16S rRNA Sequence-based Rapid and Sensitive Detection of Aquatic Bacteria by On-chip Hybridization Following Multiplex PCR

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We applied transcription *in vitro* following multiplex PCR in order to improve the sensitivity and rapidity of microbial detection in aquatic environment with an oligonucleotide microarray. Transcripts of 16S rRNA gene were fluorescently labeled and hybridized on fabricated oligonucleotide chip. The assay sensitivity was evaluated by detecting cultured bacteria inoculated into natural river water. By using our procedure, the assay was completed more rapidly (6 hr) than conventional oligonucleotide microarray assays (> 12 hr), and its sensitivity was improved: detection limit was decreased by one order of magnitude. This method might be useful for monitoring pathogenic bacteria in aquatic environments.

Key words —— oligonucleotide microarray, bacteria, monitoring, 16S rRNA, multiplex PCR, aquatic environment

INTRODUCTION

Microbial contamination of water arguably represents the most significant risk to human health on a global scale as well as toxic contamination. Monitoring of pathogenic bacteria in aquatic environment is considered as one of the most effective prevention manner of infectious disease outbreaks. Many molecular biological tools have been developed to detect bacteria, in addition to conventional culture methods. One of the most important molecular tools is polymerase chain reaction (PCR), due to its high sensitivity (less than 10 copies of a specific gene can be detected within a complex sample[1]). In addition, fluorescence in situ hybridization (FISH) can be used to analyze the bacterial community in the natural environment;[2] however, these techniques are limited due to the difficulty of simultaneous detection of various phylogenetically distinct bacteria. Although PCR allows the simultaneous amplification of several genes (multiplex PCR), the number of primer sets usually does not exceed six because the ability to resolve many amplicons in agarose gels is limited.[1] The numbers of simultaneously usage fluorochromes are limited for the detection of phylogenetically different bacteria using FISH.

Because DNA microarrays can simultaneously detect over 100000 gene targets, they are widely used by microbiologists for gene expression analysis.[3,4] Microarrays also allow the simultaneous detection of bacteria based on taxonomic marker genes, such as the 16S rRNA gene. The microbial diagnostic microarray (MDM) used for such analyses has been investigated in detail by clinical and environmental microbiologists.[5] However, MDM has significant challenges for application to bacterial monitoring with respect to sensitivity and rapidity. The sensitivity is usually 1–5% of the total abundance,[6–8] and a relatively long time is required for gene amplification (> 4 hr) and hybridization (> 6 hr) during simultaneous bacterial detection using probes targeting a wide range of sequences in the 16S rRNA.

Gene fragmentation is widely used to improve oligonucleotide microarray sensitivity. Steric hindrance caused by the secondary and tertiary structures of nucleic acids (for example, rRNA) leads to low accessibility. Shortening a nucleic acid via fragmentation of a target gene minimizes steric hindrance, and microarray sensitivity is then improved. Instead of full length of 16S rRNA gene amplification, we considered that simultaneous amplification of three or more sections of about 300 bp of 16S rRNA gene with multiplex PCR would enable

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not only 16S rRNA gene amplification but also fragmentation of 16S rRNA gene. Assay time would be saved because the multiplex PCR products are much shorter than the full length PCR product. Palmer *et al*. also reported that microarray specificity can be greatly increased by hybridizing fluorescently labelled ssRNA instead of dsDNA as the target nucleic acid.[9] We therefore postulated that transcription *in vitro* following multiplex PCR of a targeted 16S rRNA gene would improve microarray sensitivity and save time.

The objective of this assay was to prove the concept described above. First, we fabricated the oligonucleotide chip. We then determined the protocol and evaluated assay sensitivity by detecting cultured bacteria inoculated into natural river water.

MATERIALS AND METHODS

Bacterial Strains—— *Aeromonas hydrophila* ATCC 7966 and *Vibrio vulnificus* ATCC 27562 were used. Each bacterium was grown following American Type Culture Collection guidelines.

