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Molecular Analysis of Colonized Bacteria in a Human Newborn Infant Gut

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The complex ecosystem of intestinal microflora is estimated to harbor approximately 400 different microbial species, mostly bacteria. However, studies on bacterial colonization have mostly been based on culturing methods, which only detect a small fraction of the whole microbiotic ecosystem of the gut. To clarify the initial acquisition and subsequent colonization of bacteria in an infant within the few days after birth, phylogenetic analysis was performed using 16S rDNA sequences from the DNA isolated from feces on the 1st, 3rd, and 6th day. 16S rDNA libraries were constructed with the amplicons of PCR conditions at 30 cycles and 50°C annealing temperature. Nine independent libraries were produced by the application of three sets of primers (set A, set B, and set C) combined with three fecal samples for day 1, day 3, and day 6 of life. Approximately 220 clones (76.7%) of all 325 isolated clones were characterized as known species, while other 105 clones (32.3%) were characterized as unknown species. The library clone with set A universal primers amplifying 350 bp displayed increased diversity by days. Thus, set A primers were better suited for this type of molecular ecological analysis. On the first day of the life of the infant, *Enterobacter*, *Lactococcus lactis*, *Leuconostoc citreum*, and *Streptococcus mitis* were present. The largest taxonomic group was *L. lactis*. On the third day of the life of the infant, *Enterobacter*, *Enterococcus faecalis*, *Escherichia coli*, *S. mitis*, and *Streptococcus salivarius* were present. On the sixth day of the life of the infant, *Citrobacter*, *Clostridium difficile*, *Enterobacter* sp., *Enterobacter cloacae*, and *E. coli* were present. The largest taxonomic group was *E. coli*. These results showed that microbiotic diversity changes very rapidly in the few days after birth, and the acquisition of unculturable bacteria expanded rapidly after the third day.

Key words: Intestinal microbiota, newborn infant, colonized bacteria, 16S rDNA

It has been widely reported that the intestinal microbiota (population of microbes or normal flora) plays an important role in the health and well-being of the host, although a thorough scientific description and evaluation of this interaction has yet to be conducted. This has been generated, in part, by recognition of the role of intestinal bacteria in the pathogenesis of several intestinal disorders, including Crohn's disease, ulcerative colitis, and colorectal cancer (Shanahan, 2002; Gail *et al.*, 2003; Guarner 2003). During the birthing process and soon thereafter, bacterial colonization in the gastrointestinal tract of a germ-free infant begins. Several studies, which have relied almost exclusively on the use of culturing methods, have generated our current understanding of intestinal microbiology and ecology in infants (Yoshioka 1983; Ward *et al.*, 1990; Favier *et al.*, 2002). Microbial investigations based on cultivation strategies cannot be regarded as reliable in terms of their reflection of the true microbial diversity present in environments. The application of culture-independent techniques based on molecular biological methods, especially on the PCR amplification of 16S rDNA, endeavors to overcome some of the shortcomings of conventional cultivation methods (Bertilsson *et al.*, 2002; Bonnet *et al.*, 2002; Dahllof 2002). The 16S rDNA contains highly conserved sequence regions, which reflects the absence of evolutionary divergence caused by the fact that rDNA is essential for life. These sequences are interspersed with hypervariable regions, mutational changes which reflect the evolutionary divergence of the different species. The sequencing of 16S rDNA, therefore, represents a method for identifying and phylogenetically classifying intestinal bacteria. The 16S rDNAs act as an especially useful tool, as reflected by the rapidly growing number of sequences available in the databases for comparative purposes (Cho *et al.*, 2003; Cole *et al.*, 2003).

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More than 99% of the culturable fecal microbiota is represented by 30-40 bacterial species. Most of the bacteria which colonize the human intestine have strictly anaerobic metabolisms, and aerobic and facultative anaerobes are many grades of magnitude lower than the number of obligate anaerobes (Tannock, 2001; Shanahan, 2002; Fanaro *et al.,* 2003).

In this study, the development of the intestinal microbiota was investigated in terms of initial acquisition and subsequent colonization of bacteria in an infant within a few days of birth. Among the widely-available primers for amplification of 16S rDNA genes, the optimal primers were examined for culture-independent evaluation of the gastrointestinal ecosystem using the PCR-based sequencing technique.

