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Evaluation of a plasmid-based 16S–23S rDNA intergenic spacer region array for analysis of microbial diversity in industrial wastewater☆

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Abstract

A plasmid-based 16S–23S rDNA intergenic spacer region (ISR) array was developed and optimized for analysis of microbial diversity within complex environmental samples. Plasmid probes with 16S–23S rDNA ISR inserts (800–1500 bp) from industrial wastewater treatment plant (WWTP) microorganisms were arrayed onto glass slides. Hybridization of fluorescently labeled target sequences from two clones from the ISR WWTP library to arrayed probes showed that there was a good linear relationship between hybridization intensity and ISR similarity ($r^2 = 0.82$). Hybridization was highly specific (average background from arrayed probes with less than 80% similarity in ISR sequence was less than 7%). Strong fluorescence intensity corresponded to near-perfect match clones (99% or greater similarity in ISR sequence). A majority of probes (79%) showed no background hybridization. However, weak background (less than 50% for arrayed probes with 90% and 95% similarity in the 16S rRNA genes) was observed from closely related microorganisms. Background fluorescence from the negative control (plasmid vector with no insert) was similar to water and dimethyl sulfoxide (DMSO)-negative controls. Hybridization using fluorescently labeled ISR sequences from a mixed community sample produced strong fluorescent signals with no background from negative controls. A Cy5-labeled reference standard, part of the vector and present in every spotted probe, was used to normalize hybridization values. These results indicate that arrayed plasmid containing ISR probe insert sequences provides specificity and sensitivity for microbial community analysis in a high-throughput array format.
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☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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1. Introduction

In recent years, evaluation of microbial diversity in environmental systems has been aided by molecular techniques that are less selective than traditional cultural techniques and provide a more complete view of the complexity within environmental systems (Ogram and Sayler, 1988; Amann et al., 1995; Massol-Deya et al., 1995; Gurtler and Stanisich, 1996). In particular, molecular methods based on the 16S rRNA gene analysis have provided unique insights into the breadth of microbial diversity (Hugenholtz et al., 1998). This sequence is universally present in all bacteria and contains highly conserved regions interspersed with variable regions, thereby facilitating the design of primers and probes for analysis of bacterial phylogeny at different levels of specificity (i.e., family, genus, or species level)(Amann et al., 1995; Cilia et al., 1996). Many 16S rRNA-based molecular techniques [denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (tRFLP), and ARDRA (amplified rDNA restriction analysis)] have been developed for community-level analysis of differences in microbial composition associated with environmental, spatial, or temporal changes. However, these methods yield complex community profiles that provide only superficial information about community ecology (Kent and Triplett, 2002; Valinsky et al., 2002). Additionally, the size of the 16S rRNA gene is extremely constant, limiting its use in gel-based community-fingerprinting techniques, and 16S rRNA gene sequences may be too highly conserved to permit analysis of phylogenetic relationships between closely related species (Cilia et al., 1996; Mendoza et al., 1998; Aakra et al., 1999).

In contrast to the rRNA genes, the intergenic spacer region (ISR) located between the 16S rRNA and 23S rRNA genes is under less selective pressure (Buchan et al., 2001). Therefore, the 16S–23S rDNA ISR exhibits greater sequence diversity and length variation than the 16S rRNA gene (Jensen et al., 1993; Gurtler and Stanisich, 1996; Nagpal et al., 1998). This variability is due primarily to insertion and deletion events and the presence or absence of tRNA sequences. Divergence within the ISR has been used to confirm 16S rRNA gene-based phylogeny and has been shown to provide better discrimination of closely related species than 16S rRNA characterization (Cilia et al., 1996; Mendoza et al., 1998; Aakra et al., 1999; Adb-El-Haleem et al., 2002). However, ISR sequences among closely related microorganisms (species level) are conserved due to the location of the ISR between the highly conserved 16S rRNA and 23 rRNA genes and transcription of the *rrn* operon as a single pre-RNA transcript (Garcia-Martinez et al., 1999). Concerted evolution of multigene families and homologous recombination between similar ISRs also contributes to sequence conservation within the ISR despite limited evolutionary constraints (Anton et al., 1998; Gurtler, 1999). Therefore, ISR sequences are often conserved within a species, but rarely beyond the genus or family level.

The 16S–23S rDNA ISR has been used to evaluate mixed microbial communities within environmental samples via clonal library construction and community-level fingerprinting (Acinas et al., 1999; Fisher and Triplett, 1999; Ranjard et al., 2000). Conserved sequences within the 16S rRNA and 23S rRNA genes were used as primer targets for amplifying the spacer region of a broad range of microorganisms in a single amplification reaction (Jensen and Straus, 1993). Polymerase chain reaction (PCR) products, including a portion of the 16S rRNA gene, can be used for phylogenetic analysis (Acinas et al., 1999). ISR community level profiling was used to characterize community samples from Amazonian soils (Borneman and Triplett, 1997), freshwater bacterial communities (Fisher and Triplett, 1999), plant rhizosphere (Robleto et al., 1998), and Mediterranean offshore waters (Acinas et al., 1999). Microbial community profiling based on ISR size differences is limited by the fact that multiple microorganisms (either closely related strains or completely unrelated species) may possess ISR regions of identical length (Garcia-Martinez et al., 1999). Alternatively, one microorganism may possess multiple ISRs with significant length differences, so that a single microorganism may contribute multiple bands to a community profile (Fisher and Triplett, 1999).

Array technology is a high-throughput methodology that permits parallel analysis of hundreds or thousands of genetic sequences. For reviews on the application of array technologies to environmental sample analysis, see Cook and Sayler (2003) and Zhou and Thompson (2002). Alternative array methodologies have been applied for analysis of environ-

mental samples through gene expression analysis (Murray et al., 2001; Schut et al., 2001), bacterial identification (Cho and Tiedje, 2001; Loy et al., 2002; Wilson et al., 2002), and evaluation of functional genes important in environmental ecosystems (Wu et al., 2001). However, many of these arrays rely on highly conserved 16S rRNA gene sequences and/or require costly and time-consuming selection of oligonucleotide probe sequences (Guschin et al., 1997; Loy et al., 2002; Valinsky et al., 2002; Wilson et al., 2002).

This report describes the development and optimization of a plasmid-based 16S–23S rDNA ISR array for characterization of microbial diversity within an industrial wastewater treatment plant (WWTP). Conserved sequences in the 16S rRNA and 23S rRNA genes were used as primer targets to amplify ISR sequences from a mixed microbial community. Phylogenetic information was obtained through analysis of 500 bp of 16S rRNA gene sequence. The PCR products were cloned into plasmid vector and extracted plasmid was arrayed onto glass slides. Hybridization of fluorescently labeled samples against arrayed plasmid with 16S–23S rDNA ISR insert sequences provided a strong, specific fluorescent signal. This plasmid-based ISR array represents a new application of array technology in which community analysis can be achieved with a high level of specificity without the need for culture-based approaches or sequence analysis.

