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New method to characterize microbial diversity using flow cytometry

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Abstract The majority of microorganisms have yet to be cultivated and represent a vast uncharacterized and untapped resource. Here, we report the utilization of a combination of flow cytometry, cultivation, and molecular genetics to develop new methodologies to access and characterize biodiversity in microbial samples. We demonstrate that fluorescent dyes and combinations of dyes can selectively stain portions of bacterial populations that can be isolated as sub-populations using fluorescence-activated cell sorting (FACS). Microbial subpopulations obtained by FACS differ substantially from the original microbial population, as demonstrated by denaturing gradient gel electrophoresis and determination of 16S rRNA gene sequences. These sub-populations can subsequently be used to inoculate microbial growth media, allowing the isolation of different microbial species from those that can be readily cultivated from the original sample using the same microbial growth media. When this technique was applied to the analysis of activated-sludge and Yellowstone Lake hydrothermal vent samples, comparative analysis of 16S rDNA sequences revealed that FACS allowed the detection of numerous bacterial species, including previously unknown species, not readily detectable in the original sample due to low relative abundance. This approach may result in a convenient methodology to more thoroughly characterize microbial biodiversity.

Keywords Microbial diversity · Flow cytometry · Uncultivated · Cell sorting

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Introduction

It is estimated that 99% or more of all microorganisms have not yet been grown as pure cultures under laboratory conditions [1, 16, 30, 33]. Improved cultivation techniques, such as employing low substrate concentrations and extended incubation periods, has allowed an expanded range of microbial species to be cultivated but nevertheless it is clear that, in order to thoroughly investigate microbial diversity, new methods are needed [13, 16, 30]. Another means of accessing microbial diversity is to isolate total DNA from the mixed population present in environmental samples and then amplify and/or clone target DNA fragments [20, 21]. A problem with this approach is that often a few bacterial species are present in such abundance that it is extremely difficult to obtain genetic information from rare microorganisms [9, 16]. Even though it is estimated that a gram of soil contains about 10,000 bacterial species, microbial ecology investigations of soil samples using cultivation techniques and cultivation-independent (genetic) techniques typically result in the isolation/identification of ≤ 50 bacterial species [13, 14, 20, 21, 30]. While isolation/identification of greater numbers of microbial species by cultivation and cultivation-independent techniques is possible, new approaches are needed if a comprehensive characterization of microbial communities is ever to be achieved.

Flow cytometry is an extremely versatile tool that complements existing technologies and enables fundamentally new information to be obtained in microbial ecology studies [3, 10, 12, 18, 19, 23, 29, 34]. Flow cytometry used in conjunction with fluorescence-activated cell sorting (FACS) can quantify and fractionate complex bacterial communities [3]. Typically 3,000–100,000 cells/s can be characterized and sorted by flow cytometry. The type of information that can be obtained using flow cytometry concerning individual microbial cells includes size [29], shape, surface texture, viability, DNA content [6], and specific staining conferred by

fluorescent antibodies [32] or rRNA-targeted oligonucleotide probes [28, 35]. Moreover, the sorting criteria used in FACS can be adjusted to allow fractionation of bacterial populations based on any one, or a combination, of these parameters and the number of cells having certain characteristics as measured by flow cytometry can be quantified. Multiple fluorescent dyes are available that can selectively stain lipids, proteins, nucleic acids, and other cellular components [18, 25, 28, 34].

Recent studies have demonstrated that fractionation of DNA based on its GC-content followed by analysis of 16S rRNA gene fragments/sequences allows an improved characterization of microbial diversity in environmental samples [11], but this approach does not permit the isolation and cultivation of unique microbial species. However, microbial cell viability remains intact when dyes targeting cell surface molecules are used in FACS. This permits cell cultivation after FACS. The hypothesis of this investigation is that cell surfaces differ between microbial species; and these differences can be observed by the differential staining of microbial species by dyes that target compounds present on the external surfaces of cells, such as lipids, proteins, and carbohydrates. We demonstrate that flow cytometry, in conjunction with cultivation and molecular approaches, can increase the number of bacterial species that can be cultivated and the number of unique 16S rRNA gene sequences that can be recovered from environmental samples. In particular, FACS techniques are demonstrated to allow a more thorough characterization of the microbial diversity present in Yellowstone Lake hydrothermal vent and municipal activated-sludge samples than is possible using microbial cultivation and 16S rRNA analysis of unfractionated samples.

