The intestinal commensal microbiota of monogastric animals such as humans and pigs comprise hundreds of different types of microorganisms (Conway, 1995; Savage, 1977). In the last several decades, the role of the intestinal commensal microbiota in a wide range of host’s physiological functions has been increasingly recognized (Cummings and Macfarlane, 1997). Colonization by such microbiota develops a range of the functions of intestine, as evidenced by the mal-development of the intestinal functions of germ-free animals (Hooper et al., 2001; Wostmann et al., 1983) which were made artificially so that they were free from microorganisms. For example, the number of intraepithelial T lymphocytes, which play an important role in the host defenses (Yoshikai, 1999), are less than one third the number in both the jejunum and ileum of germ-free pigs than in conventional pigs (Rothkötter et al., 1999).

Since the development of the intestinal commensal microbiota interacts deeply with host physiological functions, studies that show how they are established in connection with host physiology need to be performed. The information has the potential to improve the nutritional and immunological function of the host and thus it might improve the productivity of livestock.

The development of the intestinal microbiota in the suckling piglet has been the subject of several studies, most of which depended on culture-based techniques for the bacterial analysis (Melin et al., 1997; Swords et al., 1993; Uchida et al., 1965). Since culture-based techniques only can provide information on bacteria that are readily cultivated (Langendijk et al., 1995; Suau et al., 1999), they may give a biased view of microbial diversity. The molecular biological techniques temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) have been developed in recent years, and are being used more frequently in microbial ecology (Muyzer et al., 1993). These methods are especially valuable for monitoring the shift in community structure that occurs in response to environment perturbations such as a change in diet (Satokari et al., 2001; Simpson et al., 2000; Tannock et al., 2000). And as demonstrated in this study, the application of the clustering method to the results of these techniques (gel-image) allows the statistical comparison of the differences within each banding profile that reflects the composition of the microbiota. Although the efficiency of these methods to analyze the pig fecal or intestinal microbiota has been demonstrated by several studies (Konstantinov et al., 2003; Simpson et al., 1999, 2000), the change in the intestinal microbiota in suckling piglets has rarely been analyzed by these techniques.

Short Communication

Development of the intestinal microbiota in the piglet

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The aim of the present study was to assess the development of the intestinal microbiota in the developing piglet by monitoring the change in the intestinal microbiota using TGGE. Unlike previous studies, we especially focused on the change in the intestinal microbiota composition from birth to after weaning rather than the change in a particular bacterial species, because the change in the composition reflects the acquisition and succession of microbes during the developing process which are controlled by various internal and external factors (Mackie et al., 1999).

Two conventional crossbred (Landrace × Large White × Duroc) sows were artificially inseminated and defined as sow A and B, respectively. A total of 6 piglets, 3 from each litter, were selected for this study. The selected piglets from sow A were defined as litter A, and the piglets from sow B were called litter B. Both litters were respectively kept grouped in the pen throughout the experimental period. They were injected intramuscularly with 200 mg Fe$^{2+}$ after 4 days post partum and male piglets were castrated at the age of 14 days. Powdered milk replacer (SDS No. 1; Nippon Formula Feed Co., Ltd., Yokohama, Japan) was offered to piglets from that age of 14 days but piglets were not separated from their sow at that time. The piglets were weaned at 25 days old. From 35 days old, piglets were offered a diet for weaned piglets (Koromeal GS; Nippon Formula Feed Co., Ltd.). Powdered milk replacer and weaned diets were free from intestinal microbiota modifier such as antimicrobials and probiotics, and these diets and water were given ad libitum throughout this study. Antimicrobial therapy was not conducted, because all piglets were healthy and defecated normal feces. All chemicals were obtained from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemical (Osaka, Japan) unless otherwise stated. The animals were handled in accordance with the guidelines for research with laboratory animals of the Kyoto Prefectural University Experimental Animal Committee.

From six piglets (piglets A1, A2 and A3 from litter A, and B1, B2, and B3 from litter B), a total of seventy-two rectal feces samples were collected at various ages as indicated below. Rectal feces were collected from the anus with cotton-swabs. The collections of the rectal feces were performed on piglets aged 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 35 and 49 days. The samples on days 25 and 35 were collected before the weaning and the change of the diet, respectively. Extraction of total genomic DNA from these samples was performed as described by Godon et al. (1997) with minor modifications.

