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GLUCURONIDATION OF 1-NAPHTHOL AND EXCRETION INTO THE VEIN IN PERFUSED RAT KIDNEY

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ABSTRACT:

UDP-glucuronosyltransferase is expressed in the proximal convoluted tubular cells of rat kidney. Kidney perfusion with a Krebs-Henseleit buffer containing 1-naphthol was performed to estimate the dynamics and disposition of the glucuronide conjugate formed in the epithelial cells of the renal tubules. When 1-naphthol was injected into the renal artery, and the perfusate from the renal vein was returned to a reservoir and recirculated through the kidney preparation (recirculating perfusion), most of the 1-napthol was immediately excreted into the vein as a glucuronide conjugate and its concentration increased rapidly. In contrast, the 1-napthol glucuronide appeared more slowly in the urine. 1-Naphthol was also injected during the initial 5 min of perfusion under single-pass perfusion conditions (single-pass perfusion) in situ, and the metabolite and parent compound in the venous perfusate and in urine were assayed. Under this condition, most of the 1-naphthol glucuronide was excreted into the renal vein, and not urine. Phenol UDP-glucuronosyltransferase was highly induced in the rat kidney by β-naphthoflavone treatment. Moreover, the amount of 1-naphthol glucuronide excreted in the renal vein was increased 2.7-fold in the perfused kidney of β-naphthoflavone-treated rats, but the amount in the urine was not significantly increased under single-pass perfusion conditions. These results indicate that the kidney can glucuronidate phenolic xenobiotics in epithelial cells of the tubules and excrete the resultant glucuronide into the renal vein.

Extensive oxidation, reduction, hydrolysis, and conjugation reactions of xenobiotics can occur in the kidney (Lohr et al., 1998). Moreover, renal drug metabolism can be a critical determinant of risk to chemical injury (Lash, 1994). Most drugs and environmental toxicants are eventually excreted into the urine as glucuronides. Kidney perfusion of drugs is a useful means for estimating the formation and excretion pathways of glucuronides. For example, after isolated kidney perfusion with 1-naphthol, most of the chemical was glucuronidated and excreted into the urine (Redegeld et al., 1988). Phenol UDP-glucuronosyltransferase, which glucuronidates various phenolic xenobiotics such as 1-naphthol, was shown to be expressed in epithelial cells of proximal convoluted tubules of normal rats and also in the cells of the distal tubules of β-naphthoflavone-treated rats (Yokota et al., 1997). Determination of the direction of efflux of the glucuronide formed in the cells is important to estimate the drug dynamics not only in the kidney but also in the whole body. The excretion direction depends on the locations of ATP-binding cassette (ABC) transporter members, such as multidrug resistance-associated protein (MRP), which transport drug-glucuronide out of the cells (Borst and Elferink, 2002). MRP1 and MRP3 are expressed at the basolateral membrane and MRP2 is expressed at the apical membrane in epithelial cells of renal tubules (Borst and Elferink, 2002). However, the direction of glucuronide transportation out of the cells is not known.

In this study, a 1-naphthol glucuronide, a major metabolite that is excreted into urine and veins from the perfused kidney, was analyzed, and most of the metabolite was found in veins as a glucuronide. The results indicate that excretion of 1-naphthol glucuronide out of epithelial cells of renal tubules occurs predominantly across the basolateral membrane.

Materials and Methods

Chemicals. Cholic acid, purchased from Nissui Yakuhin Co. (Tokyo, Japan), was further purified and converted to its sodium salt (Imai, 1979). UDP-glucuronic acid was obtained from Nakarai Yakuhin Co. (Kyoto, Japan). 1-Naphthol was purchased from Kanto Chemical Co. (Tokyo, Japan). High-performance liquid chromatography (HPLC)-grade acetonitrile was obtained from Labscan Ltd. (Dublin, Ireland). β-Glucuronidase was obtained from Sigma-Aldrich (St. Louis, MO). Other reagents were of the highest grade available.

Animals. Male Sprague-Dawley rats (8 weeks old, 250–280 g) were used in all experiments. The rats were housed under standard conditions and given food and water ad libitum before use. The rats were handled according to the Laboratory Animal Control Guidelines of Rakuno Gakuen University based on the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health in the United States.

Surgical Procedure and Kidney Perfusion. The rats were anesthetized by

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ABBREVIATIONS: ABC, ATP-binding cassette; MRP, multidrug resistance-associated protein; HPLC, high-performance liquid chromatography; UGT, UDP-glucuronosyltransferase.

intraperitoneal injection of 60% urethane (0.3 ml/100 g body weight). Whole kidney perfusion was performed according to methods previously reported by Bowman (1975) and de Vries et al. (1989). Briefly, after anesthesia, the abdominal cavity was exposed by a ventral incision and the right ureter was cannulated with PE-10 polyethylene tubing (BD Biosciences, San Jose, CA), and then sodium heparin (1000 I.U.) was administered by an i.v. injection into the tail vein. Subsequently, the right renal artery was cannulated with a 21 gauge needle via the superior mesenteric artery. The vascular perfusate was a Krebs-Henseleit buffer containing 6% bovine serum albumin, 5 mM glucose, 2 mM glutamine, 2 mM glutathione, and 2 mM alanine. The buffer solution was aerated by 95% O_2 + 5% CO_2, and the pH was adjusted to 7.4. Several concentrations of substrate in the solution were tested, and full metabolites were obtained at 50 μM substrates in 30 ml of solution. These buffer solutions were maintained in a water bath at 37°C. The perfusion system consisted of a peristaltic pump (MP-32N, EYELA Co., Tokyo, Japan) and silicone tubes as illustrated in Fig. 1. The perfusion flow was maintained at 10 to 13 ml/min, and perfusion pressure was monitored.

Recirculating perfusion. The isolated kidney was fixed in a recirculating apparatus containing Krebs-Henseleit buffer (30 ml) as shown in Fig. 1. The buffer solution, which was mixed with the gas (95% O_2 + 5% CO_2), was perfused into the right renal artery, and the perfusate buffer yielded from the vein was returned to a reservoir and was recirculated through the kidney preparation (recirculating perfusion). The urine excreted out of the perfused kidney was collected as shown in Fig. 1 (final volume, 1.6 μl). An equilibration period of 15 min was conducted. Then 1-naphthol was added to the buffer solution (final concentration of 50 μM in 30 ml of solution). The substrate buffer solution was injected into the artery, and the resultant perfusate from the vein was mixed with the substrate buffer solution. Recirculation was continued for 40 min.

Single-pass perfusion. The perfusion was carried out in a flow-through mode. Preliminary perfusion was done for 15 min. Then, after a 5-min interval, the substrate buffer solution was perfused, and this was followed by reperfusion of the Krebs-Henseleit buffer solution. After perfusion of the substrate buffer, the excreted urine and perfusate in the vein were sampled and assayed for metabolites by HPLC.

Viability. The viability of the perfused kidney was monitored by measuring glucose and albumin reabsorption (>95%), and by measuring creatinine clearance for glomerular filtration and *p*-aminohippuric acid clearance for tubular secretion. These parameters were all found to be within the expected normal ranges.

