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Implementation of Random Bacterial Genomic DNA Microarray Chip (RBGDMC) for Screening of Dominant Bacteria in Complex Cultures

Byoung Chan Kim · Ji Hyun Park · Man Bock Gu

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Abstract The random bacterial genomic DNA microarray chip (RBGDMC), which was fabricated using random genomic DNA fragments obtained from the fragmentation of bacterial genome by using four different pairs of restriction enzymes, was found to discriminate bacterial species in the same genus and resulted in the determination of dominant bacteria in enriched cultures. The identification of a dominant bacterial species was successfully conducted in the co-culture of three different bacteria using the RBGDMC. In addition, the analysis of the chip data could confirm if any of the selected bacteria is the most abundant or if some bacteria were enriched and became the dominant species within the consortium after the samples were prepared from the repeated cultures of real sludge in a complex medium. This study shows the successful implementation of the RBGDMC for the identification and monitoring of dominant bacteria in complex environmental bacterial communities simply without any PCR amplification of the target nucleic acids.

Keywords Random fragmentation · Genomic DNA · DNA microarray chip · Bacteria identification · Complex culture

Introduction

In complex microbial communities, the presence of dominant bacteria should be the result of interactions with neighboring bacteria and environment [1]. Diagnosis of specific or dominant bacteria in microbial communities is important for the characterization of their environmental conditions, since the dominance of certain bacteria can be used as an

B. C. Kim
Institut Pasteur Korea, 696 Sampyeong-dong, Bundang-gu, Seongnam-si, Gyeonggi-do 463-400, Republic of Korea

J. H. Park · M. B. Gu (✉)
College of Life Sciences and Biotechnology, Korea University, 5-1 Anam-dong, Seongbuk-gu, Seoul 136-701, Republic of Korea
e-mail: mbgu@korea.ac.kr

indicator of the processes status or conditions. In other words, the determination of the presence of the dominant bacterial species would provide some clues about how to control target processes that utilize complex bacterial communities and would be a step toward a systematic approach to solve the problems involved in the overgrowth of specific bacterial species [2, 3].

In this regard, various approaches for the analysis of microbial diversity in complex consortia, such as activated sludge processes and marine, sediments, soil, or fecal samples, have been studied [4–7]. These are simple morphological characterizations of the different bacteria via phase contrast microscopy and several staining procedures [4], the 16S rDNA gene-based biological and molecular techniques [8], in situ PCR, fluorescence in situ hybridization [9], denaturing gradient gel electrophoresis/temperature gradient gel electrophoresis [10, 11], restriction fragment length polymorphism [12], and real-time PCR [13]. Still, these methods have some limitations in high-throughput analysis with requirement of genome sequence information.

DNA microarray technology makes it possible to facilitate a high-throughput methodology, permitting the analysis of hundreds or thousands of genetic sequences in parallel. This technology has been adapted to the detection of microorganisms for several years [14–17]. However, most of the methods used have depended on the genomic sequences to design specific probes and primers for the target products and relied on PCR-based amplification for target DNA detection, which limits the number of probes for multiple detection [18–21]. Currently, though, the genomic sequence information available in various databases is still incomplete since the genomes of many microorganisms are only partially sequenced. To overcome this, a random genomic library-based DNA microarray chip technology has been successfully developed for the detection and discrimination of specific bacterial strains in our group [22, 23].

Therefore, the aims of the present study are to further evaluate and implement the previously developed random bacterial genomic DNA microarray chip (RBGDMC) to bacterial species discrimination and detection of the target or dominant bacterial species in both co-cultures and enriched wastewater bacterial consortia samples without the amplification of specific genes and sequence information. To the best of our knowledge, this is the first example where randomly generated DNA probes on a DNA microarray chip have been used to identify dominant bacteria in enriched cultures without amplification of target genes. Consequently, different species within the same genus were tested to check if this DNA microarray chip can differentiate between two different species from the same genus. Secondly, *Gordonia amarae*, *Paracoccus thiocyanatus*, and *Aeromonas hydrophila* were co-cultured as select model organisms to demonstrate the ability of this microarray to determine the most abundant bacteria within a mixed culture. Finally, sludge samples from a wastewater treatment plant were serially cultured in a culture medium with or without carbon sources. Samples were subjected to determine which, if any, bacterial strains were enriched in the consortium using this RBGDMC.

