Environ. Sci. Technol. **2004**, *38,* 6775−6782

Development and Evaluation of Microarray-Based Whole-Genome Hybridization for Detection of Microorganisms within the Context of Environmental Applications

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The detection and identification of microorganisms in natural communities is a great challenge to biologists. Microarray-based genomic technology provides a promising high-throughput alternative to traditional microbial characterization. A novel prototype microarray containing whole genomic DNA, termed community genome array (CGA), was constructed and evaluated. Microarray hybridizations at 55 °C using 50% formamide permitted the examined bacteria to be distinguished at the species level, while strain-level differentiation was obtained at hybridization temperatures of 65 or 75 °C. The detection limit was estimated to be approximately 0.2 ng with genomic DNA from a single pure culture using a reduced hybridization volume (3 μL). Using mixtures of known amounts of DNA or a known number of cells from 14 or 16 different species, respectively, about 5 ng of genomic DNA or 2.5×10^5 cells were detected under the hybridization conditions used. In addition, strong linear relationships were observed between hybridization signal intensity and target DNA concentrations for pure cultures, a mixture of DNA templates, and a population of mixed cells ($r^2 = 0.95-0.98$, $P < 0.01$). Finally, the prototype CGA revealed differences in microbial community composition in soil, river, and marine sediments. The results suggest that CGA hybridization has potential as a specific, sensitive, and quantitative tool for detection and identification of microorganisms in environmental samples.

Introduction

Understanding the structure, composition, and adaptive responses of microbial communities to environmental perturbations, such as toxic contaminants; climate change; and medical, agricultural and industrial practices, is critical to maintaining or restoring desirable ecosystem function and health. However, the detection, characterization, and quantification of microbial population diversity in various environments are formidable tasks. The development and application of nucleic acid-based techniques, such as small subunit (SSU) rRNA gene-based cloning methods, denatured gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), quantitative PCR, in situ hybridization, and PCR amplification, have greatly advanced our ability to provide detailed descriptions of microorganisms in natural habitats (*1*−*3*). These techniques, however, lack the high-throughput capacity and greater global resolution needed to cope with the high microbial diversity characteristic of most environments.

Reverse sample genome probing (RSGP), a whole-genome DNA−DNA membrane-based hybridization method that permits simultaneous detection and quantitation of selected bacteria from environmental samples (*4*), has been employed in oil fields (*4*−*6*), on terrestrial soils (*7*), and on intertidal salt marsh sediments (*8*) to monitor changes in the representation of sulfate-reducer and nitrogen-fixer populations, respectively. RSGP has provided valuable insight into microbial population dynamics in situ, but its parallel capacity is limited in its current format.

Numerous studies have demonstrated the utility of microarrays for analyzing gene expression and regulation on a genomic scale (*9*−*15*) and for detection of genetic polymorphisms (*16*−*18*) in both eukaryotes and prokaryotes. Recently, knowledge of the complete genomic sequence of an organism in conjunction with DNA microarray technology has allowed the comprehensive comparison of genomes among closely related species (*19*−*23*). DNA microarrays constructed with full-length open-reading frames (ORFs) from a sequenced organism, for example, can be used to reveal gene-specific differences between closely related genomes and thus provide insight into species relationships (*22*). In addition, microarrays containing probes of DNA fragments or oligonucleotides have the potential to be useful as tools for the comprehensive and quantitative characterization of complex microbial communities (*24*−*31*); however, the methodology has not been examined in terms of specificity, sensitivity, and quantitation with whole genomic DNA from multiple microorganisms using a microarray format.

We constructed a novel type of microarray consisting of whole genomic DNA isolated from 67 closely or distantly related representative bacterial strains. Because the entire genomic DNA is used as a probe for identifying microorganisms within the context of natural microbial communities, we refer to this type of microarray as a community genome array (CGA). Our results suggest that CGA hybridization can be used as a specific, quantitative, and parallel tool for the detection and identification of cultivable bacteria from different environments.

Materials and Methods

Bacterial Strains, Environmental Samples, and Genomic DNA Isolation. For evaluating the performance of CGA-based hybridization, genomic DNAs from 67 closely or distantly related representative bacterial strains were selected as probes on the basis of their phylogenetic relationships, GC content, other molecular studies, and/or the accessibility of these strains (see SI_Table 1 in the Supporrting Information; http://www.esd.ornl.gov/facilities/genomics/index.html). The

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10.1021/es049508i CCC: $27.50
Published on Web 11/12/2004

selected strains are commonly found in various environments. *Shewanella* and *Pseudomonas* reference microorganisms and aromatic compound-degrading *Azoarcus* isolates used in this study were from the laboratory culture collections of J.Z. and J.T. Genotypic and phenotypic studies describing the taxonomic classification of some of these bacteria have been reported elsewhere (*32*−*35*). Environmental isolates were collected from Washington (Pacific) continental margin sediments or deep ocean marine sediments (*36*).

To evaluate the performance of CGA-based hybridization, marine sediment samples from the Washington margin and soil samples and river sediment samples from Oak Ridge, TN, were used. Marine sediment samples from Stations W305 (997 m of water column depth) and W307 (2664 m of water column depth) (*36*) were provided by Allan Devol of The University of Washington. Two samples from each station at different depths of sediment core (1−1.5, 9−10 cm) were analyzed. Soil and river sediment samples from Oak Ridge have been described elsewhere (*37*).