Materials and Methods

Preparation of samples and DNA extraction

A full-term female infant which had been delivered by cesarean section was selected for this study. She was healthy and received routine nursery care. She was fed with formula milk 4 h after birth, and then every 3 subsequent hrs. Fresh fecal samples were collected from the baby at day 1 (MH1), day 3 (MH3), and day 6 (MH6) of life. These samples were stored at -20°C until further processing (McOrist *et al.,* 2002; Li *et al.,* 2003). DNA was extracted with the QIAamp DNA Stool Mini-kit (Qiagen, USA).

Primers and conditions of PCR

Three sets of primers were used to generate the partial and near-full-length sequences of 16S rDNA of all fecal samples (Table 1) (Marchesi *et al.,* 1998; Wang *et al.,* 2003). The partial sequence fragments using set A primer, which included two variable regions (V1 and V2) of the 16S rDNA, were 350 bp long. The near-full-length sequences of bacterial 16S rDNA using set B and set C primers, which included nine and eight variable regions of the 16S rDNA, were 1,500 bp and 1,350 bp long, respectively. The near-full-length sequence of bacterial 16S rDNA using set C primer, which included eight variable regions of the 16S rDNA, was 1,350 bp long (Fig. 1) (Martinez-Murcia *et al.,* 1999; Tung *et al.,* 2002). The constituents of the PCR mixtures were: 500 mM KCl, 100 mM Tris HCl (pH 9.0), 1% Triton X-100, 0.2 mM deoxynucleoside triphosphates (dATP, dGTP, dTTP and dCTP), 1.5 mM $MgCl_2$, 10 pmol each primer, and 1 U *Taq* DNA polymerase (Solgent, Korea). Each reaction was carried out for 5 min at 94°C, 10 to 30 cycles of 1 min at 94°C, 1.5 min at 50 - 60°C, 2 min at 72°C, and extension for 10 min at 72°C. The PCR products were electrophoresed in 2.0% agarose gels, stained with ethidium bromide, and visualized on a gel documentation system (Gel doc 2000, Bio-Rad Laboratories Inc., USA).

Construction of 16S rDNA clone libraries

PCR products were purified with a Gel Extraction kit (Nucleogen Biotechnology, Korea). The purified PCR products were ligated into the pGEM T-easy vector (Promega, USA), and were then transformed into competent *E. coli* DH5α cells. Culture tubes were incubated overnight at 37°C with shaking. Plasmid DNA was purified with a plasmid purification kit (Nucleogen Biotechnology, Korea) and was used for sequence analysis. PCR was performed using the cell lysates as the template and pGEM-T specific primers, T7 and SP6, to check the sizes of the inserts. Glycerol (100 μl of 30% glycerol in LB) was added to each culture tube, and tubes were stored at -80°C.

DNA sequencing

A purified plasmid from the 16S rDNA library was used to analyze the sequence of the cloned 16S rDNA fragments. The sequence of plasmids was determined using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) in the automated AB13700 DNA sequence analyzer (Applied Biosystems, USA).

Phylogenetic analysis

The 16S rDNA sequences were checked for chimeric con-

Table 1. PCR primers for PCR-cloning of 16S rDNA genes

Set (Size[a])	Primers	Sequences	Position[b]	Tm[c] (°C)
Set A (350 bp)	27F	5'-AgAgTTTgATCMTgg CTCAg-3'	8 - 27	58 - 60
	342R	5'-CTgCTgCSYCCCgTAg-3'	358 - 342	54 - 56
Set B (1,500 bp)	27F	5'-AgAgTTTgATCMTggCTCAg-3'	8 - 27	58 - 60
	1492R	5'-TACggYTACCTTgTTACgACTT-3'	1513 - 1492	62 - 64
Set C (1,350 bp)	63F	5'-CAggCCTAACACATgCAAgTC-3'	43 - 63	60
	1387R	5'-gggCggWgTgTACAAggC-3'	1387 - 1370	60

[a]Expected amplicon size
[b]Position based on nucleotide position of 16S rDNA of *E. coli*
[c]Tm indicates melting temperature of primer

structs using the CHECK-CHIMERA program of the Ribosomal Database Project (Cole *et al.*, 2003). Confirmed chimeric sequences were excluded from phylogenetic analysis. One partial and two near-full-length 16S rDNA sequences were directly compared to those in GenBank by BLAST search and were pivoted from the RDP-II. Sequences with 99% similarity (< 1% diversity) were designated as being of the same species. All of the other sequences were retrieved from the National Center for Biotechnology Information (NCBI). 16S rDNA sequences aligned according to secondary structure were selected from RDP-II. They were then manually edited using BioEdit. The distances for each 16S rDNA were calculated using the PHYLIP package with a Kimura 2 parameter; phylogenetic trees were also constructed by the PHYLIP package by the neighbor-joining method (Kang *et al.*, 2004).