PCRs were performed as described previously (Inoue and Ushida, 2003a) with primers U968-GC and L1401 (Nübel et al., 1996). Briefly, PCRs were performed with a Fast start Taq DNA polymerase (Roche, Tokyo, Japan). Each 50 μl PCR mixture contained 50 mM Tris-HCl (pH 8.3), KCl, (NH₄)₂SO₄, 2 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate (dNTP), 2 U Fast start Taq DNA polymerase, 0.4 μM of each primer, and 1 μl extracted bacterial DNA. The samples were amplified in a GeneAmp System 2400 (Perkin-Elmer, Branchburg, NJ, USA) by using the following program: initial denaturation at 95°C for 1 min; 40 thermal cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 50 s; and final elongation at 72°C for 7 min. The size and amounts of PCR products were confirmed by analyzing 5 μl samples by 1% agarose gel (wt/vol) electrophoresis and ethidium bromide staining. The D-code system (Bio-Rad, Tokyo, Japan) was used for the sequence-specific separation of these PCR products. Electrophoresis was performed in a 1 mm polyacrylamide gel (8% [wt/vol] acrylamide, 0.28% [wt/vol] methylene-bis-acrylamide, 1.25% Tris-(hydroxymethyl) aminomethane-acetate-EDTA [pH 8.0] (TAE) buffer, 8 M urea) with 1 × TAE as the electrophoresis buffer at a fixed voltage of 60 V for 20 h. A gradient from 67 to 70°C was applied parallel to the electrophoresis running direction (ramping rate 0.2°C/h) (Inoue and Ushida, 2003a). Gels were developed by silver staining using a Wako silver staining kit (Wako). The gel image was taken by 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, USA), and cluster analysis of TGGE band profiles was performed using hierarchical clustering analysis with Euclidean square distances by an Excel (Microsoft, Seattle, WA, USA) macro program developed by Aoki (http://aoki2.si.gunma-u.ac.jp/lecture/stats-by-excel/vba/html/clustan.html). A corresponding dendrogram was constructed with Ward linkages. Hierarchical cluster analysis is a statistical method for finding relatively homogeneous clusters of cases based on measured characteristics and widely used in various factors such as the analysis of DNA microarray (Goldstein et al., 2002). In this study, the density of each band was disregarded. If a lane had a band at a certain po-
tion, it was assigned one; if there was no band at the same position, an assignment of zero was made.

The bacterial species of two TGGE amplicon bands were identified. TGGEs were performed again with exactly the same conditions and the same PCR amplicons as described above, but the gels were developed by ethidium bromide staining. The TGGE amplicon bands were cut from the gel under UV light and stained into sterilized distilled water. They were incubated overnight at 4°C to elute the amplified DNA fragments. The DNA fragments were further PCR amplified by using the primers U968 (5'-ACGGCAAAGACTCAG-3') and L1401 (5'-CGGTGTGTAAGACCA-3'). PCRs were performed with the same conditions as described above with 18 thermal cycles. PCR products were purified and concentrated with a Wizard® SV Gel and PCR Clean-Up System (Promega, Tokyo, Japan) and then transformed into E. coli JM109-competent cells as described previously (Inoue and Ushida, 2003b). The screening of the positive clones and preparation of the plasmid DNA from them were performed in the same manner as that of Inoue and Ushida (2003b). The cloned DNA fragments were sequenced by Shimadzu Genomic Research Laboratory (Shimadzu, Kyoto, Japan). Homology searches of the GenBank DNA database for these sequences were performed with BLAST N. In this study, two E. coli clones for each band were randomly selected and sequenced.

The mean number of TGGE bands was analyzed by repeated measure ANOVA (each experimental day×sow). Significant differences were detected on each experimental day; therefore the Tukey-Kramer post hoc comparison was performed. Data were analyzed by StatView for Windows (SAS, Cary, NC, USA).