The genomic DNAs arrayed on glass slides were isolated from pure cultures using a previously described phenol−chloroform method (*38*). All genomic DNA samples were treated with RNase A (Sigma, St. Louis, MO) and analyzed on agarose gels stained with ethidium bromide prior to microarray fabrication. Community DNA from marine sediments and soils was isolated according to the grinding phenol−chloroform method described by Zhou et al. (*39*). DNA concentration was determined in the presence of ethidium bromide by fluorometric measurement of the excitation at 360 nm and emission at 595 nm using a HTS700 BioAssay Reader (Perkin-Elmer, Norwalk, CT).

Microarray Construction, Probe Labeling, and Hybridization. Initially, arrays consisting of whole genomic DNA from *Shewanella algae* BrY, *Shewanella* sp. MR-4, *Shewanella pealeana* ANG-SQ1, *Azoarcus tolulyticus* Td-15, *Escherichia coli* strain S17−1/λ_{pir}, and *Saccharomyces cerevisiae* were constructed to determine the effect of DNA probe concentration on hybridization signal intensity. Genomic DNA probes were printed on silane-modified glass slides (Cel Associates, Houston, TX) at concentrations of 10, 50, 100, 200, 300, 400, 500, 600, and 700 ng/μL. Fluorescence intensities saturated at target genomic DNA concentrations of 200 ng/μL or greater (data not shown). Genomic DNA probe concentrations of 200 ng/μL were, therefore, used for construction of the prototype CGA.

CGAs contained whole genomic DNA (probes) from 32 type strains and 35 environmental isolates (SI_Table 1). Five *S. cerevisiae* genes encoding mating pheromone α-factors (*mfα1*, *mfα2*), mating-type α-factor pheromone receptor (*ste3*), actin (*act1*), and GTP-binding protein involved in the regulation of cAMP pathway (*ras1*) were also included on the arrays as negative controls. All 72 probes (including negative controls) were arranged as a matrix of 15 rows × 5 columns (denoted columns a−e). The exact location of each genomic DNA in the matrix is listed in SI_Table 1. Genomic DNA samples were prepared for deposition and printed as described previously (*30*). Each glass slide contained three replicates of genomic DNA from individual strains. Following printing, glass slides were postprocessed and evaluated for spot quality as described previously (*30*).

Whole genomic DNA was fluorescently labeled using the random priming method and purified as described previously (*30*). All microarray experiments were performed in triplicate (a total of nine replicates per genomic DNA probe), unless otherwise noted, to enable statistical analyses. Microarray hybridization was performed essentially as described previously (*30*), with the exception of the addition of formamide. Formamide was added to the hybridization solution [denatured fluorescently labeled genomic DNA, 3× SSC, 1 μg of unlabeled herring sperm DNA (Promega, Madison, WI), and 0.3% SDS in a total standard volume of 15 μL] for experiments testing the effect of a denaturant on hybridization specificity. A reduced hybridization solution volume of 3 μL was used for testing detection sensitivity.

For microarray experiments evaluating the effect of different formamide concentrations on specificity, hybridization was performed at 55 °C in the presence of 0, 10, 20, 30, 40, 50, 60, or 70% (vol/vol) formamide. For experiments determining the effect of temperature and denaturants on signal intensity, hybridization was carried out at 45, 55, 65, or 75 °C in the presence or absence of 50% (vol/vol) formamide. For hybridization specificity analyses using diverse genomic DNA templates, hybridization (15 μL volume) was performed at 55 °C in the presence of 50% formamide. Following hybridization, coverslips were removed in washing buffer (1× SSC−0.2% SDS) and then washed sequentially for 5 min in 1× SSC−0.2% SDS and 0.1× SSC−0.2% SDS and for 30 s in 0.1× SSC at ambient temperature prior to being air-dried in the dark. For convenience, we refer to the hybridization conditions of 55 °C, 50% formamide in the hybridization buffer (15 μL total volume), and posthybridization washing in 0.1× SSC as our standard hybridization condition.

Microarray Scanning and Data Analysis. A ScanArray 5000 Microarray Analysis System (PerkinElmer, Wellesley, MA) was used for scanning microarrays at a resolution of 5 μm. Visual displays of hybridization results presented here are representative images. For detection sensitivity experiments, the laser power and photomultiplier tube (PMT) gain were both 100%. For specificity experiments, the laser power was 85% and the PMT gain was 75%. Scanned image displays were analyzed by quantifying the pixel density (intensity) of each hybridization spot using the software of ImaGene version 4.0 (Biodiscovery, Inc., Los Angeles, CA). A grid of individual circles defining the location of each DNA spot on the array was superimposed on the image to designate each fluorescent spot to be quantified. Mean signal intensity was determined for each spot. The local background signal was subtracted automatically from the hybridization signal of each separate spot. Fluorescence intensity values for all replicates of the five yeast genes (negative controls) were averaged and then subtracted from the background-corrected intensity values for each hybridization signal. Statistical analysis was performed using SigmaPlot 5.0 (Jandel Scientific, San Rafael, CA), and principal components analysis (PCA) of soil and marine sediment samples was performed using the SYSTAT statistical computing package (SYSTAT version 10.0; SPSS, Inc., Chicago, IL). Relationships between microbial genomes from the microarray hybridizations were determined using hierarchical cluster analysis (CLUSTER) and visualized with TREEVIEW (*40*). The signal-to-noise ratio (SNR) for each spot was calculated on the basis of the following formula (*41*): SNR = (signal intensity − background)/standard deviation of the background, in which the background measurement refers to the local spot background intensity and the standard deviation of the background was calculated across all pixels measured by the ImaGene software. The SNRs from nine replicate data points were then averaged to represent the SNR for a particular probe.