HPLC Analysis of Reaction Products. The perfusate and urine were filtered for removing proteins using Artkiss (Advantec Toyo Co., Tokyo, Japan). The filtered solution was stored at −80°C until analysis. Samples were analyzed by HPLC (Tosoh Co., Tokyo, Japan) according to the method described previously (Yokota et al., 1999; Daidoji et al., 2003).

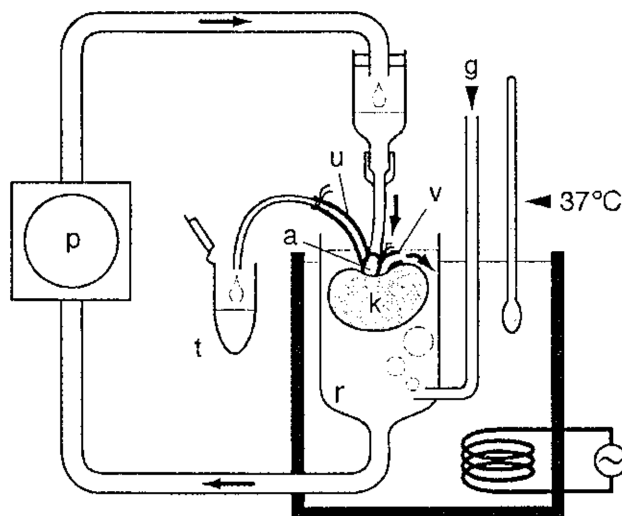

Fig. 1. Scheme of recirculating perfusion of the rat isolated kidney. The isolated kidney (k) was fixed in a tube containing Krebs-Henseleit buffer containing 6% bovine serum albumin, and the buffer solution (total, 30 ml) was pumped (p) through the kidney at 18 ml per min. The buffer solution was gassed with 95% O_2 + 5% CO_2 (g). The urine (final volume = 1.6 ml) was collected in a sample tube (t). a, artery; v, vein; u, ureter; r, reservoir.

β-Glucuronidase Reaction. For confirmation of 1-naphthol glucuronide, excreted metabolites were treated with β-glucuronidase using the method described previously (Yokota et al., 1999; Daidoji et al., 2003). The reaction was allowed to proceed for 30 min at 37°C in a mixture of 100 μl of 0.5 M acetate buffer (pH 4.5), β-glucuronidase (2.5 mg/ml), and diluted perfusate. The reaction mixture was then boiled and centrifuged for 5 min at 9000*g*. The supernatant was filtered through a disposable disk filter (HLC-DISK 3; Kanto Co., Tokyo, Japan) and analyzed by HPLC.

Treatment of Rats and Preparation of Microsomes. β-Naphthoflavone was dissolved in olive oil and orally administered at doses of 100 mg/kg of body weight daily for 4 days. The rats were killed 1 day after the final dose. The rat kidney was minced and homogenized with 4 vol of 0.15 M KCl solution containing 1 mM EDTA. The homogenate was centrifuged for 15 min at 9000*g*, and the supernatant fraction was centrifuged at 105,000*g* for 60 min to obtain microsomes.

Enzyme Analysis and HPLC. Renal microsomal UDP-glucuronosyltransferase activities toward 1-naphthol were assayed in 200 μl of 50 mM Tris-HCl buffer (pH 7.4), 5 mM UDP-glucuronic acid, 0.5 mM $MgCl_2$ containing 0.25 mM 1-naphthol at 37°C. The resultant enzyme reaction products were filtered through a disposable disk filter (HPLC-DISK 3; Kanto Co.) and analyzed by an HPLC system consisting of a Tosoh TSKgel 80TM reversed phase column (7.8 mm × 30 cm). The filtered samples were injected and eluted with an acetonitrile/H_2O/acetic acid (35:65:0.1 v/v) solution. 1-Naphthol glucuronide was determined by using authentic standards.

Results

Recirculation Perfusion. Several concentrations of substrate (1-naphthol) in the solution were tested in a pilot experiment, and quantitation time course data were obtained at a substrate concentration of 50 μM in 30 ml of solution. After injection of 1-naphthol into the rat renal artery with perfusion buffer over a period of 30 min, the concentrations of 1-naphthol glucuronide in the perfusate buffer and urine (Fig. 1) were determined by HPLC as described under *Materials and Methods*. 1-Naphthol glucuronide was detected immediately, and its concentration increased rapidly in the vein after recirculating perfusion (Fig. 2). However, glucuronide in the urine was excreted very slowly (Fig. 2). After 30 min of recirculation, the total amount of 1-naphthol glucuronide in the perfusate and in the urine was calculated to be 0.99 μmol (38 μM × 26 ml of perfusate, 66% of the total 1-naphthol dose), and 17.7 nmol (6.8 μM × 1.6 ml of urine, 0.007% of dose). These results suggested that 1-naphthol was glucuronidated in the proximal convolution tubular cells and was excreted into the renal vein, and that the glucuronide appeared in the urine only after filtration of recirculated perfusate in the kidney glomerulus. To test this hypothesis, a single-pass perfusion experiment was performed.

Single-Pass Perfusion. As shown in Fig. 3, the perfusion buffer was injected into the kidney artery in situ, and then the perfusate excreted from the renal vein was collected. The urine was also collected (single-pass perfusion), and the collected samples were analyzed by HPLC. 1-Naphthol or bisphenol A was injected into the renal artery over a period of only 5 min, and then the kidney was perfused with the buffer. The amounts of chemicals and their glucuronides were monitored, and the results are shown in Fig. 4. Large amounts of free 1-naphthol and the glucuronide were observed in the perfusate from the renal vein (80% yield) (Fig. 4A). Both were detected in the urine in very small amounts (Fig. 4A). Bisphenol A was detected only in the perfusate as unconjugated free compound (95% yield) (Fig. 4B). Renal microsomal UDP-glucuronosyltransferase activity toward 1-naphthol was induced about 3.4-fold by β-naphthoflavone treatment (Table 1) as previously described

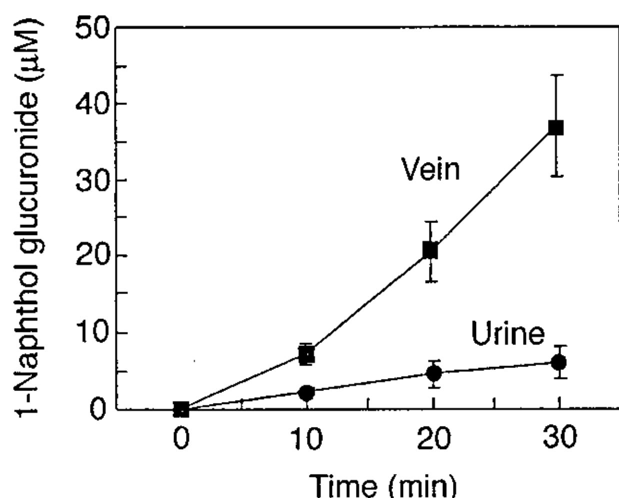

FIG. 2. 1-Naphthol glucuronide in the rat vein and urine during recirculating perfusion of the isolated kidney. The isolated kidney was fixed in a recirculating apparatus containing Krebs-Henseleit buffer as shown in Fig. 1. 1-Naphthol was added to the buffer solution (final concentration of 50 μM in 30 ml of solution). The buffer solution, which was mixed with gas (95% O_2 + 5% CO_2), was perfused into the kidney artery, and the perfusate buffer yielded from the renal vein was returned to a reservoir and was recirculated through the kidney preparation (recirculating perfusion). Amounts of 1-naphthol glucuronide formed in the renal vein (■) and in urine (●) were determined by HPCL analysis as described under *Materials and Methods*. After 30 min of perfusion, the total amount of 1-naphthol glucuronide in the perfusate and in the urine was calculated to be 0.99 μmol and 17.7 nmol, respectively.