Figure 1 shows PCR-TGGE band profiles representing the diversity of the fecal bacteria. The figures were generated from the samples taken from birth to after weaning (49 days) for six piglets. There were 6.8 amplicon bands on day 1 (mean value for 6 animals; range 4 to 9 bands). The number of TGGE bands rapidly increased to 9.7 (mean value for 6 animals; range 7 to 11 bands) on day 10, but it decreased to 8.3 (mean value for 6 animals; range 5 to 11 bands) on day 13 (Fig. 2). Thereafter, it increased again to 11.8 (mean value for 6 animals; range 8 to 20 bands) on day 22. However, the number of TGGE bands decreased again remarkably to 7.5 (mean value for 6 animals; range 6 to 9 bands) on day 25. Then it increased to 17.2 (mean value for 6 animals; range 14 to 20 bands) until day 49 (p<0.05 vs. days 1 to 28).

The six dendrograms were constructed from the TGGE band profiles for each animal from birth to the age of 49 days (Fig. 3). Most dendrograms had a cluster which was constructed by the profiles on days 25, 28, 35 and 49. But in this cluster, the profile on day 49 did not combine with the other three profiles. In the dendrogram of A1, the profile on day 49 did not construct any cluster. The profiles on days 1 and 4 construct one cluster in most dendrograms, and the other profiles (profiles on days 7 to 22) generally formed two separate clusters. In the case of A1 and A3, profiles on the days 1 and 4 did not construct one cluster. But these profiles were contained in a different cluster with the profile of day 7 in the dendrogram of A1, and the profile on day 1 did not construct any cluster with the other profiles in the case of A3 (Fig. 3, A1 and A3).

The bacterial species appeared as TGGE bands, which may typically contribute to the change in the diversity of the intestinal microbiota from days 4 to 7 and from days 22 to 25 respectively, were identified. The identified bands are shown in Fig. 1. The sequence data of two clones for each band were virtually same and hit the 16S rRNA gene of the same bacterium in the database. The sequence results for bands X and Y showed high sequence similarities to the 16S rRNA genes of Clostridium perfringens (AB075767, 99%) and E. coli strain RREC III (AF527825, 100%), respectively.

The information for the development of the intestinal microbiota has been accumulated in several animals, especially in humans, by both culture-based and molecular microbial ecological techniques (Gronlund et al., 1999; Harmsen et al., 2000; Hirayama et al., 1995; Inoue and Ushida, 2003a; Kleessen et al., 1995). However, the information about the development of the intestinal microbiota is not sufficient in the piglet, especially in the suckling piglet. Although Simpson et al. (2000) demonstrated the stability of the intestinal microbiota during the post-weaning period using DGGE, the change in the intestinal microbiota in suckling piglets has been rarely assessed. Almost all information for suckling piglets has derived by the culture-based techniques and most of those were focused on the development of selected bacterial genera with relatively long-term sampling (Melin et al., 1997; Swords et al., 1993; Uchida et al., 1965). In this study, the development of the six piglets from two litters was assessed.
by using the molecular microbial ecological techniques, TGGE, with the relatively short-term sampling (every 3 days during the suckling period). A change in the diversity of the intestinal microbiota occurred three times from birth to after weaning.

The first change occurred between day 4 and 7 in most piglets used in this study. In two piglets (A1 and A3), this change was observed at a different time but it took place at nearly the same age. The disappearance of band X seemed to contribute to this change and it showed high similarity to the 16S rRNA genes of \textit{C. perfringens} (Fig. 1). The rapid decrease in the number of \textit{C. perfringens} during the developmental process of the intestinal microbiota was also observed by Uchida et al. (1965) and Melin et al. (1997). The decrease of \textit{C. perfringens}, a potential pathogen for the piglet (Songer, 1996), might be caused by the maternal immunoglobulin A (IgA) which plays an important role in the mucosal immune system for excluding harmful antigens (Brandtzaeg, 2002). It has been reported that

Fig. 1
IgA replaces IgG within 7 days postpartum as the major immunoglobulin isotype in the breast milk of pigs (Butler and Brown, 1994). The beginning of supplantation of aerobes and facultative anaerobes by strictly anaerobes is a possible reason for the first change. According to Swords et al. (1993), aerobes and facultative anaerobes are predominant until day 5 after birth, but they start decreasing gradually afterward. The second change took place from days 7 to 22 after birth. The time of this change varied among the individuals; in some of them it occurred between day 10 and 13, but it occurred between day 13 and 16 in some of the others. There were no particular common bands among the individuals which seemed highly contributive to this change. This period corresponds with the second successional phase which has been proposed by Swords et al. (1993). The diversity of anaerobic bacteria increased in this period, and thus various minor anaerobic genera such as Eubacterium, Fusobacterium and Propionibacterium began to be detected (Swords et al., 1993). In conformity with this,
the diversity of the intestinal microbiota represented by the number of TGGE bands increased from day 16, which was just after the second change (Fig. 2). On a parallel with this result, all major short chain fatty acids in the pig colon such as acetate, propionate and butyrate remarkably increased from days 13 to 16 after birth in Murray et al. (1987). The introduction of the milk replacer on day 14 might be a potential reason of this change. However there should be other non-dietary reasons for this change because the increase of the anaerobic bacterial diversity was observed in this period even if the piglets were reared only with mater-