Materials and methods

Environmental sampling and sample preparation

Activated-sludge mixed liquor was obtained from the return sludge line of a municipal sewage treatment plant (Woodridge, Ill., USA) in May, 2003. Samples of hydrothermal vent fluid were collected from the bottom of Yellowstone Lake, using a remotely operated vehicle. Cell suspensions of environmental samples were utilized fresh or were frozen in 50% glycerol and stored at −80°C until needed. Activated-sludge samples were washed by centrifugation for 10 min at 10,000 *g* at 4°C and resuspended to a cell density of about 10^7 cells/mL in PBS buffer solution, consisting of 10 mM phosphate (pH 7), 2.7 mM KCl, and 137 mM NaCl. This buffer solution was also used as the sheath fluid in flow cytometry. For Yellowstone Lake samples, 300 mL of hydrothermal vent fluid was concentrated by centrifugation at 13,000 *g* for 15 min at 4°C and washed once in buffer solution before being resuspended in 10 mL of buffer. Cell suspensions were sonicated in a sonic bath (Fisher Scientific, Pittsburgh, Pa., USA) for 10 min to break up cell aggregates and were filtered through a 35 μm filter (Benton Dickinson Corp., Nogales, Senora, Mexico) prior to flow cytometry.

Flow cytometry and sorting

The studies reported here employed a Cytomation MoFlo MLS high-speed flow cytometer (Dako Cytomation, Fort Collins, Colo., USA) that is capable of simultaneously measuring forward-scattering intensity, side-scattering intensity , and multiple fluorescence wavelengths, enabled by three argon ion lasers. The sorting speed was maintained at 3,000–5,000 cells/s and non-overlapping sorting windows were selected (generally resulting in the loss of about 10% of the total cells) to achieve the highest degree of accuracy in the cell-sorting procedure. Sort criteria were defined by drawing polygonal gates in bivariate histograms with CyCLOPS flow cytometry software (Dako Cytomation). Cells were sorted either using intrinsic cell properties or first staining the cells with various lipid-specific fluorescent dyes [*N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, "DHPE", triethanolamine salt (catalogue number F362) or 3,3′-dioctadecyloxacarbocyanine perchlorate, "DiO", $DiOC_{18}$ (catalogue number D275); Molecular Probes, Eugene, Ore., USA]. Stock solutions of fluorescent dyes containing 1 mg/mL were prepared in ethanol. Filtered cell suspensions were stained using 1 μL of stock dye solution per milliliter of cell suspension, mixed by inversion or brief vortexing, and incubated at room temperature for 5 min. The cells were recovered by centrifugation at 6,000 *g* for 3 min to remove the unbound dye and then the supernatant was removed. The cells were washed again with PBS, centrifuged to remove all unbound dye, resuspended in 1 vol. of PBS buffer, and then analyzed immediately by flow cytometry.

Microscopy

One microliter each of the SYTO 9 and propidium iodide dye preparations of a LIVE/DEAD *Bac*Light bacterial viability kit (Molecular Probes) was added to 1 mL of prepared samples to check the viability of the microorganisms before and after flow cytometry; and the samples were stained for 5 min. Aliquots (10 μL) were placed on gelatin-coated microscope slides and examined with an Axioscope 2 Plus epifluorescent microscope equipped with a PKP510 dichromatic mirror and a LP520 suppression filter (Carl Zeiss, Germany).

Bacterial growth conditions

Microbial growth experiments employed heterotrophic aerobic media (HAM; containing per liter of distilled

water): 2 mL glycerol, 10 g peptone, 10 g tryptone, 0.2 g sodium thiosulfate, 0.2 g magnesium sulfate, and 0.5 g K_2HPO_4). Cultures were incubated at 25°C for 3–5 days to allow the growth of heterotrophic aerobic bacteria.

Genetic analyses of environmental samples

Genomic DNA was isolated from activated-sludge samples, using the FastDNA SPIN kit for soil (Qbiogene, Carlsbad, Calif., USA). The DNA was extracted from Yellowstone Lake samples by subjecting 10 µL samples to three freeze (−80°C) and thaw cycles, followed by exposure to a boiling water bath for 5 min. The samples were centrifuged at 12,000 *g* and the supernatant was used in PCR reactions. The PCR primers used in this study (Table 1) were synthesized by MWG Biotech (High Point, N.C., USA) and targeted conserved regions of bacterial and archaeal 16S rRNA genes. For analysis of activated-sludge samples, the primer BA101F included a GC clamp at the 5′ end [20] to facilitate denaturing gradient gel electrophoresis (DGGE). The amplifications were performed with a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany). Fifty microliters of PCR mixture contained 1 µL of template DNA, 400 nmol of each primer (BA101F-GC, BA518R), 1× HighFidelity buffer with Mg^{2+}, 200 µmol of each deoxynucleoside triphosphate, and 1 unit of TripleMaster enzyme mix (Brinkman Instruments, Westbury, N.Y., USA). The optimal annealing temperature for PCR was determined by a temperature-gradient PCR. Serial dilutions of original DNA templates were tested to determine the optimal DNA concentrations for PCR by visual inspection of PCR bands on ethidium bromide-stained agarose gels. The annealing temperature and template dilution producing the cleanest PCR band was used in the subsequent amplification.