2. Materials and methods

2.1. Evaluation of the ISR as a probe target for characterization of mixed microbial communities

2.1.1. DNA extraction and preparation of clonal library

DNA was extracted from industrial WWTP-activated sludge samples collected from an industrial wastewater treatment system in September 1999. A 50-ml aliquot of each sample was pelleted by centrifugation and genomic DNA was extracted from 100 mg wet weight, as described by Layton et al. (2000). 16S–23S rDNA ISR sequences from industrial WWTP microbial communities were amplified using Ready-To-Go-PCR Beads (Amersham Pharmacia, Piscataway, NJ), with 800-nM primers and 5–10 ng of template DNA. The PCR primers were 1055f (5′ ATG GCT GTC GTC AGC T 3′) (Amann et al., 1995) and 23Sr (5′ GGG TTB CCC CAT TCR G 3′) (Borneman and Triplett, 1997). The touchdown PCR program used for 16S–23S rDNA ISR analysis was as follows: 5 min at 94 °C, then 10 cycles consisting of 15 s at 94 °C, 45 s at 65 °C, followed by a 1 °C decrease for each of the 10 cycles, and 60 s at 72 °C, followed by an additional 20 cycles of 15 s at 94 °C, 45 s at 55 °C, and 60 s at 72 °C, and a final cycle of 60 s at 72 °C. Aliquots of 20 μl of the PCR product were electrophoresed in 2% agarose gels in 0.5× TBE solution (0.045M Tris–borate, 0.001 M EDTA, pH 8.0).

PCR products were cloned into the pCR® 2.1-TOPO TA vector (TA vector; Invitrogen, Carlsbad, CA). Large-scale plasmid DNA extractions were performed by using an alkaline lysis procedure from Promega Tech Bulletin .009 (Promega, Madison, WI). Contaminating RNA was removed by incubating the plasmid preparations with 10 μg of DNAase-free RNAase per milliliter of preparation followed by phenol–chloroform extraction and ethanol precipitation. Clones were characterized using phylogenetic information obtained from sequencing approximately 450 bp of the 16S rRNA using the 1492 r primer (5′ TAC GGY TAC CTT GTT ACG ACT T 3′) (Lane, 1991). All sequencings were performed at the University of Tennessee Molecular Biology Resource Facility using an Applied Biosystems 373 DNA sequencer (Perkin-Elmer, Foster City, CA).

2.1.2. Construction of a prototype nylon membrane ISR array

The 16S–23S rDNA ISR inserts were amplified from purified plasmid from 11 WWTP clones as described above, except that 0.8–1.1 ng of plasmid template DNA was used along with the primers 1492f (5′ AAG TCG TAA CAA GGT ARC CGT A 3′) and 23Sr. The amplified fragments were cleaned using the QIAquick PCR purification kit (QIAGEN, Valencia, CA). Purified PCR products (probes) were quantified using Hoechst Dye (Biorad, Hercules, CA) measured at an excitation of 346 nm and emission of 460 nm with a DyNA Quant 200 fluorometer (Amersham Biosciences, Piscataway, NJ). The PCR product was diluted to produce probes at final concentrations of 0.4, 0.2, and 0.04 pmol. The probes were blotted onto BIOTRANS nylon membranes (ICN Pharmaceuticals, Costa Mesa,

CA) using the dot blot procedure described by Dionisi et al. (2002).

2.1.3. Cross-hybridization analysis

Arrays were prehybridized in solution containing 0.5 M NaH_2PO_4, 1 mM EDTA, and 7% (wt/vol) sodium dodecyl sulfate (SDS) for 1 h at 65 °C in a shaking water bath. Hybridizations were carried out overnight following addition of ^{32}P-labeled target ISR DNA (1.0×10^6 to 3.0×10^6 cpm). Eleven separate arrays were hybridized using labeled target ISR sequences from each of the different clones. Hybridization was performed at 65 °C and membranes were washed in high stringency wash buffer (10 mM NaCl, 10 mM Tris base, 1 mM EDTA, and 0.5% SDS) at 65, 70, and 75 °C. Following each wash, the arrays were exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA) and the exposed screen was scanned on a STORM 840 PhosphorImager (Molecular Dynamics). The resulting images were analyzed by determining pixel density for each spot using ImageQuaNT™ 5.0 analysis software (Molecular Dynamics).

2.2. Evaluation of glass slide plasmid-based ISR array

2.2.1. Construction of plasmid-based 16S–23S RDNA ISR array

Samples from the same industrial WWTP were collected in July 2001 and genomic DNA was extracted from 2 ml of mixed liquor using the FastDNA kit (Bio 101, Vista, CA) as previously described (Dionisi et al., 2002). 16S–23S rDNA ISRs of microorganisms from the industrial WWTP were amplified, PCR products were cloned into the pCR® 2.1-TOPO TA vector, and plasmid DNA was extracted. The ISR array was composed of 160 plasmids from the industrial WWTP 16S–23S rDNA ISR library. Each glass slide contained triplicate ISR arrays. TA vector with no insert, dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO), and water blanks served as negative controls. Probes consisting of plasmid containing a 16S–23S rDNA ISR insert were spotted in 50% DMSO at a final concentration between 400 and 550 ng/μl. Ten microliters of plasmid probe in an equal volume of DMSO was placed into 384-well microtiter plates. Probes were arrayed at a spacing of 350 μm onto Cel Associates (Houston, TX) poly-L-lysine-coated slides in a grid of eight rows by six columns. Arrays were printed at 57% relative humidity and 22 °C using a Virtek robotic printer (Waterloo, Canada) with eight pins (TeleChem International, Sunnyvale, CA). Following printing, ISR arrays were rehydrated in a glass Petri dish containing moist filter paper (Qualitative; Whatman, Ann Arbor, MI) for 5 min and snap-dried at 80 °C for 5 s. ISR arrays were then UV cross-linked at 150 mJ in a UV Spectrolinker XL1500 (Spectronics, Westbury, NY). Arrays were blocked in a solution of 1.5% succinic anhydride (Sigma), 90% 1-methyl-2-pyrrolidinone (Sigma), and 43 mM boric acid (pH 8.0) (Sigma) for 15 min at room temperature. Following blocking, DNA on the arrays was denatured by boiling at 95 °C for 2 min. Slides were dried by centrifugation using a microarray centrifuge (TeleChem International) at $2000 \times g$ for 20 s. Two slides from each printed batch were evaluated for quality of printing by staining with Sybr Green II (Molecular Probes, Eugene, OR) diluted 1:10,000 in 0.5 × TBE. Arrays were stained for 1 min and then washed in 0.5 × TBE and sterile deionized water for 30 s each prior to scanning using the Scan Array 5000 MicroArray Analysis System (GSI Lumonics, Watertown, MA) at an emission wavelength of 522 nm.

