Short Communication

Isolation of Bifidobacteria from feces of chimpanzees in the wild

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(Received June 15, 2009; Accepted September 4, 2009)

Key Words——Bifidobacterium; chimpanzee in the wild

Intestinal bacteria in wild animals have not been thoroughly studied due to technical problems such as poor laboratory equipment in field conditions. The lack of electricity, clean water, and availability of CO2 gas makes work with anaerobic cultures difficult. In the case of wild apes in Africa, even buildings are often lacking. However, these constraints can be overcome by the use of molecular-based techniques. Indeed, bacteriological surveys based on the 16S rRNA gene can be conducted in all research fields of wild apes if fresh feces are available. In a previous study, fresh feces were collected aseptically in ethanol to fix bacteria for DNA preservation. After transportation to our laboratory in Kyoto, we successfully analyzed the bacterial 16S rRNA gene in the feces of chimpanzees in the wild (Fujita and Kageyama, 2007; Uenishi et al., 2007; Ushida, 2008). However, 16S rDNA-basis phylogenetic analyses of bacteria provide poor information on their functions in the intestine. Live bacterial cultures are essential to estimate their function in the physiology, particularly the nutrition, of the host (Gibson and Macfarlane, 1995).

In this report, we show the successful isolation of bifidobacteria from the feces of wild chimpanzees living in the tropical forest of Bossou, Guinea (Matsuzawa, 2006), though the identification of the isolates remains incomplete.

In this experiment, all equipment, anaerobic media in particular, was prepared in Kyoto and transported to Bossou. TOS-propionate agar plates (Yakult, Tokyo) and LBS agar plates (Becton Dickinson and Company, Cockeysville, MD, USA) were prepared according to the manufacturer’s instructions. Plates were placed in plastic bags (Techbarrier®, Mitsubishi Chemicals, Tokyo) with Anaeropack® (Mitsubishi Gas Chemicals, Tokyo). Plastic bags were closed by heat-sealing.

TOS-propionate stubs and LBS stubs were also prepared under O2-free CO2 using Hungate-type tubes (Bellco, Vineland, NJ, USA). Transportation to the research field took 1 week after preparation.

Fresh feces of 10 chimpanzees were successfully collected at the end of the rainy season of 2007 and rapidly transported (within ca. 30 min of defecation) to a house in Bossou Village as indicated in a previous study (Ushida et al., 2006). If chimpanzees were located at a distant location from the village, sampling was abandoned.

Collected feces were mixed with 9 volumes of a phosphate buffer (pH 6.5, 0.05 M) within a sterile 50-ml
plastic tube. Diluted feces were taken up using a sterile inoculating needle and streaked on both the TOS-propionate agar plate and the LBS agar plate. The plates were placed in a plastic kitchen container (6 plates per container) with a sac of Anaeropouch. After being tightly closed, plastic containers were placed in a polystyrene foam box. The temperature in this polystyrene foam box was maintained by air-activated disposable hand warmers as close to 37°C as possible. If the temperature increased up to 39°C, one hand warmer was removed. If the temperature decreased down to 35°C, an additional hand warmer was put into that box. The temperature was checked every 3 h (day time) to 6 h (night) with a portable digital thermometer.

After 48 or 72 h, the colonies developed on TOS-propionate plates were collected using a sterile needle and transferred to TOS-propionate stubs. Since there was no CO₂ gas supply in Bossou, the tubes were carefully manipulated in order to prevent the replacement of CO₂ in a tube with air. Since 50 tubes were transported to Bossou, 5 colonies were collected from a plate originating from each of the 10 chimpanzees. Plates were returned to a container with Anaeropouch and tightly closed with plastic tape. The containers, which contained spent agar plates, were placed in a kerosene refrigerator at ca. 4°C.

Tubes were then placed in a polystyrene foam box with hand warmers until bacterial growth became visible. Tubes were then kept in a kerosene refrigerator at ca. 4°C. Some of the pictures that explain the manipulation are available at http://www.pri.kyoto-u.ac.jp/hope/index.html (2007 12 11 Kazunari Ushida, Studies on intestinal lactic acid bacteria in great apes).

