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# Oligonucleotide Microchips as Genosensors for Determinative and Environmental Studies in Microbiology

DMITRY Y. GUSCHIN,[1] BRUCE K. MOBARRY,[2]† DMITRII PROUDNIKOV,[1,3] DAVID A. STAHL,[2]*
BRUCE E. RITTMANN,[2] AND ANDREI D. MIRZABEKOV[1,3]

*Joint Human Genome Program, Argonne National Laboratory, Argonne, Illinois 60439[1]; Joint Human Genome Program, W. A. Engelhardt Institute of Molecular Biology, Moscow 117984, Russia[3]; and Department of Civil Engineering, Northwestern University, Evanston, IL 60208-3109[2]*

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**The utility of parallel hybridization of environmental nucleic acids to many oligonucleotides immobilized in a matrix of polyacrylamide gel pads on a glass slide (oligonucleotide microchip) was evaluated. Oligonucleotides complementary to small-subunit rRNA sequences of selected microbial groups, encompassing key genera of nitrifying bacteria, were shown to selectively retain labeled target nucleic acid derived from either DNA or RNA forms of the target sequences. The utility of varying the probe concentration to normalize hybridization signals and the use of multicolor detection for simultaneous quantitation of multiple probe-target populations were demonstrated.**

Studies in environmental microbiology are often limited by the inability to unambiguously identify and directly quantify the enormous diversity of natural populations. This is now changing with increasing use of molecular techniques to directly measure different genetic features. For example, DNA probes are now commonly used to detect specific catabolic functions (9) and to resolve different populations in the environment (21, 32). In particular, the use of group-specific DNA probes complementary to the small-subunit (SSU) rRNA has provided a comprehensive framework for studies of microbial population structure in complex systems (3, 28, 29, 33). However, the scope of such studies is often limited by the experimental format, generally involving the independent hybridization of multiple environmental samples to multiple DNA probes. In addition, some detection formats require amplification of the target nucleic acid (e.g., via PCR) that may complicate quantification. Thus, there is need for experimental formats that provide for greater sample capacity and greater sensitivity. The development of oligonucleotide microchips has provided the foundation for such a format.

Oligonucleotide microchips, as used in this study, were originally developed for rapid sequence analysis of genomic DNA, i.e., sequencing by hybridization with oligonucleotides in a matrix (SHOM) (14, 35). In this approach, deoxyribonucleotide probes are immobilized within a polyacrylamide gel matrix bound to the surface of a glass slide (35). For example, a single-stranded DNA fragment about 200 bases long could be hybridized in parallel with all the 65,536 possible DNA 8-mers, with each 8-mer immobilized on a different gel element of the matrix. In principle, the DNA sequence of the fragment can be reconstructed from the pattern of hybridization to the chip. However, this method has not yet been adapted for large-scale sequencing projects because of problems resulting from inefficient discrimination between perfect and mismatched duplexes, secondary structure within the single-stranded DNA (or RNA), and the presence of tandem repeats in the DNA to be sequenced (18). Yet, it has proven to be an excellent method for sequence analysis of mutations, gene polymorphisms, and other genetic changes (8, 35). In this study, we further extend the utility of this format by demonstrating its application to studies in microbial ecology.

A large collection of SSU rRNA-targeted DNA probes has been developed for studies in determinative and environmental microbiology. These have been designed to identify phylogenetic groups of different evolutionary depths, corresponding in general to the taxonomic ranks of species, genus, family, and higher (3, 27). The development and application of these probes have been described in recent reviews and will not be elaborated here (3, 27). This collection, with little or no modification, should also be suitable for analyses using the microchip format. Either DNA or RNA recovered from the environment could serve as the target for simultaneous hybridization to a matrix array of probes. Since the rRNAs are naturally amplified, often present in thousands of copies per cell, they should provide for greater sensitivity, eliminating the need for amplification in many applications. In addition, the volume of each polyacrylamide gel pad is large enough to accommodate a large amount of probe, allowing for a wide dynamic range of measurements. For example, the sequence diversity of SSU rRNAs recovered from different microbial populations of various abundances could be analyzed by a single hybridization to the microchip.

In this study, we demonstrate the application of microchips to study samples containing nitrifying bacteria. Nitrifying bacteria have proved particularly difficult to study by use of cultivation techniques, such as most probable number (17) and selective plating (10), because of their long generation times and poor counting efficiencies. Thus, a rapid, culture-independent enumeration technique for nitrifiers could greatly facilitate research in their ecology. More generally, the format should have widespread applications in environmental, medical, and determinative microbiology.