A subarray containing probes from six 16S–23S rDNA ISR clones from the WWTP library was used for analysis of cross-hybridization and optimization of hybridization conditions for the ISR array (Table 1). The probes, as well as negative controls (TA vector with no insert and DMSO), were spotted under the conditions described above. Five replicates of the subarray were printed on each slide.

2.2.2. Preparation of fluorescently labeled target DNA

16S–23S rDNA ISR sequences from industrial WWTP samples genomic DNA extracts (2–5 ng) were amplified using the PCR conditions described above to increase overall target signal intensity. 16S–23S rDNA ISRs from plasmid insert sequences were selectively amplified using PCR to minimize background from vector. The resultant PCR products from the first reactions were purified using the Zymo DNA Clean and Concentrator-5 Kit (Zymo Research, Orange, CA). Cleaned PCR product was diluted 1:1000 and 1 μl was used as template for an incorporation reaction using the PCR bead, primers (1492f and 23Sr), and 4 μM Cy3-

Table 1
Industrial WWTP 16S–23S rDNA ISR clones used for optimization of ISR array hybridization

Clone name	Closest match (% similarity)	Phylogenetic placement	% GC	ISR size (bp)	T_m[a]
(A) Prototype ISR array clones[b]					
TED99917	*Hyphomicrobium hollandicum* Y14303 (95%)	Alpha subdivision, *Hyphomicrobium*	56	728	101
TED39909	*Magnetospirillum gryphiswaldense* Y10109 (94%)	Alpha subdivision, Rhodospirillaceae	57	829	101
TED99907	*Asticcacaulis excentricus* AJ247194 (93%)	Alpha subdivision, Caulobacter group	61	643	103
TED99915	*Leptothrix discophora* L33974 (98%)	Beta subdivision, Comamonadaceae	56	558	100
TED99903	*Thaurera aromatica* AJ315680 (99%)	Beta subdivision, Rhodocyclus branch	56	537	100
TED99928	*Pseudomonas lanceolata* AB021390 (99%)	Beta subdivision, Comamonadaceae	49	616	98
TED99910	*Methylococcus* sp. X72769 (93%)	Gamma subdivision, Methylococcaceae	54	548	100
TED99902	*Lewinella persicus* AF039295 (90%)	Sphingobacteria, Sphingobacteriales	48	876	98
TED99906	*Flexibacter tractuosus* AB078076 (91%)	Sphingobacteria, Sphingobacteriales	44	978	96
TED99901	*Nitrosphira* sp. Y14638 (98%)	Nitrospirales, Nitrospiraceae	53	511	99
TED99926	*Verrucomicrobium* sp. AF027005 (97%)	Verrucomicrobia	54	440	99
(B) ISR subarray clones[c]					
TED70103	*Leptothrix mobilis* X97071 (98%)	Beta subdivision, Comamonadaceae	55	577	100
TED70101	*L. mobilis* X97071 (99%)	Beta subdivision, Comamonadaceae	53	692	99
TED70129	*P. lanceolata* AB021390 (99%)	Beta subdivision, Comamonadaceae	54	521	100
TED70105	*Thauera aromatica* AJ315680 (99%)	Beta subdivision, Rhodocyclus branch	56	511	100
TED70113	*Rhodobacter sphaeroides* X53855 (94%)	Alpha subdivision, Rhodobacter group	54	621	100
TED70109	*Haliscomenobacter hydrossis* M58790 (90%)	Sphingobacteria, Sphingobacteriales	42	305	94

[a] Melting temperature of ISR sequence.
[b] Clones used on phototype ISR array for cross-hybridization analysis.
[c] Clones used on ISR subarray for formamide optimization experiments.

labeled dCTP (Amersham Pharmacia, Peapack, NJ). The labeled target DNA was purified by precipitation with 2.5 vol of ethanol and 0.1 vol of sodium acetate (Sigma) at −20 °C overnight.

2.2.3. Optimization of hybridization specificity and intensity using a formamide gradient

To optimize hybridization specificity and signal intensity, 16S–23S rDNA ISR subarrays were hybridized in hybridization solution containing 30%, 40%, 50%, 60%, or 70% formamide. The array was replicated five times on each slide and hybridization at each formamide concentration was replicated on three to five separate slides. Labeled target DNA was resuspended in hybridization solution containing different concentrations of formamide (Ambion, Austin, TX), 5 × SSC, 0.1% SDS, and 0.5 μg of unlabeled herring sperm DNA (Sigma) in a total volume of 60 μl. Fluorescently labeled target was denatured at 95 °C for 2 min and cooled to ambient temperature by centrifugation for 2 min. Denatured probe was deposited into an adhesive-backed Frame Seal (MJ Research, Waltham, MA) chamber placed around the arrayed DNA on the glass slide array, and the chamber was then sealed with an adhesive plastic coverslip. All hybridizations were performed in an MJ Research Twin Tower Sample Block (MJ Research) at 45 °C. Following hybridization, the arrays were washed in separate chambers with 2 × SSC and 0.1% SDS for 5 min at the hybridization temperature, and then with 0.1 × SSC and 0.1% SDS for 5 min at room temperature, followed by 0.1 × SSC for 1 min four times at room temperature. Residual salts were removed by centrifugation. Slides were scanned at a resolution of 5 μM with the ScanArray 3000 confocal laser scanner. Signal intensities for each spot were determined using QuantArray 3.0 image analysis software (Perkin Elmer, Boston, MA). ANOVA was carried out using SPSS version 11.0 (SPSS, Chicago, IL).

2.2.4. Analysis of industrial WWTP sludge library using the 16S–23S rDNA ISR array

Labeled target sequences from clones TED70103 or TED70109 (Table 1) from the industrial WWTP library were hybridized to arrayed plasmid probes on the ISR array. A formamide concentration of 50% was used in

the hybridization solution and all hybridizations were performed at 45 °C. Following hybridization, arrays were scanned and analyzed as described above. Plasmids from selected spots were sequenced for phylogenetic analysis, using the 16S rRNA gene portion of the insert (1055f primer), and for ISR similarity, using the 1492f primer. Spearman correlation coefficients (r) were calculated to find relationships between hybridization ratio and ISR or 16S rRNA gene sequences.

