

Composition of fecal microbiota of laboratory mice derived from Japanese commercial breeders using 16S rRNA gene clone libraries

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ABSTRACT. The fecal microbiota of six mice derived from three Japanese commercial breeders was analyzed by using 16S rRNA gene clone libraries to construct a database for analyzing the gut microbiota of laboratory mice. The 566 clones were obtained from the clone libraries generated from the fecal DNA samples derived from BALB/c, C57BL/6N, DBA/2 and ICR mice. Among these 566 clones, there were 446 unique 16S rRNA gene sequences. When grouped at the 98% similarity level, the 446 unique sequences consisted of 103 Clostridiales, 43 Bacteroidales, 5 *Lactobacillus* and 3 Erysipelotricaceae, as well as sequences from 11 other phyla.

KEY WORDS: 16S rRNA gene, clone library, Japanese breeder, murine fecal microbiota

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Gut microbiota contributes to basic gut physiological function [8], including protection from pathogens [9, 11, 18], proliferation and activation of colonic epithelial cells by short-chain fatty acids [20], and development of the immune system [1, 10]. Gut microbiota also influences oncogenesis [17, 24, 25] and the metabolomic profiles of the organs, blood and urine of the host [14]. Recently, relationships between obesity and gut microbiota have been reported [2, 22], and their roles in metabolic syndrome have also drawn attention [23]. Many strains of laboratory rodents, including animal models for human diseases, are available from various laboratory animal breeders. Previous studies of laboratory animal gut microbiota by culture-based methods have reported significant differences in the composition of cecal microbiota among mice from different laboratory animal breeders [6]. However, culture-based methods are not applicable to non-cultivable bacteria. To overcome the problems of culture-dependent methods, PCR-based methods, such as denaturing gradient gel electrophoresis (DGGE) [5, 7], terminal restriction fragment length polymorphism (T-RFLP) [12, 16] and next-generation sequencing (NGS), have been widely applied. In particular, analysis of the gut microbiota by using NGS has become common in recent years. However, the use of NGS is expensive and labor-intensive. By contrast, DGGE and T-RFLP are inexpensive methods for analyzing the gut microbiota composition. These methods can be used to determine the identities of the phylogenetic groups in a microbial community, if the restriction enzyme cutting sites of the corresponding bacterial strains are

known. Therefore, it is important to analyze the sequences of the phylogenetic groups in a gut microbiota community using molecular biological techniques. To identify the major phylogenetic groups of the bacteria harbored in laboratory mouse gastrointestinal tracts, we studied the murine feces derived from three major Japanese commercial breeders by creating and analyzing 16S rRNA gene clone libraries.

SPF male C57BL/6N, DBA/2 and ICR were purchased from CLEA Japan (CLEA, Tokyo, Japan), Charles River Laboratories Japan (CRJ, Yokohama, Japan) and Japan SLC (SLC, Hamamatsu, Japan), respectively. SPF male BALB/c was purchased from three suppliers (CLEA, CRJ and SLC). All mice were used at 8 weeks of age in this study. These mice were euthanized immediately after arrival, and fecal samples were collected from rectums. This study was approved by the Institutional Animal Care and Use Committee of the Central Institute for Experimental Animals (Permit No. 11034).

The DNA isolation from each fecal sample was performed using a GTC solution and a T-RFLP kit for microbiota analysis (TechnoSuruga Laboratory, Shizuoka, Japan) according to the manufacturer's instructions. Briefly, the fecal samples were suspended in a GTC solution and then homogenized in Lysing Matrix E (MP-Biomedicals, Santa Ana, CA, U.S.A.) using a FastPrep FP120 (Thermo Savant, Waltham, MA, U.S.A.). Thereafter, DNA was extracted from a bead-treated suspension using a phenol-chloroform extraction method and was purified using an UltraClean PCR Clean-up DNA purification kit (MO Bio Laboratories, Carlsbad, CA, U.S.A.).

