (Fokaiye), W7 (Yoro), W8 (Tua), W9 (Yo), W10 (Fanle), W11 (Fana), W12 (Jeje), and W13 (Velu). C1–C10: Chimpanzees from the Kyoto University Primate Research Institute in Inuyama. The individual names are given in the parentheses: C1 (Ayumu), C2 (Cleo), C3 (Puchi), C4 (Popo), C5 (Reo), C6 (Pan), C7 (Pal), C8 (Pico), C9 (Ai), and C10 (Chloe). M, marker. The 16S rDNA sequences used for the TGGE-marker were previously identified (for details see Inoue and Ushida [2003a]). Information about the individual chimpanzees is available in Biro et al. [2003] and Matsuzawa et al. [2006]. Contrast in the picture was increased about 20% using Adobe Photoshop (v. 5.0) to improve the appearance for printing. The detection of the band was performed automatically on the original image using the Gel-Pro Analyzer 3.1 (Media Cybernetics, Silver Spring, MD) for the hierarchical clustering analysis. Bands were excised for sequencing: (A and B) *Clostridium*-like sequence, (C and D) *Lactobacillus* sp.-like sequence, and (E and F) *Bifidobacterium* sp.-like sequence. For details see text.

TABLE I. Results of BLAST search on partial sequence of bacterial 16S rDNA retrieved from chimpanzee feces\*

	First match (Accession No.)	Identity%	First match with known bacterium (Access No.)	Identity%
TGGE analyses				
band A (AB250369)	Uncultured bacterium clone Eldhufec317 (AY920192)	92	<i>Clostridium</i> sp. (AB093546)	86
band B (AB250365)	Uncultured bacterium clone p-3275-A42 (AF371768)	98	<i>Ruminococcus</i> sp. (AJ318889)	91
band C (AB250366)	Uncultured bacterium (AB248944)	100	<i>Lactobacillus</i> sp. (AB158766)	100
band D (AB250370)	Uncultured bacterium clone C528 (AY985549)	99	<i>Lactobacillales</i> bacterium HY-36-1 (AY58)	95
band E (AB250367)	Uncultured bacterium clone Eldhufec 085 (AY919960)	98	<i>Bifidobacterium</i> sp. (AY287759)	98
band F (AB250368)	Uncultured <i>Bifidobacterium</i> sp. (AY373326)	98	<i>Bifidobacterium</i> sp. (AY856700)	98
ARDRA				
OTU-3 (AB239605)	Unidentified rumen bacterium 12-16 (AF018460)	100	<i>Prevotella marshallii</i> (AF481227)	94
OTU-24 (AB239609)	<i>Anaeroplasma varium</i> (M23934)	87		
OTU-26 (AB239606)	Uncultured Clostridiaceae bacterium (AB218321)	94	<i>Eubacterium dolichum</i> (L34682)	87
OTU-103 (AB239607)	Uncultured bacterium clone C466 (AY985501)	100	<i>Ruminococcus obeum</i> (X85101)	99
OTU-105 (AB239608)	Uncultured bacterium clone D674 (AY986293)	98	<i>Ruminococcus lactaris</i> (L76602)	92

\*BLAST search was done at <http://www.ncbi.nlm.nih.gov/blast/>. Details, see text.

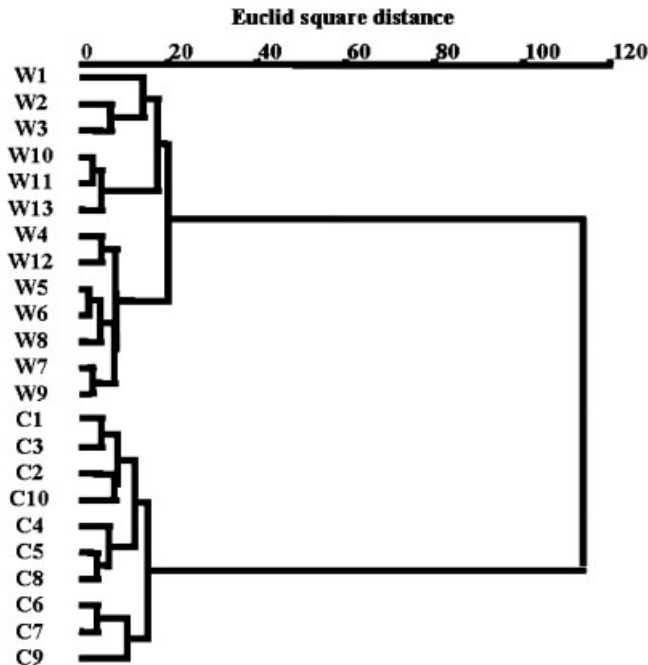


Fig. 2. Dendrogram obtained from hierarchical clustering analysis of the TGGE band profiles. The calculation was performed according to S. Aoki (Gunma University, Utsunomiya, Japan; program available at <http://aoki2.si.gunma-u.ac.jp/lecture/stats-by-excel/vba/html/clustan.html>). W1–W13 and C1–C10: see Fig. 1.

example, in the case of the Bossou chimpanzees, each of five combinations (W2 and W3, W12 and W4, W7 and W9, W5 and W6, and W10 and W11) makes a different cluster. In the case of the Inuyama chimpanzees, there were four combinations (C1 and C3, C5 and C8, C2 and C10, and C6 and C7), and each made a particular cluster. Among these combinations, W11 and W10, W5 and W6, W9 and W7, C10 and C2, and C6 and C7 were combinations of mothers and their offspring.

