

Aberrant structures of fecal bacterial community in allergic infants profiled by 16S rRNA gene pyrosequencing

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Received 8 February 2011; revised 27 August 2011; accepted 28 August 2011.

DOI: 10.1111/j.1574-695X.2011.00872.x

Editor: Eric Oswald

Keywords

infant; allergy; fecal microbiota; gastrointestinal tract; pyrosequence. 16S rRNA gene.

Abstract

We investigated the correlation between fecal bacteria composition in early infancy and the prevalence of allergic diseases in late infancy. The fecal microbiota in the first 2 months was profiled using the 16S rRNA V6 short-tag sequences in the community and statistically compared between two groups of subjects who did and did not show allergic symptoms in the first 2 years ($n = 11$ vs. 11). In the allergic group, genus *Bacteroides* at 1 month and genera *Propionibacterium* and *Klebsiella* at 2 months were more abundant, and genera *Acinetobacter* and *Clostridium* at 1 month were less abundant than in the non-allergic group. Allergic infants who showed high colonization of *Bacteroides* and/or *Klebsiella* showed less colonization of *Clostridium perfringens/butyricum*, suggesting antagonism between these bacterial groups in the gastrointestinal tract. It was also remarkable that the relative abundance of total *Proteobacteria*, excluding genus *Klebsiella*, was significantly lower in the allergic than in the nonallergic group at the age of 1 month. These results indicate that pyrosequence-based 16S rRNA gene profiling is valid to find the intestinal microbiota disorder that correlates with allergy development in later life.

Introduction

Immediately after birth, different types of bacteria begin to colonize in the previously sterilized gastrointestinal (GI) tract (Mitsuoka, 1992; Bezirtzoglou, 1997; Mackie *et al.*, 1999; Favier *et al.*, 2002; Songjinda *et al.*, 2005). This bacterial colonization in infancy is believed to have

great influence on the development of the immune system; the microbiota in this period dynamically changes from germfree to bifidobacteria-dominated flora (Mitsuoka, 1990; Kelly *et al.*, 2005; Noverr & Huffnagle, 2005; Sjogren *et al.*, 2009b; Nakayama, 2010). Previous studies found that bacterial colonization in this period was easily influenced by living conditions and environment

and that the developmental microbiota differed among individuals (Benno *et al.*, 1984; Bezirtzoglou, 1997; Sepp *et al.*, 1997; Songjinda *et al.*, 2005; Penders *et al.*, 2006; Tanaka *et al.*, 2009). From this information, it is reasonable to suspect that the hygienic lifestyle of modernized countries modulates the microbiota of the infant GI tract and has increased the incidence of allergy among infants.

Results of a series of epidemiological studies comparing Swedish and Estonian children supported the gut microbiota-mediated hygiene hypothesis (Sepp *et al.*, 1997; Björkstén *et al.*, 1999, 2001). These studies showed the difference in the fecal bacterial community structure between these two countries and also between allergic and nonallergic infants, who subsequently did and did not develop allergic diseases, respectively. After these studies, several similar prospective epidemiological studies were performed in European and Asian countries, indicating a correlation between aberrant microbial composition in infancy and the development of allergy in later life (Kalliomäki *et al.*, 2001; Kirjavainen *et al.*, 2002; Adlerberth *et al.*, 2007; Penders *et al.*, 2007; Suzuki *et al.*, 2007, 2008; Smehilova *et al.*, 2008; Vael *et al.*, 2008; Wang *et al.*, 2008; Sjögren *et al.*, 2009b; Hong *et al.*, 2010). Our group also performed a pilot study in Japanese newborns and found that the PCR count of *Bacteroidaceae* at the age of 1 month was significantly higher in a group of babies who developed any allergic disease up to the age of 2 years than in a nonallergic group (Songjinda *et al.*, 2007).

The accurate analysis of the complex bacterial community structure of GI tract microbiota has been difficult; the GI tract contains 500–1000 species, including a large variety of uncultured and/or unknown bacteria. Recent progress in molecular techniques has addressed the problem of the large unculturable fraction (Zoetendal *et al.*, 2008). Notably, a recently developed ultra-high-throughput sequencer, called a pyrosequencer, allows sequence-based 16S rRNA gene profiling of GI tract microbiota (Bailey *et al.*, 2010; Hong *et al.*, 2010; Stecher *et al.*, 2010; Nakayama, 2010). One of the advantages of this approach is that it provides a bird's eye view of infant fecal microbiota and overcomes the limitations of target-specific microbiota analyses such as quantitative real-time PCR, fluorescence *in situ* hybridization microscopy, or colony counts. Despite the limit of the read length in the pyrosequencing, tag sequences of variable region, for example, V4 and V6, provide phylogeny-linked data of bacterial composition (Nakayama, 2010). In this study, with these merits in the 16S rRNA gene pyrosequencing strategy, fecal bacterial compositions of newborns are profiled and compared between allergic and nonallergic groups.