After the experiment was over in Bossou, the plates and tubes were transported to Kyoto. Ten days were needed for the transportation to the laboratory. Bacteria grown in TOS-propionate agar stubs were transferred to a TOS-propionate agar plate in an anaerobic chamber. Plates were placed in an anaerobic culture jar (BBL, Becton Dickinson and Company) and incubated at 37°C for 48 or 72 h. Developed colonies were cloned twice by sub-culturing. A single colony was isolated to a TOS-propionate broth medium. Only 13 bacteria were obtained after this re-purification step. This low level of recovery may have been due to the tough transportation conditions from Bossou to Japan. Indeed, the temperature of tubes cannot be controlled during the transportation within Guinea. Even within a styrene foam box, it was likely that the temperature of the tubes occasionally increased to over 40°C due to the unavailability of cooling devices. Isolates were microscopically checked for their purity, Gram-staining, and cell morphologies. Two of them were Gram-positive rod-shaped bacteria. The rest were all Gram-positive cocci.

Isolates grown in the TOS-propionate broth medium were recovered to analyze their 16S rDNA (from 27 to 1492, E. coli position) according to methods described elsewhere (Takahashi et al., 2007).

A fermentation test for arabinose, xylose, mannose, maltose, cellobiose, raffinose, inulin, and dextrin was conducted using a Lactobacillus, Bifidobacterium (LB) medium (Mitsuoka, 1984). Two isolates were inoculated to this medium and incubated for 48 h at 37°C. B. angulatum JCM 1252 was used as the reference strain.

A DNA-DNA hybridization test was conducted using the method described by Tachibana et al. (2003) for one isolate (Fn1) with B. angulatum JCM 1252.

One Gram-positive rod (Isolate Fn1) was isolated from one adult female chimpanzee, and another (Isolate YL1) from one adult male chimpanzee. Isolate Fn1 (Accession No. AB489093) showed 95% identity with B. angulatum (D86182) for 16S rRNA gene. Isolate YL1 (Accession No. AB4890934) showed 96% identity with B. angulatum (D86182) for 16S rRNA gene. Figure 1 shows a neighbor-joining tree computed with sequences of isolates Fn1 and YL1 together with selected sequences of bifidobacteria. Both isolate Fn1 and YL1 were placed within a cluster of B. angulatum. Based on the results from DNA-DNA hybridization (87% identity), it is indicated that one isolate (Fn1) is likely to belong to B. angulatum. Phylogenetical analyses of these isolates remain limited in this experiment. Further phylogenetical analyses, particularly with closely-related species such as B. berycicum and B. catenulatum, are of importance.

Both isolates, together with the reference strain, fermented xylose, maltose, raffinose, and dextrin. However, the two isolates did not ferment cellobiose, while the reference strain did.

B. angulatum was detected from feces of adult humans (Scardovi and Crociani, 1974). With the molecular ecology method, B. angulatum was detected in only 2 of 48 humans in a Japanese study (Matsuki et al., 1999). Moreover, the absence of B. angulatum in 27 subjects tested has been reported in a European study.
B. angulatum is not a predominant bifidobacterium in humans because this bacterium was detected from human subjects with quite limited prevalence. At the moment, the distribution of B. angulatum for wild great apes is not known, although bifidobacteria are believed to be a common member of chimpanzees’ intestinal microbiota (Uenishi et al., 2007).

Limited sequence analyses suggest the presence of B. catenulatum-like or B. pseudocatenulatum-like bifidobacteria in the chimpanzees in Bossou (Uenishi et al., 2007). In the present study, only two strains of bifidobacteria close to B. angulatum were isolated, probably due to the insufficient control of anaerobiosis and, to some extent, to the inability to control temperature, particularly during transportation within Africa. Indeed, other isolates were aero-tolerant Gram-positive cocci, of which partial 16S rRNA showed high homologies to Enterococcus hirae, E. facium, Lactococcus lactis, and Streptococcus bovis (Accession numbers from AB489095 to AB489105). The rate of survival of strict anaerobic bacteria such as bifidobacteria is likely to be improved if we have a CO2 or N2 gas cylinder and supporting experimental facilities in the capital city in Guinea.

Nevertheless, the present study shows the principal method for the isolation of anaerobic intestinal bacteria in outdoor conditions. We believe that this method is applicable for most outdoor field conditions, although Bossou Village provided relatively superior conditions compared with other tropical rain forest sites.

Acknowledgments

Part of the study was financed by the Japan Society for the Promotion of Science core-to-core program HOPE (T. Matsuzawa) and the Grant-in-Aid for Specially Promoted Research (#16002001 and #20002001, both to T. Matsuzawa). The authors thank the Ministère de l’Enseignement Supérieure et de la Recherche Scientifique, the Direction Nationale de la Recherche Scientifique et Technologique, and the Institut de Recherche Environnementale de Bossou of the Republic of Guinea for their assistance with this study. Assistance with the sample collection in Bossou by Messers P. Goumy, J. G. Doré, B. Zogbila,
and H. Gberegbe is gratefully acknowledged.

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