Development and evaluation of 50-mer oligonucleotide arrays for detecting microbial populations in Acid Mine Drainages and bioleaching systems

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Abstract

To effectively monitor microbial populations in acidic environments and bioleaching systems, a comprehensive 50-mer-based oligonucleotide microarray was developed based on most of the known genes associated with the acidophiles. This array contained 1,072 probes in which there were 571 related to 16S rRNA and 501 related to functional genes. The functional genes in the microarray were involved in carbon metabolism (158), nitrogen metabolism (72), sulfur metabolism (39), iron metabolism (68), DNA replication and repair (97), metal-resistance (27), membrane-relate gene (16), transposon (13) and IST sequence (11).Based on the results of microarray hybridizations, specificity tests with representative pure cultures indicated that the designed probes on the arrays appeared to be specific to their corresponding target genes. The detection limit was 5 ng of genomic DNA in the absence of background DNA. Strong linear relationships between the signal intensity and the target DNA were observed ($r^2 \approx 0.98$). Application of this type of the microarray to analyze the acidic environments and bioleaching systems demonstrated that the developed microarray appeared to be useful for profiling differences in microbial community structures of acidic environments and bioleaching systems. Our results indicate that this technology has potential as a specific, sensitive, and quantitative tool in revealing a comprehensive picture of the compositions of genes related with acidophilic microorganism and the microbial community in acidic environments and bioleaching systems, although more work is needed to improve.
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1. Introduction

The Acid Mine Drainage(AMD) are incidentally generated by the deep mining and surface(opencast) mining of metal ores and coal with the increase of surface area of sulfide ores exposed to air and water. The AMD resulted in the contamination of ground and/or surface water with high concentration of sulfate and toxic metals. Despite extremely acidity and metal enrichment, a diverse range of microorganisms populated AMD environments. These organisms can form a chemoautotrophically-based biosphere in the subsurface and their activity increase the rate of acid mine drainage (AMD) formation and be responsible for the bulk of AMD generated (Hallberg and Johnson, 2001; Baker and Banfield, 2003; Silverman and Ehrlich, 1964). Moreover, a better understanding of the structure and composition of microbial communities in the AMD and their responses and adaptations to environmental perturbations may enable the development of technology to prohibit the formation of this significant form of pollution and maintain or restore desirable ecosystem functions.

Beside accelerating the generation of AMD, these acidophilic organisms populated AMD environments can also be used in ore processing and are a source of novel biomolecules (especially

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enzymes) for industrial processes (Baker and Banfield, 2003). In bioleaching systems, acidophiles are present in high numbers and are important in bioleaching of copper, uranium and gold from low grade ores as well as microbial removal of pyretic sulphur from coal (Norris and Johnson, 1998). To date, the biology of leaching bacteria becomes more and more complex (Olson et al., 2003; Edwards and Rutenberg, 2001; Rimstidt and Vaughhan, 2003). Usually, the most important acidophilic microbes involved in the bioleaching of minerals are those that are responsible for producing the ferric iron and sulfuric acid required for the bioleaching reactions, such as *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*. However, the compositions and proportions of these important acidophilic microbes may vary depending on the mineral and the conditions under which bioleaching systems are operated. The variety of these acidophiles in bioleaching systems has an important influence on the process and efficiency of bioleaching. So, a better understanding of the structure and composition of microbial communities in the bioleaching systems and their responses and adaptations to the mineral and bioleaching conditions may contribute to improve the bioleaching processing so that increase the rate of bioleaching.

Classical microbial community analysis is limited by the unavoidable need for isolation of the microorganisms prior to their characterization. Furthermore, acidophilic chemolithotrophs are not easy to grow, especially in solid media. So, a variety of molecular biology techniques, such as fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and PCR-cloning, has been widely used. But those conventional molecular methods for assessing microbial community structure and activities are labor-intensive. Rapid, quantitative, and cost-effective tools that can be operated in field scale heterogeneous environments are needed for measuring and evaluating composition of microbial community in acidic environments and bioleaching systems. The microarray, including the functional gene arrays (FGAs), community genome arrays (CGAs), and phylogenetic oligonucleotide arrays (POAs), is a powerful genomic technology that is used to study biological processes and has been used successfully to analyze the environmental studies recently (Valinsky et al., 2002; Wu et al., 2001; Loy et al., 2002; Call et al., 2001; Zhou and Thompson, 2002; Zhou, 2003). However, there were no microarrays have been extended to especially study microbial communities in the extremely environments and systems of biotechnology. In contrast, the importance of the acidic environmental studies and bioleaching systems requires experimental tools that not only detect the presence or absence of particular groups of microorganisms but also provide quantitative data to help evaluate their biological activities.

In the present study, we developed comprehensive 50-mer olionucleotide microarrays containing 16S rRNA and functional genes involved in the activities of acidophiles to monitor the composition, structure, dynamics and functional activities of microbial community in AMD and bioleaching systems, our results demonstrated that the 50-mer microarrays developed offer a rapid, powerful, high-throughput tool for detecting microbial populations in AMD and bioleaching systems.

2. Materials and methods

2.1. Bacterial strains, samples, DNA extraction, and purification

Three species of important acidophiles used for the analysis of microarray were obtained from the ATCC (Manassas, VA, USA). *A. ferrooxidans* ATCC23270, a chemolithotrophic γ-Proteobacteria with the ability of iron and sulfur oxidization. *L. ferrooxidans* ATCC35992, a chemolithotrophic *Nitrospira* with the ability of iron oxidization. These two species were most frequent in AMD and widely used for bioleaching. The third species is *Acidiphilium acidophilum* ATCC27807, a heterotrophoic α-Proteobacteria with the ability of reduce iron. This species was widely found in AMDs and bioleaching systems. Moreover, the genomic DNA from E.coli was used for the evaluation of specificity of the microarray. The genomic DNAs from these strains were extracted using the DNeasy Tissue Kit (Qiagen).

The performance of microarray on the microbial community was evaluated using 3 samples (1 from AMD, 1 from bioleaching system and 1 from the weak acidic water) in Dexing copper mine. The Dexing copper mine, located in Jiangxi Province, China, is the largest open-cast copper mine in China and the world's third largest. It has been mined for copper since the Tang Dynasty, approximately 800 years ago with recent ore production over 100,000 tons day-1. A large quantity of acid mine drainage is produced through a combination of the discharge of metal sulfide waste and microbial activity. The NAE sample was acidic water which formed through natural shaping during the oxidative dissolution of sulfide minerals. Approximately ten years ago, an irrigation-type bioleaching operation was established to recover copper in the Dexing mine. The BLS water sample was justly taken from the operation system. Moreover, the CWAE water sample was derived from the DAWU River with the weak acidic pH. The water samples taken were filtered through a 0.2-μm nylon filter and the filter was immediately transferred to a jar and kept at −20 °C prior to molecular analysis. The bulk community DNA was extracted from 5 g of filters using a protocol described by Zhou et al. (1996). The crude DNA was purified by the minicolumn purification method.

2.2. Analysis of environmental variables

For water samples, we measured environmental variables using an inductively coupled plasma-atomic emission spectroscopy (ICP-AES; Baird Plasma Spectrovac PS-6 (N+1). The pH was determined during sampling.

2.3. PCR and fractionation of 16S rRNA, iro, cbbQ, gyrB and pufM genes

To evaluate the specificity of the 50-mer oligonuleotide array hybridization, 16s rRNA, *iro*, *cbbQ*, *gyrB* and *pufM* genes were amplified from the genomic DNA of pure cultures with the specific primer (Table 1). The primers for these genes were designed based on published genome sequences from the TIGR and the NCBI GenBank.