* Corresponding author. Mailing address: Department of Civil Engineering, Northwestern University, 2145 Sheridan Rd., Evanston, IL 60208-3109. Phone: (847) 491-4997. Fax: (847) 491-4011. E-mail: d-stahl@nwu.edu.

† Present address: IMAGE, FRC 103, University of Idaho, Moscow, ID 83844.

## MATERIALS AND METHODS

**Microbial strains.** *Escherichia coli*, *Desulfovibrio vulgaris* PT2, *Nitrosovibrio tenuis* NV12, *Nitrosomonas europaea* ATCC 19718, and *Nitrosomonas* sp. strain C-56 were used as sources of nucleic acid for these experiments.

**RNA preparation.** Total cellular RNA was isolated by phenol-chloroform extraction as described previously (29). For some of the samples, a ribosome

TABLE 1. Oligonucleotide probes

| Probe[a] | Probe name in original reference | Sequence (5′–3′) | Specificity | $T_d$[b] (°C) |
|---|---|---|---|---|
| S-G-Nit-1000-b-A-15 | Nb1000 | TGCGACCGGTCATGG | Genus *Nitrobacter* | 42 |
| S-G-Nit-1031-a-A-18 | NIT3 | CCTGTGCTCCATGCTCCG | Genus *Nitrobacter* | 66[c] |
| S-*-Nso-0190-a-A-19 | Nso190 | CGATCCCCTGCTTTTCTCC | Ammonia oxidizers | 62 |
| S-*-Nso-1225-a-A-20 | Nso1225 | CGCCATTGTATTACGTGTGA | Ammonia oxidizers | 51 |
| S-G-Nsm-0156-a-A-19 | Nsm156 | TATTAGCACATCTTTCGAT | Genus *Nitrosomonas* | 46 |
| S-*-Nsv-0443-a-A-19 | Nsv443 | CCGTGACCGTTTCGTTCCG | *Nitrosovibrio*-like genera | 52 |
| S-D-Bact-0338-a-A-18 | Eubacteria | GCTGCCTCCCGTAGGAGT | Bacteria | 54 |
| S-D-NBact-0338-a-A-18 | | ACTCCTACGGGAGGCAGC | S-D-Bact-0338-a-A-18 complement | 54 |
| S-*-Univ-1390-a-A-18 | | GACGGGCGGTGTGTACAA | Almost all life | 44 |

[a] Probe names have been standardized as described by Alm et al. (1).
[b] Experimentally determined.
[c] Estimated from in situ hybridization.

enrichment was performed before RNA extraction. Forty milliliters of log-phase-grown *E. coli* or *D. vulgaris* PT2 was centrifuged at 3,500 × *g* for 10 min and resuspended in 4 ml of ribosome buffer (20 mM $MgCl_2$, 50 mM KCl, 50 mM Tris [pH 7.5], and 5 mM β-mercaptoethanol in diethyl pyrocarbonate-treated double-distilled water) at 4°C. The cell suspension was divided between four screw-top microcentrifuge tubes, and 0.5 g of 0.1-mm-diameter $ZrO_2$ beads was added. The cell suspensions were disrupted for 2 min, put on ice for 5 min, and disrupted again for 2 min. The cell suspensions were centrifuged at 14,000 × *g* for 10 min. The supernatant, which contained the ribosomes, was recovered and transferred to ultracentrifuge tubes. Ribosomes were pelleted by ultracentrifugation in ribosome buffer at 55,000 rpm (201,000 × *g* average) for 50 min in a Beckman (Fullerton, Calif.) Optima Series TL swinging-bucket rotor for a Svedberg sedimentation factor of 70S. After centrifugation, the supernatant was discarded and the RNA was recovered from the pelleted ribosomes by extraction with pH 5.1 phenol-chloroform as described above. The quality and quantity of extracted RNA were evaluated by polyacrylamide gel electrophoresis and ethidium bromide staining (25).