2.3. Hybridization of plasmid-based ISR array with DNA from mixed community

Genomic DNA was extracted from WWTP microorganisms that were inoculated into plant rhizosphere and grown for 13 days. DNA was extracted from 10 to 15 ml of plant rhizosphere suspension using the MoBio Soil Extraction Kit (Solana Beach, CA). Genomic extract (2–5 ng) was labeled with Cy3-dCTP as described above. The kanamycin gene from the pCR® 2.1-TOPO TA vector was used as reference DNA by incorporation of Cy5-dCTP (Amersham Pharmacia) using primers KanF (5′ TCA GGG CGC AAG GGC 3′; corresponding to sequence positions 963–977 of the TA vector) and KanR (5′ ACT CTT CCT TTT TCA ATT CAG AAG AAC 3′; corresponding to sequence positions 2107–2133 of the TA vector). The Cy3-labeled target and the Cy5-labeled reference standard were mixed in equal portions and hybridized to the array simultaneously. The arrays were hybridized, washed, and analyzed as described above.

2.4. Nucleotide sequence accession numbers

Clone sequences for 16S–23S rDNA ISR inserts from plasmids used for ISR array analysis were deposited into GenBank and received GenBank accession numbers AY484706 to AY484734, AY484743, and AF420301.

3. Results

3.1. Evaluation of the ISR as a probe target for characterization of mixed microbial communities

16S–23S rDNA ISR probes from a phylogenetically diverse group of WWTP clones were used in a prototype array to evaluate the feasibility of using the 16S–23S rDNA ISR as probes for characterization of mixed microbial communities in an array format. The 1492f primer was used to produce labeled target sequences to avoid amplification of conserved sequences present in the 16S rRNA portion of the ISR insert. The 23Sr primer used in these studies anneals 115 bp into the 23S rRNA. This primer was chosen because it is more conserved than previously used 23S rRNA-targeted primers that are specific for sequences closer to the ISR region (Gurtler and Stanisich, 1996). Clones were sequenced from the 16S rRNA end of the PCR product using the 1055f primer. The approximately 500 bp of partial 16S rRNA gene sequence was used to obtain phylogenetic information. The names and accession numbers of microorganisms from GenBank that most closely matched each of the 11 clones used for cross-hybridization analysis, as well as the tentative phylogenetic placement, clone number, percent GC of the ISR, ISR size, and T_m are given in Table 1A. The 16S–23S rDNA ISR library produced from WWTP mixed communities was diverse with representatives from the alpha, beta, and gamma proteobacteria as well as *Nitrospira*, *Verrucomicrobia*, and *Sphingobacteria*. ISR size varied between 440 and 978 bp. T_m for the 11 clones varied between 96 and 103 °C, with an average of 99 °C (± 2 °C).

Cross-hybridization analysis using selected clones revealed that at low stringency (65 °C wash), some cross-hybridization occurred, that is, a labeled target using clone TED39909 (*Magnetospirillum* sp.), an alpha proteobacterium, produced 35.8% background signal from spotted probe sequence TED99907 (*Asticcacaulis* sp.), another alpha proteobacterium (Table 2). This background was reduced at higher wash stringency (75 °C). At a wash temperature of 75 °C, the same labeled target (TED39909) showed only 2.1% background from the alpha proteobacterium TED99909. At the lowest stringency, wash temperature (65 °C) background averaged 10% (± 20%) over the entire group. At the highest stringency, wash temperature (75 °C) background averaged 1.5% (± 2%). Based on these results, it was determined that arrayed 16S–23S rDNA ISR provided a useful target for hybridization analysis with minimum potential for cross-hybridization given sufficiently stringent hybridization conditions.

Table 2
Characterization of percent cross-hybridization between ISRs of different phylogenetic groups

Wash temperature (°C)	Clone name	Phylogeny	Alpha proteobacteria			Beta proteobacteria			Gamma	CFB[a]		*Nitrospira*	Verruco[b]
			TED99917	TED39909	TED99907	TED99915	TED99903	TED99928	TED99910	TED99902	TED99906	TED99901	TED99926
65	TED99917	*Hyphomicrobium*	100.00	47.70	41.85	1.73	6.39	4.30	2.16	1.03	1.21	6.37	18.80
65	TED39909	*Magnetosprillum*	17.46	100.00	56.00	11.08	3.47	6.26	3.42	0.29	1.75	23.83	62.20
65	TED99907	*Asticcacaulis*	3.16	35.76	100.00	0.74	15.25	4.80	1.05	3.38	3.67	6.19	22.91
65	TED99915	*Leptothrix*	1.44	5.40	18.18	100.00	73.20	86.77	4.13	0.13	6.69	5.82	5.19
65	TED99903	*Thauera*	1.05	0.85	4.42	18.63	100.00	21.63	8.38	0.14	1.96	0.34	2.14
65	TED99928	*Pseudomonas*	0.13	0.66	1.52	0.51	14.14	100.00	0.20	0.11	1.26	− 0.02	3.68
65	TED99910	*Methylococcus*	1.10	12.24	24.29	3.89	76.01	17.37	100.00	3.19	1.42	1.81	11.20
65	TED99902	*Lewinella*	0.69	0.25	0.92	0.51	0.70	1.53	2.04	100.00	6.32	1.21	1.62
65	TED99906	*Flexibacter*	0.86	0.67	1.54	2.60	0.85	2.27	2.29	21.33	100.00	5.79	1.52
65	TED99901	*Nitrospira*	2.76	54.72	61.67	0.97	0.30	4.34	3.42	0.38	1.35	100.00	57.77
65	TED99926	*Verrucomicrobium*	1.42	38.68	40.26	0.18	8.52	1.46	1.34	0.30	0.21	15.14	100.00
70	TED99917	*Hyphomicrobium*	100.00	21.22	18.56	0.95	0.50	3.92	1.60	0.73	0.81	4.69	0.86
70	TED39909	*Magnetosprillum*	12.55	100.00	28.88	7.60	0.36	3.22	2.42	0.44	0.71	5.07	2.82
70	TED99907	*Asticcacaulis*	2.02	15.17	100.00	3.53	0.47	2.62	1.14	3.06	3.08	4.76	1.24
70	TED99915	*Leptothrix*	1.43	1.17	9.35	100.00	2.76	66.37	0.78	0.26	4.29	3.99	0.34
70	TED99903	*Thauera*	0.82	0.50	3.05	8.27	100.00	13.01	2.65	0.16	1.18	0.43	0.15
70	TED99928	*Pseudomonas*	0.02	0.38	1.24	1.31	1.20	100.00	0.22	0.25	0.34	0.21	0.05
70	TED99910	*Methylococcus*	0.79	6.58	10.93	1.89	0.32	9.73	100.00	4.41	0.58	0.82	0.64
70	TED99902	*Lewinella*	0.73	0.13	0.50	0.34	0.15	0.85	0.46	100.00	1.61	0.71	0.17
70	TED99906	*Flexibacter*	0.69	0.23	0.96	0.77	0.12	0.75	0.29	5.33	100.00	2.53	0.18
70	TED99901	*Nitrospira*	1.17	26.16	31.41	0.58	0.10	2.08	0.42	0.24	0.80	100.00	1.51
70	TED99926	*Verrucomicrobium*	0.66	15.74	20.24	0.10	0.78	0.95	0.12	0.19	0.05	3.71	100.00
75	TED99917	*Hyphomicrobium*	100.00	2.68	2.65	1.73	0.28	2.24	1.60	0.34	0.47	5.96	2.06
75	TED39909	*Magnetosprillum*	3.97	100.00	4.47	2.83	0.16	1.50	2.42	0.88	0.19	1.03	3.49
75	TED99907	*Asticcacaulis*	0.59	2.13	100.00	0.94	0.19	1.46	1.14	10.01	3.35	6.51	1.73
75	TED99915	*Leptothrix*	0.93	0.41	5.08	100.00	1.09	8.88	0.78	2.81	2.23	4.21	1.29
75	TED99903	*Thauera*	0.61	0.23	1.73	3.18	100.00	2.53	2.65	1.81	0.73	0.85	0.88
75	TED99928	*Pseudomonas*	0.03	0.12	0.80	0.14	0.47	100.00	0.22	1.77	0.22	0.50	2.03
75	TED99910	*Methylococcus*	0.40	0.76	2.51	0.87	0.14	3.26	100.00	4.98	0.44	1.07	1.22
75	TED99902	*Lewinella*	0.58	0.06	0.29	0.34	0.07	0.62	0.46	100.00	1.70	0.31	0.70
75	TED99906	*Flexibacter*	0.60	0.17	0.65	1.03	0.06	0.58	0.29	4.51	100.00	1.76	0.95
75	TED99901	*Nitrospira*	0.25	2.50	2.64	0.54	0.06	1.29	0.42	1.90	0.77	100.00	2.89
75	TED99926	*Verrucomicrobium*	0.15	1.69	1.32	0.11	0.32	0.58	0.12	0.67	0.10	1.67	100.00