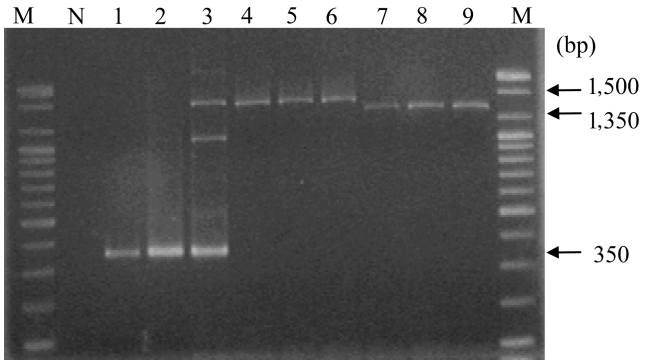

Fig. 1. PCR results using three sets (A, B, and C) of primers for 16S rDNA amplification of intestinal flora. Lanes M, 100-bp DNA ladder standard size makers; lane N, negative control; lane 1, 1-day with set A primers; lane 2, 3-day with set A primers; lane 3, 6-day with set A primers; lane 4, 1-day with set B primers; lane 5, 3-day with set B primers; lane 6, 6-day with set B primers; lane 7, 1-day with set C primers; lane 8, 3-day with set C primers; lane 9, 6-day with set C primers.

Results

PCR conditions for amplification of intestinal flora

We examined the PCR conditions, three sets of primers, cycle, and annealing temperature, for amplification of intestinal flora. Their efficacies were tested with day 1, day 3, and day 6 fecal samples.

i) PCR primers

The universal, degenerative primers (set A, set B, and set C) were successful in amplifying the 16S rDNA in day

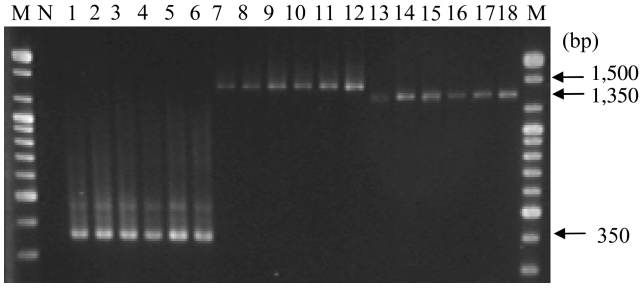

Fig. 2. PCR results with gradient PCR at 50.2°C, 52.7°C, 54.0°C, 56.9°C, 58.1°C, and 59.9°C annealing temperature for 16S rDNA amplification of intestinal flora. Lanes M, 100-bp DNA ladder standard size makers; lane N, negative control; lane 1, 50.2°C with set A primers; lane 2, 52.7°C with set A primers; lane 3, 54.0°C with set A primers; lane 4, 56.9°C with set A primers; lane 5, 58.1°C with set A primers; lane 6, 59.9°C with set A primers; lane 7, 50.2°C with set B primers; lane 8, 52.7°C with set B primers; lane 9, 54.0°C with set B primers; lane 10, 56.9°C with set B primers; lane 11, 58.1°C with set B primers; lane 12, 59.9°C with set B primers; lane 13, 50.2°C with set C primers; lane 14, 52.7°C with set C primers; lane 15, 54.0°C with set C primers; lane 16, 56.9°C with set C primers; lane 17, 58.1°C with set C primers; lane 18, 59.9°C with set C primers.

Table 2. Result of amplification and constructed clone libraries

Primer (Expected size)	Day of life	Cycles of PCR					Name of Libraries (a/b)
		10	15	20	25	30	
Set A (350 bp)	1-day	×	×	×	●	●	A-MH1 (124/300)
	3-day	×	×	×	●	●	A-MH3 (91/300)
	6-day	×	×	●	●	●	A-MH6 (110/310)
Set B (1,500 bp)	1-day	×	×	×	×	●	B-MH1 (3/15)
	3-day	×	×	×	×	●	B-MH3 (12/20)
	6-day	×	×	×	×	●	B-MH6 (6/17)
Set C (1,350 bp)	1-day	×	×	×	×	●	C-MH1 (5/23)
	3-day	×	×	×	×	●	C-MH3 (4/16)
	6-day	×	×	×	×	●	C-MH6 (1/15)

●: Amplification
×: No amplification
a : Number of clones analyzed with 16S rDNA sequence
b : Total number of clones constructed
Libraries were constructed with amplicons of 30-cycle and 50°C annealing temperature PCR conditions