Table 1
The specific primer for 16s rRNA, cbbQ, iro, gyrB and pufM genes of the pure cultures

Genes	Forward primers	Reverse primers
The cbbQ gene	5′-ATGAACGACCAGTTGACTG-3′	5′-TCAGAAGTACGTGGTGAC-3′
The iro gene	5′-CTC TGACCGGCGAATCGG G-3′	5′-CCAACCGCATCCGCATATCTT G-3′
The gyrB gene	5′-GAAGTCATCATGACCGTTCTGCA YGCNGGNGGNA ARTTYGA-3′ (Yamamoto and Harayama, 1995)	5′-AGC AGG GTA CGG ATG TGC GAG CCR TCN ACR TCN GCR TCN GTC AT-3′ (Yamamoto and Harayama, 1995)
The pufM gene	5′-ATG GCC GAA TAC CAG AAC ATC-3′	5′-CCA TGG TCC AGC GCC AGA AC-3′
The 16s rRNA	5′-GGGCGGWGTGTACAAGGC-3′ (Marchesi et al., 1998)	5′-CAGGCCTAACACATGCAAGTC-3′ (Marchesi et al., 1998)

PCR amplification was carried out using about 100 ng of DNA per μl, 1×PCR buffer (Perkin Elmer, Norwalk, Conn.), a 2 mM concentration of each of the four deoxynucleoside triphosphates, 2.5 mM MgCl$_2$, 5 pM each of the forward and reverse primers, and 0.1 U of Taq DNA polymerase per μl in a total reaction volume of 25 μl. Optimum temperature and cycling parameters were determined to be an initial denaturation step of 94 °C for 5 min, followed by 30 cycles of 94 °C (45 s), 57 °C (45 s), and 72 °C (1.5 min), with a final extension step of 72 °C for 7 min. The size and annotation of the amplified DNA products were about 1.3 kb from the 16s rRNA, about 0.7 kb from the *iro* gene, about 0.8 kb from the *pufM* gene, about 0.8 kb from the *cbbQ* gene and about 1.26 kb from the *gyrB* gene. PCR products were analyzed for correct size and the presence of a single product by agarose gel electrophoresis and ethidium bromide staining. The PCR products were then purified using the QIAquick PCR Purification kit (Qiagen, Chatsworth, Calif.) according to the manufacturer's instructions.

2.4. Microarray design and construction

The microarray was designed using the sequence information from GenBank. The oligonucleotide microarray was constructed using 16S rRNA sequences and a diverse set of functional genes involved in carbon, nitrogen, sulfur, iron metabolisms, metal resistance and other. Some sequences acquired during the construction of array came from acidophilic microorganisms or clones from AMD. Moreover, human genes were also used as negative or quantitative controls.

The names of the acidophilic microorganisms known to be in the acidic environments (according to the reference, Baker and Banfield, 2003) were used as keywords for identifying appropriate genes in the GenBank database through the national Center for Biotechnology Information Website (http://www.ncbi.nlm.nih.gov/). The gene accession numbers for the genes of interest were retrieved and used for automatically downloading gene sequences. On the choice of 16S rRNA sequences, we used all acquired sequences of acidophile from the GenBank for constructing the microarray. In terms of the functional genes, we selected some key genes involved in the important metabolic pathway of acidophilic microorganism existed at acid mine drainage, such as some genes related to nitrogen metabolism and iron metabolism of *Leptospirillum*; some genes related to sulphur metabolism and iron metabolism of *A. ferrooxidans*; some genes related to sulphur metabolism of *Acidiphilium*; some gene related to iron metabolism of *Ferroplasma*, the majority of genes relate to metal resistance, some genes related to carbon metabolism and

DNA replication and repair of acidophile, and so on. However, as for some acidophile with a few of functional genes detected in NCBI database, all functional genes of them were selected.

The 50-mer oligonucleotide probes were designed using a modified version of the software, PRIMEGENS (http://compbio.ornl.gov/structure/primegens/) (Xu et al., 2002). In brief, one primer was selected by considering GC content, melting temperature and self-complementarity. Outputs of the designed probes were imported into Microsoft Excel, and a pivot table was constructed containing the sequence information of each probe. In summary, a total of 1071 oligonucleotide probes consisting of 571 16S rRNA and 501 functional genes were designed. Each gene was spotted on the arrays in four replicates.

Table 2
The analysis of environmental variables at three sites

| (mg/L)	NAE	BLS	CWAE
Sn	0.1	0.2	0.09
Zn	1.3	3.2	0.21
Sb	0.03	0.04	0.26
Pb	0.4	0.4	0.2
B	0.5	0.6	0.01
Mn	17.1	46.4	6.79
Mg	351.4	1395	166
Al	783.7	1013	29.67
Nb	0	0	0.01
V	0.2	0.2	0.13
S	3554	3379	481
As	0.02	0.02	0.22
Mo	0.07	0.09	0.04
P	11.6	11.1	0.48
Bi	0.02	0.03	0
Ni	2.7	4	0.24
Ta	0.05	0.04	0.01
Ga	0.3	0.4	0.02
Co	3.3	4.1	0.24
Fe	1907	1677	0.21
Cr	0.4	0.4	0.04
Si	35	51.4	7.78
Na	0.5	1.1	0.02
Be	0.04	0.07	0
Ca	183.2	288.7	246.31
Cu	18.7	71.5	2.34
La	0.2	0.3	0
Sc	0.2	0.2	0.01
K	0	0	8.37
Ag	0	0	0.05
Ti	0.01	0.03	0.02
Zr	0.05	0.04	0
pH	2	2	5
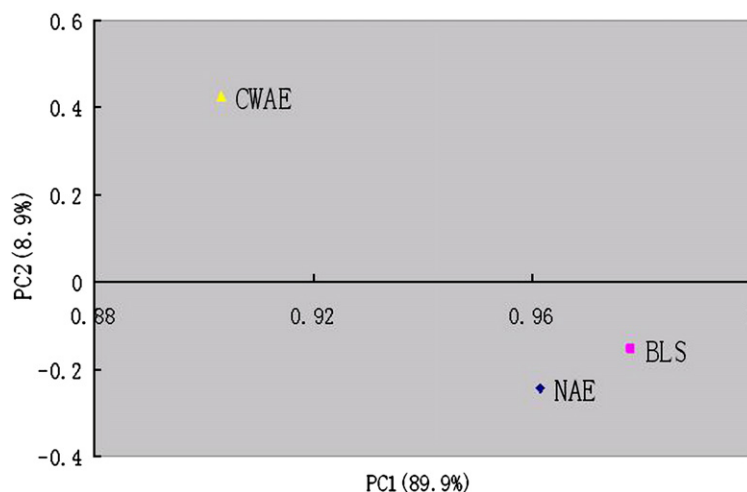
Fig. 1. Principal-component analysis (PCA) based on the environmental variables.

The 50-mer oligonucleotides were synthesized by the Sunbiotech Company (Beijing, China). The oligonucleotides were diluted to a final concentration of 50 pmol/µl in 50% dimethyl sulfoxide. The oligonucleotide probes were printed on Superamine glass slides (Cornning, Inc.) using an OmniGrid Accent Arraying System (Genomic Solutions, USA) at 55 to 58% relative humidity (Hughes et al., 2001). Each probe set was printed in 6 duplicates on a different part of the slide. The slides were then air dried at room temperature for overnight. Finally, the slides were cross-linked using an ultraviolet Stratalinker 1800 (Stratagene, La, Jolla, CA, USA) by exposing the slides to 600 mJ doses of UV irradiation prior to use.