Results

Specificity of CGA Hybridization. To examine hybridization specificity under varying experimental conditions and to determine threshold levels of genomic differentiation, a microarray was fabricated that contained genomic DNA isolated from different representative environmental organisms classified as α-, β-, and γ-*Proteobacteria* and Gram-positive bacteria. Many of the selected species are closely related to each other based on SSU rRNA and *gyr*B gene phylogenies (SI_Table 1, trees not shown) and belong

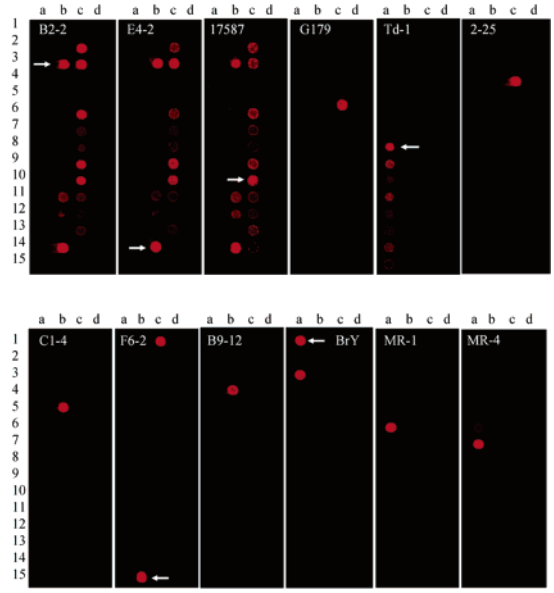

FIGURE 1. Hybridization specificity of community genome arrays. Fluorescence images display the level of hybridization specificity obtained with labeled target genomes from *P. stutzeri* B2-2, *P. stutzeri* E4-2, *P. stutzeri* ATCC 17587, *Pseudomonas* sp. G179, *A. tolulyticus* Td-1, *Marinobacter* sp. 2-25, unknown α-proteobacterium C1-4, *B. methanolicus* F6-2, *Halomonas variabilis* B9-12, *S. algae* BrY, *S. oneidensis* MR-1, and *S. oneidensis* MR-4 (bold face indicates labels in the figure). Individual genomic DNAs were labeled with Cy5 (red pseudocolor) using the random priming method, purified, and separately hybridized with the prototype CGA at 55 °C in the presence of 50% (v/v) formamide for 12−15 h. Target templates are presented in white lettering. In the case of multiple signals, white arrows indicate the hybridization signal for the target genomic DNA.

primarily to three major bacterial genera (*Pseudomonas*, *Shewanella*, and *Azoarcus*). The GC content of the genomes varies from 37% to 69.3% (SI_Table 1).

The hybridization conditions for species- and strain-specific differentiation were assessed using a toluene-degrading bacterium, *A. tolulyticus* isolate Td-21, as the target template. Increasing concentrations of formamide (ranging from 0 to 70%) in the hybridization solution clearly had an obvious impact on hybridization specificity at 55 °C (SI_Figure 1). At low formamide concentrations (0 and 10%), extensive, nonspecific cross-hybridization was observed between the target strain, *A. tolulyticus* Td-21, and the majority of the *Pseudomonas stutzeri* strains represented on the array, as well as *Azoarcus* sp. VB22^T, *Marinobacter* sp., and *Staphylococcus saprophyticus*. Hybridization was much more specific at formamide concentrations of 30−40%, with only different strains of the target species (i.e., *A. tolulyticus*) showing visible hybridization. While nonspecific cross-hybridization totally disappeared at formamide concentrations of 50−70%, all or some of the target species still hybridized to closely related nontarget strains under these conditions. The hybridization signal intensity for target strain Td-21 was comparable at formamide concentrations of 50−70%. Therefore, for experimental convenience, 50% formamide was used for all of our experiments.

CGA hybridization specificity was further examined using 12 other species and/or strains as target templates. Under hybridization conditions of 55 °C and 50% formamide, strong signals were obtained for genomic DNAs of corresponding species to the labeled target (Figure 1). Little or no cross-hybridization (~0−4% of the hybridization signals of the target strains) was observed for nontarget species as well as for negative controls (yeast genes), thus indicating that species-specific hybridizations can be achieved with CGAs under the conditions used. In addition, the hybridization signal intensities were substantially different among various *P. stutzeri* and *Shewanella oneidensis* strains. However, some strains of *P. stutzeri*, *A. tolulyticus*, *Bacillus methanolicus*, and *S. algae* could not be clearly distinguished under these conditions (Figure 1).