**Cloning of 16S rDNA and in vitro production of RNA transcripts.** DNA was extracted from *E. coli*, *D. vulgaris* PT2, *Nitrosovibrio tenuis* NV12, *Nitrosomonas europaea* 19718, and *Nitrosomonas* sp. strain C-56 cell pastes by use of a guanidine-diatom method (6). Near-complete 16S ribosomal DNA (rDNA) genes (ca. 1,500 bp) were recovered from each by PCR amplification with S-D-Bact-0011-a-S-17 (GTTTGATCCTGGCTCAG) and S-D-Bact-1492-a-A-21 (ACGGYTA CCTTGTTACGACTT) (13) as primers (1) and a premixed PCR amplification buffer (Pharmacia Biotech, Inc., Piscataway, N.J.) consisting of 0.2 mM $MgCl_2$, 2.5 mM each dATP, dCTP, dGTP, and dTTP, 0.2 mM of each amplification primer, and 2.5 U of *Taq* DNA polymerase (Pharmacia Biotech). Temperature cycling was done in an Idaho Technology (Idaho Falls, Idaho) thermocycler by using 30 cycles of 15 s at 94°C, 20 s at 50°C, and 30 s at 72°C. The PCR products were cloned in a pCR plasmid (Invitrogen, San Diego, Calif.) as described in the manufacturer's instructions. Plasmids were isolated with the Wizard kit (Promega, Madison, Wis.) and used for in vitro transcription by T7 RNA polymerase of the cloned SSU rRNA genes (19).

**DNA oligonucleotide probes.** All probes used in this study are complementary to the SSU rRNAs and were previously characterized by use of a membrane hybridization format (Table 1). Four are complementary to different groups of ammonia-oxidizing bacteria within the beta subdivision of the *Proteobacteria* (12, 20, 30, 31, 34). Probes S-G-Nso-0190-b-A-19 (Nso190) and S-G-Nso-1225-a-A-20 (Nso1225) encompass all sequenced ammonia oxidizers of the beta subclass of *Proteobacteria*, probe S-G-Nsm-0156-a-A-19 (Nsm156) identifies members of the genus *Nitrosomonas* (also including *Nitrosococcus mobilis*), and probe S-*-Nsv-0443-a-A-20 (Nsv443) is specific for the *Nitrosovibrio-Nitrosolobus-Nitrosospira* group. Probes for members of the genus *Nitrobacter* (nitrite oxidizers) were S-G-Nit-1000-b-A-15 (Nb1000) and S-G-Nit-1031-a-A-18 (NIT3) (20, 31). Other probes used were S-D-Bact-0338-a-A-18 (Bac338), which hybridizes to members of the bacterial domain (2), S-D-NBact-0338-a-S-18 (NonBac338), complementary to Bac338, and S-*-Univ-1390-a-A-18 (Uni1390), complementary to the SSU RNA of nearly all characterized living organisms, with the exception of some protists (1, 36).

TABLE 2. Location of oligonucleotides in the polyacrylamide matrices of Fig. 1

| Row | Oligonucleotide in Fig. 1 column | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| a | Nb1000 | NIT3 | | Nso190 |
| b | Nso1225 | Nsm156 | Nsv443 | |
| c | Bac338 | NonBac338 | Uni1390 | |

**RNA and DNA labeling and fragmentation.** Single-stranded DNA was prepared by asymmetric PCR as described by Ausubel et al. (4) with a 100-fold excess of the forward primer. DNA labeling and fragmentation were done as described previously (23). Briefly, DNA was partially depurinated in 80% formic acid for 30 min at 20°C and then incubated in 0.5 M ethylenediamine hydrochloride (pH 7.4) for 3 h at 37°C and subsequently for 30 min at 37°C in the presence of 0.1 M $NaBH_4$. Fluorescein isothiocyanate was incorporated into fragmented DNA by incubation in absolute dimethyl sulfoxide at room temperature for 1 h.

RNA was fragmented by base hydrolysis and dephosphorylated with bovine phosphatase. Fragmented RNA was oxidized by $NaIO_4$ and labeled either by ethylenediamine-mediated coupling of 6-carboxyfluorescein succinamide or by direct incorporation of tetramethylrhodamine-hydrazide as described previously (23).

**Microchip fabrication.** A matrix of glass-immobilized gel elements measuring 60 by 60 by 20 μm or 100 by 100 by 20 μm each and spaced 120 or 200 μm apart, respectively, was prepared as described previously (35). The polyacrylamide gel was activated by substitution of some amide groups with hydrazide groups by hydrazine-hydrate treatment. Oligonucleotides were activated by oxidizing 3′-terminal 3-methyluridine with $NaIO_4$ to produce dialdehyde groups for coupling with the hydrazide groups of the gel (14) and coupled to each micromatrix element by applying 0.5 to 1 nl of the activated oligonucleotide solution (100 pmol/ml) by use of a specially devised robot (35).