Oligonucleotide Chip Fabrication—— Oligonucleotide probes were attached to a slide glass by the procedure slightly modified from that described by Ikeda *et al*.[10] Four published probes were used in this study (Table 1).[11] AERO and VVUPA were homologous to the rRNA gene sequence of *Aeromonas* spp. and that of *Vibrio vulnificus*, respectively. M-AERO and M-VVUPA probes were used as negative control probes. All oligonucleotide probes which were modified with 5′-terminal thiol group were synthesized by Hokkaido System Science (Hokkaido, Japan). Volumes of deposited probe solutions (25 μM) on an glass slide were about 250 nl, resulting in spots with a diameter of approximately 900 μm. The slides were kept in a humidity chamber at room temperature (approximately 25°C) for 16 hr, and then washed with 2 × Sodium Chloride-Sodium Citrate Buffer (SSC) (30 mM sodium citrate, 300 mM NaCl) at room temperature for 5 min to remove unbound oligonucleotides. To block unbound EMCS, the slides were soaked in 50 mM phosphate buffer, 1 M NaCl (pH 7.0) containing 2% bovine serum albumin (BSA) (Wako Pure Chemical Industries, Osaka, Japan) at room temperature for 2 hr, and rinsed several times with distilled water, then dried in air.

Oligonucleotide Primer Design—— To amplify bacterial 16S rRNA gene, four sets of broad range bacterial primers were designed. 16 representative bacteria [*Acinetobacter baumannii*, *Aeromonas caviae*, *Aeromonas sobria*, *Aeromonas hydrophilia* (*Aer. hydrophila*), *Bacillus cereus*, *Campylobacter coli*, *Campylobacter jejuni*, *Escherichia coli*, *Mycobacterium marium*, *Pseudomonas aeruginosa*, *Plesiomonas shigelloides*, *Staphylococcus aureus*, *Tsukamurella inchonensis*, *Vibrio fluvialis*, *Vibrio parahaemolyticus* (*V. parahaemolyticus*), *Vibrio vulnificus* (*V. vulnificus*)] were selected and their

Table 1. Oligonucleotide Probes and Primers Used in this Study

Name	Sequence (5′-3′)[a]	Target	Position[b]	Reference
Primer				
8f	AGAGTTTGATCMTGG	Most *Bacteria*	8– 23	15
1492r	TACCTTGTTACGACTT	Most *Bacteria* and *Archaea*	1492–1507	15
p46f[c]	GCCTAAYACATGCAAGTCGA	Most *Bacteria*	46– 65	this study
p329r	GTGTCTCAGTYCCAGTGTG	Most *Bacteria*	311– 329	this study
p410f[c]	AAGAAGGCCTTCGGGTTGTA	Most *Bacteria*	410– 429	this study
p702r	CTACGCATTTCACCGCTACA	Most *Bacteria*	683– 702	this study
p711f[c]	AGGAACACCRGTGGCGAA	Most *Bacteria*	711– 728	this study
p1069r	AGCTGACGACAGCCA	Most *Bacteria*	1055–1069	this study
p1193f[c]	GTCAARTCATCATGSCCCTT	Most *Bacteria*	1193–1212	this study
p1512r	ACGGYTACCTTGTTA	Most *Bacteria*	1498–1512	this study
Probe				
AERO	CACATCTAACTTATCCAACCGC	*Aeromonas* spp.	585– 605	11
M[d]-AERO	CAC<u>T</u>TCTAACTTATCCAA<u>G</u>CGC	Mismatched probe		11
VVUPA	CGCTAACGTCAAATGATAGTG	*V. vulnificus/parahaemolyticus*	470– 490	11
M-VVUPA	CGC<u>A</u>AACGTCAAATGAT<u>T</u>GTG	Mismatched probe		11

a) Mixture: M = A or C, Y = C or T, R = A or G. *b*) *Escherichia coli* numbering. *c*) A 25-bp T7 promoter sequence (5′-AATTGTAATACGACTCACTATAGGG-3′) was attached to the 5′ end of forward primers p46f, p410f, p711f and p1193f. *d*) Mismatched probes have "M-" at the beginning of the names. Underlined letters in the sequences indicate mismatched nucleotides.