Materials and Methods

Bacterial Strains and Preparation of DNA Microarray Chip

The bacterial strains, *G. amarae* America Type Culture Collection (ATCC) 27808^T, *Zoogloea ramigera* ATCC 19623, *Ralstonia eutropha* ATCC 17699, *Sphingomonas terrae* Korea Culture Type Collection (KCTC) 2814, *Skermania piniformis* KCTC 9829, *Comamonas denitrificans* KCTC 12931, *Enterobacter* sp. KCTC 2701, *Thiobacillus*

thioparus ATCC 23645, *P. thiocyanatus* KCTC 2848, *Nitrobacter winogradskyi* Korea Culture Center of Microorganisms (KCCM) 41770^T, *Beggiatoa alba* KCTC 2738, *Acinetobacter calcoaceticus* KCCM 40204, and *A. hydrophila* KCTC 2358 were used to construct the RBGDMC. Additionally, *Comamonas terrigena* KCTC 2989 and *Thiobacillus denitrificans* KCCM 31224 were chosen for the cross-hybridization test since they are within the same genus with *C. denitrificans* and *T. thioparus*, respectively. For the preparation of the RBGDMC from the 13 different bacterial strains, purified genomic DNA from each bacterium was individually digested with restriction enzyme pairs to generate random genomic DNA fragments. These fragments were inserted into a vector to generate a library, and the inserted probes were amplified for the preparation of the RBGDMC. In a same row, the different fragmented genomic probes from same bacterial strains were printed. A more detailed protocol for the construction of the RBGDMC is described elsewhere [22, 23].

Preparation of the Co-culture and Enriched Activated Sludge Samples

For the co-culturing of *G. amarae*, *P. thiocyanatus*, and *A. hydrophila*, complex medium was prepared with the following composition: glucose (1.0 g), yeast extract (1.0 g), $Na_2S_2O_3 \cdot 5H_2O$ (0.5 g), KH_2PO_4 (0.15 g), Na_2HPO_4 (0.45 g), $MgSO_4 \cdot 7H_2O$ (0.01 g), and NH_4Cl (0.03 g) in 1 L of distilled water. After the mixed culture was grown at 30 °C in a rotary incubator for 2 days, the cells were collected and genomic DNA extracted, which was then used in hybridization with the RBGDMC. Also, the number of colony forming units (CFUs) was checked using strain-specific or mixed agar to confirm the DNA microarray results. The activated sludge seed from the wastewater treatment plant was cultivated for enrichment by successive transfers in two types of media. The complex medium consisted of yeast extract (2.0 g), $CaCl_2$ (0.1 g), and sodium acetate (0.5 g) per liter of distilled water with carbon sources. The synthetic medium was prepared with Na_2HPO_4 (1.2 g), KH_2PO_4 (1.8 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), $(NH_4)_2SO_4$ (0.1 g), $CaCl_2$, (0.03 g), $FeCl_3$ (0.02 g), $MnSO_4$ (0.02 g), and $Na_2S_2O_3$ (10.0 g) per liter of distilled water with no carbon sources. The pH of both media was adjusted to 7.2 before autoclave. Each medium was sterilized by autoclaving at 121 °C for 15 min. After seeding the media with sludge, they were cultivated at 30 °C in a rotary incubator, and samples were transferred into fresh media everyday for the culture grown in the complex medium and every 5 days for the culture grown in the synthetic medium. Samples were taken on the first, fifth, and tenth transfer of culture to possibly screen dominant bacteria using the DNA microarray chip. A soil DNA extraction kit (Mobio, USA) was used for genomic DNA extraction.

DNA Microarray Hybridization and Data Analysis

The RBGDMC experiment was performed according to our previous report [23]. In detail, a total of 390 ng of genomic DNA from all 13 bacterial strains (each 30 ng), a total of 390 ng of extracted DNA from the test samples (genomic DNA of co-culture or enriched sludge culture), or 30 ng of each tested bacterium genomic DNA was preheated at 100 °C for 10 min and immediately cooled on ice. The genomic DNA from all 13 bacteria was labeled with Cy5-dCTP (Amersham Bioscience, USA) for the reference signals (red fluorescence), while Cy3-dCTP (Amersham Bioscience, USA) was used to label the test samples (green fluorescence). Labeling was performed using a random priming method (Roche, Switzerland). In all, 19.5 μl of the denatured genomic DNA, 6 μl of the high priming solution, 1 μl each of dATP, dTTP, and dGTP, 0.5 μl of dCTP, and 1 μl of either