**Hybridization and image analysis.** Probe binding was quantified by measuring the fluorescence conferred by the binding of fluorescently labeled DNA or RNA (tetramethylrhodamine or fluorescein) to the individual gel elements. Hybridization and washing were controlled and monitored with a Peltier thermotable (with a working range of −5.0°C to +60.0°C) mounted on the stage of a custom-made epifluorescence microscope equipped with appropriate fluorescence filters and image analysis software as described previously (35). The microchip was hybridized at 5°C, either overnight or for 6 h, in 2 to 5 μl of hybridization buffer (33% formamide, 0.9 M NaCl, 1 mM EDTA, 1% Tween 20, 50 mM sodium phosphate [pH 7.0]) at a concentration of DNA and RNA of 0.2 to 2 pmol/ml. The hybridization mixture was replaced with 5 to 10 μl of hybridization buffer without formamide immediately prior to microscopic observation. Exposures were in the range of 0.1 to 10 s, depending on the signal intensity, but were typically around 1 s. Fluorescence was monitored either at room temperature or at a range of temperatures between 5 and 60°C.

## RESULTS

The microchip was evaluated with three different rRNA preparations (phenol extracts of cellular RNA, RNA isolated from purified ribosomes, and in vitro transcripts of cloned rDNA) and both fragmented double-stranded and single-stranded DNA. Hybridizations were performed in a formamide buffer at low temperature to enhance microchip durability and decrease RNA degradation. Although all DNA and RNA preparations could be used, the best discrimination was observed for in vitro-transcribed rRNAs under the hybridization conditions evaluated in this study.

Figure 1 shows the fluorescence of individual gel elements on the microchip following hybridization to the 16S rRNAs of *Nitrosovibrio tenuis* (Fig. 1A), *Nitrosomonas europaea* (Fig. 1B), and *E. coli*, either in vitro transcribed (Fig. 1C) or recovered