The PCR conditions for activated-sludge samples began with 5 min of denaturation at 94°C, TripleMaster enzyme mix was added to the reaction, and then a "touchdown" PCR was performed to increase the specificity of amplification and reduce the formation of spurious by-products [8, 9]. The initial annealing temperature (57°C) was set 10°C above the expected annealing temperature (47°C) and decreased by 0.5°C per cycle until a touchdown of 47°C, at which temperature ten additional cycles were carried out. Amplification was performed with 1 min of denaturation at 94°C, 1 min of primer annealing, and 2 min of primer extension at 72°C, followed by 7 min of final primer extension.

The low biomass in the Yellowstone Lake hydrothermal vent samples necessitated a nested PCR amplification of the 16S rRNA gene fragments. The DNA samples were first amplified with primer pair BA8F/UN1492R [26] for eubacteria and AR23F/UN1492R for archaea that amplified a larger segment of the 16S rRNA gene. The PCR program used was: 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 46°C for 30 s, and extension at 72°C for 1 min, and a single final extension at 72°C for 7 min. Then, 0.5 µL of these first PCR products were used as templates in nested PCR to amplify the regions of 16S rDNA with primer pairs BA101F-GC/BA518R (bacterial primer set 1) and BA114F-GC/BA1406R (bacterial primer set 2) for eubacteria and AR46F-GC/AR519R (archaeal primer set 1) and AR349F-GC/AR786R (archaeal primer set 2) for archaea, using the same PCR conditions as for direct amplification.

The PCR products were purified and concentrated, using the QIAquick PCR purification kit (Qiagen, Valencia, Calif., USA) as described by the manufacturer, and then used in DGGE analyses. The DNA concentration was determined by comparison with a DNA quantification standard (GenSura Laboratories, San Diego, Calif., USA). Four hundred nanograms of DNA were loaded onto an 8% (w/v) polyacrylamide gel in 1× TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8.0). The 8% polyacrylamide gel (40% acrylamide- *N*,*N*′-methylenebisacrylamide stock, 37:1) was made with denaturing gradients ranging from 35%

Table 1 Primers used for the amplification of 16S rRNA genes

Primer[a]	Sequence (5′ to 3′)	Position[b]	Reference(s)
BA8F	AGTTTGATCCTGGCTCAG	8–25	[26]
UN1492R	GGYTACCTTGTTACGACTT	1,474–1,492	[26]
BA101F-GC	CGCCCGCCGCGCCCCGCGC CCGTCCCGCCGCCCCCGCC CGTGGCGGACGGGTGAGTAA	101–118	[20, 27]
BA518R	CGTATTACCGCGGCTGCTGG	499–518	[27]
BA1114F	GCAACGAGCGCAACCC	1,099–1,114	[15]
BA1406R	ACGGGCGGTGTGTRC	1,392–1,406	[15]
AR23F	TGCAGAYCTGGTYGATYCTGCC	23–44	[4]
AR46F-GC	CGCCCGCCGCGCCCCGCGCCCG TCCCGCCGCCCCCGCCCGYGCA SCAGKCGCGAA	46–60	[22]
AR519R	YGGGTCTCGCTCGTTRCC	519–536	[22]
AR349F-GC	CGCCCGCCGCGCCCCGCGCCCGT CCCGCCGCCCCCGCCCGYGCASC AGKCGCGAA	349–364	[31]
AR786R	CCGGGTATCTAATCC	786–800	[31]

[a] *F* forward primer, *R* reverse primer

[b] Position in the 16S rRNA gene *Escherichia coli* [5]

to 60% (100% denaturant contained 7 M urea and 40% formamide). The DGGE was performed on a Dcode universal mutation detection System (Bio-Rad Laboratories, Hercules, Calif., USA) at 60°C overnight at 100 V. The DNA bands were visualized by silver staining; and DNA was obtained from bands in the gel by removing gel plugs with a sterile pipette tip and eluting them into 30 μL of 0.1× TE buffer at 4°C overnight. Three microliters of eluate from individual bands were used for reamplification by PCR with the primers and conditions described above, but 0.1% bovine serum albumin was added to the PCR reaction. The amplification products were run on DGGE gels to ascertain that their electrophoretic mobility was the same as that of the DNA from which they were derived and that the bands were clean. For the construction of 16S rDNA clone libraries, PCR products were purified and concentrated using the QIAquick PCR purification kit prior to ligation into a pGEM-T easy cloning vector (Promega Corp., Madison, Wis., USA). Plasmid DNA was isolated from the transformants and used as a template for reamplification of the 16S rRNA inserts with M13 forward and reverse primers. The amplified insert DNA was digested with restriction enzymes *Mbo*I and *Hae*III; and the resulting restriction enzyme fragment patterns were compared and sorted visually on 3% NuSieve agarose gel (BioWhittaker Molecular Applications, Rockland, Me., USA). Clones with similar patterns were compared in adjacent lanes of a second 3% NuSieve gel to detect small differences in the patterns. The DNA sequence data were generated (SeqWright, Houston, Tex., USA) from unique clones using M13 forward and reverse primers and analyzed. The sequence data were inspected for the presence of ambiguous base assignments and unreliable sequences were removed. The sequences were also subjected to the Check Chimera program from the Ribosomal Database Project [17] before the sequences were submitted for identity searches. Searches were done with the Blast program [2].