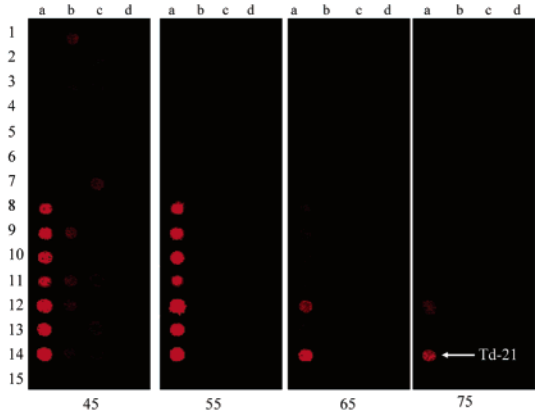

FIGURE 2. Fluorescence images showing the improvement in array hybridization specificity with increasing temperature. Genomic DNA from *A. tolulyticus* isolate Td-21 was randomly labeled with Cy5 and hybridized with the CGA in the presence of 50% (v/v) formamide at 45, 55, 65, and 75 °C. The hybridization signal for Td-21 genomic DNA is indicated (white arrow).

To determine whether strain differentiation could be achieved by adjusting the experimental conditions, microarray hybridizations were conducted at 45, 55, 65, and 75 °C in the presence or absence of 50% formamide using genomic DNA from *A. tolulyticus* Td-21 as the target template. At 55 °C, the genomic DNA probes from all eight *Azoarcus* strains hybridized well with the labeled target template (Td-21) (Figure 2). By contrast, at 65 °C, little or no hybridization was observed with nontarget strains, with the exception of strain Td-17, which exhibits 89% genome homology with Td-21 based on S1 nuclease hybridization methods (*33*). The hybridization signal ratio to Td-21 of Td-17 is about 82%, whereas the ratio of Td-21 to the other *Azoarcus* strains tested is about 30%. While the cross-hybridization of Td-17 with Td-21 was substantially decreased at 75 °C (48% of hybridization signal), the signal intensity for Td-21 also decreased. Strain differentiation could not be achieved without formamide (data not shown), even at 75 °C. These results indicated that strain differentiation could be achieved under very stringent hybridization conditions.

Detection Sensitivity and Quantitation of CGA Hybridization. The capacity of CGA hybridization to serve as a quantitative tool was explored and its detection sensitivity was determined using genomic DNA extracted from a pure culture of *P. stutzeri* B2-2. Genomic DNA was randomly labeled with Cy3 at concentrations that varied between 0.1 and 2000 ng and hybridized at 65 °C with the microarray in triplicate and in the absence of formamide. To avoid signal saturation, the slides were scanned using two different combinations of laser power and PMT gain.

Strong hybridization signals were observed with 5 ng of B2-2 genomic DNA for the target genome (row 3, column b on the array image in SI_Figure 2 of the Supporting Information). With 0.2 ng of DNA, the target hybridization signal was substantially weaker but detectable. Hybridization signals using 0.1 ng of genomic DNA, however, were barely detectable above background levels (results not shown). Therefore, the detection limit with randomly labeled pure genomic DNA under these hybridization conditions using a reduced hybridization volume was estimated to be approximately 0.2 ng.

The fluorescence intensities obtained at each DNA concentration for nine data points (three independent microarrays with three replicates on each slide) were averaged, and the log value of the concentration was compared to the corresponding log value of the mean fluorescence intensity (data not shown). Linear relationships were observed for signal intensity and target DNA concentrations ranging from 0.2 to 50 ng ($r^2 = 0.95$, $P < 0.01$) and from 50 to 2000 ng ($r^2 = 0.97$, $P < 0.01$). These results indicated that CGA hybridization is quantitative for pure bacterial cultures within a wide range of DNA concentrations.

In the above experiment, the detection limit and quantitative capability were examined with genomic DNA from a single pure culture in the absence of heterogeneous DNA templates under a nonstandard condition (3 μL hybridization volume). Generally, a larger hybridization volume is used; thus, the detection limit under the standard hybridization conditions could be lower (*30*). Also, the detection limit determined with genomic DNA from a single pure culture may not be directly applicable to real environmental samples because of the complexity of microbial communities in environmental samples. The existence of other nontarget DNAs may affect the hybridization with target DNA and hence decrease detection sensitivity, and similarly, the quantitative relationships between signal intensity and DNA concentration may also be different. To evaluate the detection sensitivity and quantitative capacity within the context of environmental applications using the standard hybridization conditions, genomic DNAs representing 16 target bacteria from different genera and species were mixed at different concentrations, fluorescently labeled, and hybridized with the CGA (see Figure 3 legend for details). Cross hybridization was not observed when the genomic DNA from each bacterium was labeled and hybridized separately. The signal intensity was significantly higher than the background level when DNA concentrations were larger than 5 ng, whereas the signal intensity was comparable to the background level when the genomic DNA concentration ranged from 0.01 to 2.5 ng (Figure 3A). These results suggested that the detection limit of CGA-based hybridization in the presence of nontarget DNAs under the standard hybridization condition is approximately 5 ng. A significant linear relationship ($r^2 = 0.98$, $P < 0.01$) was observed between signal intensity and target DNA concentration within a concentration range of 25 to 1000 ng (Figure 3A), and these results suggested that CGA hybridization could also be quantitative for mixed DNA templates.