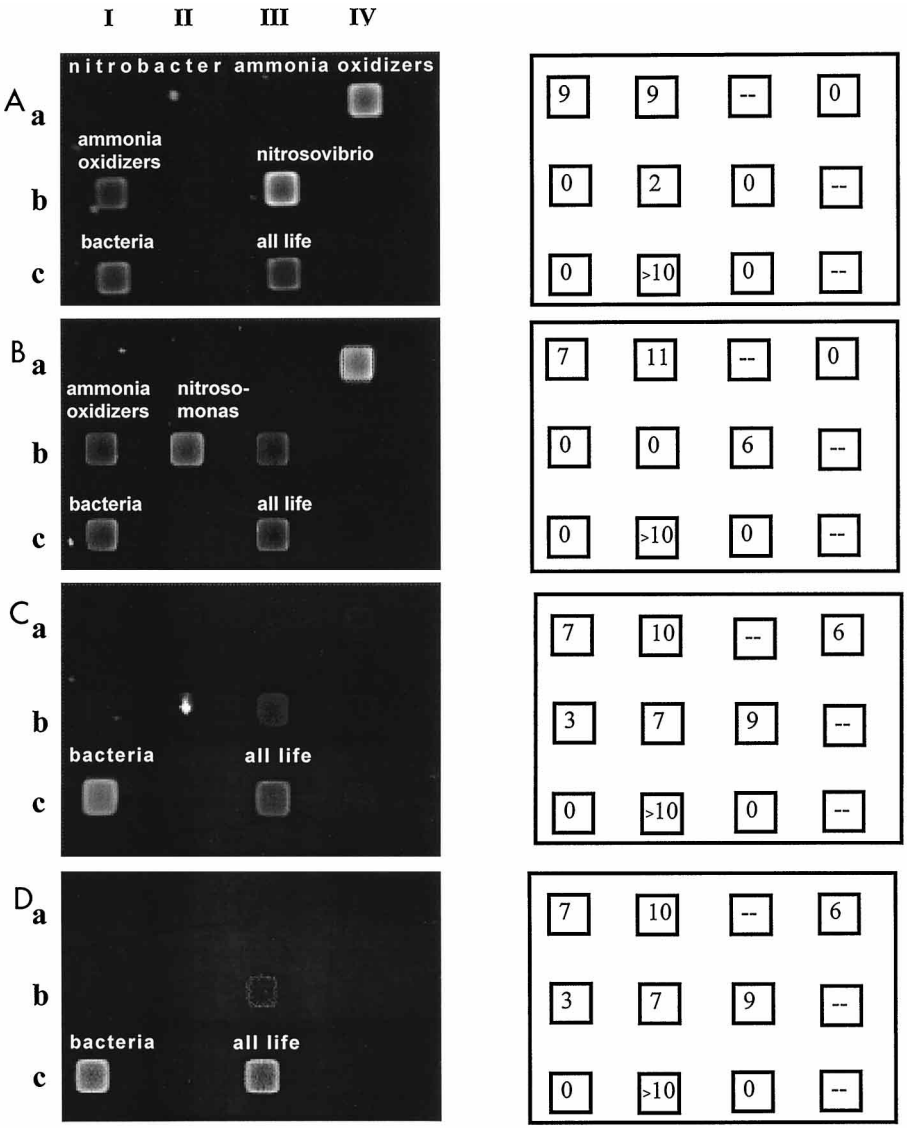


FIG. 1. Hybridization of fluorescein-labeled 16S rRNAs to the microchip. The microchip with immobilized probes (see Tables 1 and 2 for a list of probes used and an explanation of the matrices in this figure) was hybridized sequentially to in vitro-transcribed 16S rRNA of *Nitrosovibrio tenuis* (A), *Nitrosomonas europaea* (B), and *E. coli* (C) and to *E. coli* rRNA recovered from isolated ribosomes (D). The panels on the right display the number of mismatches between each probe and the RNA. – –, blank position.

from isolated ribosomes (Fig. 1D). The same microchip was used for each hybridization following washing with distilled water. Each microchip was routinely used for up to 20 to 30 hybridization experiments. The appropriate pattern of hybridization was observed for all gel elements shown, despite a significant difference in dissociation temperatures ($T_d$s) previously determined by use of membrane support hybridization (Table 1). For hybrids of comparable stability, we anticipate that discrimination can be achieved by washing at increasing temperatures (described below) or by simultaneously evaluating their melting characteristics (8), since the fluorescence analyzer can monitor hybridization signals in real time.

Figure 2 shows the results of an experiment evaluating the effect of increasing the washing temperature on target RNA retention. A mixture of *Nitrosovibrio tenuis* and *E. coli* 16S rRNA labeled with different fluorescent dyes (fluorescein and tetramethylrhodamine, respectively) was hybridized to the chip at 5°C. The hybridization solution was then replaced with washing buffer, and the retention of each RNA species was measured following each 10°C incremental increase in temperature (up to 60°C) by multicolor detection. Nonspecific hybridization of *E. coli* rRNA to Nso1225 (ammonia oxidizer probe), Nsm156 (*Nitrosomonas* probe), and NonBac338 (antisense probe) was observed following the 10°C wash. However, this nonspecific hybridization was significantly reduced following the 40°C wash. In like manner, the 16S rRNA of *Nitrosovibrio tenuis* hybridized to *Nitrosomonas* (Nsm156) at 10°C but was reduced to near background (compared to NonBac338) following the 40°C wash. A more complete correction for differences in stabilities of duplexes can be carried out by measuring the equilibrium or nonequilibrium melting curves for all microchip elements (8). This would provide a basis to compensate for the various factors influencing individual duplex stability, e.g., their length, G+C content, and competition with secondary and tertiary structures in RNA and DNA.