Nucleotide sequence accession number

Representative sequences were deposited with GenBank and are available under accession numbers AY761014–AY761063.

Results and discussion

Demonstration of flow cytometry to characterize untreated and stained cell populations

Using fluorescent dyes that have different affinities for compounds present on the external surfaces of microbial cells, such as lipids, proteins, and carbohydrates, it is possible to selectively alter the fluorescence characteristics of some of the species in mixed bacterial cell populations. This allows particular types/species of bacteria to be readily distinguished from the bulk of bacteria, so that bacterial populations of interest can be quantified and/or recovered by FACS. Panel A in Fig. 1 shows the intrinsic fluorescence of activated-sludge cells at two selected wavelengths, while panel B shows the fluorescence of the same sample of activated sludge after staining with a lipid-specific fluorescent dye, fluorescein DHPE. The quadrant labeled R18 quantifies those cells that fluoresce strongly at the wavelength monitored by fluorescence channel 1. The unstained cell population contains about 1.56% of the total cells in quadrant R18, while the stained cell sample contains about 9% of the total cells in quadrant R18. The area encompassed by these quadrants can be adjusted at will to allow any particular sub-population of cells to be quantified and/or sorted by FACS. The autofluorescent, fluorescein DHPE-stained, and the non-fluorescent cells, as shown in Fig. 1, were recovered as separate sub-populations by FACS. These sub-populations of cells were then used in cultivation and 16S rRNA gene analyses. Previous reports of the use of FACS to characterize environmental samples employed dyes/fluorescent probes that target DNA and can reduce cell viability to values as low as 2% [25]. The fluorescent dyes used in this study target lipids, proteins, or carbohydrates on external cell

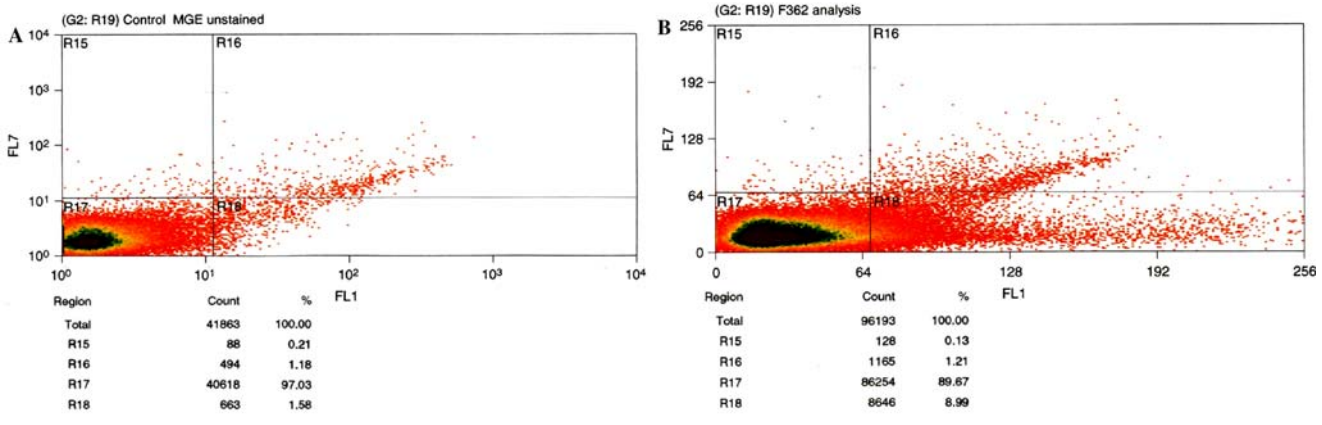

Fig. 1 Flow cytometry to characterize untreated and stained cell populations. *FL7* Fluorescence channel 7

surfaces and do not result in decreased cell viability (data not shown); and they are better suited to the cultivation of cells subsequent to flow cytometry.

The composition of cell populations is altered dramatically as a consequence of FACS

Lanes 1–5 in Fig. 2 show the results of DGGE analyses of 16S rDNA fragments detected in activated sludge, activated sludge subjected to two-phase anaerobic digestion, and activated sludge processed by FACS. The municipal wastewater treatment plant from which the activated sludge used in this study was obtained disposes of spent activated sludge by subjecting it to a two-phase anaerobic digestion process. The acidogenic phase of this process subjects the formerly aerobic activated sludge to anaerobic conditions designed to degrade organic matter into organic acids that can subsequently be degraded to methane and carbon dioxide in the methanogenic phase of the process. It is well known that the microbial communities present in activated sludge, acidogenic sludge, and methanogenic sludge are fundamentally different [24]; and the results given in lanes 1–3 in Fig. 2 show this, as the 16S rDNA fragments obtained from each sample produce unique DGGE band patterns.