Table 3. Summary of clone libraries with set A primers of intestinal microflora

Taxonomic group	Accession No. of GenBank	No. of clones		
		1-day	3-day	6-day
Citrobacter amalonaticus	AJ415574	0	0	1
Clostridium difficile	AJ132322	0	0	1
Enterobacter sp.	U39556	1	1	4
Enterobacter cloacae	AJ417479	0	0	17
Enterococcus faecalis	AY395018	0	2	0
Escherichia coli	AY430288	0	20	28
Lactococcus lactis	AB122036	83	0	0
Leuconostoc citreum	AB122037	16	0	0
Streptococcus mitis	AY005045	1	19	0
Streptococcus salivarius	AF459433	0	26	0
Unidentified bacterium	-	23	23	59
Total		124	91	110

1, day 3, and day 6 fecal samples (Fig. 1). As expected, PCR with set A, set B, and set C primers amplified approximately 350 bp, 1,500 bp, and 1,350 bp, respectively. The amplification concentrations increased with increases in time since birth.

ii) PCR cycle

The same DNA samples were used and the number of PCR cycles was increased to 30 cycles. It is possible to amplify the partial 350 bp of 16S rDNA of MH6 samples (day 6 feces) at 20, 25, and 30 PCR cycles. In the case of the amplification of near-full-length 16S rDNA, all samples were amplified at only 30 PCR cycles (Table 2).

iii) Annealing temperature

Three sets of primers were successful in amplifying 16S rDNA at 50 - 60°C an annealing temperature. To avoid the amplification of specific species, we selected an optimal annealing temperature of 50°C (Fig. 2).

Construction of 16S rDNA libraries

Libraries were constructed with 7the amplicons of PCR conditions at 30 cycles and 50°C annealing temperature. Nine independent libraries were produced by the application of three sets of primers combined with three fecal samples per day for day 1, day 3, and day 6 of life (A-MH1, A-MH3, A-MH6, B-MH1, B-MH3, B-MH6, C-

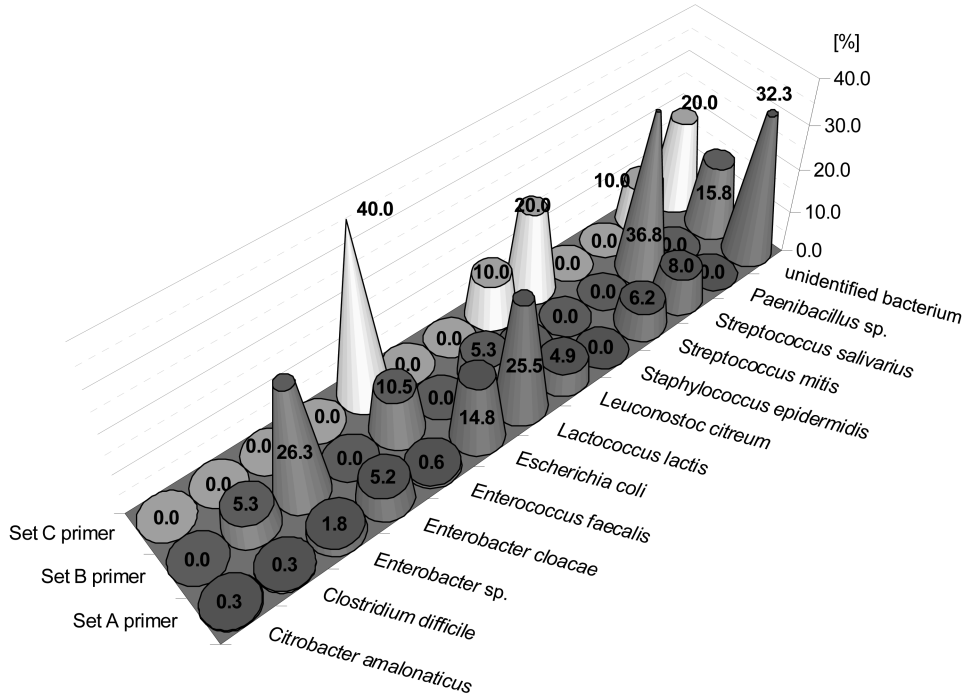

Fig. 3. Taxonomic composition of A-MH, B-MH, and C-MH libraries with set A, set B, and set C primers, respectively.