2.4.1. Preparation of fluorescently labeled DNA

A method was employed to fluorescently label DNA. For genomic DNA labeling, 0.2–750 ng genomic DNA was denatured by boiling for 2 min and immediately chilled on ice. The labeling reaction mixture contained denatured genomic DNA, 1.5 µg of random hexamers (Gibco BRL, Gaithersburg, Md.); 1 × EcoPol buffer (New England Biolabs, Beverly, Mass.); 50 µM dATP, dTTP, and dGTP; 20 µM dCTP; 10 µM Cy3-dCTP (Amersham Pharmacia Biotech, Piscataway, N.J.); 2.5 mM dithiothreitol; and 10 U of the large Klenow fragment of DNA

polymerase I (Invitrogen, USA). The reaction mixture was incubated at 37 °C for 3 h, heat treated in a 100 °C heating block for 3 min, and chilled on ice. Labeled target DNA was purified with a QIAquick PCR purification column according to the manufacturer's instructions. Finally, the labeled DNA was resuspended in an appropriate volume of distilled water.

For 16S rRNA, *iro*, *cbbQ*, *gyrB* and *pufM* genes, these genes were PCR amplified using gene-specific primers. The PCR products were purified using the QIAquick PCR purification column, mixed together and labeled by the above method of genomic DNA labeling.

Community genomic DNA was labeled by above method of genomic DNA labeling. Differently, the 5 µg community genomic DNA was used for every labeling.

2.4.2. Microarray hybridization

All hybridizations were carried out in triplicate. The hybridization solution contained 3 × standard saline citrate (SSC) (1 × SSC contained 150 mM NaCl and 15 mM trisodium citrate), 1 µg of unlabeled herring sperm DNA (Promega, Madison, WI, USA), 0.30% SDS, and 50% formamide in a total volume of 15 µL. To avoid bubbles, the hybridization solution was deposited directly onto the microarrays. The microarray (probe-side

Table 3
Summary of probes designed

Function	No. of genes	Total no. of probes synthesized	Representative genes for overall diversity evaluation
Carbon metabolism	191	158	CbbQ gene, pufM gene
Nitrogen metabolism	82	72	Glutamine syntheyase gene
Sulfur metabolism	49	39	Sulfide:quinone oxidoreductase (sqr) gene
Iron metabolism	93	68	Fe(II)-oxidizing enzyme (*iro*)
Inheritance	110	97	*gyr*B gene
Metal-resistance	39	27	Mercuric ion reductase gene
Membrane-relate gene	19	16	*aha*I gene
Transposon	19	13	ISAfe 1 transposase
IST sequence	14	11	internal transcribed spacer 1 (ITSI)
16s rDNA	2440	571	16s rDNA from *Acidithiobacillus ferrooxidans*
Functional genes (total)	616	501	
In all	3056	1072	

Table 4
Number of probes design from different acidophilic microorganisms

Microorganisms	16S rRNA	Functional genes
Actinobacteria	10	6
Nitrospira	11	52
σ-Proteobacteria	14	31
β-Proteobacteria	2	1
γ-Proteobacteria	30	209
δ-Proteobacteria	14	29
φ-Proteobacteria	3	3
Acidobacteria	377	9
Fimicutes	88	147
Iron mountain clones	22	14
Total	571	501

down) was placed on the coverslip (22×22 mm) and then into a hybridization chamber (Corning® hybridization chamber; Corning, Acton, MA, USA). Fifteen microliters of $3 \times$ SSC were dispensed into the hydration wells on each side of the microarray slide, and hybridization was carried out for 12–15 h. The hybridization was carried out at 50 °C. Following hybridization, the arrays were washed with $1 \times$ SSC, 0.2% SDS and $0.1 \times$ SSC, and 0.2% SDS for 5 min each at ambient temperature and then with $0.1 \times$ SSC for 30 s (ambient temperature) prior to being air dried in the dark.

For evaluate the specificity of 50-mer functional gene array hybridization, same concentrations of PCR products from pure cultures containing 16S rRNA, *iro*, *gyrB*, *cbbQ* and *pufM* genes were mixed together, directly labeled with cy3 using a random priming method and then hybridized on the microarrays as described above.

2.4.3. Image processing and data analysis

The microarrays were scanned with a GenePix 4000B Array Scanner (AXON instruments, Inc, USA). The emitted fluorescent signal was detected by a photomultiplier tube (PMT) at

570 nm (Cy3). The scanned images were saved as 16-bit TIFF files, and each spot was quantified using GenePix Pro 6.0 software (AXON instruments, Inc, USA). 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. The mean signal intensity was determined for each spot. The local background signal was subtracted automatically from the hybridization signal of each separate spot also computed for each spot to discriminate true signals from noise. The SNR ratio was also calculated based on the following formula (Verdnick et al., 2002): SNR = (signal intensity-background)/ standard deviation of background, in which the background measurement refers to the local spot background intensity and the standard deviation of background was calculated across all pixels measured by the ImaGene software. The SNRs from four replicate data sets were then averaged to represent the SNR for a particular probe. A commonly accepted criterion for the minimum signal (threshold) that can be accurately quantified is SNR ≥ 2 (Loy et al., 2002; Franke-Whittle et al., 2005). Spots that appeared to be lower than the threshold value were removed from the data set.

3. Statistical methods

Principal-component analysis (PCA) was performed by using the SYSTAT statistical computing package (version 13.0; SPSS, Inc., Chicago, Ill) for each sampling site. PCA simultaneously considers many correlated variables and identifies the lowest number to accurately represent the structure of the data. These variables are then linearly combined with the eigenvectors of the correlation matrix to generate a principal component axis. In the present study, PCA was used to group or separate stations, which were similar or different, based on the geochemical parameters.

The relationships of the genes detected in various environmental samples were determined using hierarchical cluster analysis (CLUSTER) and visualized with TREEVIEW (Eisen et al., 1998).
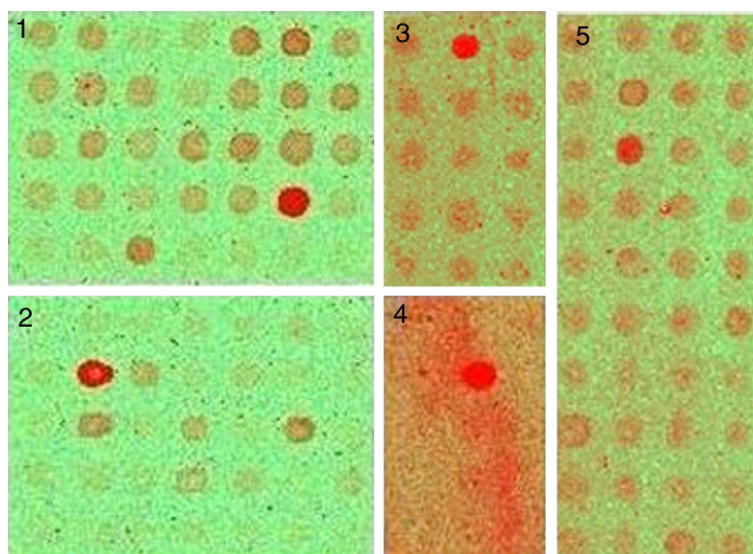

Fig. 2. Images showing hybridization specificity. The number 1, 2, 3, 4 and 5 were respectively representatively of pufM, cbbQ, iro, qyrB genes and 16s rDNA. The target DNAs were labeled with Cy3 and hybridized with the microarrays containing 1072 probes at 50 °C. Specific hybridizations are shown in the red colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Results

4.1. Environmetnal variables of three water samples and PCA analysis

The environmental variables shown in Table 2 were differed in three water samples from the Dexing copper mine. Altogether, the contents of elements contained in the NAE and BLS samples were higher than that of the CWAE. The contents of elements in the BLS sample were highest among all three samples. At the CWAE sample, the concentration of Fe and S was only 0.21 and 481 mg/L, respectively. This was far lower than that of the BLS sample (1677 and 3379 mg/L, respectively) and NAE sample (1907 and 3554 mg/L, respectively). Moreover, as far as pH value, the CWAE sample was highest (pH 5.0) among three samples, compared with the BLS and NAE samples (pH 2.0).