When DNA is extracted from cell populations before and after FACS and then analyzed by DGGE, it is easy to see that the composition of these samples is dramatically different. By comparing lanes 1 (unsorted activated sludge), 4 (FACS sample of autofluorescent activated-sludge cells), and 5 (FACS sample of activated-sludge cells lacking fluorescence) in Fig. 2, it is obvious that the composition of the cell populations in the sorted cell samples is different, both from each other and with respect to the control (Fig. 2, lane 1). The species composition of these sorted cell populations differs substantially from the original activated-sludge sample (Fig. 2, lane 1), demonstrating that FACS allows access to the biodiversity present within environmental samples. These changes in DGGE profiles of cell subpopulations obtained by FACS versus the DGGE profile of untreated environmental samples are reproducible and are not merely due to microbial population changes

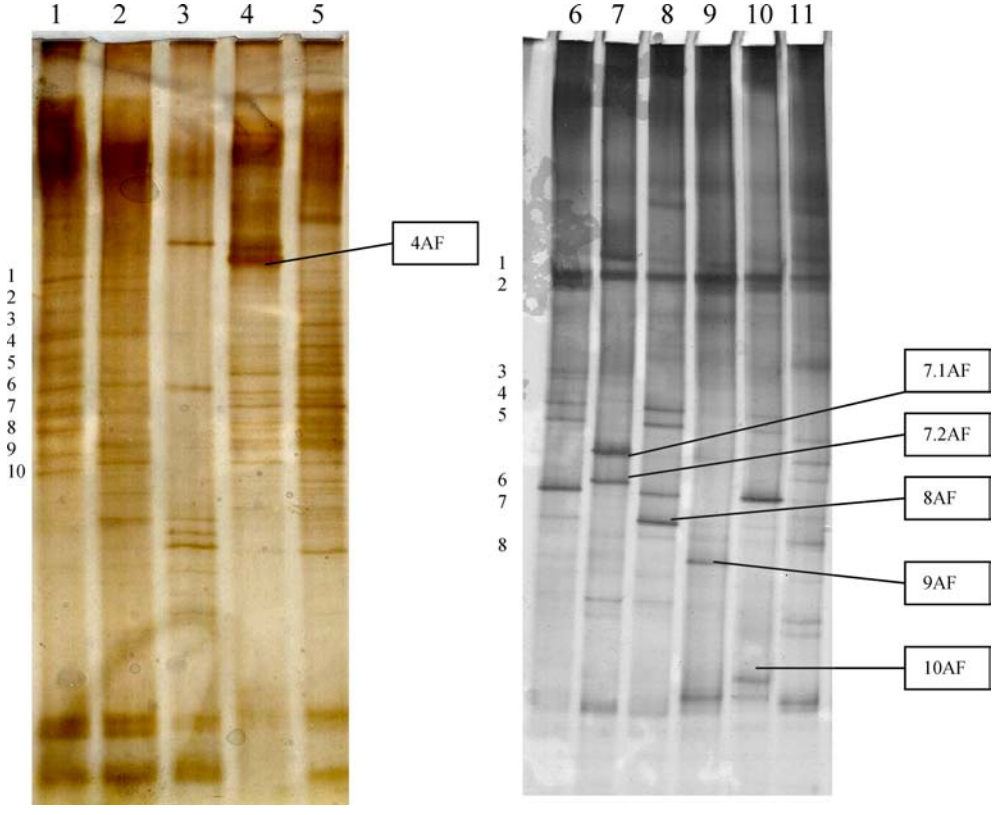

Fig. 2 DGGE analysis of activated-sludge sample before and after FACS. *Lane 1* activated sludge before FACS, *lane 2* acidogenic-phase sludge before FACS, *lane 3* methanogenic-phase sludge before FACS, *lane 4* FACS sample of top 10% of autofluorescent cells from activated sludge, *lane 5* FACS sample of the least fluorescent 80% of autofluorescent cells from activated sludge, *lane 6* activated sludge cultivated in HAM, *lane 7* FACS sample of most fluorescent 10% of cells after staining with fluorescein DHPE from activated sludge cultivated in HAM, *lane 8* FACS sample of most fluorescent 10% of cells after staining with DiO from activated sludge cultivated in HAM, *lane 9* FACS sample of top 10% of autofluorescent cells from activated sludge cultivated in HAM, *lane 10* FACS sample of the least fluorescent 80% of cells after staining with fluorescein DHPE and cultivation in HAM, *lane 11* FACS sample of the least fluorescent 90% of cells after staining with DiO and cultivation in HAM. *Framed band designations* indicate bands from which nucleotide sequence data were determined

resulting from cells subjected to flow cytometry (data not shown). There are several prominent DGGE bands in lanes 4 and 5 in Fig. 2 that are either not detectable or far more abundant, as compared with lane 1. Band 4AF in lane 4 of Fig. 2 was sequenced and shown to be a *Bacteroides* sp. that was not detectable in the original sample (lane 1 in Fig. 2) either in DGGE bands or in DNA fragments cloned directly from PCR amplifications of the activated-sludge sample (data not shown).