MH1, C-MH3, and C-MH6) (Table 2). A total of 356 sequences from nine libraries were subjected to sequence analysis. Approximately 100 clones constructed (A-MH1, A-MH3, and A-MH6) by set A primer were sequenced. On the basis of sequence similarities, the clones were classified into two groups, known and unknown species (Table 3). Approximately 220 clones of all 325 isolated clones were characterized as known species; the other 105 clones were found to be unknown species.

Comparison of partial and two near-full 16S rDNA libraries using three sets of primers

We analyzed the precision of a partial sequence (set A primers) and two near-full-length sequences (set B and set C primers) for the screening of the intestinal microbiota. An estimation of the bacterial diversity in each library was made by taxonomic distribution of the analyzed clones. The results showed that the two clone libraries of amplicons from set B and set C primers had similar overall diversities, while the clone library of amplicons by set A primers had more diversity (Fig. 3).

A-MH (day 1, day 3, day 6), B-MH (day 1, day 3, day 6), and C-MH (day 1, day 3, day 6) libraries contained 11, 6, and 5 different sequences, respectively. The frequency distributions of the unique sequences varied markedly among the libraries. The relative proportions of each of the taxons clearly varied between the libraries, with the largest proportion of *L. lactis* in the A-MH library, while this taxon was completely absent from the B-MH and C-MH libraries. These results demonstrated that the diversities of the three sets of primer-derived libraries were different; the set A primers library displayed increased diversity (Fig. 3). The data indicated that primers used during PCR amplification may affect the diversity of the amplified 16S rDNAs. The set A primers were better suited for this type of molecular ecological analysis.

Bacterial colonization in gut of a newborn infant

For analysis of bacterial colonization in the gut of a newborn infant, three clone libraries (A-MH1, A-MH3, and A-MH6) were constructed with amplicons (350 bp) using set A primers. The 124, 91, and 110 respective sequences

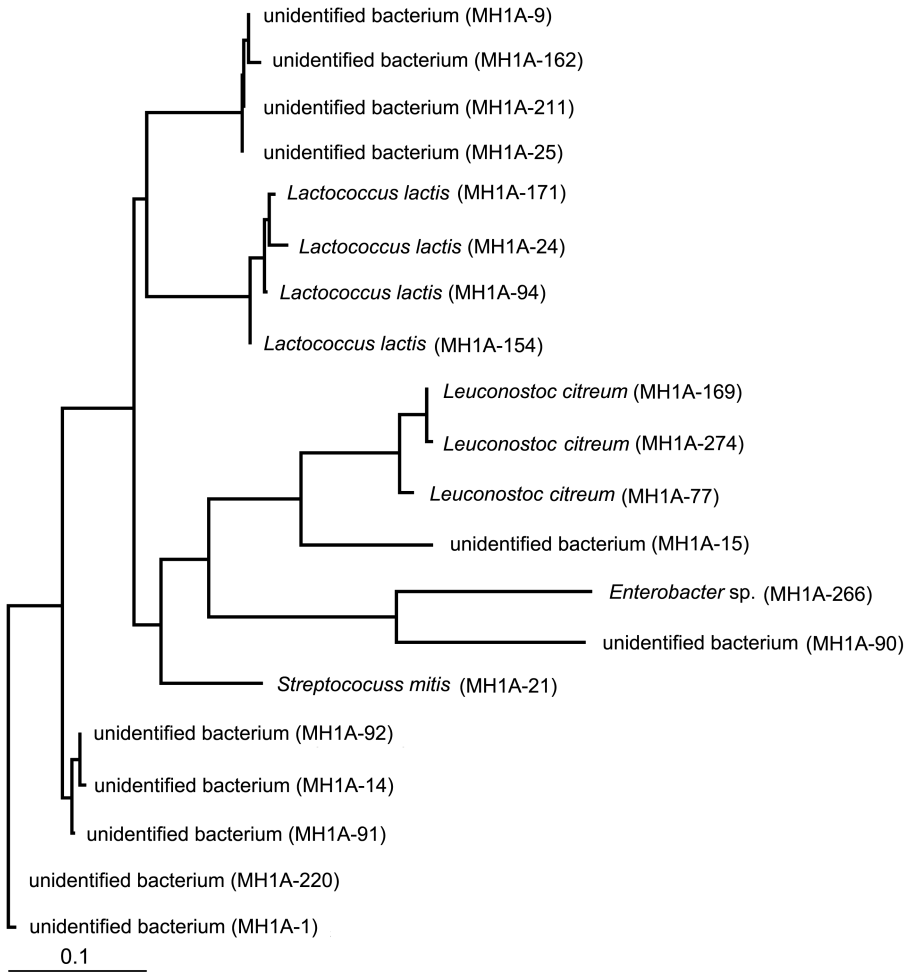

Fig. 4. Phylogenetic tree derived from 16S rDNA sequence data of 16S rDNA sequences from clone library constructed with set A primers at 1-day. The tree was constructed by use of the neighbor-joining method based on 16S rDNA sequences.