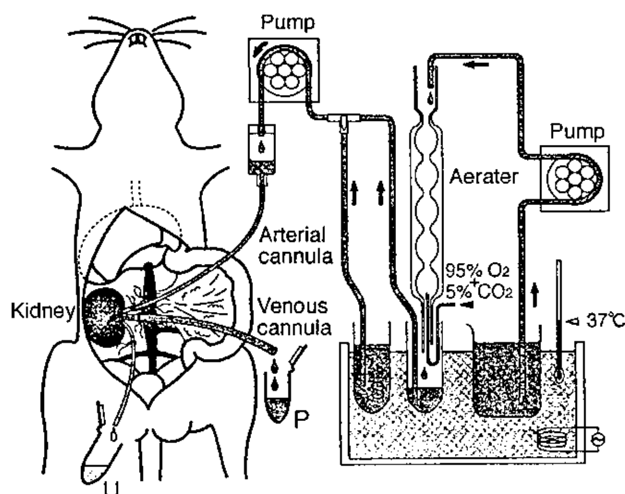

FIG. 3. Scheme of in situ single-pass perfusion of the rat kidney. Krebs-Henseleit buffer containing 6% bovine serum albumin that was mixed with gas (95% O_2 + 5% CO_2) was injected into the kidney artery in situ, and then the perfusate excreted from the renal vein was collected. The urine was also collected (single-pass perfusion), and the collected samples were analyzed by HPLC. 1-Naphthol or bisphenol A was injected into the renal artery over a period of only 5 min, and then the kidney was perfused with the buffer. S, substrate solution; K, Krebs-Henseleit perfusion buffer.

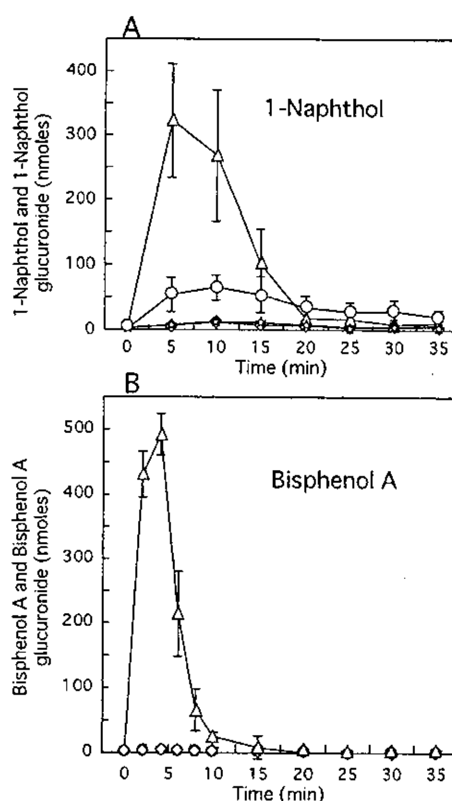

FIG. 4. Metabolites of 1-naphthol and bisphenol A after single-pass perfusion. Kidney perfusion with 1-naphthol (A) and bisphenol A (B) was performed as described under *Materials and Methods* and shown in Fig. 3. Parent drug and the metabolites in the renal vein and urine were assayed by HPLC. Amounts of free 1-naphthol (△ in the vein and ◇ in the urine) and 1-naphthol glucuronide (○ in the vein and ☆ in the urine) are shown in panel A. Amount of free bisphenol A (△ in the vein and ◇ in the urine) and bisphenol A-glucuronide (○ in the vein and ☆ in the urine) were shown in panel B. About 80% and 98% of the perfused chemicals, 1-naphthol and bisphenol A, respectively, were recovered.

TABLE 1

UDP-glucuronosyltransferase activities toward 1-naphthol in kidney microsomes and the amounts of 1-naphthol glucuronide in the vein and urine during 40 min of single-pass perfusion

Rats were administered β-naphthoflavone intraperitoneally, and renal microsomal UDP-glucuronosyltransferase activity toward 1-naphthol was assayed as described under *Materials and Methods*. Single-pass perfusion of the kidney with 1-naphthol was performed, and the resultant glucuronide in the vein and urine was assayed by HPLC. Total amounts of the glucuronide during 40 min of perfusion are shown as nanomoles. Data are presented as means ± S.E. of three animals.

Treatments	UGT Activity	Total Excreted Glucuronide	
		Vein	Urine
	nmol/min/mg	*nmol*	
Control	8.85 ± 0.80	143 ± 33.5	35 ± 0.9
β-Naphthoflavone	29.95 ± 5.93	393 ± 67.6	59 ± 19.5

(Yokota et al., 1997). 1-Naphthol was perfused in kidneys from β-naphthoflavone-treated rats, and the results are shown in Fig. 5. After single-pass perfusion, the amount of 1-naphthol glucuronide excreted in to the vein was increased in the kidney of the rat treated with β-naphthoflavone; however, the glucuronide in the urine was not significantly affected by the treatment (Fig. 5). Total amounts of 1-naphthol glucuronide in the renal vein and urine after perfusion for 40 min are shown in Table 1. About 80.3% and 93.8% of the total amount of the glucuronide formed was excreted into the renal veins of control and β-naphthoflavone-treated rats, respectively (Table 1). The amounts collected from the renal veins of treated rats were about 2.8-fold greater than that from the veins of control rats (Table 1).

Discussion

UDP-glucuronosyltransferase, has been shown to be present in epithelial cells of proximal convoluted tubules of the rat kidney (Yokota et al., 1997). UGT1A6, an isoform of the enzyme that glucuronidates phenolic substances such as 1-naphthol, but not

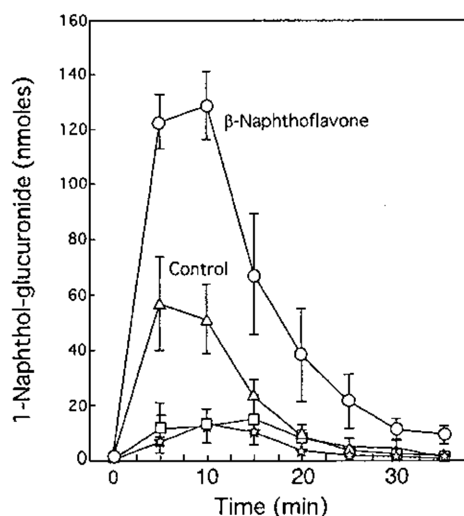

FIG. 5. Amounts of metabolites of 1-naphthol after single-pass perfusion of the kidneys of control and β-naphthoflavone-treated rats. β-Naphthoflavone was administered to rats as described under *Materials and Methods*, and then kidney perfusion with 1-naphthol was performed by the single-pass method. Amounts of 1-naphthol glucuronide in the vein (△, control rats; ○, β-naphthoflavone-treated rats) and in the urine (☆, control rats; □, β-naphthoflavone-treated rats) are shown.