Figure 2B shows the ratios of hybridization intensities of

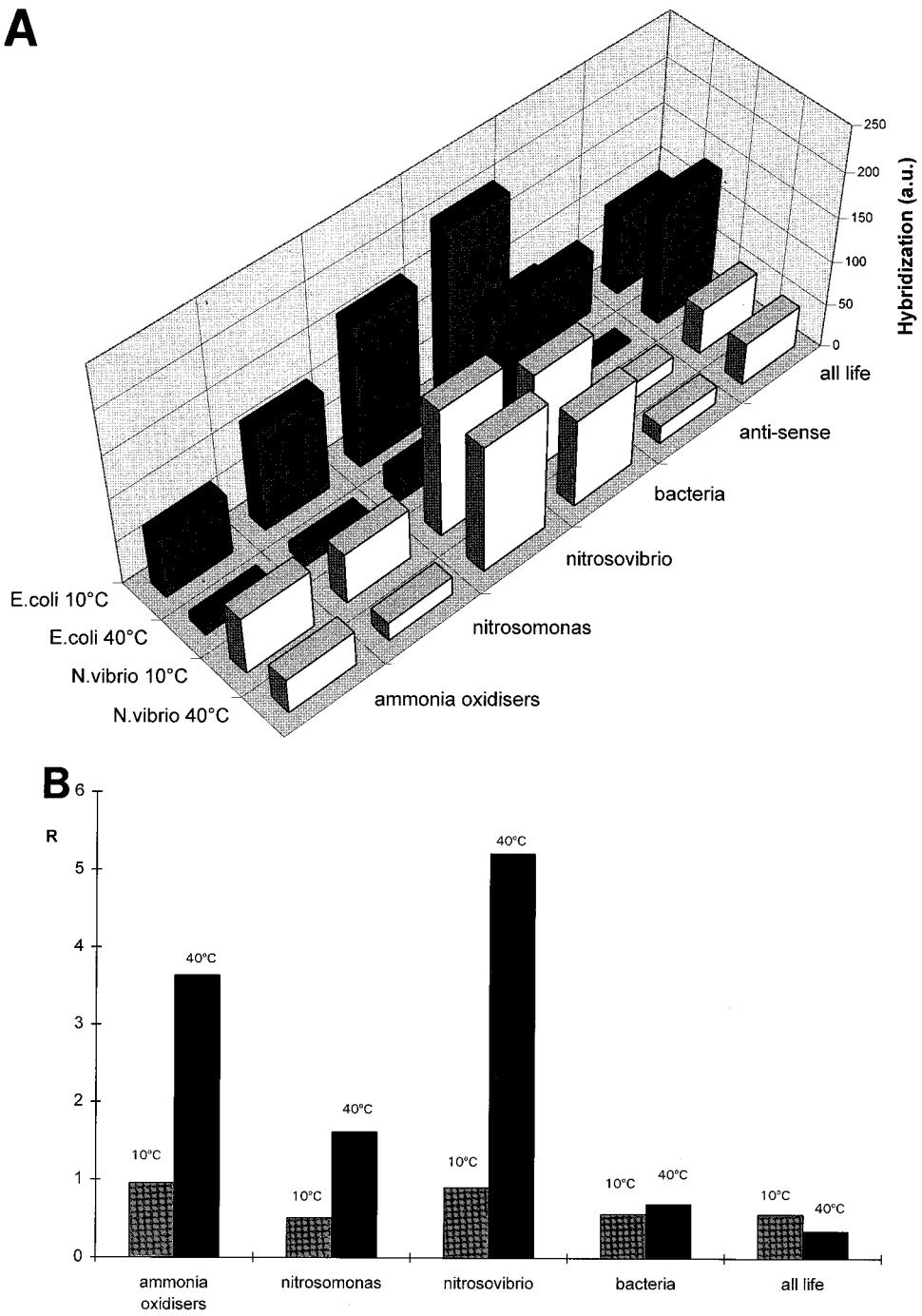


FIG. 2. Hybridization of the mixture of differently labeled *E. coli* and *Nitrosovibrio tenuis* rRNAs to the microchip at 10 and 40°C, measured simultaneously by multicolor detection. (A) The microchip (see Fig. 1) was hybridized with a mixture of fluorescein-labeled *Nitrosovibrio tenuis* and tetramethylrhodamine-labeled *E. coli* 16S rRNA and washed serially at the indicated temperatures. a.u., arbitrary units of fluorescence intensities. (B) The ratio of the hybridization intensities of *Nitrosovibrio tenuis* ($I_{Nt}$) to *E. coli* ($I_{E.\ coli}$) 16S RNA measured at 10 and 40°C. R = ($I_{Nt}/I_{E.\ coli}$).

fluorescein-labeled *Nitrosovibrio tenuis* to tetramethylrhodamine-labeled *E. coli* with different microchip oligonucleotides at 10 and 40°C (the ratios are derived from the data presented in Fig. 2A). These ratios were not changed significantly for oligonucleotides specific to bacteria and all living organisms between 10°C and, for more stringent conditions, 40°C. However, the ratio is dramatically increased at 40°C (compared to 10°C) for oligonucleotides specific to ammonia oxidizers and nitrosovibrios. This increase reflects the greater duplex stability of *Nitrosovibrio tenuis* RNA with the complementary oligonucleotides compared with that of *E. coli* RNA. Although the *Nitrosomonas* ratio increases, the signal originating from each labeled RNA is near background. This experiment demonstrates that the inclusion of second dye-labeled RNA, either isolated from cells or synthesized (8), could be used as an internal standard for quantitative assessments of hybridization patterns.