The results of the PCA of environmental variables, which represented 98.8% of the total variance, revealed a profile among the three samples examined. BLS and NAE samples were close together and were distant from CWAB sample. Thus, the geochemical properties at BLS and NAE samples were similar but different from those at the CWAB sample. The overall profiles (Fig. 1) showed that the environmental variables at the NAE sample were more similar with the NAE sample than that of the CWAE sample.

4.1.1. Probe design

Altogether, 1071 probes were designed based on 3056 sequences downloaded from NCBI database. A probe was designed for any sequence with <85% nucleic acid sequence identity to any downloaded sequences. The target genes of these probe are involved in carbon metabolism (158), nitrogen metabolism (72), sulfur metabolism (39), iron metabolism (68), DNA replication and repair (97), metal-resistance (27), membrane-relate gene (16), transposon (13), IST sequence (11) and 16s rRNA (571) (Table 3). Almost all of the probes are affiliated with 35 species of acidophilic microorganisms and 9 of related subclass, in addition of some iron mountain clones, detected in the natural acid environment and bioleaching systems. Number of probes designed from different acidophilic microorganisms was listed in Table 4.

4.2. Specificity evaluation with different genes and genomic DNAs from pure cultures

To determine the specificity of microarray hybridization, 50-mer olionucleotide microarray across 1072 probes consisting of 16s rRNA, Fe(II)-oxidizing enzyme gene (*iro*), the gyrB gene
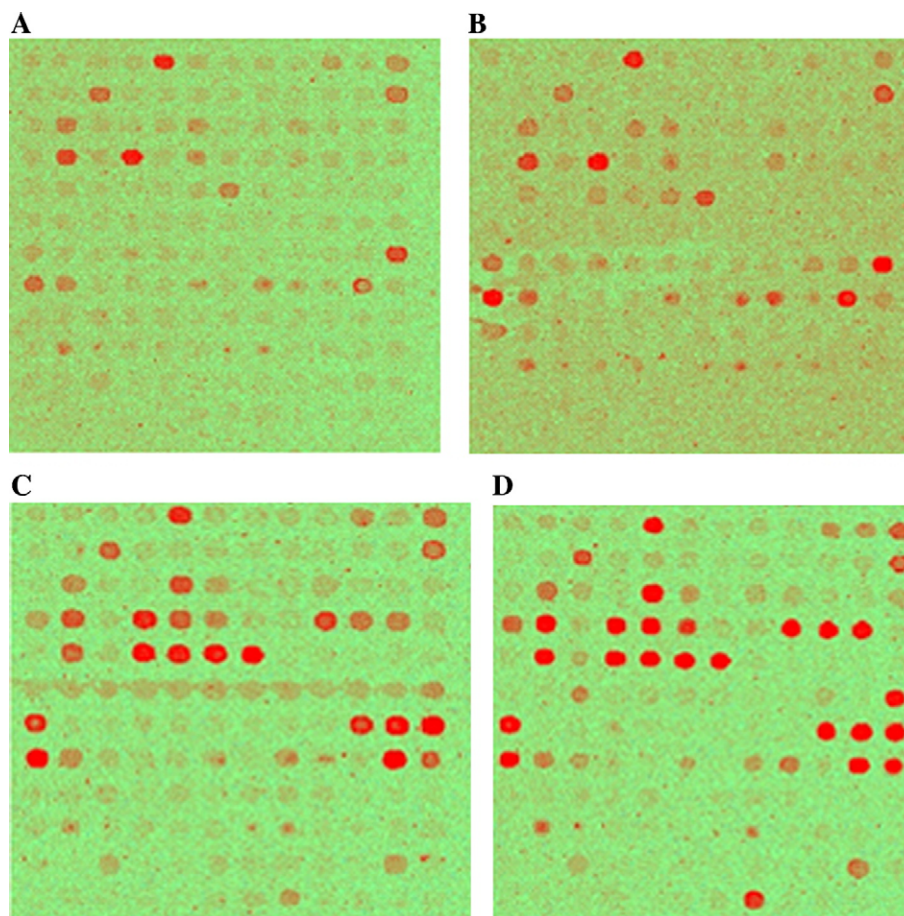

Fig. 3. Array hybridization images showing the detection sensitivity with labeled pure genomic DNA. Genomic DNA from a pure culture of *A. ferrooxidans* was labeled with Cy3 using the random primer labeling method. The target DNA was hybridized to the microarrays at total concentrations of 0.2 ng (A), 1 ng (B), 5 ng (C) and 25 ng (D).

encodes the β-subunit of DNA gyrase B subunit (*gyrB*), M subunit of photosynthetic reaction center complex (*pufM*) and the putative post-translational RubisCO activator CbbQ (*cbbQ*) gene were used. These genes were amplification by PCR method and were purified using the QIAquick PCR purification column. All genes were mixed together and hybridized to the oligonucleotide microarrays at 50 °C to determine the specificity of individual probes. When the labeled target genes from five different functional gene groups were combined and hybridized to the oligonucleotide arrays, strong hybridization was observed with their corresponding probes sequences from the same strain (Fig. 2). As expected, no cross-hybridization with the nontarget genes was observed. These results indicated that the 50-mer olionucleotide microarray appear to be specific to their corresponding target genes.

The specificity of hybridization with the 50-mer oligonucleotide array across 1072 probes was also determined using the genomic DNA of *A. ferrooxidans* ATCC23270 labeled with Cy3 as described above (Materials and methods). Probexs corresponding to most known genes reported in the strain *A. ferrooxidans* had strong hybridization signals to the responding genomic DNA of *A. ferrooxidans*. Altogether, 155 of 161 probes from the *A. ferrooxidans* had SNRs of ≥2. In contrast to the data for *A. ferrooxidans*, the probes from the other species showed the lower cross-hybridization. In addition, the ferredoxin gene (Accession number, NC_005023) from the strain *A.ferrooxidans* 23270 had the most strong cross-hybridization (SNR=47.09).The results suggested that the designed probes were specific for their corresponding target genes from the genomes examined. Moreover, genomic DNA from E.coli without any probes in the microarray was used for evaluating the hybridization specificity. When the *E. coli* genomic DNA mixed with the PCR product of 16S rRNA, *iro*, *cbbQ*, *gyrB* and *pufM* genes hybridized with the microarray, only these probes corresponding to the four functional genes showed the strong cross-hybridization on the microarray.