For detecting microorganisms in environmental samples, bulk community DNA must first be extracted from environmental matrixes and then purified to remove contaminants prior to microarray hybridization. The existence of residual contaminants may affect sensitivity, and it can be a source of variation of quantitation. Some portion of the community DNA could be lost during the extraction process, and hence the detection sensitivity and quantitative capacity could also be affected. To further mimic the environmental application processes of molecular ecology, bacterial cells from 14 different species were mixed in different quantities (see Figure 3B legend for details) and seeded into autoclaved soils in triplicate. The bulk DNA was extracted from the seeded soils, purified, labeled with Cy dyes, and hybridized with the CGA. The hybridization signal intensity was significantly higher than the background signal when the cell number was larger than 2.5×10^5 cells. However, the signal intensity was not significantly different from the background level when cell numbers ranged from 1×10^4 to 1×10^5 (Figure 3B). These results suggested that the detection limit of CGA-based hybridization is approximately 2.5×10^5 cells of an individual target genome in the presence of other bacterial cells under the experimental conditions and protocols used. A linear relationship ($r^2 = 0.94$, $P < 0.01$) was observed between signal intensity and initial bacterial cell numbers between 5×10^4 and 2×10^8 cells (Figure 3B). These results indicated that CGA-based hybridization could be a potentially

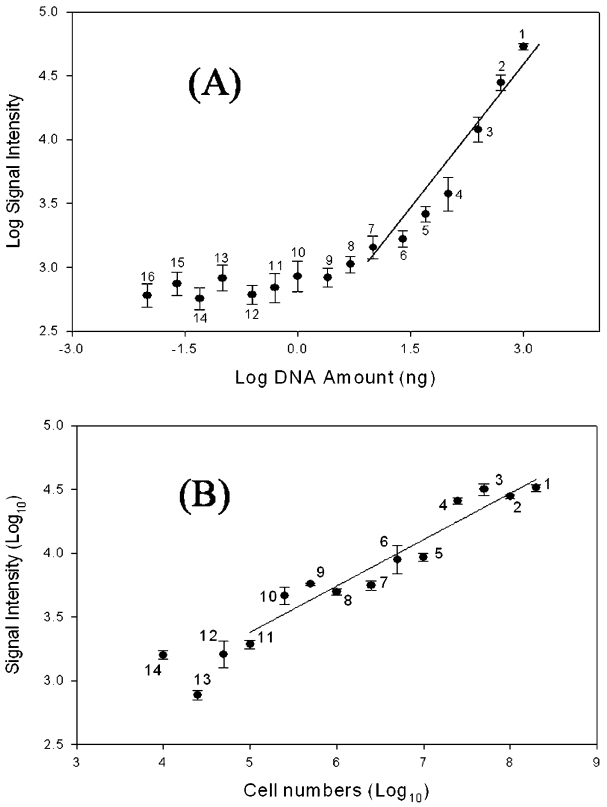

FIGURE 3. Evaluation of quantitative potential of community genome arrays. (A) Quantitative analysis of community genome arrays using a mixed genomic DNA population. Sixteen different genomic DNAs were mixed together at the following concentrations and labeled with Cy3: (1) *P. stutzeri* B2-2, 1000 ng; (2) *A. tolulyticus* Td-21, 500 ng; (3) *S. oneidensis* MR-1, 250 ng; (4) *H. variabilis* B9-12, 100 ng; (5) *Pseudomonas* sp. G179, 50 ng; (6) *S. algae* Bry, 25 ng; (7) *E. coli*, 10 ng; (8) an unknown α-proteobacterium C1-4, 5 ng; (9) *B. methanolicus* F6-2, 2.5 ng; (10) *Marinobacter* sp. E1-7, 1 ng; (11) *Shewanella amazonensis* SB2B, 0.5 ng; (12) *S. saprophyticus* D3-16, 0.25 ng; (13) *Shewanella woodyi* MS32, 0.1 ng; (14) *Marinobacter* sp. D5-10, 0.05 ng; (15) *Shewanella* sp. A8-3, 0.025 ng; and (16) *Marinobacter* sp. C10-5, 0.01 ng. The log of each DNA amount in the mixture is plotted against the log of the average signal intensity corresponding to each probe. DNA concentrations below 25 ng were outside the linear portion of the curve and therefore were not included in the regression line. The r^2 value was 0.98 in the DNA range of 25−1000 ng. The data points represent the average values derived from three independent microarray slides, with three replicates on each slide (a total of nine data points per genome probe). The standard deviation among these replicate data points are shown by the error bars. (B) Relationship of CGA hybridization signal intensity to target cell number using a mixed population of bacterial strains. The following 14 distantly related bacterial strains were seeded at different cell numbers into soil that was autoclaved three times prior to cell seeding: (1) 2×10^8 *Pseudomonas putida* ATCC 12633; (2) 1×10^8 *S. algae* BrY; (3) 5×10^7 *Marinobacter* sp. D5-10; (4) 2.5×10^7 *P. stutzeri* B2-2; (5) 1×10^7 *Pseudomonas fluorescens* ATCC 13525; (6) 5×10^6 *S. saprophyticus* D3-16; (7) 2.5×10^6 *Pseudomonas* sp. G179; (8) 1×10^6 *Pseudomonas aeruginosa* ATCC 15692; (9) 5×10^5 *S. oneidensis* MR-1; (10) 2.5×10^5 *H. variabilis* B9-12; (11) 1×10^5 *Pseudomonas chlororaphis* ATCC 17811; (12) 5×10^4 *E. coli*; (13) 2.5×10^4 *B. methanolicus* F6-2; and (14) 1×10^4 α-proteobacterium. Cell number was determined using serial dilution plating. Bulk genomic DNA was isolated from seeded soil using a previously described method (*15*), labeled, and hybridized to community genome arrays in triplicate.

useful tool for quantifying microorganisms in environmental samples.