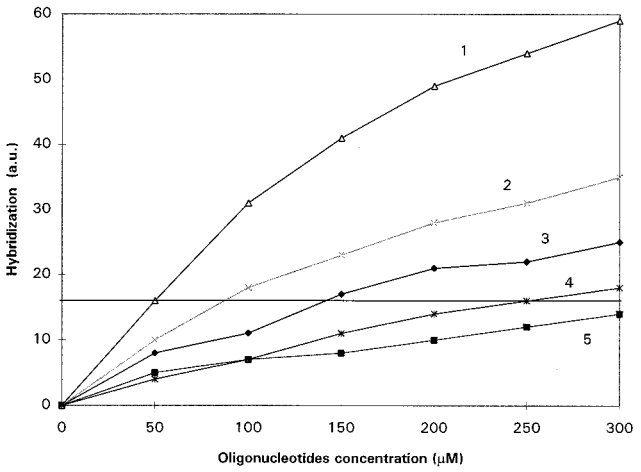


FIG. 3. Concentration effect of the immobilized oligonucleotides on hybridization intensity. A microchip with different concentrations of immobilized oligonucleotides was hybridized with *Nitrosovibrio tenuis* 16S rRNA labeled with fluorescein and washed at 20°C. Curves 1 to 5 1 correspond to the Nsv443 (nitrosovibrio-like), Bac338 (bacterial domain), Nso1225 (ammonia oxidizer), Uni1390 (all living organism), and Nsm-156 (nitrosomonas) probes, respectively. a.u., arbitrary units of fluorescence intensities.

Variable hybridization to the different gel elements is the expected consequence of using a single hybridization condition to evaluate an array of probes, each having different kinetics of association and dissociation. To some extent, these differences can be normalized by varying the concentration of oligonucleotides in the individual gel elements. For example, the relatively low hybridization signals of Nso1225 and Uni1390 compared to Nsv443 (Fig. 1A and Table 2) could be elevated by increasing the amount of the corresponding oligonucleotide probes immobilized in the gel. We evaluated this approach by synthesizing a microchip with selected probes immobilized at several different concentrations, up to six times higher than that used in the experiments described previously. This was accomplished by multiple applications of the standard loading solution (100 pmol/μl of probe) to each gel element. Comparable hybridization of Nso1225 (ammonia oxidizer probe) and Nsv443 (nitrosovibrio-like probe) was achieved following three applications of the Nso1225 probe (Fig. 3). Similarly, two applications of Bac338 (bacterial domain probe) and five of Uni1390 (all living organism probe) resulted in hybridization comparable to that of Nsv443.

## DISCUSSION

The use of microchips for determinative studies provides several advantages over conventional hybridization formats. Hundreds or even thousands of different oligonucleotides can be immobilized on a single microchip, allowing for the simultaneous detection of a great variety of different microorganisms in a single sample. In addition, a microchip can be used up to 20 to 30 times without noticeable deterioration of the hybridization signal. A theoretical sensitivity of about 10 amol of tetramethylrhodamine-labeled target per 60- by 60-μm microchip element should be sufficient for the direct analysis of many environmental populations and therefore not require prior amplification of the target nucleic acids. A further decrease of the microchip pad size to 5 by 5 μm could additionally enhance the sensitivity of the measurements to about 0.1 amol of the target nucleic acid, assuming that quantitative hybridization is possible with an array element of this size.

The gel array also offers several advantages over formats using an in situ synthesis of the oligonucleotide array. The synthetic oligonucleotides are purified by gel electrophoresis or high-performance liquid chromatography prior to immobilization on the microchip. This provides for stringent quality control of oligonucleotide purity and ensures high specificity. The polyacrylamide gel support has a higher capacity of immobilized oligonucleotides (from 3 fmol to 300 fmol per 100- by 100-μm gel pad). This provides for improved quantification and better discrimination between perfect and mismatched duplexes (15).