UGT2B1, an isoform that glucuronidates bisphenol A, was recently reported to be expressed in the rat kidney (Shelby et al., 2003). These findings agree with our results showing that formation and excretion of 1-naphthol glucuronide, but not bisphenol A glucuronide, was observed in the renal vein and urine from the kidney perfusion model. 1-Naphthol was principally metabolized to 1-naphthol glucuronide and excreted in large amounts into the urine following recirculating perfusion of the kidney (Redegeld et al., 1988; de Vries et al., 1989). We also obtained the same results with recirculating perfusion of the kidney (Fig. 2). In contrast, most of the 1-naphthol glucuronide was detected only in the renal vein after single-pass perfusion, suggesting renal formation and preferential excretion of the glucuronide across the basolateral membrane. However, it is possible that some of the glucuronide excreted into the tubular filtrate side may be reabsorbed by tubular epithelial cells and diffuse into the renal vein. UDP-glucuronosyltransferase was induced and detected in the epithelial cells not only of the proximal convoluted tubules but also of the straight portion of the distal tubules after β-naphthoflavone treatment of rats (Yokota et al., 1997). The amount of the resultant glucuronide formed in the perfused kidney was increased in proportion to the enzyme activity of renal microsomal UDP-glucuronosyltransferase in β-naphthoflavone-treated rats. An additional cause of an increase in 1-naphthol glucuronide in the renal vein was thought to be induction of the transporters mediating the glucuronide transport. These results indicate that 1-naphthol glucuronides are transported into the renal vein from epithelial cells of proximal and distal tubules.

The membrane proteins belonging to the ABC transporter family play an important role in the extrusion of many substrates and metabolites from cells (Borst and Elferink, 2002). Members of the ABC family of MRPs, such as MRP1, MRP2, and MRP3, accept glucuronide conjugates. In the liver, MRP2 mediates the efficient biliary excretion of glucuronides and is expressed on the bile canalicular membrane (Borst and Elferink, 2002). MRP2 is expressed not only in the liver but also in the kidney, where it is located in the apical membrane of epithelial cells (Schaub et al., 1997). Significant disturbance of renal clearance of 1-naphthol glucuronide was found in isolated perfused kidneys of TR^- (transport-deficient) rats, which are deficient in MRP2 (de Vries et al., 1989), indicating that 1-naphthol glucuronide is a substrate of MRP2. Thus, MRP2 could be responsible for the secretion of 1-naphthol glucuronide into the urine (13–20% of total glucuronide). MRP1 and MRP3 are expressed at basolateral membranes of distal convoluted tubular epithelial cells (Kool et al., 1999; Van Aubel et al., 2000; Scheffer et al., 2002), but neither of them appears to be expressed in the proximal tubule (Peng et al., 1999). MRP1 and MRP3 are thought to transport 1-naphthol glucuronide across the basolateral membranes of distal convoluted tubular epithelial cells into the renal vein in β-naphthoflavone-treated rats, but the transporter in proximal tubular epithelial cells in which UDP-glucuronosyltransferase is expressed under normal conditions is unknown. Since the direction and speed of glucuronide excretion depends on the location and properties of the transporter in epithelial cells, it is important to identify the transporter.

Based on the results described above, it is concluded that 1-naphthol injected into the renal artery is absorbed into epithelial cells and glucuronidated. The resultant glucuronide is transported primarily across the basolateral membrane into the vein and circulates through the whole body. The glucuronide is then filtrated through the glomerulus of the kidney into urine.

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Maturation of the murine cecal microbiota as revealed by terminal restriction fragment length polymorphism and 16S rRNA gene clone libraries

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Abstract

The maturation of murine cecal microbiota was determined by terminal restriction fragment polymorphism (T-RFLP) and 16S rRNA gene clone libraries. Cecal microbiota in specific pathogen free (SPF) mice aged four to 10 weeks were collected. The cluster of samples in 4-week-old mice was different from those of other ages based on T-RFLP profiles. The majority of clones obtained in this study belonged to the *Clostridium coccoides* (*C. coccoides*) group, the *Bacteroides* group or the *Lactobacillus* group. Phylogenetic analysis showed characteristic clusters composed of new operational taxonomic unit (OTU) of the *C. coccoides* and *Bacteroides* groups. The existence of a large number of yet unidentified bacteria inhabiting the murine cecum was demonstrated by 16S rRNA gene clone libraries. T-RFLP analysis data were more complex and more sensitive than the patterns generated by computer simulation of 16S rRNA gene clone library analysis data. T-RFLP revealed development with maturation of cecal microbiota including unidentified bacteria of SPF mice.

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Keywords: Unidentified cecal microbiota; Maturation; SPF mice; T-RFLP; 16S rRNA gene

1. Introduction

The microbial ecology of intestinal tract in animals is composed of a large number and variety of bacteria. The indigenous intestinal microbiota play an important role in the health of the host animal. It is also known that intestinal microbiota can significantly be influenced by diets and other factors, such as the host and the environment. Therefore, many efforts to study microbial communities in the intestinal tract have focused on host-microbial relation. Experimental animal studies are indispensable when formulating basic concepts of the microbial ecology of the intestinal tract. Many reports have been published on the intestinal microbiota of mice using culture-based methods [1,2]. However, culture-based methods are inapplicable to non-cultivable bacteria and are selective for readily cultivated bacteria. Consequently, culture-independent methods have become indispensable tools for this type of research, among which PCR is a core technique. Terminal restriction fragment polymorphism (T-RFLP) allows the assessment of complex bacterial communities, rapid comparison of the community structure and diversity of different ecosystems [3]. T-RFLP analysis was applied to characterize human fecal microbiota among individuals [4,5] and to compare mucosal microbiota in the terminal ileum of mice [6]. Recent studies of murine intestinal microbiota using molecular methods dependent on 16S

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rRNA gene sequences revealed the existence of many previously unidentified microorganisms in intestinal tract of mice [7,8] In this regard, the cecum is known to contain the largest number and the greatest diversity of bacteria in the intestinal tract of mice [9] however, the cecal microbiota of mice is still ill-defined. As far as we use mice in experiments, we need to know the structure of microbiota of mice. The purpose of this study was to elucidate the complexity and bacterial diversity of the murine cecal microbiota with maturation by T-RFLP and 16S rRNA gene libraries.