4.2.1. Detection sensitivity of 50-mer oligonucleotide arrays hybridization

The detection sensitivity of hybridization with the array (1072 genes) was determined using genomic DNA from pure cultures *A. ferrooxidans* (Fig. 3). Genomic DNA (at concentrations of 0.2, 1, 5, 25, 50, 100, 250, 500 and 750 ng) from *A. ferrooxidans* 23270 was randomly labeled with Cy3 as described. Strong hybridization signals were observed with 25−750 ng of DNA for all genes corresponding to the *A. ferrooxidans* 23270 (Fig. 3D), whereas the fluorescence intensity was measurable for the most genes with 5 ng of genomic DNA although little genes had weaker hybridization signals (Fig. 3C). Little or no hybridization was observed at 0.2 and 1 ng of genomic DNA for most genes (Fig. 3A,B). Therefore, the detection limit with randomly labeled pure genomic DNA under these hybridization conditions was estimated to be approximately 5 ng.

4.2.2. Microarray hybridization-based quantization

To evaluate whether microarray hybridization can be used as a quantitative tool, the relationship between target DNA concentration and hybridization signal was examined. Genomic DNA from a pure culture of *A. ferrooxidans* 23270 was fluorescently labeled with Cy3 as described and hybridized with the microarray at total concentrations ranging from 0.2 to 750 ng. The fluorescence intensities obtained at each DNA concentration (three independent microarrays with four replicates on each slide) were averaged, and the log of the concentration was compared to the corresponding log value of the mean fluorescence intensity. For the Fe(II)-oxidizing enzyme gene (*iro*), strong linear relationships were observed between the signal
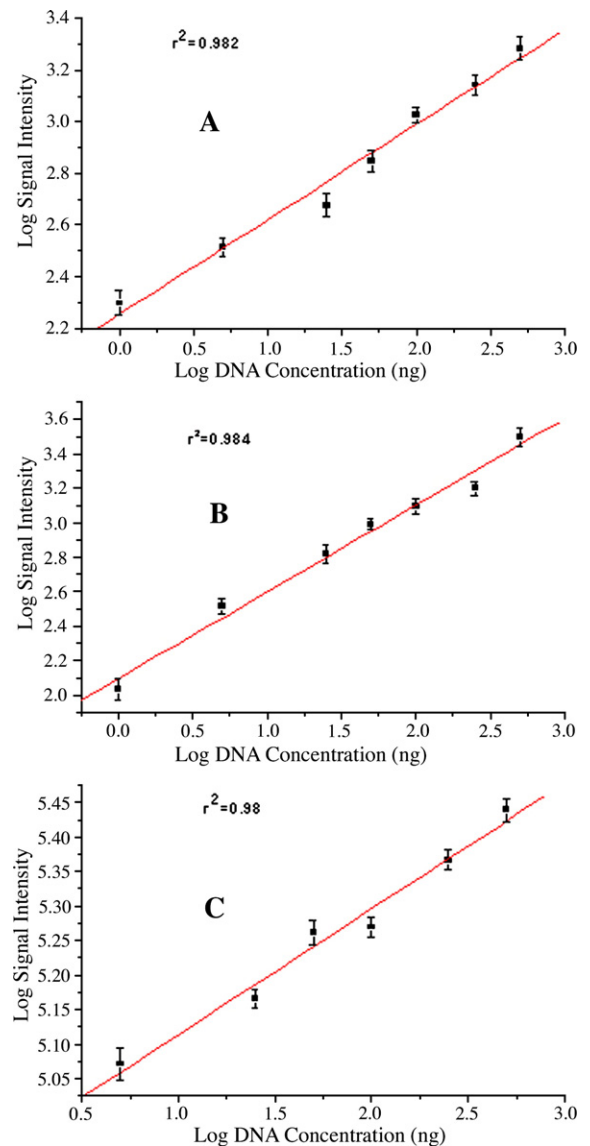

Fig. 4. Evaluation of quantitative potential of 50-mer FGA-based hybridization. The log ratios of hybridization signals between the target genes were calculated and plotted against the log of the concentration of genomic DNA. Quantitative relationship of 50-mer FGA-based hybridization with pure genomic DNA. Genomic DNA of *A. ferrooxidans* strain ATCC 23270 was serially diluted in 1×Tris–EDTA buffer, and the diluted genomic DNAs at concentrations of 0.2 to 750 ng were labeled with Cy3 using a random-primer labeling method. The labeled DNAs were hybridized with the microarrays. The quantitative relationship shown is that of the hybridization signals from the gene for (A) Fe(II)-oxidizing enzyme gene (iro) (Accession number, X57324), (B) 16s rRNA gene of *A. ferrooxidans* (Accession number, AJ621559) and (C) the correlation between the total signal intensity from *A. ferrooxidans* and DNA concentrations.
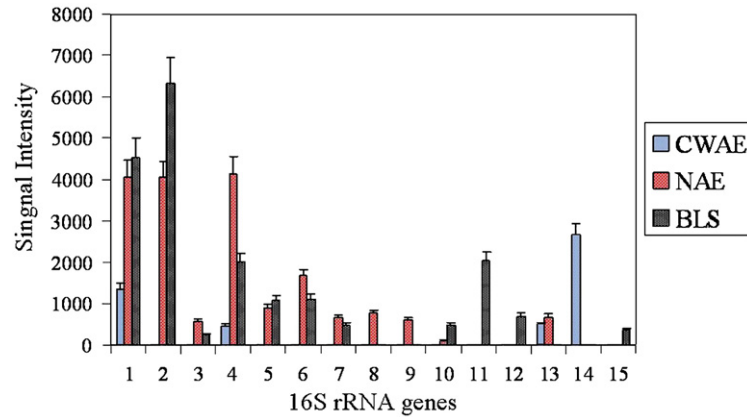
Fig. 5. The normalized distribution of signal intensity levels for important bioleaching bacterium 16s rRNA genes as determined by microarray-based analysis of community DNA from CWAB, NAB and BLS. The data represents the mean values from twelve replicates after subtracting background hybridization signals. Bars represent the major important function genes. Bar 1 represent *A. ferrooxidans*, bar 2 represent *Leptospirillum* sp., bar 3 represent *A. thiooxidans*, bar 4 represent *Sulfobacillus* sp., bar 5 represent *Acidiphilum* spp., bar 6 represent *Holoplaga* sp., bar 7 represent *Metallosphaera* sp., bar 8 represent *Alicyclobacillus* spp., bar 9 represent *Ferroplasma* spp., bar 10 represent *Thiomonas* sp., bar 11 represent *A. tenuissima*, bar 12 represent *Acidianus* sp., bar 13 represent *A. naeslundii*, bar 14 represent *Desulufosarcina variabilis*, bar 15 represent *Thermoolasmataceae archeon*.

intensity and target DNA concentrations from 1 to 500 ng (r^2=0.982) (Fig. 4A). Significant correlations between the signal intensity and DNA concentrations from 1 to 500 ng were also observed for 16s rRNA of *A. ferrooxidans* (r^2=0.984) (Fig. 4B). In addition, significant correlations between the total signal intensity from *Acidtithiobacillus ferrooxidans* and DNA concentrations ranging from 5 to 500 ng were also observed (r^2=0.98) (Fig. 4C). The results suggested that the microarray hybridization is quantitative for pure bacterial cultures within a limited range of DNA concentration (Fig. 4).