[a] CFB = Cytophaga, Flavobacterium, Bacteroides group.
[b] Abbreviation for *Verrucomicrobium*.

3.2. Construction of plasmid-based 16S–23S rDNA ISR array

Plasmid probes extracted from a clonal library of 16S–23S rDNA ISR sequences from industrial WWTP microorganisms were spotted onto glass slides at a concentration of 400–550 ng/μl. This concentration of starting material was necessary to achieve saturating concentrations of spotted probe since approximately one-fourth of the plasmid is probe ISR sequence, the rest being vector sequence. No effect of this concentration of starting material was observed (i.e., there was no evidence of the "comet effect" typically seen when excessively high concentrations of probe are spotted) (Eisen and Brown, 1999). TA vector with no insert was spotted onto the array and its intensity following hybridization was subtracted from all other background-subtracted intensity values to eliminate the effect of nonspecific hybridization to the plasmid. Initial background levels from TA vector were minimal (5–7%) and were only slightly higher than values for the DMSO control (2–3%).

3.3. Optimization of hybridization specificity and intensity using a formamide gradient

Hybridization specificity was optimized using a subarray of six probes from the WWTP ISR array. Spotted probe 16S–23S rDNA ISR insert sequences matched the labeled target 16S–23S rDNA ISR sequence (TED70103) between 58% and 100% (Fig. 1). The names and accession numbers of microorganisms from GenBank that most closely matched each of the six plasmid probes spotted onto the subarray, as well as their tentative phylogenetic placements, clone number, percent GC of the ISR, ISR size, and T_m are given in Table 1B. The subarray also contained TA vector with no insert. The array was replicated five times on each slide and hybridization at each formamide concentration was replicated in three to five separate slides. Replicate spots from each array were averaged to obtain one value per probe per slide. Final fluorescence intensity (I') for each spot resulted from subtraction of local mean background and negative control (TA vector with no insert) values from the initial fluorescence intensity (I). The hybridization

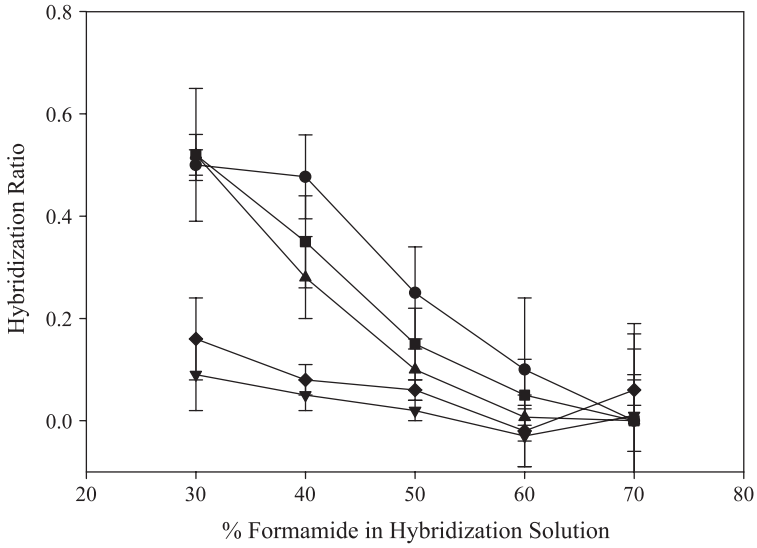

Fig. 1. Effect of formamide concentration on hybridization specificity. Each subarray consisted of five replicate arrays and three to five separate subarrays were tested at each formamide concentration. The hybridization ratio is derived from the ratio of final fluorescence intensity (I') for each spot in the subarray to the final fluorescence intensity of the 100% 16S rRNA and ISR matched probe (TED70103). TED70101, 98% 16S rRNA, and 80% ISR match to target sequence (●); TED70129, 94% 16S rRNA, and 74% ISR match to target sequence (■); TED70105, 90% 16S rRNA, and 70% ISR match to target sequence (▲); TED70113, 80% 16S rRNA, and 64% ISR match to target sequence (▼); and TED70109, 80% 16S rRNA, and 58% ISR match to target sequence (♦).

ratio for each array spot was measured as the ratio of I' for the test spot to I' for the control spot (the spot matching the labeled target 100%; hybridization ratio= I' test/I' control).