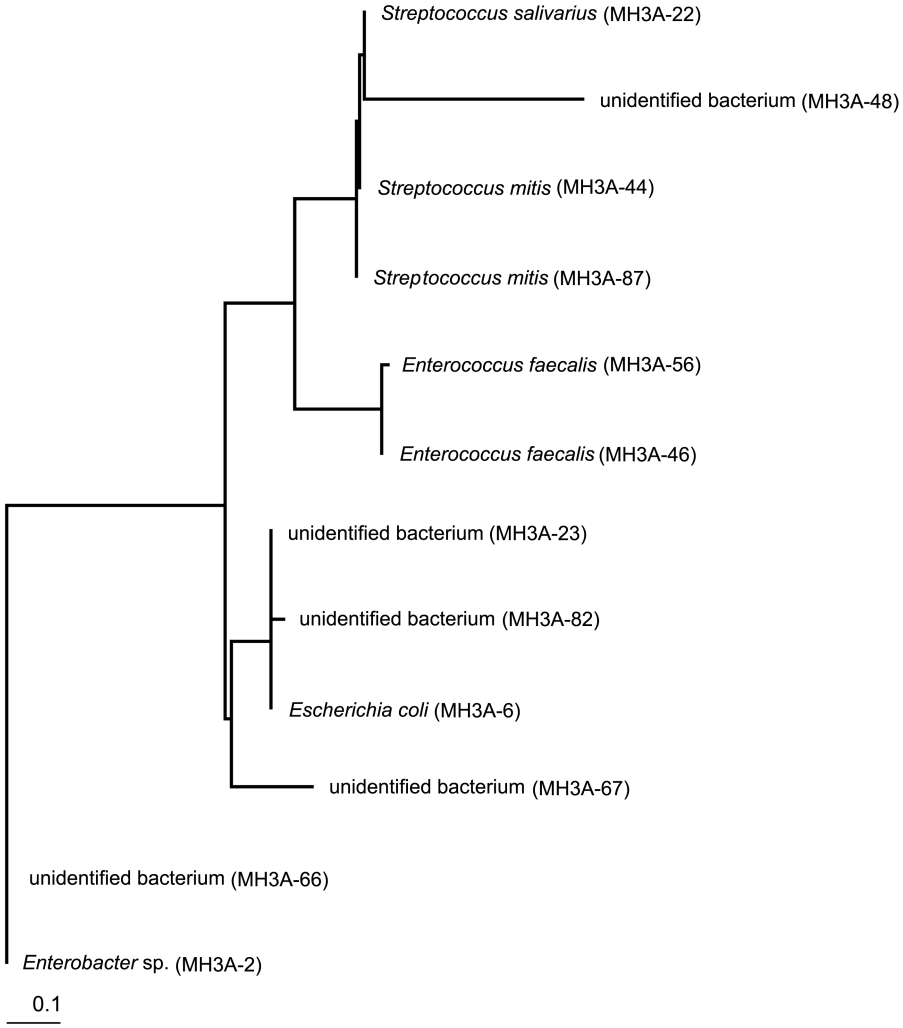

Fig. 5. Phylogenetic tree derived from 16S rDNA sequence data of 16S rDNA sequences from clone library constructed with set A primers at 3-day. The tree was constructed by use of the neighbor-joining method based on 16S rDNA sequences.

from A-MH1, A-MH3, and A-MH6 libraries were subjected to sequence analysis. Table 3 shows the taxonomic composition of clones constructed at day 1, day 3, and day 6 of life.

On the first day of life, *Enterobacter*, *L. lactis*, *L. citreum*, and *S. mitis* were present. The 124 clones of the A-MH1 library were classified into five molecular species (at least 99% sequence similarity). The largest taxonomic group, representing 66.9% of the total 124 library clones, was *L. lactis*. *L. lactis* and *L. citreum* disappeared after day 3.

On the third day of life, *Enterobacter*, *E. faecalis*, *E. coli*, *S. mitis*, and *S. salivarius* were present. The 91 clones of the A-MH3 library were classified into six molecular species (at least 99% sequence similarity). The largest taxonomic group, representing 28.5% of the total 91 library clones, was *S. salivarius*.