Future tests with pure cultures of *Bacteroides* species are needed to determine whether this microbial culture is autofluorescent, to determine more completely the full diversity of autofluorescent species present in activated-sludge samples, and to determine the biochemical basis of their autofluorescence. Nonetheless, these results clearly demonstrate that intrinsic properties of cells can be used in conjunction with FACS to obtain unique sub-populations of microbial cells derived from environmental samples and that the analysis of these sub-populations enables a more thorough characterization of the microbial ecology of environmental samples.

FACS allows an expanded range of bacterial species to be grown in the laboratory

The DGGE results shown in lanes 6–11 in Fig. 2 were all obtained by analyzing the bacterial population that grew in the same bacterial growth medium, HAM, when activated sludge, or activated sludge after fractionation by FACS, was used as an inoculum. The results indicated that, while some bacterial species were abundant in each growth experiment (band 1 in lanes 6–11 in Fig. 2, which proved to be *Comamonas testosteroni*), each inoculum derived from FACS yielded DGGE bands that were not detectable in the original sample, or were greatly enriched compared with the original sample (bands 7.1AF *Pseudomonas* sp., 7.2AF *Aeromonas* sp., 8AF *Acidovorax* sp., 9AF *Agrobacterium* sp., 10AF *Enterobacter* sp.). Similar results were obtained when activated sludge or activated sludge after fractionation by FACS was used to inoculate other nutrient solutions, such as sulfate-reducing bacteria media (data not shown).

These results demonstrate that even though the same media composition and growth conditions were employed, each sub-population of cells obtained by FACS resulted in the growth of unique assemblies of microbial species, despite the fact that each sub-population was derived from the same activated-sludge sample. The phenomenon of competitive exclusion is one likely explanation for these results [7]. When an environmental sample is used to inoculate a given nutrient solution and incubated under a given set of conditions, those microbial species present that are best suited to that particular growth condition are reproducibly isolated. While painstaking cultivation experiments can isolate 100 or more unique species from environmental samples, typically, fewer than 50 species are isolated/detected in microbial growth experiments employing a single nutrient solution and incubation condition, even though the environmental sample may contain 10,000 or more microbial species [14, 16, 33]. While a larger number of microbial species may be capable of growth under the chosen conditions, the most rapidly growing species consume nutrients so that they are unavailable to the slower-growing species. Thus, competition for nutrients between microbial species results in the proliferation of a comparatively few fast-growing species and the exclusion of other species. However, if the faster-growing species can be separated from some of the slower-growing species, using dilution culture for example [7], then the dynamics of competitive exclusion can be at least partially avoided and an expanded range of species can be cultivated using a given set of growth conditions [7]. Selective staining of some members of a microbial community and fractionation of the community by FACS can produce multiple sub-populations, separating pairs of competing microbial species in a more thorough manner than can be accomplished using dilution culture techniques.

The results shown above are only a portion of the results obtained by the authors in demonstrating that FACS can be used to fractionate activated sludge and other environmental samples which can subsequently be used to cultivate a wider range of bacterial species than can be cultivated from the original unfractionated environmental sample. The ability to obtain an expanded assortment of cultivated bacterial cultures from complex environmental samples will greatly assist microbial ecology studies.

Molecular analysis of Yellowstone Lake hydrothermal vent samples

Five Yellowstone Lake samples (unsorted, autofluorescence-positive, autofluorescence-negative, dye DiO-positive, dye DiO-negative) were obtained by FACS and the DNA from these cell populations was amplified by PCR to detect both eubacteria and archaea. These samples were analyzed using DGGE and the results are shown in Fig. 3, which shows that each FACS-derived sub-population yields a unique DGGE band pattern. Libraries of archaeal and bacterial 16S rRNA genes were also prepared from these five samples, using multiple combinations of PCR primers to more thoroughly characterize the biodiversity present in these samples. The results of DNA sequence analysis of the DNA fragments obtained from DGGE gels or cloned archaeal and bacterial 16S rRNA gene fragments obtained from each of the five cell populations derived from the Yellowstone Lake hydrothermal vent sample are shown in Tables 2 and 3, respectively.

Table 2 shows that the original/unfractionated Yellowstone Lake hydrothermal vent sample yielded seven archaeal 16S rDNA fragments, using two sets of PCR primers, for which DNA sequences were determined;

and each of these DNA sequences was unique. When the four sub-populations of cells obtained by FACS were analyzed using two sets of PCR primers, an additional 17 unique DNA sequences were obtained. Nearly all of the archaeal 16S rDNA sequences were most similar to species that are only known from previous molecular genetic analyses of environmental samples, rather than as cultivated microbial cultures. A further illustration of the ability of FACS to enable a more thorough characterization of microbial diversity is the data in Table 2 which show that the average percentage identity to known archaeal 16S rRNA gene sequences obtained using PCR primer set 1 from the unfractionated Yellowstone Lake sample was 99%, whereas the average identity for unique DNA sequences obtained after fractionating the cell population by FACS was 95%.