The purified DNA was amplified with a TaKaRa PCR Thermal Cycler Dice (Takara Bio, Otsu, Japan) using two universal primers 8f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1391r (5'-GACGGGCGGTGTGTRCA-3') [3]. PCR reactions were performed in a total volume of 20 μ l containing 1x *Taq* buffer, 250 μ M dNTPs, 1.5 mM MgCl₂, 0.4 μ M of each primer, 10 ng of fecal DNA and 0.5 U of

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HotStarTaq DNA Polymerase (Qiagen, Venlo, Netherlands). The PCR amplification program included preheating at 95°C for 15 min; followed by 25 cycles consisting of 95°C for 30 sec, 50°C for 30 sec and 72°C for 2 min; and a final extension step at 72°C for 10 min.

The amplified 16S rRNA genes were cloned into *Escherichia coli* TOP10 cells using a TOPO TA Cloning Kit (Life Technologies, Carlsbad, CA, U.S.A.), and transformants were randomly selected and subcultured. The plasmid DNA was purified using a QIAprep Spin Miniprep Kit (Qiagen). The inserted PCR products were confirmed by restriction enzyme (*EcoR* I; Takara Bio) analysis. The purified plasmid DNA was outsourced for sequencing analysis (Operon Biotechnologies, Tokyo, Japan). All of the sequences obtained were analyzed using GENETYX-MAC ver. 13 (GENETYX, Tokyo, Japan) to identify identical clone sequences. The phylogenetic classifications of the obtained 16S rRNA gene sequences were estimated using Classifier provided by the Ribosomal Database Project, and the closest relative was determined by the nucleotide BLAST program provided by the National Center for Biotechnology Information. To eliminate chimeric sequences, the 16S rRNA gene sequences were analyzed with the chimera check program on the Greengenes website [4]. A phylogenetic tree was constructed based on the neighbor-joining method using MEGA software [21]. The nucleotide sequences determined from the clone libraries have been deposited into the DDBJ with accession numbers AB702715 to AB702926.

A total of 566 clones were obtained from the 6 fecal DNA samples. Among these 566 clones, there were 446 unique 16S rRNA gene sequences. The remaining 120 clones were removed, because 77 clone sequences represented chimeric sequences and 43 clones shared a completely identical sequence with another clone. When grouped at the 98% similarity level, the 446 unique sequences consisted of 103 Clostridiales, 43 Bacteroidales, 5 *Lactobacillus*, 3 Erysipelotricaceae and sequences from 11 other phyla (data not shown). Using representative sequences, which were submitted to DDBJ, a phylogenetic tree was constructed for Clostridiales (Lachnospiraceae and Ruminococcaceae) and Bacteroidales (Figs. 1, 2 and 3). As shown in Fig. 1, numerous sequences belonged to Lachnospiraceae, particularly the *Clostridium coccoides* group. Numerous sequences obtained from this study were classified into characteristic clusters. In addition, a number of representative sequences were classified into other characteristic clusters. Within the Ruminococcaceae, numerous sequences obtained from this study were classified into two new characteristic clusters