On the sixth day of life, *Citrobacter amalonaticus*, *C. difficile*, *Enterobacter* sp., *E. cloacae*, and *E. coli* were present. The 110 clones of the A-MH1 library were classified into six molecular species (at least 99% sequence similarity). The largest taxonomic group, representing 25.4% of the total 110 library clones, was *E. coli*.

Phylogenetic analysis

Fig. 4, 5, and 6 show the phylogenetic analysis of the clones in the A-MH1, A-MH3, and A-MH6 libraries. A-MH1 libraries constructed using set A primers on day 1 were classified into seven clusters (Fig. 4). A-MH3 libraries constructed using set A primers on day 3 were was classified into six clusters (Fig. 5). A-MH6 libraries constructed using set A primers on day 6 were classified into nine clusters (Fig. 6).

Discussion

Microbial colonization and infection of the gut can profoundly influence the status of specific and nonspecific cellular and humoral elements of the gut mucosal immune system during the lives of neonates (Cebra, 1999). Thus, it is important to understand bacterial colonization in the

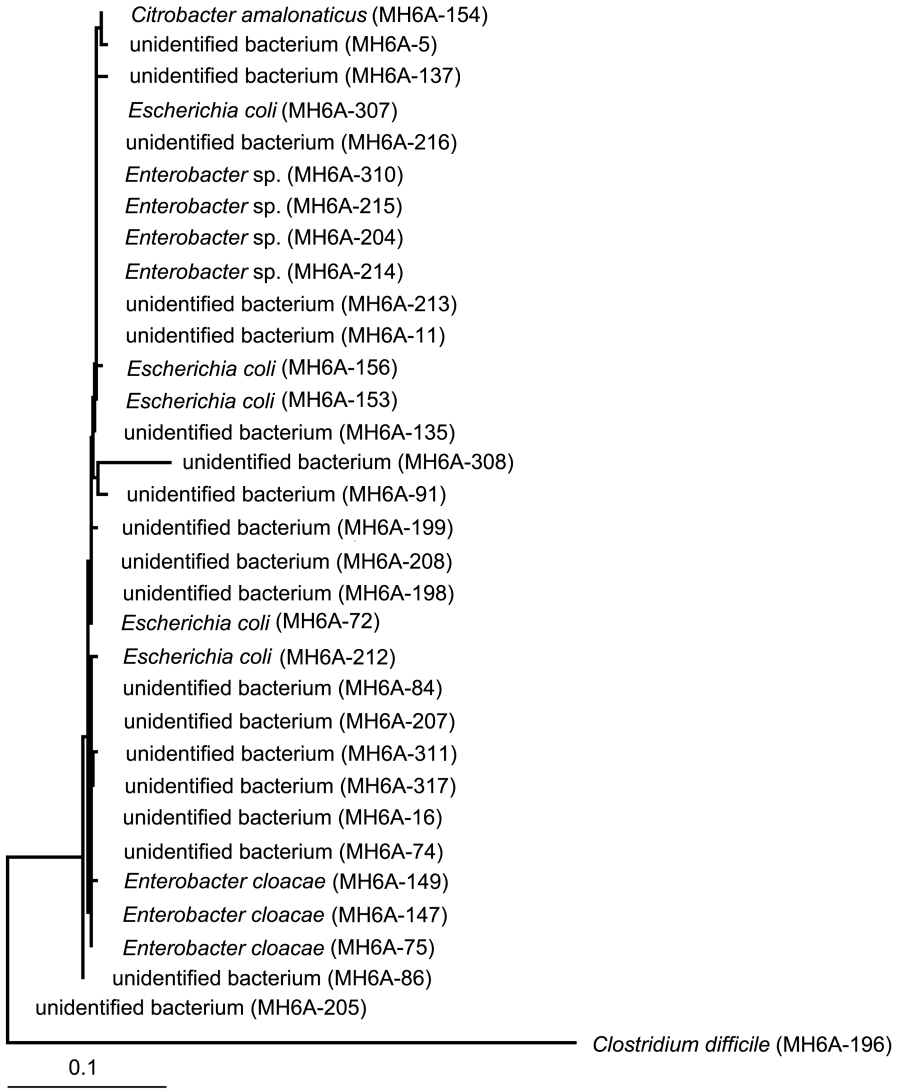

Fig. 6. Phylogenetic tree derived from 16S rDNA sequence data of 16S rDNA sequences from clone library constructed with set A primers at 6-day. The tree was constructed by use of the neighbor-joining method based on 16S rDNA sequences.

guts of newborn infants. The intestinal microbiota of the infant is a complex ecosystem composed of numerous genera, species, and strains of bacteria. Most studies of intestinal microbiota of infants have been based on fecal samples which use conventional culture techniques on specific media. The limitations of these methods must be taken into account when evaluating the varied results of the different studies (Fanaro *et al.*, 2003; Hur *et al.*, 2004). Human intestinal microbiota is analyzed mainly by the culture-based method, though 60 to 80% of the microorganisms of the entire intestinal microbiota have not yet been cultivated (Rondon *et al.*, 1999). It has now been recognized that culture-dependent techniques can strongly bias and underestimate the diversity of microbial populations.