4.3. Analysis of microbial community composition in acidic environmental samples applying the 50-mer oligonucleotide arrays

The developed microarray could potentially be used as a generic profiling tool that would reveal differences among various microbial communities. To evaluate such potential, bulk community DNAs from the natural acidic bio-niche (NAE) (AMD sample) and the bioleaching system (BLS) were acquired from the acidic water (NAE was the natural bio-niche with pH 2.0; BLS was the bioleaching system with pH 2.5). For comparison, DNA from a weak acidic water sample − CWAE with higher pH 5.0 was used as a reference. Five micrograms of the purified bulk community DNA from the three samples was directly labeled with Cy3 using random-primer labeling. The Cy3-labeled DNAs were hybridized with the microarrays. All spots with SNRs of ≥2 were considered positive signals. Overall, the numbers of 16S rRNA probes with statistically significant positive signals were 80 (37 in the BLS sample, 42 in the NAE sample and 23 in the CWAE sample). These 16S rRNA probes were mainly related to 15 different genus or species, including *A. ferrooxidans*, *Leptospirillum* sp., *Acidithiobacillus thiooxidans*, *Acidiphilum* spp., *Sulfobacillus* sp., *Holophaga* sp., *Metallosphaera* sp., *Alicyclobacillus herbarius*, *Ferroplasma* spp., *Thiomonas* sp., *Alicyclobacillus* spp., *Alternaria tenuissima*, *Thermoplasmataceae archaeon*, *Desulfosarcina variabilis* and *Acidianus* sp. (Fig. 5). However, the composition and richness as determined by microarray-based analysis of microbial community was different in all three
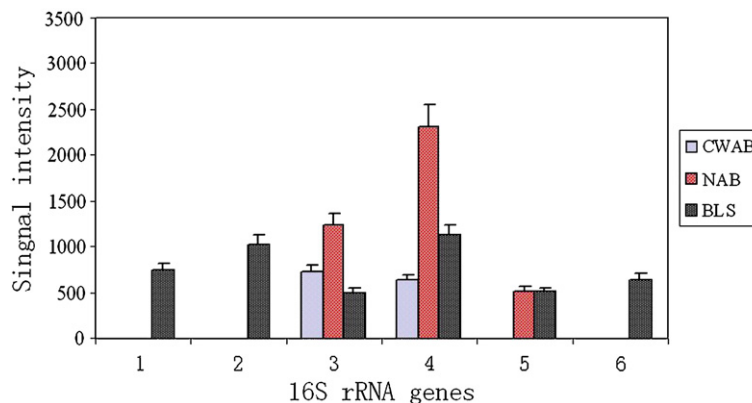

Fig. 6. The normalized distribution of signal intensity levels for 16s genes of different *A. Ferrooxidans* strains as determined by microarray-based analysis of community DNA from CWAB, NAB and BLS. Bar 1 represents strain Tf 49, bar 2 represents strain ATCC 19859, bar 3 represents strian DSm 2392, bar 4 represent strains TFU18089, bar 5 represents an unmarked strain and 6 represents strain SSP.
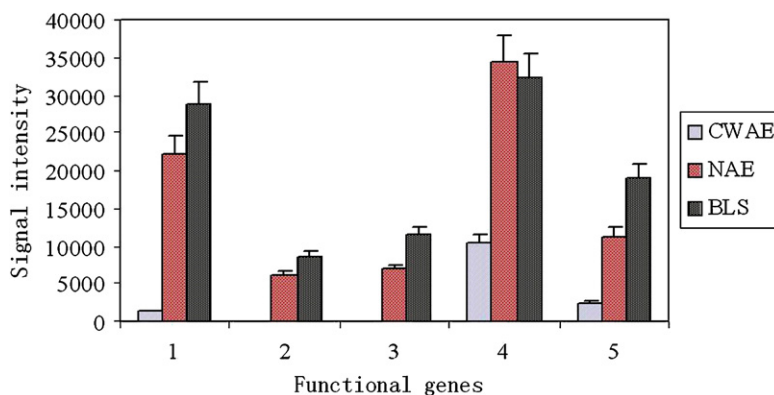
Fig. 7. The normalized distribution of signal intensity levels for key functional genes as determined by microarray-based analysis of community DNA from CWAB, NAB and BLS. Bar represented the key important function genes. Bar 1 represented the genes related to iron metabolism, bar 2 represent the genes related to sulfur metabolism, bar 3 represented the genes related to metal resistance, bar 4 represented the genes related to carbon metabolism, bar 5 represented the genes related to nitrogen metabolism.

sites (Fig. 5). For example, *A. ferrooxidans*, *Leptospirillum* sp. and *sulfobacillus* sp. were dominant in NAE and BLS samples and the signal intensity levels was far higher than that of the CWAE sample. However, there was a distinctly heterogeneity for these dominant genus or species at the NAE and BLS samples. For example, as far as *Acidithiobacillus ferrooxdians*, there was higher heterogeneity of *A. ferrooxidans* strains at the BLS sample compared with the NAE sample. These *A. ferrooxdians* strains at the BLS sample were affiliated with Tf-49, ATCC19859, DSM 2392, TFU18089, SSP and unmarked strains, respectively (Fig. 6). Of all *A. ferrooxdians* strains at the BLS sample, the stains ATCC19859 and TFU18089 showed the rather stronger hybridization signal intensity. By compared with the BLS sample, only three different strains (DSM 2392, TFU18089, and an unmarked strain) were detected at the NAE sample and the strain TFU18089 showed the much strongest hybridization signal intensity. Furthermore, as far as the distribution of *Leptospirillum* sp and *A. ferrooxidans*., richness of BLS sample was higher than that of the sample NAE. Some

bacteria, such as *A. tenuissima*, *T. archaeon* and *Acidianus* sp., were only detected at the BLS sample. Another bacterium related to *Ferroplasma* spp. and *Alicyclobacillus* sp. only occurred at the NAE sample. On the other hand, only *A. ferrooxidans*, *Actinomyces naeslundii*, *D. variabilis* and *Sulfobacillus* sp. were discovered in the CWAE sample and the other bacteria were not detected in this site (Fig. 5).

4.4. Analysis of functional gene applying the 50-mer oligonucleotide arrays

A total of 150 functional gene probes mainly involved in nitrogen metabolism, carbon metabolism, sulfur metabolism, iron metabolism and metal-resistance produced significant positive signals ($SNR \geq 2$) in all three samples. The number of detected functional gene probes was 35, 118 and 116 in CWAE, NAE and BLS samples, respectively. These functional genes from CWAE sample clearly displayed lower abundance relative to the other two environmental samples. As shown in
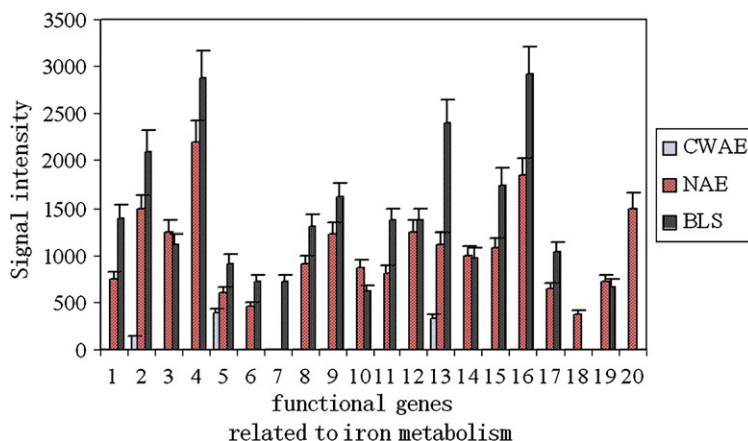