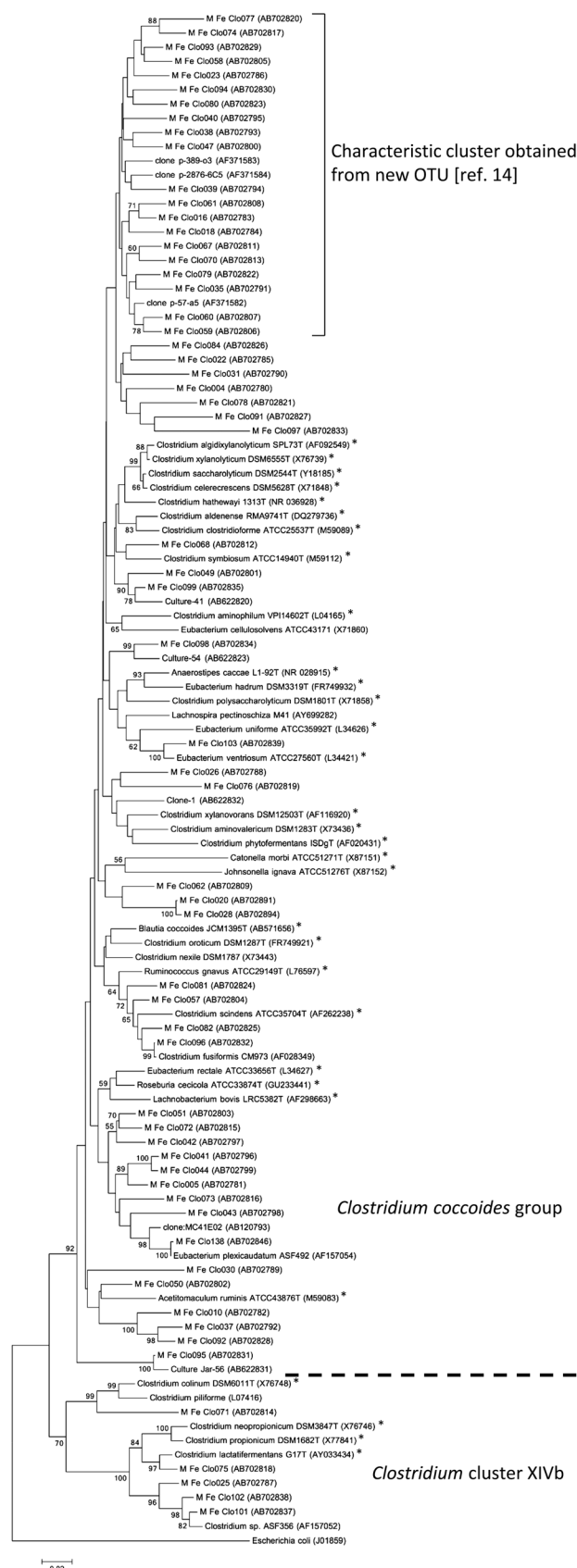


Fig. 1. Phylogenetic tree showing the relationship between representative 16S rRNA gene sequences of Lachnospiraceae from the clones of the feces of six laboratory mice and published sequences. The tree was constructed using the neighbor-joining method. Bootstrap values, based on 1,000 replications, at the nodes of the tree show >50% confidence. Scale bar=0.02 substitutions/nucleotide position. Accession numbers for each of the published sequences are given. *Escherichia coli* is used as the outgroup for rooting the tree. *, Type strains.