Cy3-dCTP or Cy5-dCTP were mixed to give a total volume of 30 µl. The random priming reaction was done at 37 °C for 1 h under optimized conditions and then the fluorescently labeled genomic DNA was denatured directly at 100 °C for 5 min. The Cy5- and Cy3-labeled genomic DNA probes were mixed and purified using a QIAquick PCR purification kit (Qiagen, USA). The labeled genomic DNA was then concentrated after purification using a Microcon-30 filter (Millipore, USA). The hybridization buffer was prepared with 27.5 µl of fluorescently labeled genomic DNA, 6× standard saline citrate (SSC), 0.2% sodium dodecyl sulfate (SDS), 5× Denhardt's solution, and salmon sperm DNA in a total volume of 50 µl. Before hybridization, the DNA chip was incubated at 65 °C for 1 h in the prehybridization buffer containing 0.4 g bovine serum albumin (Sigma, USA), 5× SSC, and 0.1% SDS. After prehybridization, the DNA chip was washed with distilled water for 1 min, then with isopropanol for 1 min at room temperature and finally dried in a centrifuge at 6,000 rpm for 5 min. The DNA microarray chip was placed in the slide chamber with 3× SSC added to the side wells to maintain the humidity. The hybridization solution was loaded between the slide glass and cover glass in the slide chamber after the DNA was denatured at 100 °C for 3 min. The slide chamber was incubated in a water bath at 65 °C for 16 h. After hybridization, the DNA chip slide was removed from the slide chamber and washed in 2× SSC and 0.1% SDS at 65 °C for 2 min to remove the slide glass. The slide was then washed in fresh buffer with the same composition at 65 °C for 5 min. Afterward, the DNA microarray chip was washed in 0.1× SSC and 0.1% SDS for 10 min at room temperature and finally four times in 0.1× SSC buffer for 1 min each. The DNA microarray chip was then immediately dried by centrifuging at 6,000 rpm for 5 min. The hybridized DNA microarray chip was scanned in a GenePix 4000B scanner (Axon Instruments, USA) at excitation wavelengths of 532 and 635 nm to detect the Cy3- and Cy5-labeled genomic probes, respectively. The hybridization signal intensities were analyzed using the GenepixPro 3.0 software (Axon Instruments, USA) after subtracting the background and nonspecific signals. In the presentation of intensity data, the sum of the intensity (green signal) of all probes in each lane is used for the indication of the presence of target bacteria, since each DNA probe has different sizes via fragmentation with different enzyme pairs, the intensity in each spot after hybridization could be different. The highest summation of the intensity compared to other lines strongly supports the presence of target bacteria (or target genomic DNA). The background intensity in array analysis was almost 100 in arbitrary unit; therefore, we selected the positive signals over 200 in arbitrary unit. Each positive probe has different intensity from 200 to around 1,500 because each probe has a different size and the priming reaction is varied depending on the probe size. Therefore, we assumed that over 10,000 in intensity (green signals) indicate the presence of target bacteria.

Results and Discussion

In our previous studies, it was demonstrated that random genomic probes could be used to detect the presence of specific bacteria quantitatively and qualitatively without cross-hybridization [23]. Since the possibility exists that cross-hybridization might occur when using probes from different species under the same genus, the first concern of this study was to determine whether the random genomic DNA probes used to fabricate the DNA microarrays could discriminate the different species within the same genus. In order to verify the capabilities of the RBGDMC for the discrimination at the species level, therefore, two bacteria, *C. terrigena* and *T. denitrificans*, were selected as counter-partners for *C. denitrificans* and *T. thioparus*, respectively. The latter two bacterial probes are spotted on

the RBGDMC. The 16S rRNA sequence of *C. terrigena* shows 95% similarity with that of *C. denitrificans*, and the 16S rRNA sequence of *T. denitrificans* shows 96% similarity with that of *T. thioparus* in Blast 2 sequence analysis (NCBI). Although their 16S rRNA sequences are very similar, hybridization tests with genomic DNA (gDNA) from *C. terrigena* resulted in no hybridized signals on the array (Fig. 1b). Control hybridization