Materials and methods

Sampling

The protocol of this study was approved by the ethics commission of the Faculty of Medicine of Kyoto University in Japan. Eighty-three vaginally delivered infants, born in 2002–2004 at a hospital in Fukuoka City, Japan, were registered as participants of this study. Informed consent was obtained from all parents of the infant subjects. The infants were born between the 36 and 41 weeks of gestation (mean, 39 weeks). All of the infants were raised in the same hospital in the first week after birth, and a follow-up for the first 2 months supported that all of the infant subjects were healthy. Fecal samples were collected at 5 days, 1 month, and 2 months after birth, except for the lack of sample of subject number 28 at 5 days. Freshly evacuated feces in diapers were gathered into collection tubes and refrigerated until they were transported to the laboratory. All of the samples were stored at -80°C until further processing.

A questionnaire was used to investigate the history of medical diagnosis of allergies and symptoms of atopic dermatitis, asthma, and food allergy during the first 2 years. Answers to the questionnaire were obtained for 73 of the 83 subjects, and 13 of the 73 infants were found to have an allergic disease. Two allergic infants were excluded from this study because they had received orally administered antibiotics immediately after birth. The remaining 11 infants who had not received antibiotics were eventually selected as allergic subjects for this study (male/female = 6/5; food allergy/atopic dermatitis/asthma = 3/5/4, including one infant who showed symptoms of both food allergy and atopic dermatitis). Eleven infants who were not administered antibiotics during the first 2 months and did not have any allergy for the first 2 years were randomly selected as nonallergic subjects for the negative control group. The type and onset of allergy disease in each subject was described in the Supporting Information, Data S1.

DNA extraction from fecal samples

DNA was extracted from fecal samples by using the QIA-amp DNA Stool Mini-Kit (Qiagen, Hilden, Germany) in combination with the bead-beating method (Wang *et al.*, 2004; Songjinda *et al.*, 2007; Tanaka *et al.*, 2009). Approximately 50 mg (wet weight) of feces was washed twice with 1 mL phosphate-buffered saline (PBS; pH 7.4) and then suspended in 900 μL PBS. The suspension was transferred to a 2.0-mL screw-capped tube containing 0.3 g of zirconium beads (diameter, 0.1 mm; As One

Corporation, Osaka, Japan). Thereafter, 300 µL of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) was added to the suspension. The tube was shaken at 2700 r.p.m. for 3 min in a bead-beater instrument (Multi-beads Shocker; Yasui Kikai, Osaka, Japan). The treated sample was centrifuged at 20 400 g at 4 °C for 2 min. Subsequently, bacterial DNA was isolated from the upper layer by using the QIAamp DNA Stool Mini-Kit according to the manufacturer's instructions. The DNA was eluted into a final volume of 50 µL buffer AE from the column supplied in the kit. The concentration of nucleic acids was measured using Spectrophotometer ND-1000 (Nano Drop Technologies, Wilmington, DE), and 10–50 ng of the DNA template was subjected to the following PCR to amplify the V6 region of 16S rRNA gene.

Bacteria composition analysis by 16S rRNA gene V6 short-tag pyrosequencing

The V6 fragment of 16S rRNA gene was amplified from the total bacterial DNA isolated from each fecal sample by using the universal primer Q-968F (5'-WACGCGAR GAACCTTACC-3') and the reverse primer Q-1046R (5'-CGACRRCCTGCANCACCT-3'; Nakayama, 2010). PCR was performed in 50 µL of a solution containing 25 µL Premix Ex Taq polymerase (Takara Bio, Shiga, Japan), 1 µL of the template solution containing 10–50 ng of the isolated DNA, and 10 pmol of each primer. The PCR condition was as follows: 94 °C for 3 min; 20 cycles at 94 °C for 30 s, 52 °C for 45 s, and 72 °C for 1 min; and finally 72 °C for 2 min. Subsequently, a barcode sequence tag was added to the amplicon by a second PCR that was performed using the primers Q-968F-# (5'-GGTTBNHYW-ACGCGARGAACCTTACC-3') and Q-1046R-# (5'-GGTT-BNHYCGACRRCCTGCANCACCT-3'; # indicates a series of 48 barcode sequence tags underlined in the sequence). The second PCR was performed in a 100-µL volume containing 50 µL Premix Ex Taq polymerase, 2 µL of the reacted solution from the first PCR, and 20 pmol of each primer. The PCR condition was as follows: 94 °C for 3 min; 20 cycles at 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 1 min; and finally, 72 °C for 2 min. The amplicons were purified using a QIAquick PCR purification Kit (Qiagen), and the DNA concentrations were measured using Spectrophotometer ND-1000. A total of 65 samples were divided into 39-sample and 26-sample groups and subjected to the pyrosequencing separately. In each group, equal amounts (200 ng) of amplicons from each sample were pooled and purified using the ethanol precipitation method. Each amplicon mixture was applied to the Genome Sequencer FLX system using 1/8 region of 350 000-well pico titer plate (Roche Diagnostics, Tokyo, Japan).