2. Materials and methods

2.1. Animals

C57BL/6 mice kept under specific-pathogen-free (SPF) conditions, at the Department of Animal Experiments, Brain Science Institute, RIKEN, Saitama, Japan, were used in all experiments. They were housed in metal cages with TEK-FRESH (Edestrom Japan) as bedding and given RO water and γ-radiation sterilized commercial pellets (CLEA Japan) ad libitum.

2.2. Sampling

Six female mice from four, five, six, seven and ten weeks of age, and seven female mice from eight weeks of age were used. Since the results of four weeks of age were the most characteristic, 15 female mice from four weeks of age were added and used in different terms. All samples were collected randomly from several cages to avoid potential litter and cage effects. Animals were sacrificed by cervical dislocation, and their cecal contents were removed and were stored at −80 °C as the sample of intestinal microbial community.

2.3. Cell lysis and DNA isolation from cecal samples of mice

The DNA was isolated from cecal samples using Ultra Clean™ Soil DNA Isolation Kit (Mo Bio Laboratories). The sample (0.05 g) was suspended in bead solution containing 5 $mg\,ml^{-1}$ lysozyme and 0.08 mg ml^{-1} *N*-acetylmuramidase, and incubated for 30 min at 37 °C for cell lysis. DNA extraction and purification were based on the methods described by Clement and Kitts [10] with some modification.

2.4. PCR amplification for T-RFLP analysis

A pair of universal primers, 27f (5′-AGAGTTTGA TCCTGGCTCAG-3′) and 1492r (5′-GGTTACCT TGT TACGACTT-3′) [11], were used for PCR amplification. 27f was labeled with 6-FAM (6-carboxyfluorescein, Applied Biosystems Japan). PCR was performed with a Thermocycler T Gradient (Biometra) in a reaction mixture (50 μl) containing 5 μl of dissolved DNA (100 ng), 1.25 U of TaKaRa *Ex Taq*™ (Takara), 10 × *Ex Taq*™ buffer, 4 μl of dNTP mixture (2.5 mM each), and 10 pmol of each primer. The amplification program used was as follows: preheating at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1.5 min; and finally a terminal extension at 72 °C for 10 min. Amplified DNA was verified by 1.5% agarose gel electrophoresis. PCR products were purified by the PEG precipitation method [4].

2.5. T-RFLP analysis

Purified PCR products were digested with 20 U of either *Hha*I or *Msp*I (Takara) in a total volume of 10 μl at 37 °C for 3 h. The lengths of the terminal restriction fragments (T-RFs) were determined with the standard size marker GS500 ROX and 1000 ROX (Applied Biosystems) using ABI PRISM™ 310 genetic analyzer (Applied Biosystems) and GeneScan® Analysis Software (Applied Biosystems). Dendrogram analysis was performed using T-RFLP patterns by software BioNumerics (Applied Maths). Dendrogram analysis was based on the similarity coefficient, for the objective interpretation of the difference of T-RFs patterns. The distances between samples were represented graphically by constructing a dendrogram based on the Pearson's correlation coefficients and Jaccard matching coefficients of T-RFLP profiles. Unweighted pair-group method with arithmetic mean (UPGMA) was used for establishing the dendrogram type.

2.6. PCR amplification of 16S rRNA gene sequences, cloning

The samples used for cloning and sequencing were from two mice 4 weeks of age and two mice 8 weeks of age. The 16S rRNA gene was amplified using two universal primers 27f and 1492r [11]. PCR was performed with a Thermocycler T Gradient (Biometra) in a total volume of 100 μl containing 5 μl of dissolved DNA, 2.5 U of TaKaRa *Ex Taq*™, 10 × *Ex Taq*™ buffer, 8 μl of dNTP mixture (2.5 mM each), and 10 pmol of each primer. PCR conditions used were preheating at 95 °C for 3 min; 15 cycles of denaturation (30 s at 95 °C), annealing (30 s at 50 °C), and extension (1.5 min at 72 °C), with a final extension at 72 °C for 10 min. These PCR conditions avoid preferential amplification [12]. The amplified 16S rRNA genes were purified with an UltraClean PCR Clean-up Kit (Mo Bio Laboratories). Purified amplicons were cloned into pCR®2.1 vectors (Invitrogen), and One Shot® INVaF' (Invitrogen) competent cells were transformed. Transformants were ran-

domly picked and purified with Montage™ Plasmid Miniprep 96 Kit (Millipore).

2.7. DNA sequencing and phylogenetic analysis

Cycle sequencing was performed with the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), 27f or 520r primers, and ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems). Additionally, sequences which belonged to new OTU were determined with 520f or 920r primers. All sequences were examined for possible chimeric artifacts by the CHECK CHIMERA program of the Ribosomal Database Project (RDP) [13] and compared with similar sequences of the reference organisms by BLAST search [14] and SIMILARITYRANK program [13]. Previously determined 16S rRNA sequences were used for comparisons in this study and were retrieved from the DDBJ, EMBL, and GenBank nucleotide sequence databases. Sequence data were aligned with the CLUSTAL W package [15] and corrected by manual inspection. Nucleotide substitution rates were calculated [16] and the phylogenetic tree was constructed using the neighbor-joining method [17]. A bootstrap resampling analysis [18] for 100 replicates was performed to estimate the confidence of tree topologies. The term "phylotype" is used for a cluster of clone sequence that differed from known species by approximately 2%, and these clusters were at least 98% similar to numbers within a cluster of clone sequence [19].

2.8. Nucleotide sequence accession number

The sequence data determined in the present study have been assigned DDBJ, EMBL and GenBank Accession Nos. AB120782 to AB120845, respectively.

3. Results and discussion

3.1. Comparison of cecal microbiota of mice of different ages by T-RFLP analysis

In the present study, samples collected from 4- to 8- and 10-week-old mice were used, because these age groups were studied by many researchers. Common and unique T-RFs were identified in T-RFLP profiles of mice cecal samples. We performed dendrogram analysis based on the Jaccard matching coefficients and the Pearson's correlation coefficients of T-RFLP profiles. UPGMA was used as the dendrogram type (Fig. 1). Table 1 summarizes the differences between age groups shown as similarity indices and p values of paired comparisons. The p values indicate significance of the similarity of the samples within each age group.