Table 2. Bacterial Mixture Used for DNA Extraction in Order to Determine Detection Limits

River water[a)]	Bacterial cells (cells)			Ratio (%)	
	indigenous cells	*A. hydrophila*	*V. vulnificus*	*A. hydrophila*	*V. vulnificus*
Takiue	1.0×10^6	0	—	0	—
	1.0×10^6	1.0×10^1	—	0.001	—
	1.0×10^6	1.0×10^2	—	0.01	—
	1.0×10^6	1.0×10^3	—	0.1	—
	9.9×10^5	1.0×10^4	—	1	—
	9.0×10^5	1.0×10^5	—	10	—
Juhachijo	1.1×10^7	—	0	—	0
	1.1×10^7	—	1.1×10^2	—	0.001
	1.1×10^7	—	1.1×10^3	—	0.01
	1.1×10^7	—	1.1×10^4	—	0.1
	1.1×10^7	—	1.1×10^5	—	1
	9.9×10^6	—	1.1×10^6	—	10

a) The density of bacteria (cells per milliliter), determined by SYBR Green II staining, in river waser at Takiue and Juhachijo were 1.1×10^5 and 2.1×10^6.

16S rRNA genes were aligned by using Clustal X.[12)] Conserved regions of 16S rRNA gene were chosen to design the 4 sets of primers used in this study. Sequence specificity of each pair of primers was confirmed in silico by BLAST analysis against all available microbial sequence databases in GenBank. A 25-bp T7 promoter sequence (5′-AATTGTAATACGACTCACTATAGGG-3′) was attached to the 5′ end of all forward primers.

Determination of Oligonucleotide Chip Detection Limit——To evaluate the oligonucleotide chip detection limits, *Aer. hydrophila* or *V. vulnificus* were inoculated into natural river waters. Surface river water samples were collected at two locations [Juhachijo (industrial area) on the Kanzakigawa river; and Takiue (forested area) on the Minohgawa river] from the northern part of Osaka, Japan.[12, 13)] The contents of bacterial mixture were shown in Table 2.

Genomic DNA Extraction and PCR——Genomic DNA was extracted by using the method described previously.[11)]

Monoplex PCR: 16S rRNA gene fragments were amplified with primers 8F and 1492R,[15)] which are specific for universally conserved bacterial 16S rRNA gene sequences. The PCR mixture, containing 1.25 U Ampli Taq Gold (Applied Biosystems, Foster City, CA, U.S.A.), 50 pmol each primer, 150 nmol $MgCl_2$, 25 nmol each deoxyribonucleoside triphosphate, 10 µl of 10x PCR buffer, 1 µl of 2.5 mg ml^{-1} 8-methoxypsoralen (Sigma Aldrich Chemie GmbH, Steinheim, Germany; dissolved in dimethyl sulfoxide), and 2 µg BSA (Takara Bio, Shiga, Japan), was made up to 98 µl with DNA-free water. A DNA suspension was added finally in a 2-µl volume after irradiation of the PCR mixture with UV light: the tube containing the PCR mixture was irradiated from above at a distance of 1 cm with UV (wave length: 312 nm) at room temperature.[16)] Thermal cycling was performed as follow; after an initial incubation at 94°C for 9 min, 80°C for 1 min, 42°C for 1 min, and 72°C for 3 min, amplification step then proceeded with 33 cycles of 94°C for 1 min, 42°C for 1 min, and 72°C for 3 min. The final extension step was performed at 72°C for 7 min.

Multiplex PCR: 16S rRNA genes were amplified with 4 sets of primers designed in this study. The multiplex PCR mixture, containing 1.25 U Ampli Taq Gold, 50 pmol each primer, 150 nmol $MgCl_2$, 25 nmol each dNTP, 10 µl of 10x PCR buffer, 1 µl of 2.5 mg/ml 8-methoxypsoralen and 2 µg BSA, was made up to 98 µl with DNA-free water. A DNA suspension was added finally in a 2-µl volume after irradiation of the multiplex PCR mixture with UV light. Thermal cycling was performed as follow; after an initial incubation at 94°C for 9 min, amplification step proceeded with 34 cycles of 94°C for 30 sec, 53°C for 30 sec, and 72°C for 30 sec. The final extension step was performed at 72°C for 7 min.