PCR is routinely used in the amplification and cloning of rRNA genes from environmental DNA samples for studies of microbial community structure and identification of novel organisms. Greater bacterial diversity was detected by PCR than by cultivation. Several bacteria which had never been isolated from human feces were detected by PCR (Schabereiter-Gurtner *et al.*, 2001; Gail, 2003). Nevertheless, the drawbacks of assessing microbial diversity using PCR-based rDNA analysis have been described in several studies (von Wintzingerode *et al.*, 1997; Shafikhani, 2002). The choice of a primer for universal PCR amplification of 16S rDNA from complex microbiota may influence the recovery of target sequences (Wilson, 1997; Hunter-Cevera, 1998).

Various PCR primers for the amplification of 16S rDNA genes are widely available (Marchesi *et al.*, 1998; Martinez-Murcia *et al.*, 1999; Tung *et al.*, 2002; Wang *et al.*, 2003). Set A (27F-342R) and set B (27F-1492R) primers have been used in many investigations on micro-

biota (Wang *et al.,* 1997; Suau *et al.,* 1999). Marchesi *et. al.*(1998) reported that primers 63F and 1387R were used successfully and consistently to amplify 16S rDNA genes. We studied the efficacy of the above three sets of universal primers for investigating intestinal microbiota. We found that set A (27F - 342R) primers produced more diverse clones than did primers of sets B and C. Libraries with set A, set B, and set C contained 11, 6, and 5 different sequences, respectively (Fig. 3). Set A primers were better suited for this type of molecular ecological analysis. We analyzed a few clones from libraries with set B and set C primers. We are constructing more clones with set B and set C primers in order to conduct a more sophisticated analysis. The choice of primer for universal or taxon-specific PCR amplification of 16S rDNA from complex microbiota may influence the recovery of target sequences (von Wintzingerode *et al.,* 1997).

Fanaro *et al.*(2003) reported that the intestinal microbiota in fecal samples of infants consisted of aerobic bacteria, i.e. *Enterobacter*, *Enterococcus*, and *Staphylococcus*, and anaerobic bacteria, i.e. *Bacteroides*, *Bifidobacterium*, *Lactobacillus,* and *Clostridium*. Upon culture-based investigation, some results showed differences in the presence of anaerobic bacteria, *Bacteroides*, *Bifidobacterium*, *Lactobacillus,* and *Clostridium* (Yoshioka *et al.,* 1983). It is difficult to cultivate anaerobic bacteria because they require anaerobic conditions for growth and survival. In our study using PCR-based sequencing, aerobic and anaerobic bacteria were detected (Fig. 3). The composition of the intestinal microbiota is profoundly influenced by the diet of the infant. In breast-fed infants, the microbiota is rapidly dominated by *Bifidobacterium*, and a more diverse microbiota develops only after dietary supplementation is initiated. In contrast, the intestines of formula-fed infants are colonized by members of a variety of bacterial genera, including enterobacterial genera, *Streptococcus*, *Bacteroides*, and *Clostridium*, in addition to members of the genus, *Bifidobacterium* (Favier *et al.,* 2002). Colonization begins immediately after delivery, and enterobacteria and *Streptococcus* appear in the feces on the first day of life. *Enterobacter*, *L. lactis*, *L. citreum*, and *S. mitis* were also found to be present in our study on the first day of life. *C. difficile* appeared on the sixth day of life (Table 3). According to Mackie *et al.* (1999), healthy newborn infants often harbor *C. difficile* and its toxin with no apparent consequences. In this study, 220 clones of all 325 isolated clones were characterized as known species, while the other 105 clones were unknown species. The use of PCR-based sequencing in this study of infant intestinal diversity has successfully revealed the existence of many novel organisms which cannot be isolated by traditional culture-dependent techniques. In conclusion, we found optimal universal primers for investigation of intestinal microbiota and detected new species that have not yet been characterized.

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