Fig. 3. Dendrogram based on TGGE profiles of bacterial 16S rRNA of each piglet.
The cluster analysis employed hierarchical clustering with Euclidean square distances using an Excel macro program. The numbers given at the top of each dendrogram indicate Euclidean square distances and the indicated day numbers represent the age of the piglets. The introduction of milk replacer began at 14 days after birth and weaned diet was given from day 35 after birth. Piglets were separated from their sow on day 25. The combinations of capital letters and numbers at the upper left of each dendrogram represent the piglet number.
nal milk (Swords et al., 1993).

The third change occurred between day 22 and 25. Although the piglets were separated from their sow at day 25, the rectal feces were collected before the separation. Therefore, this change in the intestinal composition and diversity were not caused by the separation from the sow. Around this period, the proportion of anaerobic bacteria such as Bacteroides spp. began to increase in the study of Swords et al. (1993), whereas those of aerobic bacteria such as E. coli and Coliforms got extremely low. In this study, the TGGE band (band Y) which showed high similarity to the 16S rRNA genes of E. coli also visually disappeared from most profiles from days 22 to 25. These indicated that the supplantation of aerobic and facultative anaerobic bacteria by the anaerobic bacteria which might begin from the time of the first change was almost completed by this age. The causes for this change remain controversial but since the piglet wasn’t taking the weaning diet yet, it is unlikely that it was caused by diet. The immunological maturation of the intestine is the other reason for this change. Until the age of 28 days, CD4+ cells occupy the position in the core of the villi seen in the mature intestine (Bailey et al., 2001). And 90% of plasma cells for IgA and IgM secretions in the lamina propria of the adult pig predominate also until 28 days after birth (Butler and Brown, 1994). Matured immune functions such as secreting self IgA may affect the development of the intestinal microbiota (Bollinger et al., 2003; Kirjavainen and Gibson, 1999). The ingestion of the weaning diet on day 35 also affected the development of the intestinal microbiota. The diversity of the intestinal microbiota increased after the weaning (day 25), the same as in the rodent experiments (Fig. 2) (Inoue and Ushida, 2003a). Moreover, the TGGE profiles of day 49 tended not to be combined with the other profiles on the suckling period in all piglets (Fig. 3). Such changes in diversity and composition were due to the change in the diet that occurs at weaning. The diverse bacteria in the intestine may proliferate dramatically by the introduction of weaning foods that have complex chemical compositions as demonstrated in many previous studies (Favier et al., 2002; Inoue and Ushida, 2003a; Katouli et al., 1997; Mackie et al., 1999).

This study suggested that the development of the intestinal microbiota in the piglet is a gradual and sequential process. It was also suggested from this study that the development of the intestinal microbiota relates to non-dietary factors as well as dietary factors. Most of these factors, the morphological and immunological functions of the intestine for instance, may develop along with the aging of the host interacting with the development of the intestinal microbiota (Inoue and Ushida, 2003a). Since this study especially aimed to assess the major change of the intestinal microbiota which is probably a common event for piglets in the suckling period, the individual differences in the development of the intestinal microbiota were not focused on. Simpson et al. (2000), however, reported the composition of the intestinal microbiota in piglets differed individually even though all piglets were offered same compositions of diet. From these observations, it is suggested that non-dietary host factors may contribute not only to the major change of the intestinal microbiota but also to the minor changes which cause the individual differences in the intestinal microbiota. Further studies will be required to reveal the interactions between the development of the intestinal microbiota and these factors. The results of this study are practical for those studies since this study could suggest closer times when the intestinal microbiota change in the developing process than previous studies because of using TGGE.

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References