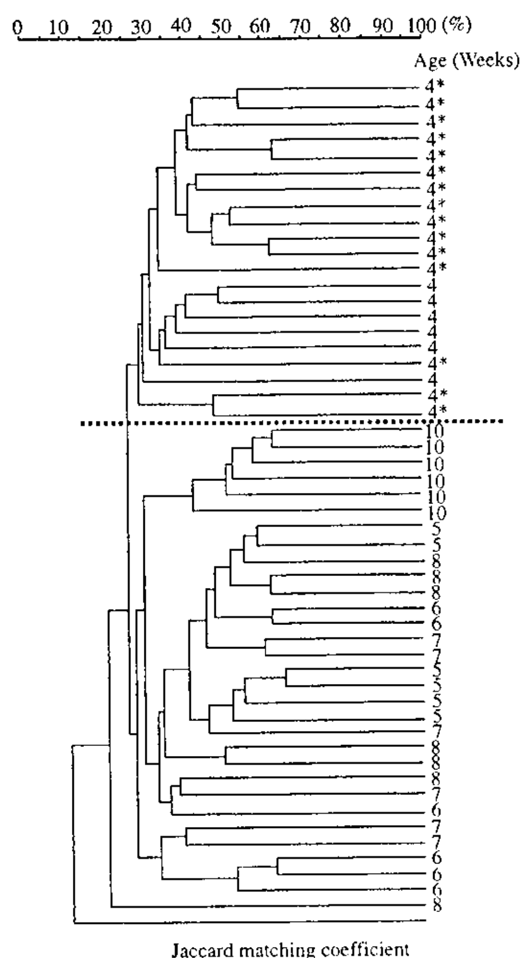

Fig. 1. Dendrogram analysis of cecal microbiota of mice based on T-RFLP (Jaccard matching coefficients and UPGMA). Dotted line represents the boundary between the cluster of 4-week old and the cluster of other ages. 4*: samples of second sampling from four-week age. The scale bar represents similarity indices (%).

Analysis of T-RFLP profiles of cecal microbiota of different age showed significant differences between age groups. Cecal microbiota in samples collected from 4-week-old mice were different from those of others in both dendrograms. Moreover, samples from 10-week-old mice also formed different groups. There was no significant group separation among samples from 5- to 8-week-old mice. However, similarity indices between the same age showed that samples collected from mice of all ages (except 4-week-old mice) were closely similar. Differences between samples of 4-week-old mice and those from other age groups were the most significant because the similarity indices between the same age were often higher than those between different ages. The p values were correspondingly higher ($p < 0.001$) between samples from 4-week-old mice and others. The p values were also high for samples from 10-week-old mice. The results of the present study

Table 1
Comparison of cecal microbiota in mice of the same and different age

	Similarity indices in same age	Age (weeks)	Age (weeks)					
			4	5	6	7	8	10
Jaccard matching coefficient								
4–4	34.4 ± 7.7	4		27.2 ± 4.0***	27.3 ± 5.4***	26.3 ± 4.7***	25.6 ± 6.0***	23.3 ± 4.3***
5–5	55.1 ± 13.5	5	***		33.4 ± 8.2***	37.5 ± 6.7***	37.5 ± 9.2***	30.6 ± 5.6***
6–6	38 ± 13.5	6	***			31.2 ± 6.7*	31.0 ± 8.7*	26.1 ± 5.8**
7–7	36.9 ± 7.4	7	***		**		32.8 ± 7.8*	28.5 ± 4.3***
8–8	37.9 ± 11.9	8	***		*	*		29.3 ± 6.0
10–10	49.7 ± 7.6	10	***	***	***	***	***	
Pearson correlation coefficient								
4–4	46.7 ± 18.8	4		34.2 ± 12.4***	31.1 ± 12.6***	27.2 ± 12.0***	25.9 ± 12.4***	33.8 ± 14.3***
5–5	71.7 ± 10.2	5	***		45.5 ± 13.4***	57.7 ± 10.6***	52.4 ± 18.5***	48.1 ± 12.2***
6–6	53.4 ± 15.2	6	***			43.2 ± 16.0*	38.6 ± 18.5**	35.7 ± 17.6**
7–7	55.5 ± 10.8	7	***		**		47.7 ± 19.7	39.8 ± 10.8***
8–8	50.5 ± 22.0	8	***					45.3 ± 9.4
10–10	60.7 ± 13.2	10	***	**	***	***	***	

Similarity indices (mean ± SD) are given. Similarity indices were calculated from comparison between T-RFs patterns in each age group.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

showed that cecal microbiota in samples collected from 4- and 10-week-old mice are different from those of mice of other ages, as determined by T-RFLP. These results were confirmed by comparison of two dendrograms, the similarity indices and p values. These results suggest that cecal microbiota changes drastically with maturity after weaning, although there were also some differences between the same age, i.e., the individual specificity difference exists in mice of the same age. According to previous reports by culture and histological technique [2,9,20], cecal microbiota increased in number from the second day to 4 weeks of age and remained stable thereafter. T-RFLP results confirmed the presence of marked variation in cecal microbiota of 4- to 5-week-old mice, in agreement with previous studies [2,9,20].

3.2. Sequence and phylogenetic position of 16S rRNA gene of cecal microbiota

Two samples each from 4 weeks and 8 weeks of age were used for 16S rRNA gene clone library analysis (MC41, MC42, MC81 and MC82, respectively). A total of 186 clones from four cecal samples were analyzed. The partial sequencing with the primer provided about 520 bp or 920 bp of the 16S rRNA gene (*Escherichia coli* position 27–520 and 27–920) [11]. Although no exact 16S rRNA gene similarity limits exist for defining specific taxa such as genus and species, species definition in general requires sequence similarities greater than 98% [21]. All clones were divided into 87 species or phylotypes, and only seven species (17 clones) had greater than 98% sequence

Table 2
Comparison of the number of clones in 4- and 8-week age of mice

Phylogenetic group	No. of clones (%)				Total (%)
	4-week age		8-week age		
	MC41	MC42	MC81	MC82	
Clostridium coccoides	25 (52.0)	14 (29.7)	4 (8.3)	8 (18.6)	51 (27.4)
Clostridium leptum	2 (4.1)	6 (12.7)	6 (12.5)	2 (4.6)	15 (8.0)
Clostridium botulinum	1 (2.0)	0 (0)	1 (2.0)	0 (0)	2 (1.0)
Clostridium thermocellum	0 (0)	0 (0)	1 (2.0)	0 (0)	1 (0.5)
Unclassified *Clostridium*	0 (0)	0 (0)	1 (2.0)	0 (0)	1 (0.5)
Lactobacillus	8 (16.6)	13 (27.6)	2 (4.1)	17 (39.5)	40 (21.5)
Bacteroides	12 (25.0)	12 (25.5)	10 (20.8)	7 (16.2)	45 (24.1)
Mycoplasma and relatives	0 (0)	2 (4.2)	21 (43.7)	6 (13.9)	26 (13.9)
"*Deltaproteobacteria*"	0 (0)	0 (0)	2 (4.1)	2 (4.6)	4 (2.1)
"*Epsilonproteobacteria*"	0 (0)	0 (0)	0 (0)	1 (2.3)	1 (0.5)
Total	48	47	48	43	186

similarity to known species. The remaining nine (35 clones) and 71 (134 clones) sequences belonged to "known phylotype" and "unknown phylotype", respectively. "Known phylotype" represents a clone with more than 98% sequence similarity to the GenBank database. "Unknown phylotype" represents a clone with sequence similarity that is not closely related to any organisms and whose 16S rRNA sequences are currently deposited in the GenBank database. Sequences from the sample of 4-week-old mice were classified into six phylogenetic groups and those from 8-week-old mice were classified into 10 phylogenetic groups. Comparisons of the number of clones from each sample are shown in Table 2. The majority of clones obtained from this study belonged to the *Clostridium coccoides* group, the *Bacteroides* group or the *Lactobacillus* group. Most clones in samples of 4-week-old mice were included in *C. coccoides* group, although the clones isolated from 8-week-old mice were small in number. Clones that belonged to the *Mycoplasma* and relatives group were detected at higher incidence in samples from 8- than 4-week-old mice. The "*Deltaproteobacteria*" group and the "*Epsilonproteobacteria*" group were not detected in samples from the 4-week-old mice. The existence of a large number of previously unidentified bacteria in the cecum was detected by 16S rRNA gene clone libraries. Moreover, these results considered that the number of group diversity of microbiota increased with age. However, obvious differences in the species structure of cecal bacteria between 4- and 8-week-old mice could not be confirmed by this method. Godon et al. [22] reported the relationship between number of sequences and cumulative number of operational taxonomic unit (OTUs). In this study, only 48 clones per sample were analyzed. There is probably a need to analyze more clones in order to find differences in the composition of cecal microbiota in individual mice.