Pyrosequencing data analysis

The batch sequence data were sorted into each sample batch using the barcode tag, and the primer sequences were then trimmed from each sequence in the Pipeline Initial Process at the Ribosomal Database Project (RDP) II website (<http://pyro.cme.msu.edu/>). The result obtained was 321–882 reads (an average of 584 reads) for each sample from 37 978 total reads. The RDP classifier (<http://rdp.cme.msu.edu/classifier/classifier.jsp>) was used to convert the batch sequence data of each sample into bacterial population data at hierarchical levels, from the genus to phylum (Wang *et al.*, 2007). For the RDP classifier, the confidence threshold was set at 50%. The relative abundance of each taxonomic group was determined by dividing the number of classified reads by the total read number of each sample. The data were provided to Data S2. Sequence match analysis of the V6 sequence was performed for the RDP-II database of cultured bacteria using the SEQMATCH program (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

Statistical analysis

A heat map showing the relative abundance of each genus in each sample was generated using R version 2.9.2 (R Foundation for Statistical Computing). The statistical analyses were performed using the Kolmogorov–Smirnov test in the web site (http://www.physics.csbsju.edu/stats/KS-test.n.plot_form.html), the *F*-test, Welch's *t*-test, and Student *t*-test in EXCEL2007 software, and the Mann–Whitney *U*-test in R version 2.9.2. Principal component analysis (PCA) was performed using MVSP 3.1 (Kovach Computing Services, UK).

Results

Comparison of fecal bacterial composition between allergic and nonallergic subjects

Three different-aged samples (5 days, 1 month, and 2 months after birth) were collected from 11 allergic and 11 nonallergic subjects, and their bacterial compositions were profiled using 16S rRNA gene V6 short-tag sequences (the data provided in Data S2). The relative abundance of each genus in each infant subject was statistically compared between the allergic and nonallergic groups at the same age. To survey possible candidates of bacteria that correlated with the later allergy development, both parametric and nonparametric analyses were performed. At 5 days after birth, only genus *Acinetobacter* showed significant differences ($P < 0.05$ in Mann–Whitney *U*-test, Supporting Information). The data from month 1 and month 2 are expressed as a heat map in Fig. 1. The differences were



Fig. 1. Heat map representation of the relative abundances of bacterial genera in feces from infants aged 1 and 2 months and comparison between the allergic and nonallergic groups. The relative abundance was determined by dividing the number of the V6 short-tag reads by the total read number of each sample. Both parametric Welch's t -test and nonparametric Mann-Whitney U -test were used to compare the relative abundances of each genus between the two groups. The number above the map indicates subject number. Statistical significances are represented by symbols on the right side of the heat map under p^u (Mann-Whitney U -test) and p^t (Welch's t -test) as follows: $>$ and $>$ (blue character), allergic group was lower than the nonallergic group with $P < 0.05$ and $P < 0.1$, respectively; $<$ and $<$ (red character), allergic group was higher than the nonallergic group with $P < 0.05$ and $P < 0.1$, respectively.

most remarkable at 1 month after the birth, when *Bacteroides* and *Klebsiella* were more abundant in the allergic group than in the nonallergic group, whereas *Clostridium*, *Lactobacillus*, *Lactococcus*, and some genera of *Proteobacteria* were less abundant in the allergic group than in the nonallergic group. At 2 months after the birth, the difference in the relative abundance of *Klebsiella* was more evident than at 1 month. In addition, at 2 months, the relative abundance of *Propionibacterium* was significantly higher in the allergic group than in the nonallergic group, according to the results of the nonparametric analysis. *Bifidobacterium* appeared as a predominant bacterium at 1 month in all of the subjects, except subject number 79, and no significant difference was found in the relative abundance between the allergic and nonallergic group throughout the sampling period.

Taxonomic analysis of V6 short-tag sequences of the genera showing the population difference between the allergic and nonallergic groups

The V6 short-tag sequences of the genera showing the population differences in the comparative analysis were placed into operational taxonomic unit (OTU) with 100% identity and listed in Table 1. The reliability of genus classification of each OTU was evaluated with the value of classification of reliability in the RDP classifier. Also, their V6 sequences were searched in the database of cultured bacteria.