Application of CGA Hybridization for Profiling Microbial Communities in Environmental Samples. To evaluate the potential applicability of CGAs for profiling microbial community structure, bulk community DNA was isolated from 2 g of three surface soil, three river sediment, and four marine sediment samples. Two micrograms of the purified bulk community DNA were directly labeled with Cy5 using the random primer labeling method and hybridized with the pilot CGAs in triplicate. All spots having SNRs larger than 3 were considered positive signals. Overall, the proportions of the arrayed genomic DNA probes (53 in total) that showed statistically significant positive signals were as follows: 33−43%, 28−56%, and 22−72% in soils, river sediments, and marine sediments, respectively. The percentage of positive signals in some marine sediment samples (W305, 1−1.5 cm) was apparently higher than those in soils. No hybridization signal was observed for the five yeast control genes. The average variation in signal intensity for all of the gene replicates in the samples was 14.6% with a standard deviation of 7.3%. The signal variation was lower for the soil sample (13.2% ± 7.1) than for the marine sediment (15.9% ± 8.7) and river sediment (14.8% ± 8.2) samples.

About 3−18% and 1−16% of the genomic DNA probes showed hybridization with DNA from soil samples and river sediment samples, respectively. While about 20% of the probes hybridized well with DNA from the top layer (1−1.5 cm) of the shallow marine sediment sample, W307 (997 m), none of the probes showed hybridization with DNA from the deep layer of the sediment core (9−10 cm) of W307 or with DNA from both layers of the sediment core from Station W305 (2664 m). These results were consistent with the site geochemical observation that the deep sea sediments were carbon-limited, and the total microbial biomass was lower. In addition, the microarray data revealed considerable differences for some abundant microbial populations (with hybridization signal intensity well above the average signal intensity) among different types of samples. For instance, *Marinobacter* species similar to the strains D3-15, D5-10, and E1-7, and *Shewanella* species similar to *S. algae* and *S. oneidensis* were abundant in the top layer of W307 (1−1.5 cm), but they were not observed in soil and river sediments. The bacteria similar to the "*Pseudomonas*" sp. G179 in α-*Proteobacterium*, a typical *nir*K-containing denitrifier, were abundant in soil samples but not in the river and marine sediment samples.

To evaluate whether CGA-based hybridization can be used to reveal microbial community differences, principal component analysis (PCA) of the hybridization intensity data (Figure 4A) and physical and chemical data in marine sediments (Figure 4B), soils, and river sediments (Figure 4C) was performed. The PCA analysis of the hybridization data was able to reduce the data to two principal components that explained a large amount (92%) of the variation in the hybridization patterns (Figure 4A). Three distinct clusters based on the habitat types (soils, river sediments, and marine sediments) were identified. The marine sediment samples clustered together and were well-separated from the river sediment samples. Although both soil and river sediment samples were from the same geographical region, two separate clusters were identified on the basis of habitat types. The soil samples formed a separate cluster and were located between river and marine sediment clusters, but some soil samples were more close to the river sediment cluster than the marine sediment cluster. This is understandable, because the soil and river sediment samples were geographically very close. Similarly, two separate clusters of the soil and river sediments were also observed from PCA analysis of their geochemical data (Figure 4C).