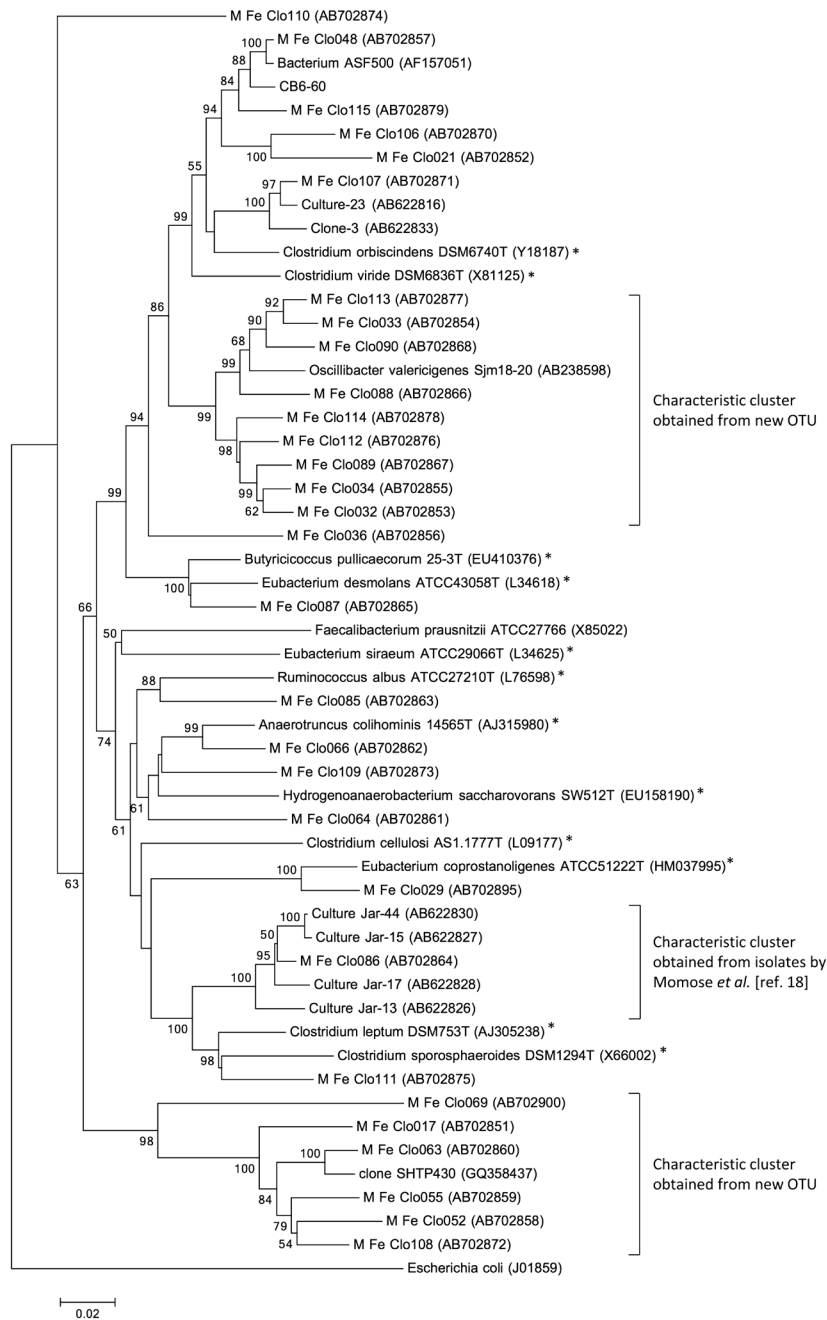


Fig. 2. Phylogenetic tree showing the relationship between representative 16S rRNA gene sequences of Ruminococcaceae from the clones obtained from the feces of six laboratory mice and published sequences. The tree was constructed using the neighbor-joining method. Bootstrap values, based on 1,000 replications, at the nodes of the tree show >50% confidence. Scale bar=0.02 substitutions/nucleotide position. Accession numbers for each of the published sequences are given. *Escherichia coli* is used as the outgroup for rooting the tree. *; Type strains.

(Fig. 2). Within the Bacteroidales, numerous sequences obtained from the mouse feces belonged to Porphyromonadaceae (Fig. 3). This characteristic cluster, including the majority of the sequences identified as Bacteroidales in this study, was classified as Porphyromonadaceae, except for *Parabacteroides*.

The laboratory mice have been bred in strictly controlled environment and have a stable quality. Therefore, it was estimated to be a preferred way for our study aimed at construction of a database for analyzing the gut microbiota of laboratory mice, although we only analyzed the feces of six mice using clone library methods. The majority of the clones