Except for *Propionibacterium*, more than one phylotype were found in each genus. For instance, eight OTUs were found in the genus *Bacteroides*, and these corresponded to six different species. This suggests that allergy

Table 1. List of OTUs classified to genus showing different abundance between allergic and nonallergic groups at the indicated age

OTU	Accession No.	Classified genus*	Age†	Count‡	Averaged population (%)§		No. of carrier¶		Identified or closest species**
					Nonallergy	Allergy	Nonallergy	Allergy	
m1-4	AB636666	<i>Bacteroides</i> (88)	m1	101	0.06	1.88	1	2	<i>Bacteroides dorei</i>
m1-69	AB636667	<i>Bacteroides</i> (97)	m1	99	0.00	1.67	0	4	<i>Bacteroides thetaiotaomicron</i>
m1-18	AB636668	<i>Bacteroides</i> (96)	m1	92	0.02	1.66	1	2	<i>Bacteroides ovatus</i>
m1-17	AB636670	<i>Bacteroides</i> (91)	m1	63	0.02	1.00	1	2	<i>Bacteroides uniformis</i>
m1-596	AB636671	<i>Bacteroides</i> (65)	m1	48	0.02	0.78	1	1	<i>Bacteroides thetaiotaomicron</i>
m1-6	AB636674	<i>Bacteroides</i> (85)	m1	29	0.04	0.50	1	2	<i>Bacteroides vulgatus</i>
m1-256	AB636675	<i>Bacteroides</i> (53)	m1	20	0.00	0.40	0	2	<i>Bacteroides uniformis</i>
m1-285	AB636676	<i>Bacteroides</i> (96)	m1	20	0.02	0.35	1	2	<i>Bacteroides thetaiotaomicron</i>
m1-202	AB636672	<i>Acinetobacter</i> (68)	d5	30	0.44	0.02	6	1	<i>Acinetobacter lwoffii</i> ,
			m1	43	0.81	0.00	4	0	<i>Acinetobacter septicus</i>
			m1	38	0.62	0.07	4	3	<i>Acinetobacter rhizosphaerae</i>
m1-39	AB636665	<i>Clostridium</i> (79)	m1	187	2.54	0.01	4	1	<i>Clostridium perfringens</i>
m1-91	AB636669	<i>Clostridium</i> (97)	m1	76	1.01	0.12	3	2	<i>Clostridium butyricum</i>
m2-4	AB636677	<i>Klebsiella</i> (58, 95 ^{††})	m2	909	2.16	13.01	7	9	<i>Enterobacter cloacae</i> , <i>Klebsiella pneumoniae</i> , <i>Klebsiella variicola</i> , <i>Klebsiella singaporensis</i> , <i>Escherichia coli</i>
m2-57	AB636678	<i>Klebsiella</i> (50, 96 ^{††})	m2	211	1.16	2.40	7	7	<i>Klebsiella pneumoniae</i> , <i>Klebsiella oxytoca</i>
m2-34	AB636679	<i>Propionibacterium</i> (83)	m2	152	0.50	1.81	2	5	<i>Propionibacterium avidum</i> ***, <i>Propionibacterium propionicum</i> ***

OTU, operational taxonomic unit; d5, 5 days old; m1, 1 month old; m2, 2 months old.

*Genus classified by RDP classifier with the nucleotide sequence of the OTU. The number in parenthesis indicates the classification reliability using bootstrapping in the RDP classifier.

†Age at which the significant difference in the abundance of the classified genus was found.

‡Read count of the OTU at the indicated age. OTUs with more than 20 counts are shown in this table.

§The relative abundance of the OTU in total population.

¶The number of subjects from whom the OTU was detected.

**The sequence of each OTU was subjected to SeqMatch search in the RDP website. Species having identical V6 sequence except for ones marked by triple asterisk showing one-base mismatch.

††Sequence of V6–V8 region corresponding to *Escherichia coli* 16S rRNA gene position 986–1389. The details about this experiment and result were provided to Data S3.

development in the later years was not correlated with one specific species of *Bacteroides*. Two OTUs associated with the genus *Clostridium* correspond to *C. perfringens* and *C. butyricum*, respectively. Regarding *Proteobacteria*, two OTUs were found in *Acinetobacter* and two in *Klebsiella*. These OTUs could not be identified to a single species because they were identical to those of different species in the database. Especially, the two OTUs, m2-4 and m2-57, which were classified to *Klebsiella* but with low reliabilities, 58% and 50%, respectively, were identical to different genera other than *Klebsiella*. To examine the taxonomy of these OTUs more precisely, two OTUs corresponding to m2-4 and m2-57 were found in a small sequence library of V6–V8 amplicons, and their sequences were subjected to the RDP classifier (See the details in Data S3). As a result, m2-4 and m2-57 were classified to genus *Klebsiella* with the score of 95% and 96%, respectively. It was noticeable that m2-4 existed as a predominant phylotype in the majority of allergic subjects.