ISR insert from the 100% matched clone (TED70103) was labeled with Cy3-dCTP and hybridized to the subarray in hybridization solution with formamide concentrations ranging between 30% and 70%. Specificity was found to increase with formamide concentration (Fig. 1). ANOVA suggested that hybridization specificity significantly increased at a formamide concentration of 50% or greater ($P < 0.05$; Fig. 1). At low hybridization stringency, the closest sequence matches exhibited some background hybridization. At a formamide concentration of 70%, there was no detectable background from other clones; however, overall intensity was also lower (30% lower than the intensity at the lowest concentration of formamide). At a formamide concentration of 50%, background hybridization was less than 25%, and overall intensity was 95% of the intensity at the lowest concentration of formamide. Based on the level of background hybridization and overall intensity, all further experiments were conducted at 45 °C in hybridization solution containing 50% formamide.

3.4. Analysis of industrial WWTP sludge library using the 16S–23S rDNA ISR array

The 16S–23S rDNA ISR inserts of two clones from the industrial WWTP library were used as labeled targets to investigate the occurrence of these sequences in the arrayed portion of the industrial WWTP library and to evaluate the specificity of hybridization. A common clone (TED70109; 16% of sequenced clones in the library matched 99% in ISR sequence) was chosen to evaluate hybridization specificity. A beta proteobacterium (TED70103) was less common in the sequenced portion of the WWTP library, but was chosen to evaluate the potential for cross-hybridization between closely related microorganisms. Fig. 2A shows the relationship between hybridization ratio, 16S rRNA sequence similarity, and ISR sequence similarity for 69 sequenced clones hybridized against labeled target sequences from clones TED70103 and TED70109. Spearman correlation analysis of these data showed a positive correlation between hybridization ratio and 16S–23S rDNA ISR sequence ($r = 0.63$, $P < 0.0005$). Slide-to-slide variation in signal intensity for replicate spots averaged 8.0% with a standard deviation of 5.5%. Given that labeled targets are complementary to the ISR portion of arrayed probes, it was not surprising to find that the linear relationship between hybridization ratio and ISR sequence similarity ($r^2 = 0.82$) was better than that for hybridization ratio and 16S rRNA gene sequence similarity ($r^2 = 0.77$). Two of four ISR subarrays from the ISR array, hybridized with labeled target from TED70103 and TED70109, are shown in Fig. 2B and C, respectively.

Hybridization of labeled target from clone TED70109 to the array resulted in 10 clones with a hybridization ratio greater than 0.85 (Fig. 2A). These clones were all greater than 99% matches to the labeled target sequence in terms of ISR and 16S rRNA gene sequence. A cluster of four clones had 99% 16S rRNA gene sequence similarity to clone TED70109, but had low hybridization ratios (between 0.60 and 0.38) (Fig. 2A). This discrepancy was due to the low level of ISR sequence similarity (84%) between the 100% match clone and this cluster of clones. Differences in the ISR sequence were due to insertions and single base-pair mismatches in comparison to the ISR sequence of the perfect match clone.

Hybridization of the less common clone (TED70103) to the array produced only two matches with a hybridization ratio greater than 0.55; both were 98% similar in 16S rRNA gene sequence, but only 78–80% similar in ISR sequence to the perfect match (Fig. 2A). Only one other 98% 16S rRNA gene match to TED70103 was identified in the sequenced portion of the library. The clone had a hybridization ratio of 0.36. This finding was consistent with a significantly longer ISR region (692 bp versus 577 bp for clone (TED70103) containing several long insertions. Despite fewer matched clones, hybridization with TED70103, a beta proteobacterium, resulted in greater background hybridization by related beta proteobacteria than that observed for the *Sphingobacterium* clone TED70109, a more common clonal library sequence (Fig. 2B and C). Background hybridization ratio was less than 0.50 for arrayed probes matching between 90% and 95% in 16S rRNA gene sequence (less than 80% ISR match) and less than 0.10 for arrayed probes with less than 90% match in 16S rRNA sequence. More than 79% of the probes pro-