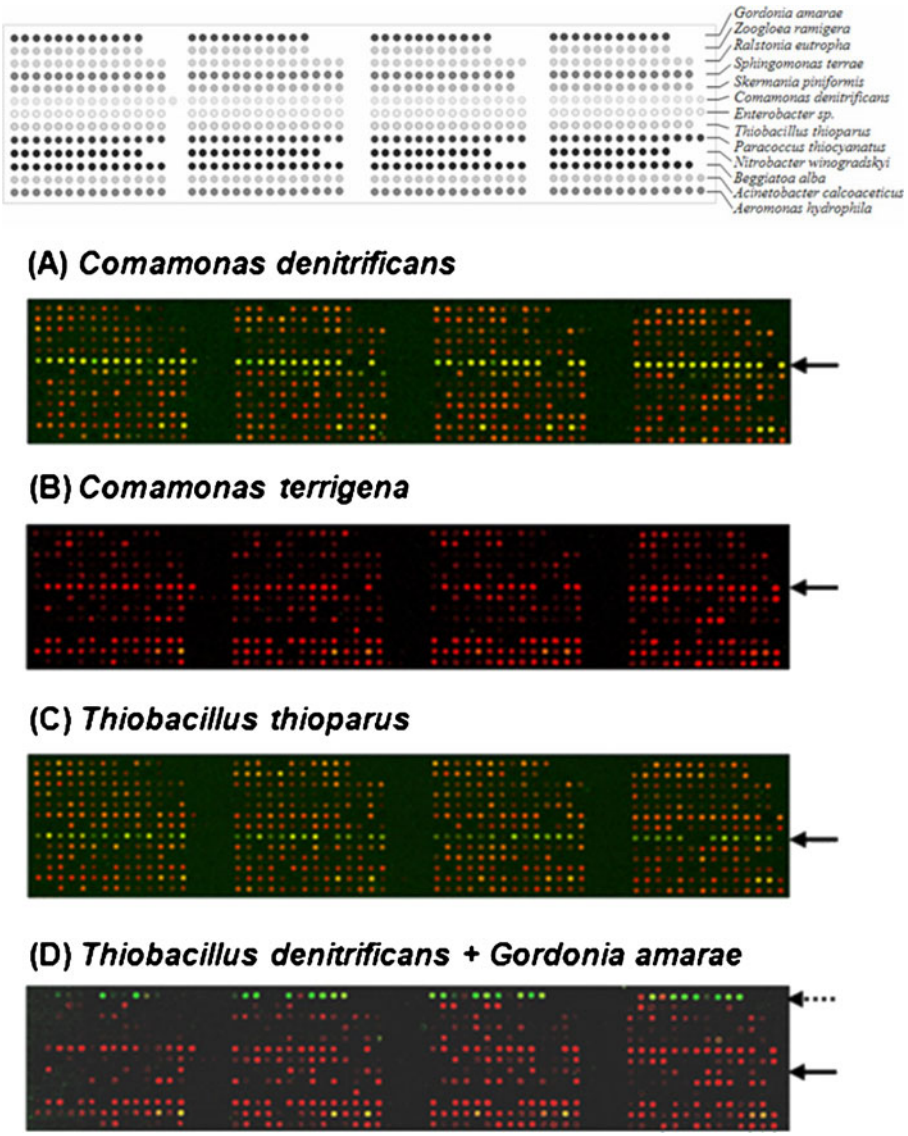

Fig. 1 Cross-species test with DNA microarray chip. The hybridization figures are the results with DNA from cultures of **a** *C. denitrificans*, **b** *C. terrigena*, **c** *T. thioparus*, and **d** *T. denitrificans* and *G. amarae* mixed together. The *solid arrows* indicate the row containing the probes of interest as listed in the upper panel. The *broken arrow* in **d** denotes the probes corresponding to *G. amarae*. The *upper panel* indicates the arrangement of the random genomic DNA probes on DNA microarray chip for each of the 13 bacterium, which can lead to clear comparisons among strains

tests with *C. denitrificans* gDNA are shown in Fig. 1a for comparison. Similar hybridization tests were performed with *T. denitrificans* gDNA or *T. thioparus* gDNA along with an equal amount of *G. amarae* gDNA, which was added as an internal control. Whereas the *T. thioparus* gDNA clearly hybridized to its own probes, test with *T. denitrificans* gDNA showed no cross-hybridization with any probes of *T. thioparus* (Fig. 1c, d, respectively). Likewise, the strong responses from the *G. amarae* probes demonstrate that the experiments were conducted successfully. These results strongly demonstrate the capability of this RBGDMC in the species-level discrimination and suggest