Principal component analyses showing aberrancy in fecal bacteria composition of the allergic infants

A principal component analysis (PCA) was performed using the relative abundance data of 106 genera detected by the pyrosequencing from the fecal samples at 1 and 2 months, and the results showed two clusters composed of allergic subjects (clusters I and II in Fig. 2). Cluster I and II were shifted to the PC1-positive and PC2-positive direction, respectively, compared to a widely distributed nonallergic cluster centered on the PC1-negative/PC2-negative area. In the loading plot of bacterial genera, *Bacteroides*/*Parabacteroides* and *Klebsiella*/*Propionibacterium* showed strong positive loading on PC1 and PC2, respectively, indicating that these genera correlate with allergy development. Indeed, samples in cluster I and cluster II were abundant in *Bacteroides* and *Klebsiella*, respectively. Cluster I widely ranged between the loading vectors of *Klebsiella* and *Escherichia*. Indeed, samples in the upper and lower parts of cluster I were abundant in *Klebsiella* and *Escherichia*, respectively. It was obvious that most major genera of *Proteobacteria*, for example, *Escherichia* and *Kluyvera*, had negative loading on PC2, whereas *Klebsiella* had an opposite contribution.

Accordingly, PCA was performed using the relative abundance data of major four phyla + one genus; phylum *Proteobacteria* was separated into two groups, genus *Klebsiella* and the others (Fig. 3). Similar to the results of the PCA at the genus level, two allergic clusters were found in the directions of the *Bacteroidetes* and the *Klebsiella* loading vector, respectively, and a nonallergic

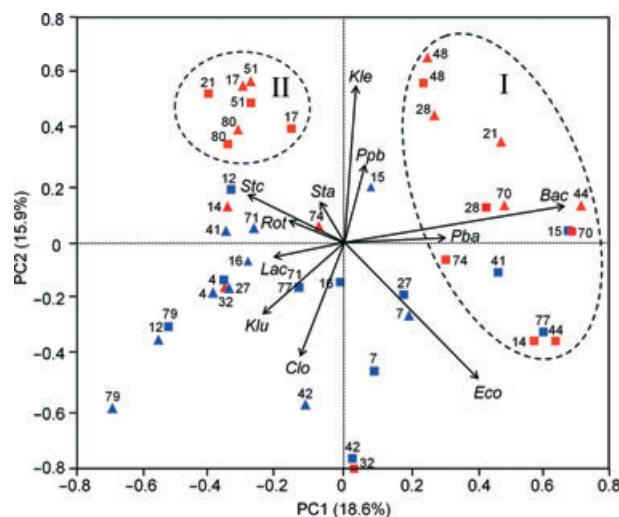


Fig. 2. Principal component analysis (PCA) of the genus compositions of 44 fecal samples from infants aged 1 or 2 months. The relative abundance of 106 bacterial genera detected in 44 samples was $\log_2$ transformed and used for this PCA. The genus component loadings > 0.1 are shown by arrows, the directions of which indicate the relative loading on the first and second principal components. *Kle*, *Klebsiella*; *Ppb*, *Propionibacterium*; *Sta*, *Staphylococcus*; *Stc*, *Streptococcus*; *Rot*, *Rothia*; *Lac*, *Lactococcus*; *Klu*, *Kluyvera*; *Clo*, *Clostridium*; *Eco*, *Escherichia*; *Pba*, *Parabacteroides*; *Bac*, *Bacteroides*. Red triangle, allergic subject aged 1 month; blue triangle, nonallergic subject aged 1 month; red square, allergic subject aged 2 months; and blue square, nonallergic subject aged 2 months. The numbers besides the symbols indicate subject numbers.

cluster was located in the direction of *Firmicutes* and non-*Klebsiella* *Proteobacteria*.

Population changes in *Klebsiella* and other *Proteobacteria* in the allergic and nonallergic groups

Figure 4 shows the relative abundances of *Proteobacteria*, excluding *Klebsiella*, and the genus *Klebsiella*. As shown in Fig. 4a, the relative abundance of the non-*Klebsiella* *Proteobacteria* at the age of 1 month was significantly lesser in the allergic group than in the nonallergic group. However, the relative abundance of *Klebsiella* was greater in the allergic group than in the nonallergic group, even though the difference was not statistically significant (Fig. 4b). The difference was more evident at the age of 2 months than at 1 month.