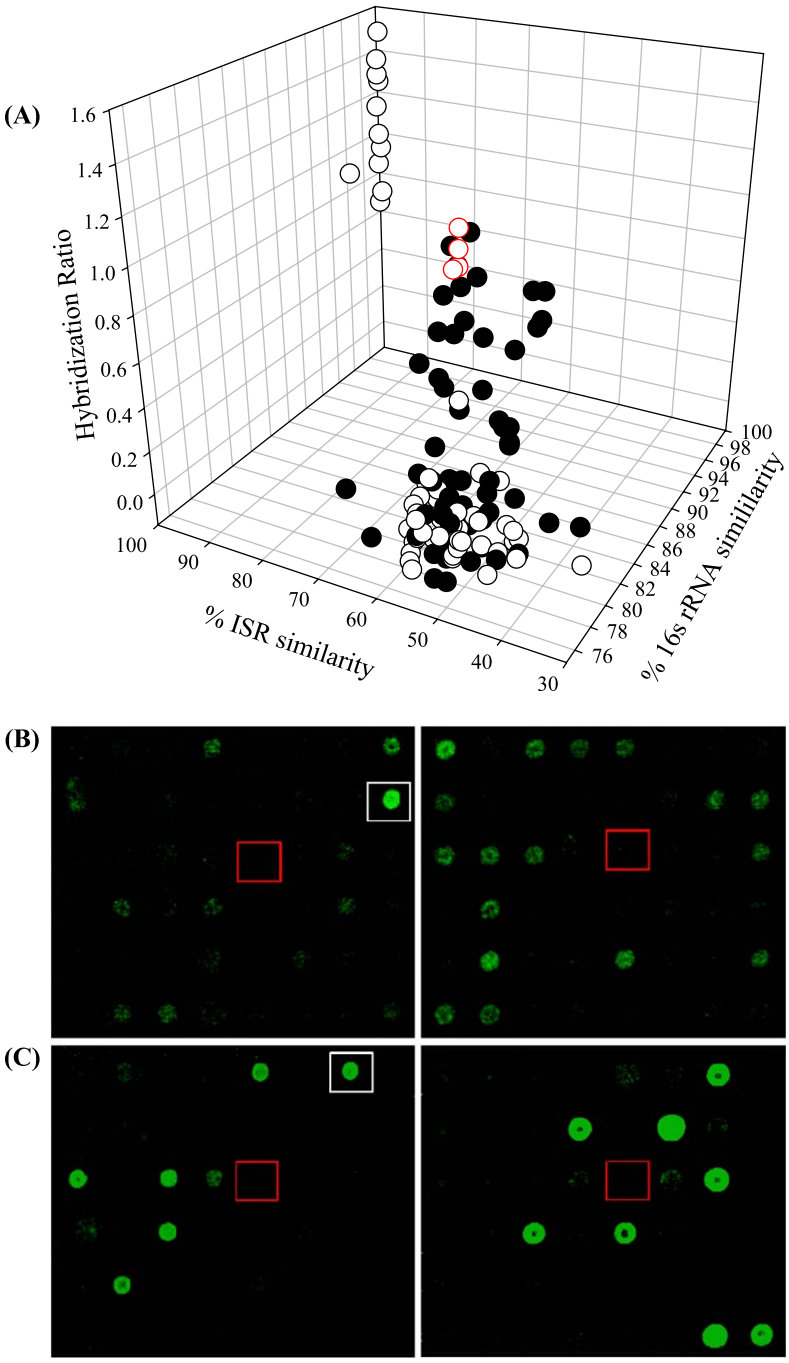

Fig. 2. (A) Relationship between hybridization ratio, ISR sequence similarity, and 16S rRNA sequence similarity. ISR sequences from two clones, TED70103 (●) and TED70109 (○), were labeled with Cy3-dCTP and hybridized to separate arrays in triplicate. Cluster of four clones with 99% 16S rRNA sequence similarity to clone TED70109, but low ISR similarity and low hybridization ratios (○). (B) Two subarrays of the ISR array hybridized with Cy3-dCTP-labeled ISR from clone TED70103. Red boxes indicate TA vector with no insert (negative control); white boxes indicate the 100% match to the labeled target sequence. (C) Two subarrays of the ISR array hybridized with Cy3-dCTP-labeled ISR from clone TED70109. Red boxes indicate TA vector with no insert (negative control); white boxes indicate the 100% match to the labeled target sequence.

duced little or no signal (hybridization ratio less than 0.20) in comparison to the hybridization intensity of the perfect match probe for both of the labeled targets used for hybridization.

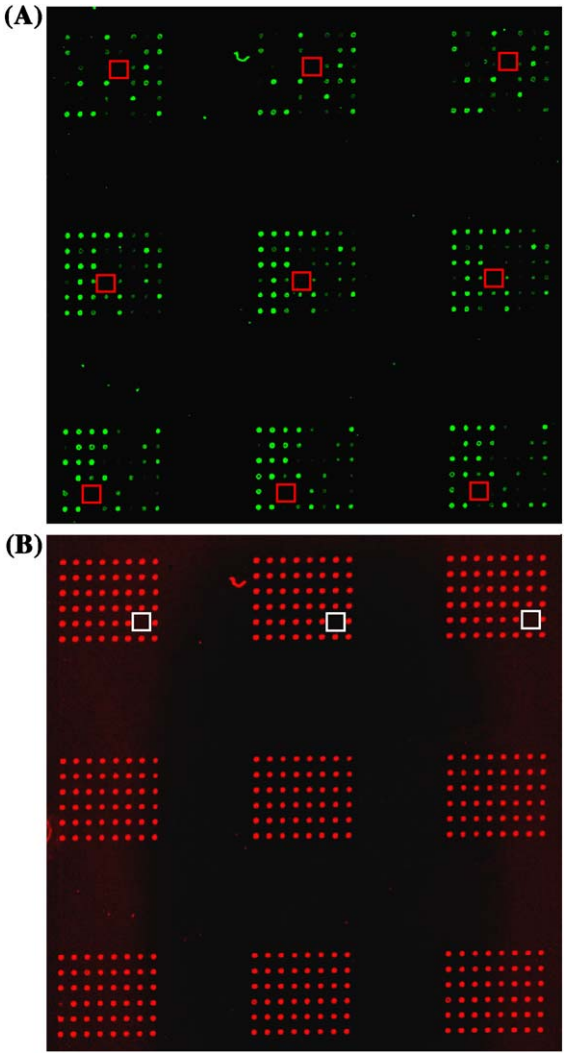

Fig. 3. Reproducibility of hybridization to triplicate arrays on one slide. (A) Hybridization of Cy3-dCTP-labeled ISR from mixed community to one array. The figure shows three subarrays (horizontal row) of the array and triplicate arrays (vertical rows) that are present on each slide. Red boxes indicate TA vector with no insert (negative control). (B) Hybridization of Cy5-dCTP-labeled kanamycin gene to the same array. The kanamycin gene is present in the TA vector portion of every spot on the array. White boxes indicate dropped spot.

3.5. Hybridization of plasmid-based 16S–23S rDNA ISR array with DNA from mixed community

Hybridization using fluorescently labeled target DNA from sludge organisms inoculated into plant rhizosphere resulted in a strong fluorescent signal from matching probe sequences. Hybridization to ISR arrays was reproducible among triplicate arrays on each slide and hybridization produced strong, distinctive hybridization patterns that corresponded to the overall diversity of organisms present in the sample (Fig. 3). Normalization of spot intensity from the sample (Cy3-dCTP-labeled ISR) to the intensity of the reference sequence (Cy5-dCTP-labeled kanamycin) present on every spotted plasmid on the array minimized slide-to-slide variability due to differences in the amount of arrayed probe material and some differences in hybridization. Additionally, this normalization procedure reduced within slide variability (for replicate spots) due to differential hybridization occurring over the surface of the slide. The kanamycin hybridization also provided a visible measure of spot quality (i.e., a spot dropped during printing was detected in each of the replicate arrays seen in Fig. 3, white boxes). As expected, hybridization with labeled target from the environmental samples resulted in no background from plasmid vector sequences (vector control intensity less than or equal to DMSO and water blanks; Fig. 3A). Coefficient of variation (CV) between replicate spots on the slide was fairly high (40%) with a high standard deviation (54%). However, the CV for spots with strong hybridization signals (hybridization ratio of 0.75 or greater) was very low (5 ± 4%). These results indicate that variability was highest among spots with low overall fluorescence, but was very consistent among strongly positive responses.

4. Discussion

In this study, we report the use of a plasmid-based 16S–23S rDNA ISR array for analysis of microbial diversity within complex environmental samples. The ISR array utilizes the sequence diversity of the 16S–23S rDNA ISR to obtain greater specificity than traditional 16S rRNA gene-based methods. The array format eliminates the need for length-based evaluation of ISR differences and provides a high-throughput,

rapid, and cost-effective means for simultaneous analysis of hundreds or thousands of phlyotypes from clonal libraries or environmental samples.