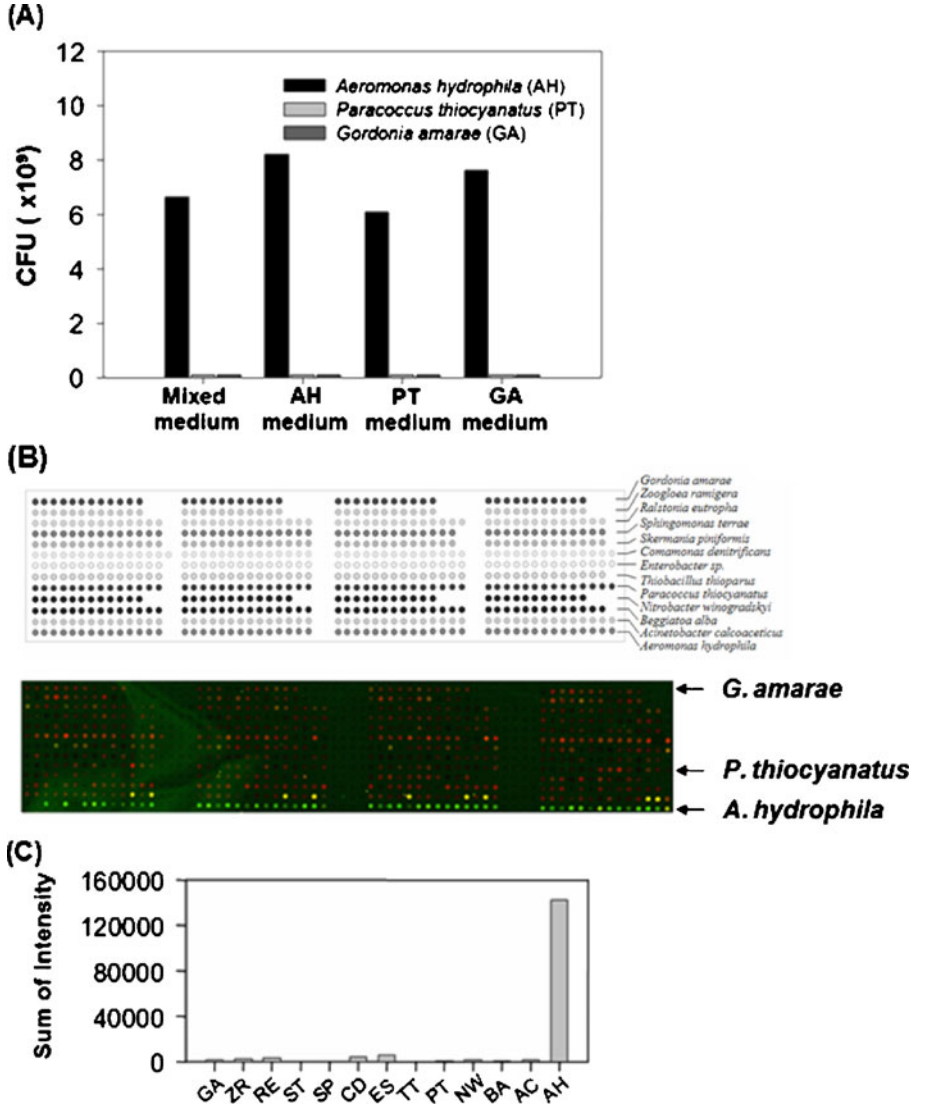

Fig. 2 Genomic DNA hybridization results from a co-culture of *G. amarae*, *P. thiocyanatus*, and *A. hydrophila*. **a** Colony enumeration results for *G. amarae*, *P. thiocyanatus*, and *A. hydrophila* from agar plate. **b** DNA microarray chip scan image showing the strong hybridization signals at the *A. hydrophila* probe located line. **c** Sum of hybridization signal intensity of scanned images from **b**

that it is possible to use random genomic DNA fragments as probes to detect specific bacterial species in complex microbial consortia without concerning the serious cross-hybridization, which may lead to false-positive test results.