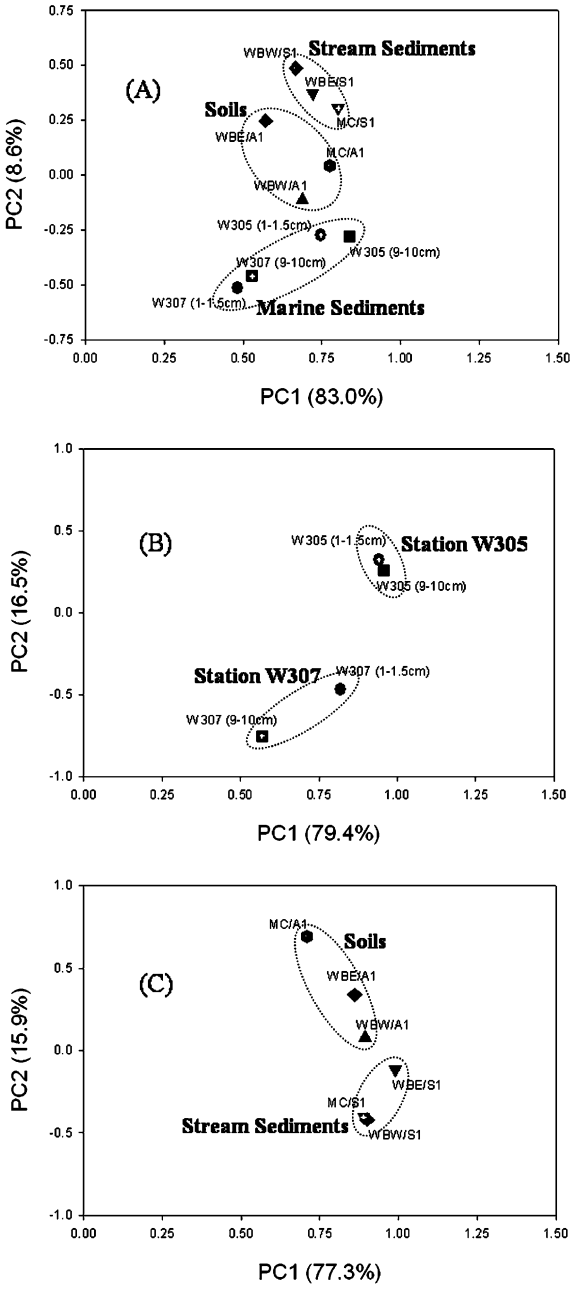

FIGURE 4. Principal component analyses of (A) CGA-based hybridization intensity data, (B) biogeochemical properties in marine sediments [organic carbon, total nitrogen, CN ratio, NH_4^+, NO_3^-, denitrification rate, PO_4^+, Fe(II), and temperature], and (C) chemical and physical data of soil and river sediment properties (moisture, pH, sand, silt, clay, carbon, and nitrogen). Values in parentheses indicate percent of total variances of PCA derived from hybridization data and biogeochemical data. Sample designation (*37*) for soil and river sediments are A = A-Horizon; S = stream sediment; WBE: Walker Branch East (ORNL); WBW: Walker Branch West (ORNL), MC, Mossy Creek in the Great Smoky Mountains National Park.

Within the marine sediment cluster, the samples from different depths of the same stations were more closely clustered together (Figure 4A), indicating that the overall microbial communities were more similar between different stations than different depths of the sediment cores. Similar distribution patterns were also observed on the basis of the geochemical data of these sediment samples (Figure 4B), suggesting that there is an apparent overall correlation between microbial community structure and site geochemistry. Similarly, a correlation between denitrifying community

structure and geochemistry was observed in continental margin sediments within the oxygen-deficient zone off the Pacific Coast of Mexico (*42*).

The overall consistency between microbial community structure based on CGA hybridization and geographical locations as well as site geochemistry indicated that CGA-based hybridization was able to reveal, to some extent, apparent differences in microbial community structure. However, the resolving power of the pilot CGA appears to be low. For instance, it is expected that the marine sediment microbial communities will be dramatically different from soil and river sediments, but the separation distances of these samples in both PC1 and PC2 were not very large. Also, considerable discrepancy was observed for WBW/S1 and WBW/A1 in the ordinate plots of PCAs based on the hybridization intensity data and physical and chemical properties. This could be because the pilot CGA contained only a small number of useful genomic DNA probes, and they are not representative of the microbial communities examined.

Discussion

Microarrays offer the advantage of a large capacity of gene probes and parallel analyses and when adapted to RSGP could reduce labor and increase the capacity, speed, and quantitation of microbial community structure analyses. CGA hybridization differs from membrane-based RSGP in that the nonporous surface has advantages of miniaturization, hybridization kinetics, sample volume, reagent absorption, signal detection approaches, and reproducibility (*43*). The capability of accurate and precise miniaturization with robots on nonporous substrates is one of the two key advances of microarray-based genomic technologies. The use of fluorescence-based detection is the other key advance of microarray-based genomic technologies, which offers significant advantages over RSGP, which uses radioisotope detection, in terms of speed, throughput, data quality, and user safety (*43*). RSGP, however, appears to be 10 times more sensitive than CGA (*6*), which is most likely due to the use of radioisotope labeling and the larger probe-binding capacity of porous membranes.

Like RSGP, the main disadvantage of the CGA is that only the cultured components of a community can be monitored, because the construction of this type of array requires the availability of individual pure isolates, although CGA-based hybridization itself does not require culturing (*44*). With the recent advances in environmental genomics, high molecular weight DNA from uncultivated microorganisms could be accessed through bacterial artificial chromosomes (BAC). BAC clones could also be used to fabricate CGAs, thus allowing the investigation of uncultivated components of a complex microbial community. Because the size of BAC clones is generally 50−100-fold less than that for an entire genome, it is expected that microarrays fabricated with high molecular weight BAC clones should have similar performance characteristics as CGAs.

Due to conserved genes and the complicated nature of surface hybridization, one would expect low levels of cross-hybridization to nontarget strains. The central question is how to distinguish true hybridization signals from nonspecific background noise. One common approach is to determine signal-to-noise ratios (SNRs) and discard values below 3.0 (*41*). Our studies showed that the average SNR for hybridizations with different species within a genus is about 3.35 ± 0.32, which is substantially lower than hybridizations with different strains from the same species (Table 1). These results also suggest that CGA-based hybridization appears to be species-specific. However, it should be noted that the commonly used SNR threshold was not defined within the context of hybridizations with whole-genome DNA. This threshold value could vary with the genome complexity of species, the types of slides, and the hybridization conditions used. Future work is needed with species possessing a wide range of genomic complexity and GC contents to establish a universal threshold SNR value for whole-genome DNA-based hybridization.

TABLE 1. Summary of Signal-to-Noise Ratios for Hybridizations with Species at Different Levels of Relatedness[a]

groups compared	signal-to-noise ratio		
	n	mean	SD
target strains	14	81.1	30.1
strains from the same species	67	25. 8	19.2
species from a genus	71	3.4	0.32
species from different genera and above	594	0.74	1.15
negative controls	79	−0.33	0.23

[a] Hybridizations were carried out at 55 °C with 50% formamide, followed by 0.1× SSC washing.