Fig. 8. The normalized distribution of signal intensity levels for functional genes related to iron metabolism as determined by microarray-based analysis of community DNA from CWAB, NAB and BLS. Bar represented the key function genes. Bar 1–20 represented the iro gene, Fe(II)-oxidizing enzyme gene, the rustA gene, the petA2 gene, the two different petB2 genes (Bar 5 and 6), the petC gene, the cycA gene , the rus gene, the ferredoxin gene, 2Fe–2S ferredoxin gene, the Ferredoxin gene, the Putative ferredoxin gene, the Polyferredoxin gene, Ferric uptake regulator protein, the Fer1 gene, the cytochrome oxidase gene, the doxC gene, the putative electron transfer protein, and the Fe–S oxidoreductase gene, respectively.
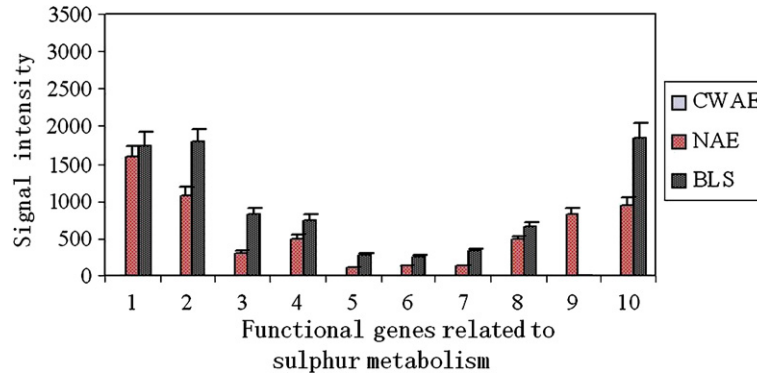
Fig. 9. The normalized distribution of signal intensity levels for functional genes related to sulphur metabolism as determined by microarray-based analysis of community DNA from CWAB, NAB and BLS. Bar represented the key function genes. Bar 1–10 represented the sulfide:quinone oxidoreductase (sqr) gene, the two different cydA genes (Bar 2 and 3), the putative thiosulfate sulfur-transferase gene, the three different thioredoxins genes, the sulfur reductase molybdopterin subunit, the dissimilatory sulphite reductase subunit A, and the sulphite reductase gene, respectively.

Fig. 7, microarray analysis of the functional genes indicated that the NAE and BLS samples were distinctly different from the CWAE sample. The NAE and BLS samples resulted in higher signal intensity of functional genes related to carbon metabolism, nitrogen metabolism, iron metabolism and metal-resistance. However, the hybridization signal intensity of these functional genes was significantly reduced when the extract DNA of the CWAE was used in the hybridization process (Fig. 7).

As shown in Fig. 8, microarray analysis of the composition of NAE and BLS samples indicated that these genes related to iron metabolism were abundant and extensive diverse relative to the CWAE sample. For example, the gene related to ferrous oxidizing enzyme (Bar 2) originally found in *A. ferrooxidans* was the most abundant in the BLS sample, and second most abundant in the NAE sample. The ferredoxin gene of *Leptospirillum* sp. (Bar 12) was observed in BLS and NAE samples but not detected at the CWAE sample. However, some genes related to electronic transport of iron metabolism were only detected at certain samples. For example, petC of *A. ferrooxidans* (Bar 7) was only observed at the BLS sample but the doxC gene (Bar 18) was only found at the NAE sample. Altogether, at the CWAE sample, there were only four functional genes of iron metabolism detected compared with the NAE (19) and BLS (18). Some genes of iron metabolism originally from the *A. ferrooxidans* and *Leptospirillum* sp. at the NAE and BLS samples showed the stronger signals hybridization intensity relative to the CWAE sample. Therefore, *A. ferrooxidans*-type and *Leptospirillum*-type microorganisms may be the most active microbial groups involved in the iron metabolic pathway. Moreover, similar to what is observed in the distribution of *Leptospirillum* sp. and *A. ferrooxidans*, the most of functional genes of iron metabolism showed the highest hybridization signal intensity with labeled DNA from the BLS samples.

These genes related to sulphur metabolic pathway from *A. ferrooxdians* (Bar1-4) were abundant in the BLS and NAE samples (Fig. 9). Interestingly, there were not any genes related to sulphur metabolic pathway in the CWAE sample. Compared to that of *A. ferrooxdians*, the genes associated with sulphur metabolic pathway of *Leptospirillum* sp. (Bar 5–7) showed very lower signal hybridization intensity.

4.5. Cluster analysis of 16S rRNA and functional genes from three environmental samples

16S rRNA and functional genes derived from three environmental samples were analyzed by cluster analysis using CLUSTER and TREEVIEW software (Figs. 10 and 11). Clustering analysis based on 16S rRNA revealed that the BLS sample was more closely clustered with the NAE sample than the CWAE sample (Fig. 10), suggesting that the microbial community structure in the BLS sample was more similar to that the NAE sample than that of the CWAE sample. Moreover, functional genes detected in the BLS sample also more closely clustered with the NAE sample than with the CWAE sample (Fig. 11), suggesting that the community structure in terms of components of the functional genes was also most similar to that of the CWAE sample. In the cluster analysis of functional analysis, some genes related to Fe and S metabolisms clustered together and showed strong hybridization signals in the NAE and BLS sample. For example, the genes coded with ferredoxin, Fe(II)-oxidizing enzyme, ferric uptake regulator, and Fe–S oxidoreductase were clustered together. However, these genes can not been found any meaningful hybridization signals in the CWAE sample.

5. Discussion

Over the past decade, DNA microarray technology (also known as DNA microchip, DNA chip, or gene chip) has catapulted into the limelight, promising to accelerate genetic and microbial analysis (Emily et al., 2003). This technology provides tremendous potential for microbial community analysis, pathogen detection and process monitoring in both basic and applied environmental sciences (Guschin et al., 1997; Cho and Tiedje, 2002; Bodrossy et al., 2003). In the present paper, we constructed a microarray containing 1071 probes from the acidophilic microorganisms and showed it could be used to analyze the microbial community and microbial activities with specificity, sensitivity, and quantitative characterization.