Many phylotypes in the *C. coccoides* group and the *Bacteroides* group were found in the present study. The results of phylogenetic analysis are shown in Fig. 2 (the *C. coccoides* group) and 3 (the *Bacteroides* group). Characteristic new clusters shared by both age-groups were detected from these bacterial groups in this study. The genus *Clostridium* is extremely heterogeneous, with many species phylogenetically intermixed with other spore-forming and non-spore-forming genera [23]. Fusiform bacteria, which are present in very large numbers in the normal microbiota of rodents [24], also belong to the *Clostridium* group [25]. Their presence was documented to affect the cecal physiology [26]. Bacteria found in this study may also affect the normal physiology of the host. *Bacteroides* species, including *Bacteriodes distasonis* and *Bacteroides acidifaciens*, have been isolated from the cecum of mice [27–29]. Salzman et al. [8] reported new OTUs obtained from the murine intestine and these were designated as mouse intestinal bacteria (MIB). We confirmed that a characteristic cluster in this report belongs to MIB (data not shown).(See Fig. 3)

3.3. Comparison between T-RFLP analysis and 16S rRNA gene clone libraries

To compare the results of T-RFLP and 16S rRNA gene clone libraries, we identified sequences obtained from all samples by computer simulation of T-RFs with *Hha*I or *Msp*I. Computer simulation analysis of the sequences correlated with the T-RFLP profiles. The *Bacteroides* group in the database was distributed as genus specific T-RFs using both restriction enzymes, about 100 bp (*Hha*I) and about 90 bp (*Msp*I) though T-RFs of the *C. coccoides* group were not specific. T-RFLP patterns included sequences of T-RFs except for only four clones. Previous studies reported a lack of diversity when fecal DNA samples were amplified using a large number of PCR cycles [12,30]. Although PCR-related bias affects almost all molecular methods, it is not an exception in the T-RFLP analysis [31,32]. Whereas it is impossible to remove all biases in the PCR, because all samples were analyzed consistently, any biases affecting one sample should be seen also in all samples. The amplification in T-RFLP was performed for 30 cycles, while in clone libraries it was performed for 15 cycles. Therefore, differences in the T-RFs pattern between T-RFLP and clone libraries may appear. In addition, T-RFLP patterns were more sensitive than about 50 clones sequence per sample for the assessment of overall species diversity. We considered that the minority of microorganisms, which were not detected by 16S rRNA gene clone libraries in this study, appeared as small T-RFs by T-RFLP analysis. Molecular approaches relying on 16S rRNA gene sequences are now commonly used for the identification and classification of bacterial species in complex microbiota. However, analysis of individual 16S rRNA gene clones is an expensive and extremely inefficient approach. We recommend T-RFLP for the detection of overall changes in individual cecal microbiota.

Previous studies using culture methods and histological examination reported that many unclassified bacteria and strictly anaerobic bacteria inhabit the cecum of SPF and normal mice [2,9]. Furthermore, it has been reported that microbiota can be significantly altered by diets, different breeders and other factors [33]. Because variation of intestinal microbiota in experimental animals can influence experimental results, standardization of intestinal microbiota is required. In previous studies, researchers have directed their efforts towards standardization of intestinal microbiota of germ-free mice with cecal bacteria of normal mice