When using CGAs for detecting bacterial populations in a mixed microbial community, stringent hybridization conditions (e.g., 55−65 °C plus 50% formamide) should be used to minimize potential cross-hybridization among closely related species and strains. Also, it might be necessary to determine SNR threshold values for individual communities of interest, because these values could also vary with community composition and complexity besides the factors mentioned above. However, determining appropriate SNR threshold values for community analysis will be more challenging, because the composition and structure of the community studied in an environmental sample is generally unknown. One possible way to determine SNR threshold values for environmental samples is to spike these samples with reference microorganisms that are not present in the community of interest based on SSU rRNA sequence information, followed by community DNA extraction and hybridization. On the basis of the hybridization patterns with other nontarget probes, one should be able to determine an SNR threshold value appropriate to the community of interest.

Sensitivity is another important issue for environmental studies. On the basis of experiments with mixtures of a known amount of DNA or a known number of cells, about 5 ng of genomic DNA or 2.5×10^5 cells can be detected using our standard hybridization conditions. If these values can be directly applicable to real environmental samples, the level of CGA detection sensitivity should be sufficient for detection of at least the more dominant members of a microbial community. However, it is still not sensitive enough to detect less abundant microbial populations. Other approaches for increasing hybridization sensitivity are needed to detect rare populations in natural environments. We are currently exploring ways to enhance the level of detection sensitivity.

The quantitative capability of microarray-based hybridizations has not been well-established. The quantitative accuracy of microarray-based hybridizations is uncertain, due to inherent high variations associated with array fabrication, labeling, target concentration, and scanning as well as sample variations. A linear relationship was observed between hybridization signal intensity and target DNA concentration for a pure culture, a population of mixed DNA templates, and a population of mixed cells in soil. The quantitative feature of microarray hybridization reported here is similar to our previous studies (*24, 30*) and is consistent with the findings of microarray studies on gene expression (*45*). However, like other molecular approaches, the quantitative accuracy of CGA-based hybridization will depend on probe specificity.

To obtain more accurate results, the probe must be highly specific to the target microorganisms. Any cross-hybridization from closely related species could distort quantification. Because very low cross-hybridization was observed among different species examined with the pilot CGA, by subtracting the background noise from the hybridization data set, it is expected that CGA-based hybridization will be quantitative for organisms at the species level or higher. This is supported by our experimental results with the mixed DNA templates and mixed cells in soil, but it will be difficult to quantify microorganisms at the strain level with CGA-based hybridization due to potential cross-hybridization. In addition, since potential cross-hybridization is always a concern, especially when dealing with environmental samples of unknown composition, it is important to use CGAs for relative comparisons. In general, relative changes in microbial communities can be measured by the hybridization signal ratios of treatment samples to a common reference or control sample. The effects of cross-hybridization can be canceled out when the hybridization intensity signals from treatment samples are divided by the hybridization intensity signals from the common reference samples under the assumption that the community composition is similar between the treatment and reference samples. Thus, using hybridization ratios will help to minimize the effects of cross-hybridization on quantitative accuracy. Finally, multiple hybridizations with replicate samples are always important for statistically assessing the reliability of the hybridization data and for obtaining reliable quantitative results with high confidence.

The cluster patterns of the PCA analysis of CGA application to the surface soils, river sediments, and marine sediments based on hybridization intensities were consistent with that of the geochemical characteristics of the samples (Figure 4A−C), indicating that CGA hybridization has the capability of revealing differences in community composition and structure. The percentage of positive signals in some marine sediment samples (W305, 1−1.5 cm) in this study was apparently higher than those in soils. However, this does not necessarily indicate that the diversity of the microbial community in this marine sediment sample was higher than those in soil samples. Most likely, this is because about 30% of the genomic DNA probes were from bacteria that were directly isolated from those marine sediments or from similar environments.

In summary, a prototype microarray containing whole-genomic DNA was constructed and evaluated in terms of specificity, sensitivity, and quantitation. Our results suggest that CGA hybridization could be used as a species-specific, sensitive, and potentially quantitative tool for bacterial detection and identification. The potential applicability and usefulness of CGA hybridization to environmental samples will depend not only on the arrayed probes, specificity, and representation but also on the genome diversity and structure complexity of microbial communities in natural environments. Compared to the membrane-based RSGP, CGAs offer high-throughput capacity for diverse groups of microorganisms, have broader screening capacity, and easier construction.

Acknowledgments

We thank Lynn Kszos for editorial assistance. This research was supported by the United States Department of Energy under the Natural and Accelerated Bioremediation Research, the Biotechnology Investigations-Ocean Margins, and Microbial Genome Programs of the Office of Biological and Environmental Research, Office of Science, and by Laboratory Directed Research and Development Program, Oak Ridge National Laboratory. Oak Ridge National Laboratory is managed by University of Tennessee−Battelle LLC for the U.S. Department of Energy under Contract DE-AC05-00OR22725. L.W. and D.K.T. contributed equally to this work.

Supporting Information Available

A list of locations of reference strains on the community genome array (SI_Table 1), fluorescence images showing the effect of different formamide concentrations on hybridization specificity of community genome arrays (SI_Figure 1), and a figure showing the detection sensitivity of CGA hybridization using labeled pure genomic DNA (SI_Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review March 31, 2004. Revised manuscript received July 14, 2004. Accepted August 13, 2004.

ES049508I