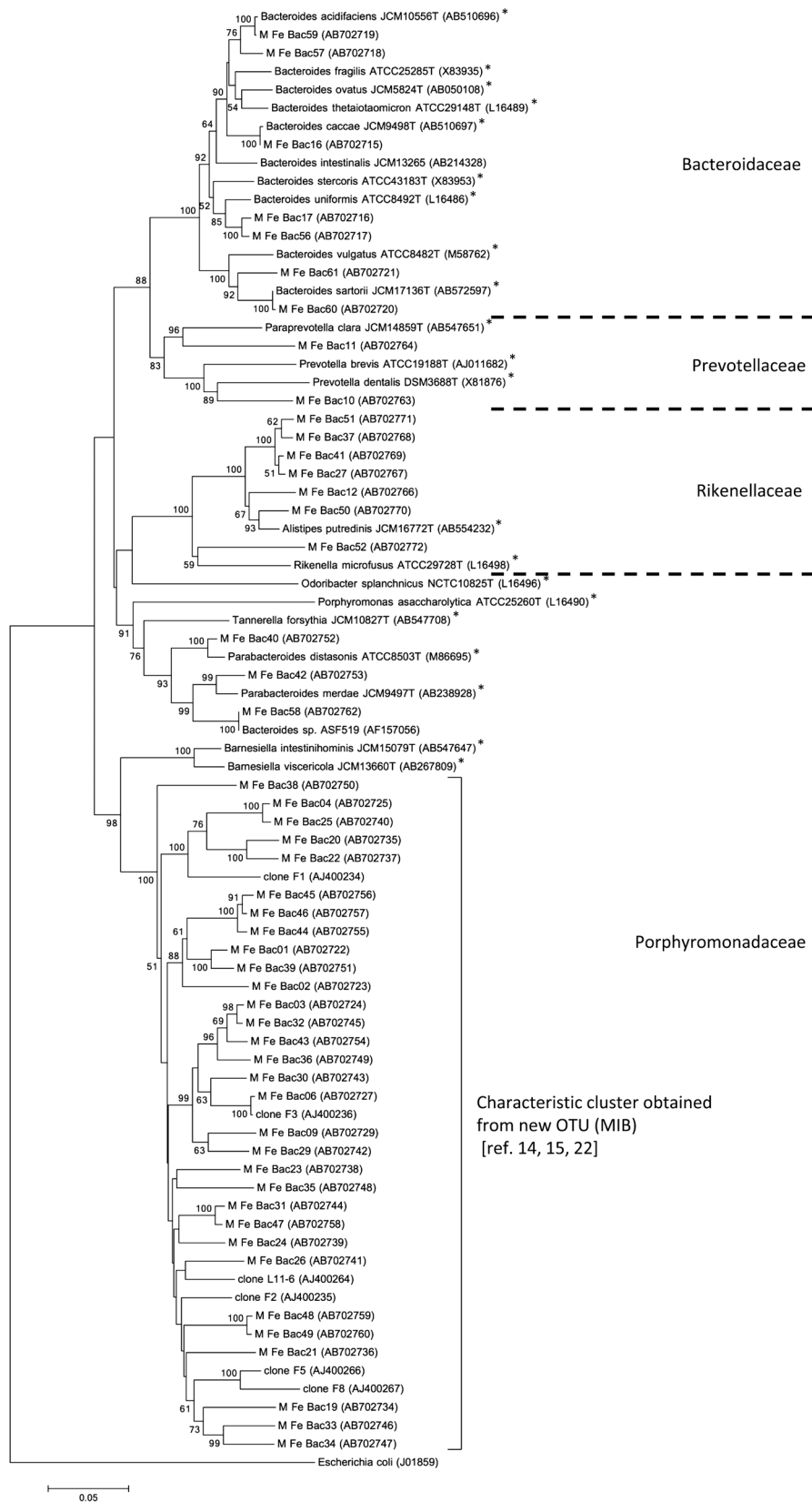


Fig. 3.

obtained from this study were classified as Lachnospiraceae, Porphyromonadaceae and *Lactobacillus*. Previous phylogenetic studies of Clostridiales and Bacteroidales in murine gut microbiota have been reported by Momose *et al.* [15], Salzman *et al.* [19] and Kibe *et al.* [12, 13]. The presence of the *C. coccoides* group, belonging to Lachnospiraceae, in this study is consistent with the cluster reported by Kibe *et al.* [12]. By contrast, the clustering of the *C. leptum* subgroup, belonging to Ruminococcaceae, in this study differed from the clusters reported by Momose *et al.* [15]. The Porphyromonadaceae detected in this study included an operational taxonomic unit (OTU) previously reported to be mouse intestinal bacteria (MIB) by Salzman *et al.* [19] and Kibe *et al.* [12, 13].

In conclusion, the fecal microbiota of mice derived from three Japanese commercial breeders consisted mainly of Clostridiales, Bacteroidales and *Lactobacillus*. Among Clostridiales and Bacteroidales, a high diversity of Lachnospiraceae and Porphyromonadaceae (other than *Parabacteroides*) was detected in this study.

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Fig. 3. Phylogenetic tree showing the relationship between representative 16S rRNA gene sequences of Bacteroidales from the clones obtained from the feces of six laboratory mice and published sequences. The tree was constructed using the neighbor-joining method. Bootstrap values, based on 1,000 replications, at the nodes of the tree show >50% confidence. Scale bar=0.02 substitutions/nucleotide position. Accession numbers for each of the published sequences are given. *Escherichia coli* is used as the outgroup for rooting the tree. *, Type strains.

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