Similarly, Table 3 shows the results obtained from DNA sequence determinations of bacterial 16S rRNA genes obtained from unfractionated and FACS-fractionated samples of Yellowstone Lake hydrothermal vent fluid. Only two unique DNA sequences were obtained from the unfractionated sample. However, FACS resulted in the identification of an additional 20 unique

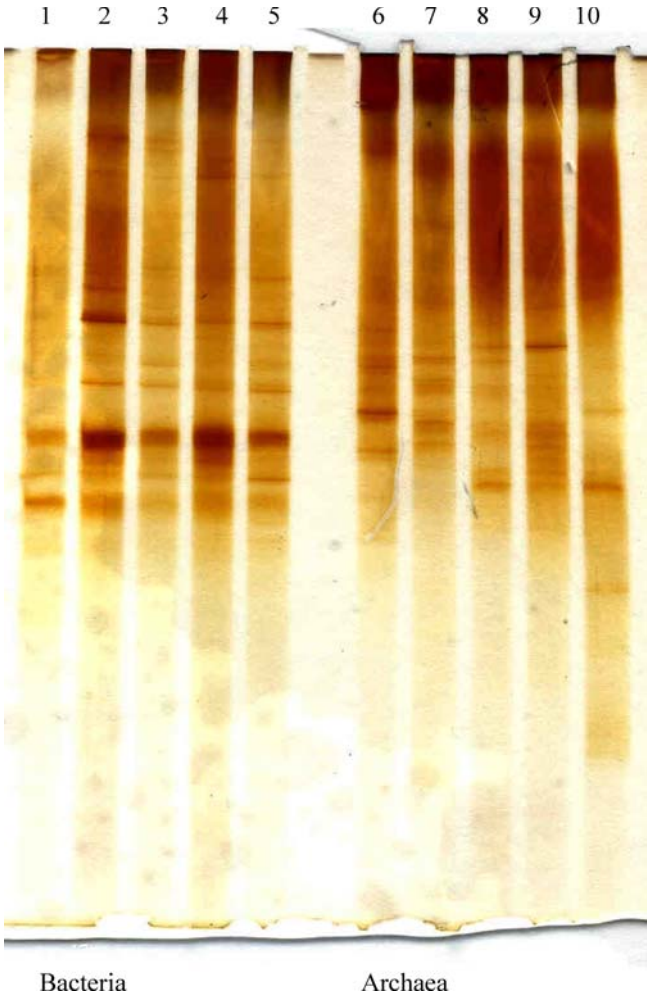

Fig. 3 DGGE analysis of Yellowstone Lake hydrothermal vent bacterial (*lanes 1– 5*) and archaeal (*lanes 6– 10*) 16S rRNA. *Lanes 1, 6* Yellowstone lake sample before FACS, *lanes 2, 7* FACS sample of the top 10% autofluorescent cells from Yellowstone Lake, *lanes 3, 8* FACS sample of the least fluorescent 80% of autofluorescent cells from Yellowstone Lake, *lanes 4, 9* FACS sample of the 10% most fluorescent cells from Yellowstone Lake after staining with fluorescein DHPE, and *lanes 5, 10* FACS sample of the least fluorescent 80% of cells from Yellowstone Lake after staining with fluorescein DHPE

Table 2 Archaeal 16S rDNA sequence analysis of Yellowstone Lake FACS unsorted and sorted sample

Sample[a]	Most closely related Archaea	Accession number	Similarity (%)
Nested primer set 1			
UN-1	Uncultured archaeon CR-PA15a	AF180725	99
UN-2	Uncultured crenarchaeote clone SU10h3g	AY157658	99
UN-3	Unidentified crenarchaeote	UCU6334	99
UN-4	Unidentified archaeon LMA229	UAU87519	99
AU-P2	Unidentified crenarchaeote	UCU63345	98
AU-P3	Uncultured crenarchaeote	AY157656	99
AU-P5	Uncultured crenarchaeote clone SU10h3g	AY157658	99
AU-N1	Unidentified archaeon gene	AB007307	88[b]
DiO-P1	Uncultured crenarchaeote	UCR535136	89
DiO-P2	Unidentified crenarchaeote	UCU63345	95
DiO-P3	Uncultured crenarchaeote clone SU10h3g	AY157658	99
DiO-P5	Unidentified crenarchaeote	UCU63345	97
DiO-P6	Crenarchaeotal sp. clone pJP 89	CNBRG16F	98
DiO-P9	Uncultured crenarchaeote clone SU10h1d2	AY157656	99
DiO-P10	Uncultured Front Range soil crenarchaeote	AY016494	89
DiO-N2	Uncultured archaeon gene	AB095124	95[b]
DiO-N3	Unidentified archaeon LMA229	UAU87519	99
DiO-N4	Uncultured archaeon	AB095124	94
Nested primer set 2			
UN-1	*Methanofollis aquaemaris* strain N2M9704	AY186542	96
UN-5	Uncultured archaeon VC2.1 Arc36	AF068824	93[b]
UN-7	Uncultured archaeon clone pCIRA-N	AB095124	96[b]
AU-P4	Unidentified crenarchaeote	U63345	96
AU-P5	Uncultured archaeon 19b-34	AJ294865	94
AU-N8	Uncultured archaeon VC2.1 Arc36	AF068824	93[b]
DiO-P9	Unidentified crenarchaeote	U63345	96
DiO-P11	Unidentified crenarchaeote clone FHMa6	AJ428028	93
DiO-P13	*Crenarchaeotal* sp. clone pJP 89	L25305	100[b]
DiO-N2	Uncultured archaeon clone pCIRA-N	AB095124	96[b]

