The Journal of Microbiology (2012) Vol. 50, No. 4, pp. 684–688
DOI 10.1007/s12275-012-2115-4
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NOTE

Evaluation of a Fosmid-Clone-Based Microarray for Comparative Analysis of Swine Fecal Metagenomes[§]

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(Received March 2, 2012 / Accepted April 24 ,2012)

Glass slide arrayed with fosmid clone DNAs generated from swine feces as probes were fabricated and used as a metagenome microarray (MGA). MGA appeared to be specific to their corresponding target genomic fragments. The detection limit was 10 ng of genomic DNA (ca. 10^6 bacterial cells) in the presence of 1000 ng of background DNA. Linear relationships between the signal intensity and the target DNA (20–100 ng) were observed (r^2=0.98). Application of MGA to the comparison of swine fecal metagenomes suggested that the microbial community composition of swine intestine could be dependent on the health state of swine.

Keywords: microarray, fosmid clone, gut, community analysis

The intestinal microbiome of animals is composed of a diverse and complex community of bacteria. Because of its impact on physiological, developmental, nutritional, and immunological processes, the microbial community of animals markedly influences health status and performance of the animal (Richards *et al.*, 2005). Thus, in order to verify the effect of probiotics and of other feeding materials, which are likely to influence the composition of the intestinal microbiome and to explain the mechanisms of their actions, it is essential to develop methodological strategies and useful tools to characterize the microbiome of animals.

Due to the development and application of nucleic acid-based techniques, such as fluorescent *in situ* hybridization (Pernthaler *et al.*, 2002; Wagner *et al.*, 2003), terminal restriction fragment length polymorphism (Liu *et al.*, 1997; Dunbar *et al.*, 2001), and denaturing gradient gel electrophoresis (Muyzer *et al.*, 1993; Campbell *et al.*, 2009), cultivation-dependent methods are not frequently used for microbial detection. In addition, the use of these molecular approaches have greatly advanced our understanding of microbial communities in natural habitats (Amann *et al.*, 1995; Kim *et al.*, 2005; Chang *et al.*, 2008). Furthermore, compared to other nucleic acid-based techniques, microarray-based hybridization has the advantages of high throughput and parallel detection of microorganisms. Various environmental microarray formats employing oligonucleotides (Peplies *et al.*, 2003; Taroncher-Oldenburg *et al.*, 2003), cDNA (Musarrat and Hashsham, 2003), and whole microbial genome (known as GPM or CGA) (Wu *et al.*, 2004; Bae *et al.*, 2005) as probes have been developed and evaluated for analysis of microbial communities in environmental samples. Although the 16S rRNA gene is a valuable marker for determining phylogenetic relationships among different bacteria, it is dependent on bias-prone PCR amplification. In addition, it provides poor resolution at the species level (Dahllof, 2002; Kakinuma *et al.*, 2003) and insufficient sequence information for determining positive signals when used as a short oligonucleotide probe (Small *et al.*, 2001; Call *et al.*, 2003; Chandler *et al.*, 2003; Wang *et al.*, 2004). Due to the low sensitivity of the developed DNA microarrays employing oligonucleotides or cDNAs as probes, these prior microarray techniques could be used for detection of functional genes from only dominant members of microbial communities or be dependent on PCR amplification of conserved genes (Wu *et al.*, 2001; Zhou and Thompson, 2002; Zhou, 2003; Rhee *et al.*, 2004). CGA (GPM) is more sensitive than other probes and Chang *et al.* (2008) recently extended CGA for detection of uncultivated microorganisms by employing a digital multiple displacement amplification (MDA) technique. Thus, due to the extremely high diversity of microbial communities in environmental samples, sensitive profiling of metagenomes using microarray is still a great challenge. Recently, fosmid clone-based metagenome microarray (MGA) was developed and used to screen target gene-containing fosmid clones (Park *et al.*, 2008). Since the fosmid clone inserts (ca. 35 kb) are much longer than oligonucleotide or cDNA probes and contain many genes, MGA could provide stronger hybridization signals and greater genetic information. In this study, we applied the MGA format to profile metagenomes

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[§]Supplemental material for this article may be found at http://www.springer.com/content/120956

of fecal microbial communities of swine for diagnosis of intestinal microbial communities.