The signal intensities depend on the many factors that influence the kinetics of the hybridization reaction, including concentration of the target nucleic acid and the immobilized oligonucleotides, duplex length, G+C content, and secondary structure (28). Since the concentration of the oligonucleotides in each gel element (and therefore the associated hybridization signal) can be varied over a wide range, this provides a basis to normalize their hybridization signals without changing probe design (14). For example, the relatively low hybridization signals observed for oligonucleotides Nso1225 and Uni1390 compared with those of Nsv443 and Bac338 were increased to comparable levels by increasing their concentrations on the microchips by approximately three and five times, respectively (Fig. 3). This method of normalization, by providing more direct quantitative interpretation, should simplify microbial analyses. We have yet to evaluate other methods to reduce differences in hybridization efficiency for different probe-target duplexes. These include the addition of tetramethylammonium chloride (16) or betaine (24) to equalize the A:T and G:C base pair stability.

Quantification of individual target populations should also be facilitated by use of multicolor fluorescence monitoring. As shown in Fig. 2, two-color detection allowed us to carry out measurements simultaneously for two different RNA populations. In this way, pure culture microorganisms, purified target nucleic acid, or even synthetic oligonucleotides could be added as internal standards (8), serving to evaluate the efficiency of nucleic acid isolation or the absolute amount of target nucleic acid recovered.

The hybridization of the microbial microchips was carried out with five different preparations of target nucleic acid. rRNA and total RNA were recovered from cells. RNA transcribed in vitro as well as single- and double-stranded PCR-amplified 16S rDNA were obtained from plasmids containing the cloned 16S rRNA gene. All of these sample types provided comparable identification of the microorganisms and could be used individually for different purposes. For example, the rRNA provides a naturally amplified target. Also, since cellular ribosome content is well known to vary with the growth rate, it is generally thought that direct quantification of rRNA serves to identify the more active environmental populations (22). In contrast, analysis of PCR-amplified rDNA should provide a more general measure of all microorganisms present in a sample (11). Alternatively, these measures could be combined. For example, the RNA and DNA components of an environmental sample could be labeled with different fluorescent dyes. Following their combined hybridization, the resulting ratio of RNA and DNA hybridizing to an individual gel element could be used to infer some aspects of the physiological status of the corresponding microbial population.

The resolution of probe-based analyses ultimately depends upon the discrimination between perfect duplexes and those containing one or more mismatches. Due to the dependence of duplex stability on length and G+C content, the greater stability of long G+C-rich duplexes with mismatches can sometimes contribute to signal intensities comparable to those of completely

matched, but shorter and G+C-poor, duplexes (e.g., when the same wash conditions are used). Such confusion can be significantly diminished by measuring the hybridization at the proper $T_d$ for each oligonucleotide. Since the fluorescent analyzer monitors the hybridization signals in real time (0.1- to 1-s response time), dissociation measurements can be made simultaneously for all microchip oligonucleotides to define the optimum conditions for discrimination. For example, we used this approach to demonstrate improved discrimination for several probes by increasing the washing temperature from 10 to 40°C (Fig. 2). The discrimination between perfect and mismatched duplexes can be further enhanced by measuring the melting curves simultaneously for all duplexes formed on the microchips (8).

In summary, these studies have documented the utility of the gel array microchip for use in determinative and environmental microbiology. Continuing studies are examining the utility of alternative methods of nucleic acid preparation and labeling in relationship to sensitivity and accuracy of quantitation. The method offers the remarkable capacity of simultaneous hybridization as well as other noted advantages of the gel element format. Although applications to diagnostic and determinative studies are clear, we also suggest that it should provide a powerful format for the systematic exploration of natural microbial diversity. It is increasingly evident that only a small fraction of the microbial world has been characterized, although there is little agreement about the full extent of undescribed diversity (5, 7, 26). However, the development of rRNA-targeted probes that selectively target characterized phylogenetic groups now provides a format to systematically address this question. These probes are fully compatible with the microchip format. In addition to their use in identifying described phylogenetic groups, the great capacity of the microchip array could be used to evaluate sequence motifs that have yet to be identified and so point out habitats that contain novel microbial populations. Thus, we anticipate that the microchip will have application not only to determinative microbiology but also to ongoing studies of global diversity.

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