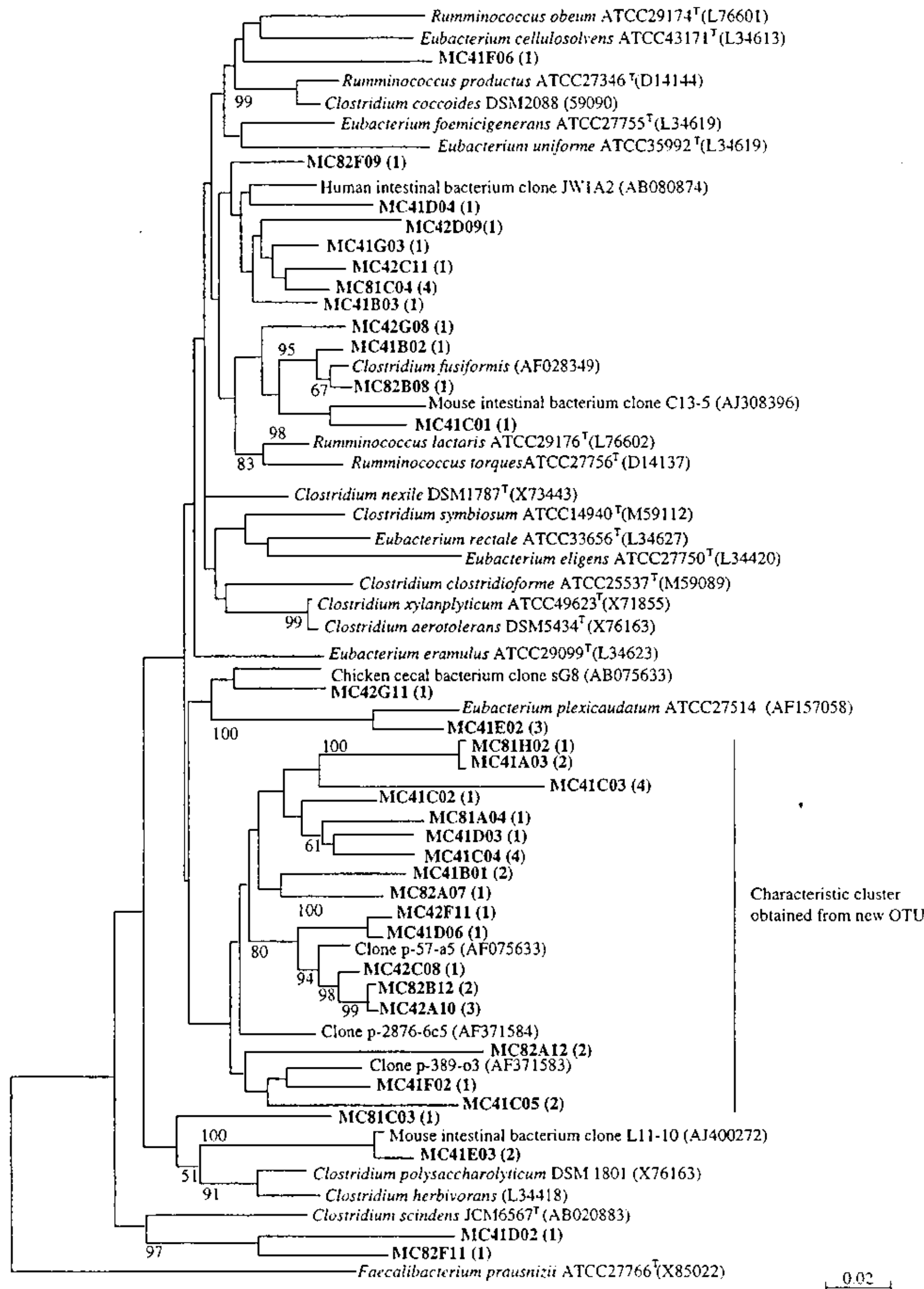

Fig. 2. Phylogenetic tree showing the relationship between 16S rRNA genes sequences in cecal samples of mice within the *C. coccoides* group. Approximately 500 bp 16S rRNA genes sequence were used to draw the tree. The tree was constructed using neighbor-joining analysis based on 16S rRNA sequences. Bootstrap values ($n = 100$ replicates) of $\geqslant 50$ are reported as percentage. Scale bar = 0.02 substitutions/nucleotide position. Clones obtained from samples appear as bold letters. Accession numbers for each of the published sequences are given. Numbers in parenthesis represent the number of clones detected in same age groups. *Faecalibacterium prausnitzii* is used as the outgroup for rooting the tree.

[34,35]. SPF mice are used in many studies as experimental animals. However, we do not know the composition of microbiota of SPF mice except for specific microorganisms and parasites. Therefore, there is a need to know the microbiota status including unidentified microorganisms of each experimental animal in order to choose the most suitable animals and to achieve reliable results. The present study reported the development with maturation on cecal microbiota of SPF mice by T-RFLP, and our results were in agreement with previous

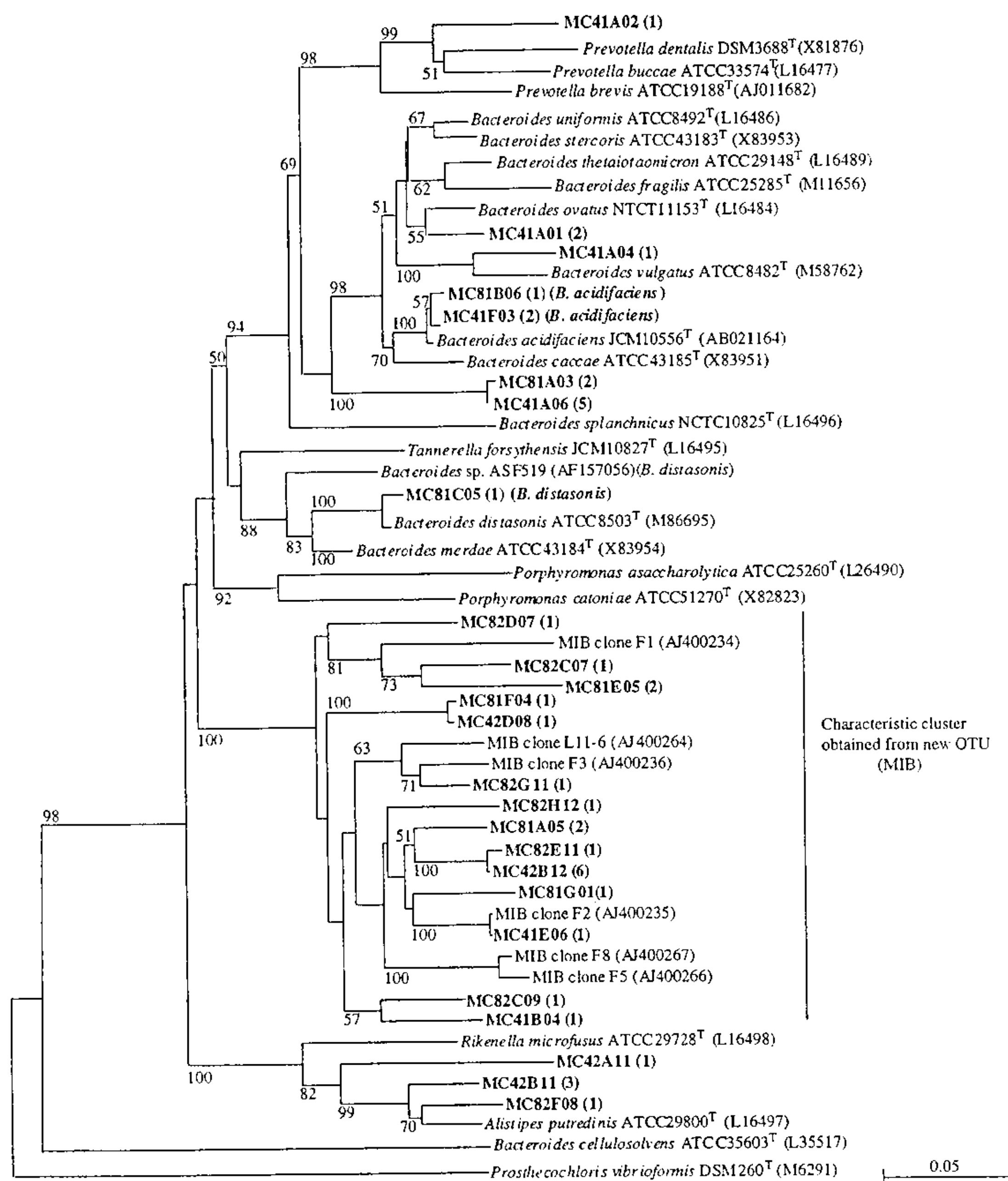

Fig. 3. Phylogenetic tree showing the relationship between 16S rRNA genes sequences of cecal samples of mice within the *Bacteroides* group. Approximately 500 bp 16S rRNA genes sequence were used to draw the tree. The tree was constructed using neighbor-joining analysis based on 16S rRNA sequences. Bootstrap values (n = 100 replicates) of ≥50 are reported as percentage. Scale bar = 0.05 substitutions/nucleotide position. Clones obtained from samples appear as bold letters. Accession numbers for each of the published sequences are given. Numbers in parentheses represent number of clone detected from the same age groups. *Prosthecochloris vibrioformis* is used as the outgroup for rooting the tree.

studies [2,9,20]. Therefore, T-RFLP analysis, which is a simple and rapid method, is very useful for monitoring the cecal microbiota to know the state of microbiota in experimental mice before they are used for research.

Additionally, 16S rRNA gene clone libraries was used to examine the influence of maturation on cecal microbiota based on the results of T-RFLP analysis, since T-RFLP analysis does not identify the bacterial species responsible for the age-dependent changes. Consequently, this study confirmed that a large number of yet unidentified microorganisms inhabit the cecum of mice and that the diversity of microbiota increased with maturation.

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