Construction of fosmid-clone-based microarray

Fecal samples were collected from five- to six-month old Yorkshire-Landrace-Duroc crossbred pigs from a livestock farmhouse located in South Korea, which bred more than 1,000 heads of swine at the time of collection. Swine were weaned three or four weeks after birth and fed a commercial maize-soybean based diet *ad libitum*. For this study, we selected four pigs for healthy and runty swine, respectively. The criterion for demarcating healthy and runty swine is based on market weight (100–110 kg) (Kim *et al.*, 2005). Excreted fecal samples were immediately stored in portable ice coolers with dry ice on sites and carried to the lab in 2 h and stored at -80°C until analysis. Metagenomic DNA was extracted using a Power Soil™ DNA isolation kit (Mo Bio Laboratories, USA) as described by Rhee *et al.* (2004).

Two fosmid clone libraries were prepared with total genomic DNA extracted from fecal samples of healthy and runty swine, respectively, using the procedure described by Quaiser *et al.* (2002). From the libraries two hundreds fosmid clones were randomly selected for further study. The fosmid DNA was extracted from each clone after cultivation in 3 ml of LB containing chloramphenicol and an inducer using a Plasmid DNA Extraction Kit according to the manufacturer's protocol (QIAGEN, Germany). The fosmid DNA samples were resuspended in deionized water at final concentration of 200–300 ng/μl. A two-fold concentrated carbonate solution [0.2 M sodium carbonate (pH 9.0) with 0.02% SDS] was selected as the spotting solution for the ideal size and quality of spot. In the 384-well microplate, the DNA samples were diluted 1:1 (v/v) in 2× carbonate spotting solution.

The fosmid DNA samples were arrayed on glass slides using a Micro Grid II Compact (Genomic Solutions, USA). The fosmid clones prepared with genomic DNA of a marine bacterial strain, *Thioalbus denitrificans* Su4 (Park *et al.*, 2011), were printed and used as a position marker on the slide as well as a positive control probe. Genomic DNA (20 ng) of the strain *T. denitrificans* Su4 was intentionally included in the labeling of 1 μg of metagenome samples. In this microarray experiment, the term 'probe' was used for the fosmid clone arrayed (spotted) on a glass slide and the term 'template' was used for the metagenomic DNA fluorescently labeled and for hybridization. Each fosmid DNA set was printed in three replicates on a different position of the glass slide and the slides were subjected to post-treatment as described by Bae *et al.* (2005).

Hybridization specificity

Specificity is one of the most critical parameters for all assay techniques, including microarray, which are used to track microorganisms in samples. The specificity of microarray hybridization of fosmid clone probe in MGA was already demonstrated using a gene or its fragment as templates for specific detection of genes in fosmid clones (Park *et al.*, 2008). In this study, the specificity was more carefully investigated because metagenomes used as templates had more diversity compared with single or several genes in MGA. To test the possibility of cross hybridization of metagenomic DNA to fosmid DNA probes, genomic DNAs originating from pure cultures [a marine bacteria strain (*T. denitrificans* Su4) and an archaeal strain (*Natronomonas* sp.)]were used as templates for hybridization (Supplementary data Fig. S1). Fluorescent labeling of the genomic DNA was performed using the BioPrime DNA Labeling kit (Invitrogen, USA) as described by Rhee *et al.* (2004). One microgram of template DNA was routinely used for labeling and hybridization. The labeled template was purified using a QIAquick PCR purification kit (QIAGEN), concentrated in a Speedvac for 1 h, resuspended in 4.35 μl of deionized water, and then stored at -20°C.