Antagonism among genera *Clostridium*, *Bacteroides*, and *Klebsiella* and its correlation to allergy development

Figure 5 shows a two-dimensional plot of the relative abundance of *Clostridium* vs. that of *Klebsiella* or *Bacteroides*

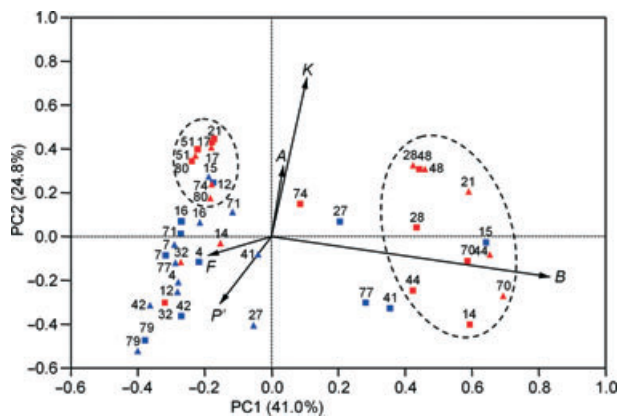


Fig. 3. PCA of the composition of five bacterial groups in 44 fecal samples from infants aged 1 or 2 months. The relative abundances of five bacterial groups, phylum *Actinobacteria* (A), phylum *Bacteroidetes* (B), phylum *Firmicutes* (F), phylum *Proteobacteria* excluding *Klebsiella* (P), and genus *Klebsiella* (K), were log₂ transformed and used for this PCA. The directions of the arrows indicate the relative loading on the first and second principal components. Indications of symbols and numbers are the same as in Fig. 2.

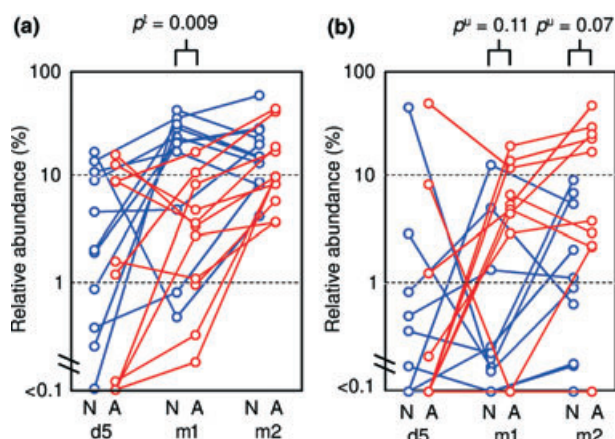


Fig. 4. Succession of the relative abundances of phylum *Proteobacteria* excluding genus *Klebsiella* (a) and genus *Klebsiella* (b) in feces from 5 days to 2 months after birth. The relative abundances were statistically compared between the allergic (A) and nonallergic (N) groups by using the Mann–Whitney *U*-test (p^u) for the datasets non-normally distributed ($P > 0.05$ in Kolmogorov–Smirnov test) or the Student *t*-test (p^t) for the datasets normally and equally distributed ($P < 0.05$ in Kolmogorov–Smirnov test and $P < 0.05$ in *F*-test). d5, 5 days old; m1, 1 month old; m2, 2 months old. Red circle, allergic subject; blue circle, nonallergic subject.

in each subject. The plot clearly indicates a strong antagonism in the GI tract between these genera and indicates that all of the subjects who showed high colonization of *Bacteroides* developed allergy, whereas all of the subjects who showed high colonization of *Clostridium* did not

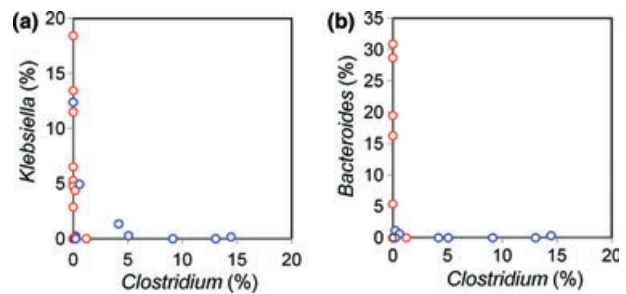


Fig. 5. Two-dimensional plot of the relative abundances of *Clostridium* vs. *Klebsiella* (a) and *Clostridium* vs. *Bacteroides* (b). Red circle, allergic subject; blue circle, nonallergic subject.

develop allergy. In addition, *Klebsiella* carriers developed allergy, with the exception of two subjects.