To test the ability of this RBGDMC in the detection of the dominant bacteria, *G. amarae*, *P. thiocyanatus*, and *A. hydrophila* were co-cultivated for 2 days after seed inoculation in a mixed medium, which was composed of all of the nutrients needed for the growth of these three bacterial strains, or in a medium specific for the cultivation of each individual bacterium. The mixed cultures were plated on the strain-specific or mixed agar plates, and the CFUs were enumerated. The results found that only *A. hydrophila* grew on the mixed agar plates or specific agar plates (Fig. 2a). Consequently, the gDNAs were extracted from the cultures grown using the "mixed" medium and hybridized with the RBGDMC. The only significant hybridization signals were observed with the *A. hydrophila* genomic probes (Fig. 2b, c), thereby verifying the agar plating results. This suggests that as long as the RBGDMC includes gDNA probes appropriate for target consortia, it can be used to provide information about which bacteria are dominant in co-cultures without any PCR amplification of the nucleic acid pools or colony enumeration.

Since this RBGDMC was shown to identify the most dominant in mixed consortia, only one was retained to apply the RBGDMC to environmental samples. Consequently, activated sludge samples from a wastewater treatment plant were cultivated in either a complex or synthetic medium. The bacterial consortium was continuously cultivated by transferring a portion of the culture into the fresh media repeatedly. Samples from the first, fifth, and tenth day of the growth were collected to identify the enriched bacteria at each

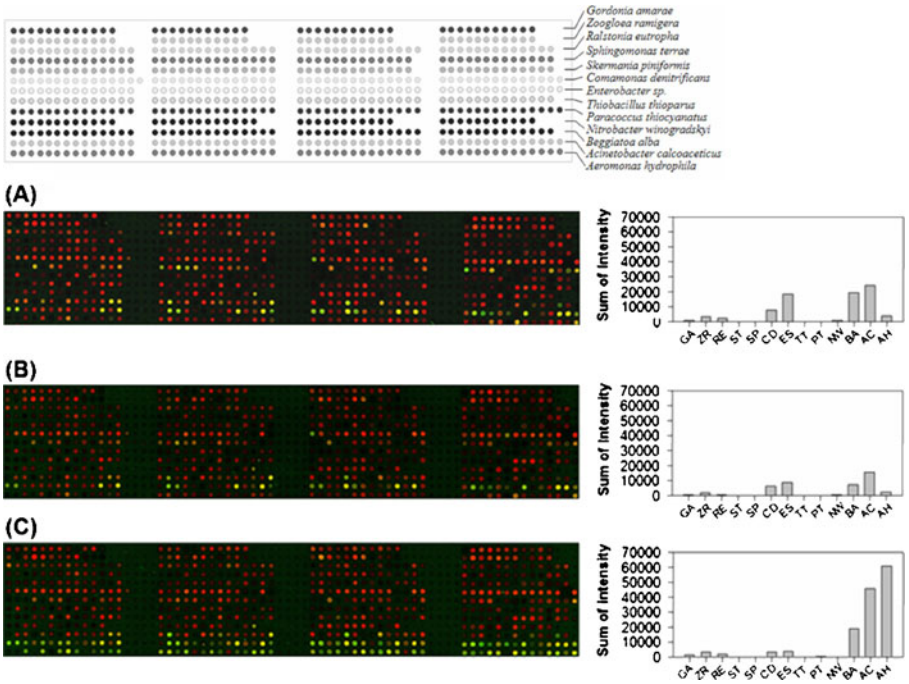

Fig. 3 Detection of the target sludge bacterial strains cultivated in the complex medium. The DNA microarray scan images and signal intensities are shown for **a** the first subcultured sample of the activated sludge seed, **b** the fifth subcultured sample, and **c** the tenth subcultured sample

sampling point. When the activated sludge was grown in a complex medium, the signals corresponding to several bacteria, including *B. alba*, *A. hydrophila*, and *A. calcoaceticus*, were increased in their intensities as the culture passages progressed (Fig. 3). On the other hand, when the sludge was cultivated in the synthetic medium, which has no carbon sources, only very weak signals were observed with the RBGDMC for all of the sampling times (Fig. 4). This may be attributed to the lack of carbon source, which is important to bacterial growth. Whereas some of the bacterial gDNA (*C. denitrificans* and *Enterobacter* sp.) were detected at the first day after seeding the activated sludge, their probe intensities were decreased as culture passage progressed. This indicates that the RBGDMC identified the portion of the bacterial genomic DNA in complex cultures and reported the presence of enriched bacteria.