The fluorescently labeled template was mixed with hybridization solution: 4.35 μl of labeled DNA, 8.75 μl of formamide (50%, v/v), 3× SSC (1× SSC contained 150 mM NaCl and 15 mM trisodium citrate), 1.25 μg of unlabeled herring sperm DNA (Promega, USA), and 0.3% sodium dodecyl sulfate (SDS) in a total volume of 17.5 μl (Rhee *et al.*, 2004; Park *et al.*, 2008). The hybridization was performed in a hybridization chamber at 42°C. Image processing of the microarray slides was performed using a ScanArray 4000 Microarray Analysis system (Perkin-Elmer, USA). Each spot was of scanned images quantified using GenePix version 6.0 software (Molecular devices Co., USA). The signal-to-noise ratio (SNR) was calculated using the following formula (Verdnick *et al.*, 2002) as a basis: SNR = (signal intensity – background)/standard deviation of background. The SNRs from three replicate data sets were then averaged to represent the SNR for a particular fosmid clone. Since acommonly accepted criterion for the minimum signal (threshold) that can be accurately quantified is an SNR of 3 (Verdnick *et al.*, 2002), the spots with SNR values of >3 were used for further analysis. For normalization, the signals were divided by the signal of the positive control that originated from *T. denitrificans* Su4.

We did not observe any significant hybridization signal above 3 SNR from the swine fosmid DNA probes with genomic DNAs of the pure cultures (see the image in the Supplementary data Fig. S1). This result was in agreement with the findings of a previous study, where the signal intensity of fosmid clone probes obtained from marine sediments was negligible (an SNR below 0.1) when the genomic DNA of *E. coli* DH5α was hybridized to the probes (Park *et al.*, 2008).

Sensitivity and quantification of hybridization

DNA fragments with different sizes could be spotted and cross-linked on a slide as microarray probes. Fosmid clones were arrayed for higher sensitivity in hybridization due to its large size compared with oligonucleotide-based or cDNA-based microarrays. In fecal samples, the amount of genomic DNA of each microorganism, which is the target of the corresponding probe, is dependent on the diversity of microbial communities. Non-target DNAs in the metagenomic DNA may also interfere with the hybridization of target DNA decreasing the detection sensitivity (Rhee *et al.*, 2004). To evaluate the detection sensitivity in the presence of heterogeneous non-target DNAs, genomic DNA of the marine bacterial strain *T. denitrificans* Su4 in the range of 1 to 1,000 ng

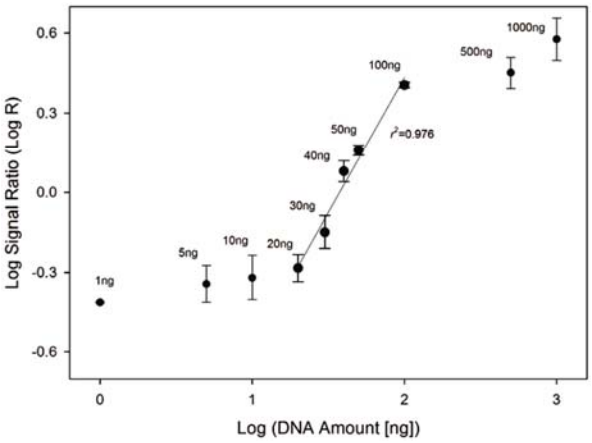

Fig. 1. Evaluation of the detection sensitivity and quantitative potential of MGA-based hybridization. Quantitative relationship of MGA hybridization was examined with pure genomic DNA in the presence of heterogeneous background DNA. Genomic DNA of strain *T. denitrificans* Su4 was serially diluted in 1× Tris-EDTA buffer. The diluted genomic DNAs at concentrations ranging from 1 to 1,000 ng were mixed with 1 μg of the negative control genomic DNA from *Natronomonas* sp., and labeled with Cy5 using a random-primer labeling method. The labeled DNAs were hybridized with fosmid clone probes. The quantitative relationship representsthe hybridization signals derived from the fosmid clone probe of strain *T. denitrificans* Su4.