***In vitro* Transcription**——PCR products were purified with a MinElute PCR purification kit (QIAGEN GmbH, Hilden, Germany) as recommended by the manufacturer and eluted with 10 µl nucleases-free water, and the 16S rRNA transcripts were generated *in vitro* with the purified PCR products and T7 RNA polymerase from a T7 RiboMAX

express kit (Promega, Madison, MI, U.S.A.).

Transcripts Labeling and Hybridization—— The RNA in the reaction mixture was purified with an RNeasy MinElute Cleanup Kit (QIAGEN GmbH) and eluted with 14 µl nucleases-free water. The purified RNA was labelled with Alexa Fluor 546 at guanine by using the ULYSIS Alexa Fluor 546 nucleic acid labeling kit (Invitrogen, Eugene, OR, U.S.A.) and the manufacturer's recommended procedures. After removing of unreacted fluorophore with the Cleanup Kit, the hybridization solution was prepared as follows: Fluorescently labeled RNA, 15 µl of 20 × Sodium Chloride-Sodium Phosphate-EDTA Buffer (SSPE) [1 × SSPE: 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA (pH 7.7)], and 5 µl of 5% (w/v) sodium dodecyl sulfate solution were mixed, and adjusted to a total volume of 50 µl with the nucleases-free water. Afterwards, the hybridization solution was heated at 95°C for 5 min and subsequently cooled on ice. 40 µl of hybridization solution was poured on the oligonucleotide micropchip and the hybridization was performed for 30 min at 45°C. Then, the chip was washed with 2 × SSPE for 20 min at 48°C and rinsed with distilled water.

Data Acquisition and Analysis—— Air-dried slides were imaged at a resolution of 10 µm with a GenePix4000 microarray scanner (Molecular devices, Sunnyvale, CA, U.S.A.) at the same laser power and sensitivity level of the photomultiplier for each slide. Fluorescent spot intensities were quantified using GenePix Pro 5.1 software (Axon) after normalizing the data by subtracting local background from the recorded spot intensities. The average value for each set of duplicate spotted probes was compared to the corresponding mismatched probes, and probes that had a signal-to-noise fluorescence ratio of greater than 2.0 were considered positive.

RESULTS AND DISCUSSION

Microarrays have great potential for simultaneously detecting various phylogenetically different bacteria. However, they have hitherto been unsuitable for microbial water monitoring, because of insufficient sensitivity and being time consuming. We evaluated the effect of *in vitro* transcription following multiplex PCR on hybridization sensitivities.

First, we confirmed the hybridization sensitivities of the full-length 16S rRNA gene transcripts generated *in vitro* transcription following monoplex PCR. Concentration of *V. vulnificus* and *V. parahaemolyticus* were below the detection limit in the river water from Juhachijo, as were *Aeromonas* spp. in the river water from Takiue. When full-length 16S rRNA gene transcripts were hybridized on the oligonucleotide chip, the lowest detectable levels (intensity ratio ≥ 2) of *V. vulnificus* inoculated into Juhachijo river water and of *Aer. hydrophila* inoculated into Takiue river water were 0.1% (10^4 cells) and 1% (10^4 cells), respectively (Fig. 1).

Multiplex PCR were performed with four sets of newly designed primers (Table 1). These primers successfully amplified DNAs extracted from phylogenetically diverse bacteria (Fig. 2). Hybridization sensitivity was improved by using *in vitro* transcription following multiplex PCR. Hybridization signals of *V. vulnificus* against VVUPA probes (homologous to *V. vulnificus*) were detectable at levels ranging from 0.01% (10^3 cells) to 10% (10^6 cells) of samples from inoculated Juhachijo river water. In Takiue river water samples inoculated with *Aer. hydrophila*, ≥ 0.1% (10^3 cells) of this organism was positive against AERO probes homologous to