Specificity is one of the most critical parameters for any technique used to detect and monitor microorganisms in natural
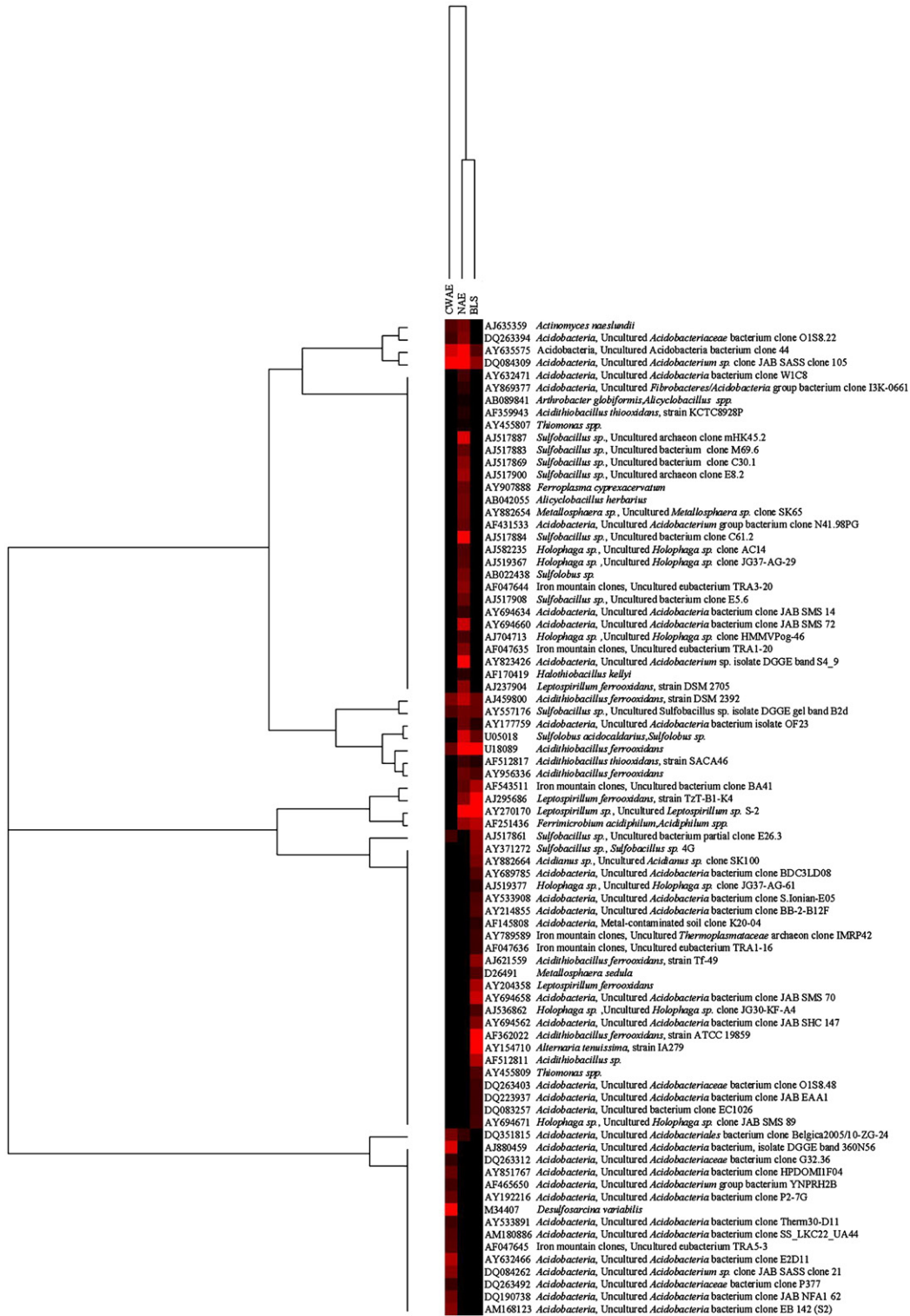
Fig. 10. Hierarchical cluster analysis of community relationships based on hybridization signal intensity ratios for 16S rRNA genes showing SNRs of ≥2, The figure was generated using hierarchical cluster analysis (CLUSTER) and visualized with TREEVIEW. The hybridization signals of genomic DNAs from each of the three water samples were divided by the hybridization signals from by the hybridization signals from corresponding genomic DNA. Black represents no detectable difference in the hybridization signal, while red represents a significant hybridization signal. The columns correspond to the hybridization patterns obtained with Cy3-labeled genomic DNA from the following water samples: BLS, NAE and CWAE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

environments (Wu et al., 2001). Short oligonucleotide probes generally offer greater specificity than cDNA or PCR products to distinguish single-nucleotide-polymorphism (SNP). Howev-

er, it is noted that short oligonucleotide probes often have poor hybridization properties and the resulting signal intensities are often much lower than for longer oligonucleotides(>30 nt)

Fig. 11. Hierarchicical cluster analysis of community relationships based on hybridization signal intensity ratios for function genes showing SNRs of ⩾2, The figure was generated using hierarchical cluster analysis (CLUSTER) and visualized with TREEVIEW. The hybridization signals of genomic DNAs from each of the three water samples were divided by the hybridization signals from by the hybridization signals from corresponding genomic DNA. Black represents no detectable difference in the hybridization signal, while red represents a significant hybridization signal. The columns correspond to the hybridization patterns obtained with Cy3-labeled genomic DNA from the following water samples: BLS, NAB and CWAB. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Emily et al., 2003; Guo et al., 1994; Relogio et al., 2002). Unfortunately, as the length of oligonucleotide probes increase, specificity is lost. Therefore, in this study, we used the 50-mer

olionucleotide probes to construct the microarray. The results showed the microarray with the 50-mer olionucleotide probes had the strong specificity. For example, when the gyrB gene

from *Leptospirilum ferriphilium*, pufM gene from *Acidiphilium acidophilum* ATCC27807 and cbbQ gene from *A. ferrooxidans* ATCC23270 were researched, strong hybridization was observed with their corresponding probes sequences from the same strain (Fig. 2). The results by Wu et al also suggested that the use of 50-mer oligonucleotide probes hybridized at 50 °C was considered to represent strong specificity (Wu et al., 2001).

Sensitivity is another critical parameter for acidic environmental application. With the 50-mer oligonucleotide arrays, the most of genes related to *A. ferrooxidans* ATCC23270 could be detected with 5 ng of pure genomic DNA. If the value can be directly applicable to real acidic environmental samples, then the level of the 50-mer-based functional gene array detection sensitivity should be sufficient for detecting composition and activities of dominant microbial populations, but it may not be sensitive enough to detect less abundant microbial populations. The quantitative aspects of the 50-mer oligonucleotide arrays have been well established. In present study, a linear relationship was observed between hybridization signal intensity and target DNA concentration for a pure culture (Fig. 4), suggesting that DNA microarrays may potentially be used for quantitative analysis. However, the accuracy of microarray-based quantitative assessments is uncertain due to inherent variations associated with array fabrication, labeling, target concentration, and scanning (Wu et al., 2001). The quantitative accuracy of the 50-mer-based FGA hybridization like other molecular approaches will depend on probe specificity (Rhee et al., 2004). To obtain more accurate results, the probe must be highly specific for the target genes. In our study, the probes were designed using the software PRIMEGENS with modified parameters. One primer was selected by considering GC content, melting temperature and self-complementarity (Xu et al., 2002). Such probes had been confirmed to be highly specific for the target genes. So, the linear relationship reflected by our data was enough for quantitative analysis.

In the present study, a total of 80 16S rRNA genes probes and 150 functional genes probes were detected in the three samples. However the observed numbers and groups of the 16S rRNA genes probes and functional genes probes detected differed among the sites. In term of the microbial community composition and functional genes detected, the NAE sample was more similar with BLS sample than with CWAE sample. Although there was not a significant relationship between environmental variety and microbial community composition or functional gene distribution, the general trend based on the PCA analysis and cluster analysis showed that the sample with the similar environmental variables had the similar microbial community composition and functional gene distribution. Our results also showed that there were clearly differences in the richness of *Leptospirillum* sp. and *A. ferrooxidans* from three environmental samples. The difference was possible due to variety of available substrates (iron and sulfur) and pH value. Some results in previous studies had also suggested that microbial community structure is strongly controlled by many environmental factors including pH (Paul, 1996), nutrient content (Compton et al., 2004), availability of electron acceptors (Kopke et al., 2005), carbon resources (Zhou et al., 2002), and temperature (Fey and Conrad, 2000). In our microarray analysis from the environmental

samples, the strains from the dominant microorganisms in different samples showed the high strain heterogeneity. The strain heterogeneity was possible correlated with their original geographical sites. The previous studies also showed that there was the existence of a certain degree of correlation between the heterogeneity of strains such as *A. ferrooxidans* and mineralogical characteristics of their habitats (Grigorii et al., 2003). Altogether, the changes that occur in environmental variables often lead to changes in structure of microbial community and microbial activity which in turn can significantly affect the component of geochemical variables.

In summary, this work evaluated the specificity, sensitivity, and quantitation of the 50-mer oligonucleotide-based microarrays. These results suggest that the 50-mer oligonucleotide array can be used as a specific and quantitative parallel tool for studies of the acidic environments and bioleaching systems. However, more development is needed for sensitivity, quantization, and the biological meaning of a detectable specificity before it can be used broadly and interpreted meaningfully.

Acknowledgements

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