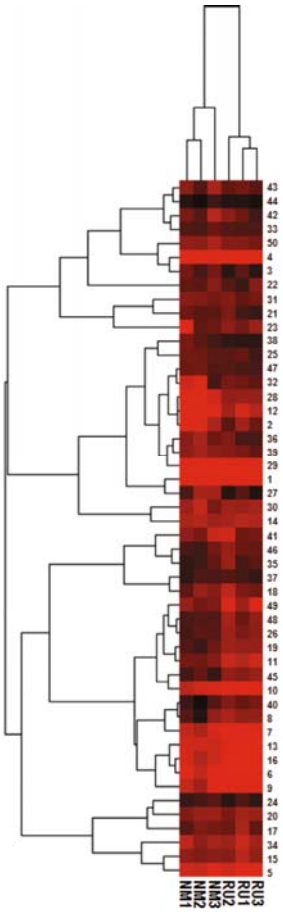

Fig. 2. Hierarchical cluster analysis of fecal metagenome relationships based on hybridization signal intensity ratios for fosmid probes showing SNRs of >3. The figure was generated using hierarchical cluster analysis and visualized with TREEVIEW (http://rana.stanford.edu/). The hybridization signals of fecal metagenomic DNAs from each of the three normal and runty swine are shown in each column (NM, normal; RU, runty). Each row represents the hybridization signal observed for each fosmid clone when the fecal metagenomic DNA from the swine indicated belowthe column was used for hybridization. Red represents a significant hybridization signal.

were mixed with genomic DNA of *Natronomonas* sp. (used as non-target DNAs) and adjusted to be total 1,000 ng as the final template DNA before randomly labeling with Cy5. Hybridization signal was measurable with more than 10 ng of target genomic DNA, but the signal intensity was barely detectable at 5 ng (Fig. 1 and Supplementary data Fig. S2). Since the genome size of *T. denitrificans* is 2 Mb, 10 ng of genomic DNA was assumed to correspond to about 2×10^6 cells. This result indicates that our microarray format could detect at least more than 2% of the bacterial constituents (2×10^6 cells/μg-DNA) present in fecal samples. Individual populations in many environmental samples generally consisting of less than 10% (less than 10^7 cells/μg-DNA) were not suitable for direct detection using the 50-mer oligonucleotide-based microarray, which has a detection sensitivity ranging from 50 to 100 ng (Rhee *et al.*, 2004).

In addition, while the signal intensity was saturated at more than 100 ng of genomic DNA, significant correlations between the signal intensity and DNA concentrations ranging from 20 to 100 ng were observed (r^2=0.98) (Fig. 1). Consequently these results suggested that the fosmid clone-based hybridization in our format could be useful for quantitative determination.

Amplification of metagenomic DNA

In the microarray hybridization of environmental samples, the most important technical considerations include obtaining a sufficient amount of high-purity DNA. It was not feasible to extract at least 3 μg of metagenomic DNA directly from swine fecal samples for the triplicate hybridization experiments, which should also be pure enough for random primer labeling. It was recently demonstrated that metagenomic DNA could be amplified without suffering from problems associated with biases and artifacts for analysis of microbial communities in environmental samples by employing MDA (Hutchison *et al.*, 2005; Raghunathan *et al.*, 2005). Metagenomic DNA was amplified by a MDA technique using the REPLI-g® Mini kit (QIAGEN) following the manufacturer's protocol. Appropriate amounts of genomic DNA (10 ng to 100 ng) were mixed thoroughly with 40 μl of reaction buffer containing random hexamers, deoxynucleotides, and 1 μl of an enzyme mixture. Reactions were stopped by heating the mixtures at 65°C for 3 min, and the amplified products were quantified as described above and visualized on 0.8% (w/v) agarose gels.

This approach has been used for the routine preparation of sufficient amounts of metagenomic DNA (>3 μg) from

Table 1. Effect of DNA template concentration on representative amplification of fecal metagenomic DNA from normal swine

Parameter	Amount of fecal metagenome template		
	10 ng	50 ng	100 ng
Total no. of positive signals[a]	44	44	44
$F_{2.0}$[b]	1.6	1.4	0.9
$F_{3.0}$[b]	0.23	0	0

[a] Different amounts of fecal metagenomic DNA from normal swine were amplified for 4 h in triplicate. Amplified metagenomic DNAs were labeled with Cy5 in triplicate and hybridized with MGA.
[b] $F_{2.0}$ and $F_{3.0}$, percentages of clone probes whose hybridization ratios of amplified DNA to nonamplified genomic DNA are more than 2.0- and 3.0-fold, respectively.