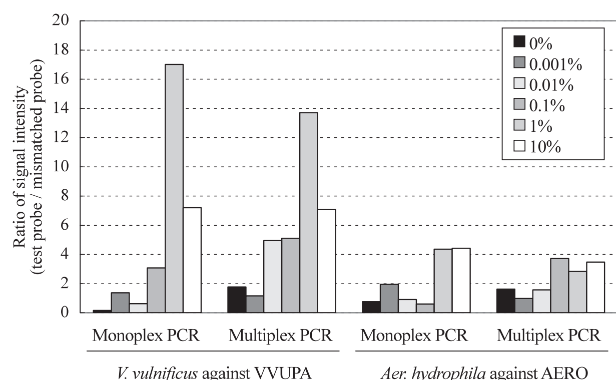

Fig. 1. Hybridization of Fragments or Full Length of 16S rRNA on Oligonucleotide Chip

Results are shown as fluorescence intensity ratio of probe relative to that of the mismatched probe.

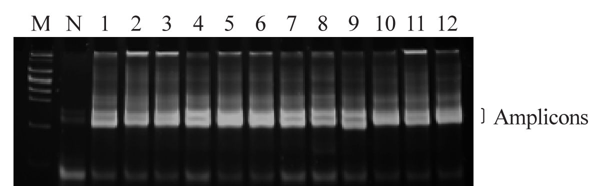

Fig. 2. Specificity of Newly Designed Primers for Multiplex PCR

M, molecular size marker (pHY); N, no template control; 1, *Aci. baumannii*; 2, *Aer. caviae*; 3, *Aer. hydrophila*; 4, *B. cereus*; 5, *E. coli*; 6, *Ple. shigelloides*; 7, *Ps. aeruginosa*; 8, *S. aureus*; 9, *T. inchonensis*; 10, *V. fluvialis*; 11, *V. parahaemolyticus*; 12, *V. vulnificus*.

Aeromonas spp. (Fig. 1).

Steric hindrance caused by the secondary structure of targeted ssDNA or RNA inevitably leads to low probe accessibility and low hybridization efficiency.[17] Peplies *et al.* demonstrated that fragmentation of the targeted bacterial DNA or RNA decreases steric hindrance and improves hybridization efficiency. Genes are often fragmented using chemical or enzymatic means after DNA amplification.[18, 19] Here, we used multiplex PCR to simultaneously amplify genes and fragment a targeted 16S rRNA gene. The results showed that the detection limit determined by hybridization of the fragmented 16S rRNA generated via multiplex PCR was 10 times lower than that of the full length 16S rRNA. The lower detection limit can be explained by the assumption that 300-base RNA fragments formed relatively simple secondary structures compared with those formed by 1.5-kbase RNA. Moreover, 10^3 cells were detected by using either *Aer. hydrophila* or *V. vulnificus* as targeted bacteria.

The probe-targeted sequences inevitably scatter on 16S rRNA when various genera of bacteria are simultaneously targeted.[10] Therefore, amplification of the full length of the 16S rRNA gene is usually required to improve microarray sensitivity. However, the long extension time (3 min per cycle[20]) involved in a 1.5-kbp amplification cycle is a critical problem which should be solved to rapidly detect bacteria (total: 5 hr for full-length amplification). In this study, 300-bp multiplex PCR using four sets of broad-range bacterial primers amplified four regions (about 300 bp each) of the 16S rRNA gene, so almost the entire 16S rRNA gene was amplified within 1.5 hr. The total assay (from DNA extraction to fluorescence intensity measurement) was completed within 6 hr (DNA extraction, 1 hr; multiplex PCR and *in vitro* transcription, 3 hr; fluorescent labelling, 30 min; hybridization and washing, 1 hr; fluorescent intensity measurement, 10 min).

The present study demonstrated that using multiplex PCR of the 16S rRNA gene in an amplification strategy improved the sensitivity of an oligonucleotide microarray and also decreased the time required to amplify the 16S rRNA gene. This method might be useful for monitoring pathogenic bacteria in aquatic environments.

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