According to ARDRA, 596 *E. coli* clone groups (operational taxonomic unit (OTU)) were detected. Among them, 119 *E. coli* clone groups were detected in at least two individuals. Therefore, one representative *E. coli* clone from each of the 119 groups was sequenced. Among them, 59 clone groups harbored a partial sequence of 16S rDNA of Firmicutes, such as *Clostridium*, *Eubacterium*, *Lactobacillus*, *Ruminococcus*, and *Bacillus*. They were followed by clone groups harboring a partial sequence of 16S rDNA of Bacteroidetes (48 clone groups), such as *Prevotella* and *Bacteroides*. Partial sequences of 16S rDNA of Proteobacteria (eight clone groups), such as *Succinimonas*, *Succinivibrio*, and *Acinetobacter*, and of Actinobacteria (four clone groups), such as *Bifidobacterium* and *Slackia*, were detected. One clone group harbored a partial sequence of 16S rDNA of Spirochaetes, genus *Treponema*.

The major OTUs detected in the Bossou chimpanzees were OTU–3, –24, –26, –103, and –105. The percentage of particular clones in the whole clone library attributable to OTU–3, –24, –26, –103, and –105 were 3.63, 2.18, 5.35, 4.34, and 2.30, respectively. The results from the BLAST search are shown in Table I. Both a clone group harboring a partial sequence of 16S rDNA of the *Eubacterium* sp.

-like bacterium (AB239606) and another clone group harboring a sequence from the *Ruminococcus obeum*-like bacterium (AB239607) were shared by 12 of 13 Bossou chimpanzees. Both a clone group harboring a sequence from the *Anaeroplasma varium*-like bacterium (AB239609) and another clone harboring the *Ruminococcus* sp.-like bacterium (AB239608) were shared by 10 of 13 individuals. A clone harboring a sequence from the *Prevotella* sp.-like bacterium (AB239605) was shared by nine of 13 individuals.

## DISCUSSION

The ARDRA results show the predominant presence of plant polymer and sugar-fermenting bacteria, such as clostridia, ruminococci, and eubacteria, in chimpanzees in the wild. These bacteria are usually detected in a wide range of herbivorous and omnivorous animals, including humans [Holdeman et al., 1977; Salyers et al., 1977]. The *C. coccoides* group (Clostridium rRNA cluster XIVa), including *Eubacterium* sp., and ruminococci, such as *R. obeum*, have been found predominantly in human feces [Akkermans et al., 2000; Suau et al., 1999; Zoetendal et al., 2002b]. Among them, at least three sequences with the highest homology to *R. obeum* and *Eub. hallii* and *Fusobacterium prausnitzii* were present in all human subjects in one study, and their universal role in the human gastrointestinal tract was suggested [Akkermans et al., 2000]. The ARDRA results from the present study indicate a similarity in the predominant components of the intestinal microbiota in humans and chimpanzees.

A cluster analysis of the TGGE band profiles of intestinal microbiota indicated a difference in the composition of the intestinal microbiota between wild chimpanzees and those in captivity. Intestinal microbiota are affected by various factors in the host, such as food, age, and stress [Benno et al., 1984; Conway, 1995; Holdeman et al., 1976]. During the sampling period, the Bossou chimpanzees consumed fruits, such as figs (*Ficus* spp.), musanga (*Musanga cecropioides*), and spondia (*Spondias mombin*). However, in the rainy season the consumption of fruit is limited compared to that in the dry season [Takemoto, 2004; Yamakoshi, 1998]. The chimpanzees often consumed gum exudates of *Albizia* spp. [Ushida et al., 2006] to fulfill their nutritional requirement for minerals. The daily consumption of these plant materials depends on the accessibility of the food [Takemoto, 2004]. In contrast to wild conditions, the feeding of animals in captivity is highly controlled, and chimpanzees regularly consume a commercial monkey chow supplemented with various kinds vegetables and fruits. Further research is required to reveal the actual differences between bacteria in wild and captive chimpanzees. Such information may contribute new ideas concerning diet and environmental enrichment for captive chimpanzees.

Despite these differences between chimpanzees in the wild and those in captivity, there were still several intestinal bacteria common to both groups. The detection of bacteria commonly shared by chimpanzees in the wild and in captivity is interesting because these bacteria should have a tight physiological relationship with host chimpanzees. These bacteria may be so essential that they have not been washed out by the modification of environmental conditions. These bacteria in chimpanzees, which are suggested to belong to the genera *Clostridium*, *Lactobacillus*, and *Bifidobacterium* by the partial sequences of 16S rRNA gene, may be considered to be common intestinal bacteria that have been transmitted vertically over the generations. The *Clostridium leptum* subgroup (Clostridium rRNA cluster IV) includes the bacteria that are predominantly detected in humans [Suau et al., 1999]. *L. gasseri*-like bacteria are also commonly



detected in the human gastrointestinal tract [Zoetendal et al., 2002a]. Bifidobacteria are a major component of human intestinal microflora [Moore & Holdeman, 1974]. Among them, species in the *B. catenulatum* group have been demonstrated to be the predominant bifidobacteria in humans [Matsuki et al., 2004]. The present TGGE results also indicate a similarity of the intestinal microbiota in humans and chimpanzees.

Bifidobacteria are traditionally considered to be beneficial to humans [Macfarlane & Cummings, 1999]; therefore, it is plausible that this bacterium could be beneficial to the health of chimpanzees. Further research is under way to isolate these bacteria from feces of chimpanzees.

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