DNA microarray technologies have emerged as a promising tool for the high-throughput biosensor and analysis of specific bacteria and complex communities in environmental samples [24, 25]. However, DNA microarrays with probes have typically relied upon the complementary hybridization of the known sequences. The use of the sets of random genomic DNA probes may present several advantages over the use of 16S rRNA gene probes when trying to determine what bacteria are present dominantly in environmental samples. First, random genomic probes from pure strains are easy to generate and require no sequencing information. A second noteworthy finding is that the random probe sets were able to discriminate different species under the same genus. This is important since the randomly generated probes in same genus may share similar sequences, which would lead to cross-hybridization with the probes spotted on the DNA microarray. However, no serious

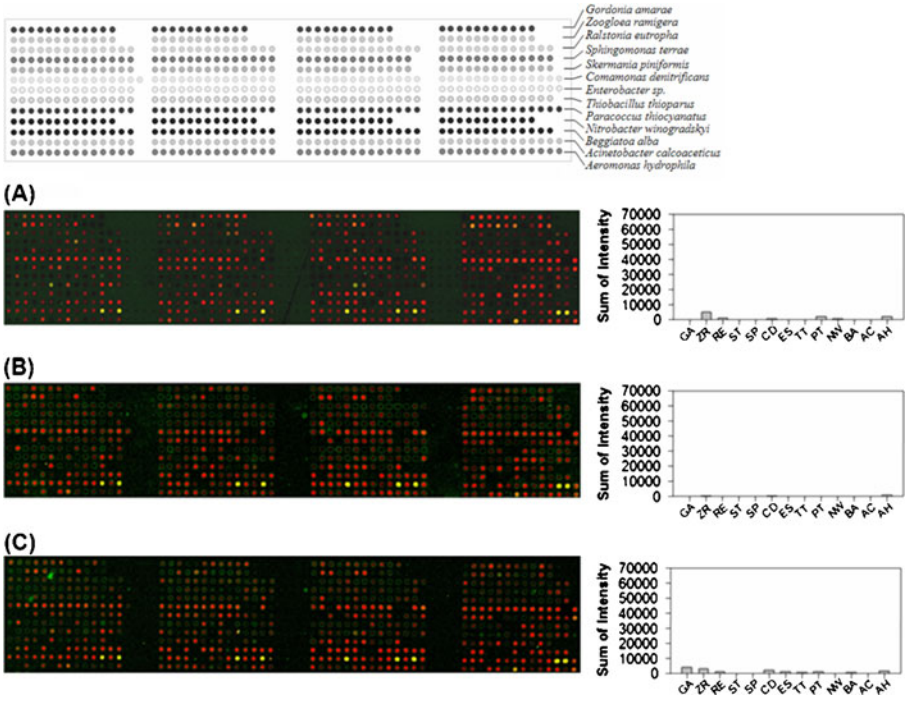

Fig. 4 Detection of the target sludge bacteria cultivated in the synthetic medium. The DNA microarray scan images and signal intensities are shown for **a** the first subcultured sample of the activated sludge seed, **b** the fifth subcultured sample, and **c** the tenth subcultured sample

cross-hybridization was observed in this study. This may be due to the small number of probe sets (about 50 for each bacterial strain) used, which represent only a small fraction of the whole genome. Another benefit of this protocol is that the DNA hybridization was performed directly, i.e., without PCR amplification. If only a few target genes are used, an amplification process is needed to increase the intensity of the signals, which is dependent upon the amount of extracted nucleic acid samples that are fluorescently labeled. However, this study focused on the application of RBGDMC in the detection of naturally enriched bacteria. It could be true that some minor environmental constituents cannot be the major constituent even after the enrichment process. Considering the fact that the detection of those minor environmental constituents was not the objective of this study, however, we could suggest that the amplification of genomic DNA of those minor constituents would be a possible solution to address detectable signals in real environments. This study shows that the RBGDMC can be used as a tool to successfully detect target bacteria in activated sludge samples with enrichment. In addition, it should be noted that during the enrichment of the bacterial community, the growth and fraction of these bacterial strains may be affected by other members in the community since they are experiencing competition, mutualism, and antagonism in mixed consortia [1]. It is important, therefore, to highlight that this type of microarray chip can be applied to analyze the population or dominant species present during these interactions.

Conclusion

The present study showed a successful example of a specific implementation of the RBGDMC to screen for dominant bacterial strains and the capability to discriminate bacteria at the species level. The use of random genomic probes, supported by a DNA microarray chip platform, confirms the features of this high-throughput sensing format to identify and screen for dominant bacteria in complex environmental conditions without genomic amplification.

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