swine fecal samples. The amplification bias was determined with a series of dilutions of fecal metagenome of a runty swine (amounts ranging from 10 ng to 100 ng). Very robust amplification of genomic DNA (more than 5 μg) was obtained using this approach [Supplementary data Fig. S3(A)]. Although the detection sensitivity has been shown to be as low as 10 fg, there was significant problems in representativeness when the amplification of low amounts of genomic DNA (<10 ng) was performed (Wu *et al.*, 2006; Park *et al.*, 2008). However, it was not necessary to use this low range of DNA concentration in this study since approximately 100 ng of highly pure DNA could be obtained using a DNA extraction kit. There was no significant differences between the amplified metagenomic DNA from >10 ng of extracted DNA and the nonamplified original samples in microarray hybridization (Table 1) as reported by Wu *et al.* (2006). Representative amplification was also supported by the observation of hybridization signal ratios which were aligned along a line corresponding to ratios close to 1:1 for the the fecal metagenome of runty swine [see Supplementary data Fig. S3(B)].

Comparison of metagenomes between healthy and runty swine feces

The constructed microarray format developed in this study holds great promise for use as a generic metagenome profiling tool and to identify differences among various metagenomes from swine feces. To evaluate the potential of using the microarray format for such applications, fecal metagenomic DNAs isolated from three normal and runty swines, respectively, were amplified and labeled with Cy5 using the random-primer labeling method as described above. All spots with SNRs of 3 were considered positive signals. Fecal metagenomes from runty swine were more closely clustered with those from other runty swine feces (Fig. 2). Images of differential hybridization of fecal metagenome from normal and runty swine are shown in Supplementary data Fig. S4. Some fosmid clone probes were specific for normal or runty swine with $P<0.05$.

End-sequencing of those fosmid clone probes (see Supplementary data Table S1) provided the following phylogenetic information. The pCC1^{TM}/pEpiFOS^{TM} Forward (5′-GGAT GTGCTGCAAGGCGATTAAGTTGG-3′)/Reverse (5′-CTCG TATGTTGTGTGGAATTGTGAGC-3′) Sequencing Primers (Epicentre, USA) were used to determine both end sequences of a fosmid clone. The sequences obtained in this study were deposited in the GenBank database under the accession numbers JQ696876-JQ696951. The DNA sequences were compared with related gene sequences in GenBank by performing BLAST (http://www.ncbi.nlm.nih.gov). End-sequence information of fosmid clone probes is described in Supplementary data Table S1. The fosmid clone probes (No. 8, 11, 19, 26, 45, and 49) specific for the fecal metagenomes of runty swine were affiliated with *Butyrivibrio* (*Clostridia*), *Bacteroides* (*Bacteroidia*), *Ruminococcus* (*Clostridia*), and *Kineococcus* (*Actinobacteria*). The fosmid clone probes (No. 2, 32, and 38) specific for the fecal metagenomes of normal swine were affiliated with *Desulfovibrio* (*Deltaproteobacteria*), *Coprococcus* (*Clostridia*), and *Mycobacterium* (*Actinobacteria*). Most of these bacteria are frequently observed in fecal samples. This result showed that the collection of probes specific for fecal metagenomes of various health states of swine and a database of their hybridization profiles could be used for diagnosis of swine health. Even though next generation sequencing technology is widely used for microbial diagnosis, our microarray format still has several advantages, including cost and processing time, which can be completed in two days.

In summary, the specificity, sensitivity, and quantitation of MGA for comparative analysis of fecal metagenomes was evaluated in this study. Although our results demonstrated that the developed microarray could potentially be used as a tool for comparing metagenomes of swine feces, their usefulness and power should be further evaluated with more probes and samples from swine under various physiological states.

This research was financially supported by the Ministry of Education, Science Technology (MEST) and National Research Foundation of Korea (NRF) through the Human Resource Training Project for Regional Innovation (I00108) and Small & Medium Business Administration (S1073083), South Korea.

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