Discussion

In this study, we profiled the fecal bacteria compositions in allergic and nonallergic infants by using the 16S rRNA gene short-tag pyrosequencing approach and correlated some anomalies in the microbiota with allergy development in later years. The comparative analysis of genus-level composition data identified population differences in some genera between the allergic and nonallergic groups. Interestingly, in genera *Bacteroides*, *Clostridium*, and some genera of *Proteobacteria*, the differences were most remarkable at the age of 1 month among the three different sampling ages during the first 2 months. At the age of 1 month, the GI tract microbiota is in a transition stage, before the establishment of stable flora dominated by *Bifidobacteria* (Mackie *et al.*, 1999). Our results suggested that this transition stage in the infant intestinal microbiota appears to be critical to allergy development in later years.

Fecal microbiota including highly colonized *Bacteroides* was noted in some allergic subjects. This finding coincides with our previous quantitative PCR data (Songjinda *et al.*, 2007) and agrees with some previous studies that found that allergic infants, before weaning, showed higher colonization of *Bacteroides* than did nonallergic infants (Kirjavainen *et al.*, 2001; Mah *et al.*, 2006; Adlerberth *et al.*, 2007; Suzuki *et al.*, 2008; Vael *et al.*, 2008). In addition, some animal and *in vitro* studies have indicated the influence of *Bacteroides* on the development of inflammation or allergy (Onderdonk *et al.*, 1981; Rath *et al.*, 1996; Odama *et al.*, 2007). On the contrary, some studies have suggested that *Bacteroides* is a key regulator of the human mucosal immune system and of bowel development (Xu *et al.*, 2003; Kelly *et al.*, 2004). It has also been reported that polysaccharide A produced by *Bacteroides fragilis* suppressed a series of inflammatory reactions (Mazmanian

et al., 2008). This inconsistency may be attributable to the difference in species or strain, discrepancies between *in vivo*/animal models and human biological characteristics, and may be related to the timing of colonization. In general, *Bacteroides* is a commensal organism that appears after weaning as a predominant member. However, as indicated by this study, some infants showed colonization of *Bacteroides* even in early infancy. Sjogren *et al.* (2009a) indicated that such early *Bacteroides* colonization was associated with decreased lipopolysaccharide (LPS) responsiveness in later life owing to the downregulation of TLR4, inflammatory cytokines, and chemokines, for example, IL-6 and CCL4. The influence of hyper- and early colonization of *Bacteroides* on immune system development could correlate with allergy development in later life. It should be also noted that no specific species of *Bacteroides* was correlated with allergy development in later years in this study. Further molecular- and cellular-level studies to address the precise mode of action of *Bacteroides* on allergy development are necessary to clearly understand the correlation between early colonization of *Bacteroides* and allergy development in later years.

PCA indicated that *Klebsiella* is also an allergy-related genus. Notably, *Klebsiella* showed a positive contribution in the allergic group, whereas a majority of the other genera in phylum *Proteobacteria* showed negative contributions. This adverse effect was also observed in the comparative analysis shown in Fig. 1, which indicated that *Acinetobacter*, *Pseudomonas*, *Enhydrobacter*, *Yersinia*, and *Duganella* were less abundant in the allergic group than in the nonallergic group at the age of 1 month, whereas *Klebsiella* showed an opposite trend at 1 and 2 months. These data suggest that *Klebsiella* has a contradictory mode of action among *Proteobacteria* in terms of correlation with allergy development in later years. To our knowledge, no report has shown a direct relationship between *Klebsiella* and allergy diseases. However, Rhoads *et al.* (2009) described infants with colic who were a few months old and showed higher colonization of *Klebsiella* spp. than did infants without colic, suggesting that *Klebsiella* induces an inflammatory reaction. In their study, as in our study, other *Proteobacteria*, including *Enterobacter* or *Pantoea*, were detected more in the healthy control subjects.

In general, *Proteobacteria* are considered nonbeneficial GI tract bacteria because of the immunotoxicity of their LPS. However, an interesting study found that the LPS level in the environment surrounding children was inversely related to the occurrence of allergies (Braun-Fahrlander *et al.*, 2002). Moreover, a *Proteobacteria* species, *Acinetobacter lwoffii*, was identified as a potential allergo-protective agent in a cowshed dust sample (Conrad *et al.*, 2009; Debarry *et al.*, 2010). It is quite interesting that *A. lwoffii*-like bacteria, represented by the OTUs, m1-202, and

m1-353 in Table 1, were also found at a higher frequency in our nonallergic subjects. Taken together, this suggests that exposure to LPS in early infancy is of great importance to the development of a nonallergic immune system. However, it should be noted that *Bacteroides* and *Klebsiella* also carry LPS in their cell walls. It is known that *B. fragilis* LPS shows a proinflammatory effect via the TLR4 pathway, as does a dose of enterobacterial LPS, but its potency is 100- to 1000-fold lower (Mancuso *et al.*, 2005). Further studies on the molecular and cellular mechanisms how host cells recruit, interact, and recognize these bacterial components are required to correctly understand the correlation between colonization of *Proteobacteria* and allergy development in later years.