Specificity is a critical aspect of array experiments since parallel hybridization of hundreds or thousands of arrayed probes increases the potential for cross-hybridization between unrelated genetic sequences. Probes must have sufficiently high specificity and uniformity for simultaneous hybridization in an array format. Cross-hybridization analysis using a prototype ISR array showed that the 16S–23S rDNA ISR used as probe provides specificity over a broad range of closely and distantly related microorganisms (Table 2). These results agree with those of other researchers that have found that the ISR provides species to strain level differentiation of bacteria (Leblond-Bourget et al., 1996; Mendoza et al., 1998; Buchan et al., 2001). The arrayed probes must also exhibit uniformity in terms of optimal conditions for hybridization. The size range of ISRs of selected microorganisms resulted in probes with T_m values within a relatively narrow range (Table 1). Based on these data, 16S–23S rDNA ISR probes appear to provide specificity and uniformity for hybridization in a high-throughput array format.

Arraying plasmids eliminated the need for costly and time-consuming PCR amplification steps. Often, multiple PCR reactions must be combined and purified to provide product for arraying. Arrayed plasmid remained tightly bound to the slide (i.e., no comet effect) and produced strong hybridization signals with no need for modification (i.e., partial digestion, nicking, tailing, or amination of nucleotide sequences). Plasmid vector with no insert was spotted in each subarray to permit subtraction of fluorescence contributed by the vector (Figs. 2 and 3). For most of these studies, ISR insert from plasmid was used as the labeled target. Even in this case, background from the vector was minimal and only slightly greater than that for other negative controls (DMSO and water). When hybridization was carried out with a labeled probe from a mixed community extract, background from the vector control was less than or equal to that from other negative controls. These results suggest that arrayed plasmid containing probe insert sequences provides the same specificity as the more laborious and costly arraying of PCR product.

Given adequate probe specificity, successful analysis of mixed communities using array technology depends on sufficiently stringent hybridization conditions. In this study, formamide concentration was adjusted to optimize hybridization specificity and sensitivity. Formamide is normally adjusted in conjunction with temperature. However, elevated hybridization temperatures resulted in probe removal from glass slides in a nonlinear and unpredictable fashion (data not shown). As a result, formamide concentrations were adjusted and a constant hybridization temperature of 45 °C was employed. As expected, hybridization specificity increased as a function of formamide concentration (Fig. 1). This was consistent with previous work, which showed that formamide addition increases hybridization specificity and intensity, thereby increasing the dynamic range of the array (Urakawa et al., 2002). Our final hybridization conditions, 50% formamide in the hybridization solution and 45 °C hybridization temperature, were sufficiently stringent to permit high specificity without diminishing signal intensity.

There was a linear relationship between hybridization intensity and ISR similarity ($r^2 = 0.82$) and results were highly reproducible (8% difference among replicates ± 5.5%). Strong hybridization intensity (hybridization ratio of 0.85 or greater than that of the perfect match probe) corresponded to near-perfect match clones (99% or greater similarity in ISR sequences). Weak background was observed from closely related microorganisms (i.e., beta proteobacteria with greater than 95% similarity) to the labeled target. This background was probably due to hybridization within conserved $tRNA^{ile}$ and $tRNA^{ala}$ sequences, which were the only regions of extensive similarity between clones with 96% or less 16S rRNA sequence similarity to the perfect match sequence. The tRNA sequences represent the most highly conserved regions within the ISR, but generally make up less than 50% of the length of the ISR (Garcia-Martinez et al., 1999).

The linear relationship between hybridization intensity and 16S rRNA similarity was not as robust ($r^2 = 0.77$). This was expected because the ISR is known to be more variable than the 16S rRNA gene. For example, there were three sequences that matched the hybridized target by 98% in 16S rRNA gene sequence, but exhibited very low ISR hybridization ratio values. These sequences contained insertions or deletions resulting in an ISR match to the labeled target

of less than 80%. Evidence suggests that there can be significant variation in the number of *rrn* operons within a single microorganism and variation in ISR sequence between multiple *rrn* operons within a single microorganism (Brosius et al., 1981; Anton et al., 1998; Nagpal et al., 1998; Boyer et al., 2001). This may be an issue in array analysis, since only the arrayed 16S–23S rDNA ISRs would be represented. Additionally, there may be PCR-based biases affecting the number or kind of ISRs amplified. For example, Boyer et al. (2001) found that ISRs lacking tRNA sequences were preferentially amplified. Shorter templates and those without secondary structure also may be preferentially amplified (Fisher and Triplett, 1999). Gurtler and Stanisich (1996) found that differences in primers specific for 23S rRNA gene sequences affected the detection of spacer variation. For these reasons, it may be prudent to improve the resolution of the array by including clonal libraries from diverse environments using primers specific for different regions in the bacterial 16S rRNA or 23S rRNA genes (Gurtler and Stanisich, 1996; Garcia-Martinez et al., 1999), or primer sets targeting specific taxa (Berridge et al., 1998; Jiang and Fu, 2001).

Hybridization using fluorescently labeled 16S–23S rDNA ISRs from a mixed community sample produced strong fluorescent signal with no background from negative controls (Fig. 3). Simultaneous hybridization with a Cy5-labeled standard targeting the kanamycin gene served as a reference for normalizing hybridization signal. This is important as differences in signal intensity may result from overall differences in the amount of the microorganism in the sample population, as well as variations in the amount of DNA probe deposited during arraying (Wu et al., 2001; Cho and Tiedje, 2002). Using the kanamycin gene present in the TA vector and therefore part of every arrayed probe, it was possible to normalize for differences in the amount of arrayed material and to account for some differences in hybridization conditions.

The ISR array format described here permits the use of 16S–23S rDNA ISR sequences from mixed microbial communities as probe targets for characterization of community diversity. The method is based on PCR amplification for labeling of target sequences, making it semiquantitative and prone to PCR biases. However, for detection of target microorganisms or for analysis of microbial community dynamics or community characterization, PCR amplification of target sequences diminishes issues relating to array sensitivity. Detection of target sequences in environmental samples is limited by low concentrations of biomass and high concentrations of inhibitors and by the limited loading capacity of glass slide arrays (Hoheisel and Vingron, 2000; Cho and Tiedje, 2002; Zhou and Thompson, 2002). Target labeling through PCR amplification thereby enhances the ability to effectively characterize mixed populations of microorganisms by increasing sensitivity.

Using the 16S–23S rDNA ISR, it was possible to obtain clone probes from a mixed population of microorganisms in one PCR reaction, to hybridize with a high level of specificity, and to obtain uniform hybridization due to the narrow T_m range of ISR sequences. The ISR array provides a sensitive, rapid, and cost-effective method for analyzing mixed microbial communities. These arrays are now being used for analysis of microbial community dynamics within microbial populations exposed to temporal and environmental perturbations.

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