The difference in the relative abundance of the genus *Clostridium* between the allergic and nonallergic groups was also notable. This finding is different from that of previous studies in which *Clostridium* was more abundant in allergic infants (Bjorksten *et al.*, 2001; Kalliomaki *et al.*, 2001; Smehilova *et al.*, 2008). This discrepancy may be attributable to species differences, because *Clostridium* contains a highly diverse group of bacteria. A sequence search in the 16S rRNA gene database indicated that the major phylotype from our healthy subjects was identical to *C. perfringens* and the minor one was identical to *C. butyricum*, with both belonging to the *C. perfringens* group. It is interesting that subjects who showed high colonization of *Clostridium* did not carry *Bacteroides* and *Klebsiella*, both of which were significantly correlated with allergy development. *Bacteroides* is known to be sensitive to short-chain fatty acids, especially in low pH conditions (Duncan *et al.*, 2009), which suggests that the observed antagonism is attributable to a short-chain fatty acid produced by *Clostridium*.

In conclusion, aberrant structures of fecal bacterial community in allergic infants were precisely profiled using 16S rRNA gene tag pyrosequencing. Although we used V6 short hypervariable sequences in this study, it is now possible to expand the target region to V6–V8, and this will enable determination of the bacterial composition at a highly precise taxonomic level (Nakayama, 2010). Further studies with this technique will provide a number of candidate bacterial risk factors that correlate with allergy development in later years. In conjunction with molecular and cellular biologic studies, we will soon be able to clearly and correctly understand the correlation between infant developmental intestinal microbiota and allergy development in later years.

Acknowledgements

We thank Dr Kunio Izuchi and the staff in his hospital for their cooperation with this study. We also thank all

the families who provided fecal samples. This research was supported in part by a Research Grant for Research on Allergic Disease and Immunology from the Ministry of Health, Labour, and Welfare of Japan (to T.S.), grants-in-aid for Exploratory Research (to J.N.), and JSPS Fellows (to S.T.) from the Japan Society for the Promotion of Science, a grant for a Research for Promoting Technological Seeds from the Japan Science and Technology Agency (to J.N.), and grants from the Danon Institute for promotion of health and nutrition, the Morinaga Hoshikai, and Mishima Kaiun Memorial Foundation (to J.N.).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

- Data S1.** Type and onset of allergy disease in each subject.
- Data S2.** Relative abundance (%) of each bacterial genus in each subject at the age of 2 months, 5 days and 1 month.
- Data S3.** Taxonomic analysis of OTUs m2-4 and m2-57 using the V6–V8 sequence.

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Supplemental Data S3: Taxonomic analysis of OTUs m2-4 and m2-57 using the V6-V8 sequence.

To examine the taxonomy of OTUs m2-4 and m2-57 more precisely, the DNA samples, nos 17, 21, 28, 44, 48, 51, 70, 74, and 80, were selected and the V6-V8 fragment of 16S rRNA gene was amplified from each sample and was then subjected to the pyrosequencing according to the method described by Nakayama et al., 2010. From the obtained 3831 reads, OTU1 and OTU844, each consisting identical 459 and 177 reads, respectively, were found to correspond to m2-4 and m2-57. As a result of the RDP classifier analysis (<http://rdp.cme.msu.edu/classifier/classifier.jsp>), OTU1 and OTU844 showed 95% and 96%, respectively, in the classification reliability.

OTU1:

```
TGGTCTTGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGT
GCTGCATGGCTGTCGTCAGCTCGTGTGTTGTGAAATGTTGGGTAAAGTCCCGCAACGAGCGCAACCC
TTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGG
AAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGG
CATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGA
TTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACG
GTGAATACGTTCCCGGGCCTT
```

OTU844:

```
TGGTCTTGACATCCACAGAACTTA-GCAGAGATGCTTT-GGTGCCTTCGGGAACTGTGAGACAGGT
GCTGCATGGCTGTCGTCAGCTCGTGTGTTGTGAAATGTTGGGTAAAGTCCCGCAACGAGCGCAACCC
TTATCCTTTGTTGCCAGCG-GTTAGGCCGGGAACTCAAAGGAGACTGCCAGT-GATAAACTGGAG
GAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATG
GCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGG
ATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGA-ATGCTAC
GGTGAATACGTTCCCGGGCCTT
```