[a] *UN* Unsorted, *AU* autofluorescent, *DiO* fluorescent dye 3,3′-dioctadecyloxacarbocyanine perchlorate, *P* positive (high fluorescence), *N* negative (no fluorescence)

[b] These archaeal 16S rRNA gene sequences were most closely related to sequences recovered from marine hydrothermal vent samples

Table 3 Bacterial 16S rDNA sequence analysis of Yellowstone Lake FACS unsorted and sorted sample

Sample[a]	Most closely related Bacteria	Accession number	Similarity (%)
Primer set 1			
UN-1	*Bacterium* H12	AY345556	96[b]
UN-2	Uncultured Comamonadaceae bacterium clone C-15	AF523046	99
AU-P1	β-Proteobacterium EC4	AY337598	98
AU-P2	*Flavobacterium xinjiangense* AS1.2749	AF433173	98
AU-P3	Uncultured β-proteobacterium clone CLs95	AF529357	97
AU-P4	Uncultured Crater Lake bacterium CL500-29	AF316678	99
AU-N1	*Flavobacterium limicola* gene	AB075232	98
AU-N2	*Bacterium* strain 82348	AF227863	99
DiO-P1	*Janthinobacterium* sp. An8	JSP551147	94[b]
DiO-N1	Antarctic bacterium R-7515	UBA440977	98
DiO-N2	Unidentified eubacterium	UEU224988	98[b]
DiO-N3	Antarctic bacterium R-7579	UBA440981	96
DiO-N4	*Cytophaga* sp. An36	CSP551174	99
DiO-N5	Uncultured bacterium clone GOUTB7	AY050594	97
Primer set 2			
AU-P3	Uncultured β-proteobacterium clone NE70	AJ575690	99
AU-P4	Uncultured bacterium clone KD6-37	AY218757	99
AU-P5	*Vogesella indigofera* strain ATCC 19706T	AB021385	96
AU-N3	*Herbaspirillum seropedicase* strain Z78	AY191275	96
AU-N4	*Bacterium* H12	AY345552	98[b]
AU-N5	*Bacterium* strain 82348	AF227863	100
DiO-P3	*Bacillus* sp. 17-1	AB043843	100
DiO-N2	Uncultured yard-trimming-compost bacterium	AY095380	98
DiO-N3	*Vogesella indigofera* strain ATCC 19706T	AB021385	96
DiO-N4	*Bacterium* strain 82348	AF227863	100

[a] *UN* Unsorted, *AU* autofluorescent, *DiO* fluorescent dye 3,3′-dioctadecyloxacarbocyanine perchlorate, *P* positive (high fluorescence), *N* negative (no fluorescence)
[b] These bacterial 16S rRNA gene sequences were most closely related to sequences recovered from marine hydrothermal vent samples

bacterial 16S rRNA gene sequences. Analysis of 16S rRNA gene sequence data reported in Tables 2 and 3 revealed that only a few of the sequences from Yellowstone Lake hydrothermal vent samples were most closely related to 16S rRNA gene sequences recovered from deep-sea hydrothermal vent samples. Since even among these related 16S rDNA genes the sequence identity was generally less than 97%, these data suggest that the microbial populations of the freshwater hydrothermal vents in Yellowstone Lake are substantially different from marine hydrothermal vent microbial populations, but further research is required to enable a comprehensive comparison of these two types of microbial populations. The data in Tables 2 and 3 support the hypothesis that FACS could enable a more thorough characterization of microbial diversity in environmental samples than would be possible using conventional procedures. Because of the large number of fluorescent dyes available with affinity for various cell components, such as lipids, proteins, carbohydrates, and nucleic acids, FACS can be used to fractionate environmental samples into a vast array of unique sub-populations that can then be used in molecular or cultivation experiments. The results reported here employed two dyes with affinity for lipids (fluorescein DHPE, DiO) and demonstrated that these dyes can selectively stain microbial species present in environmental samples. These dye-stained cells can be selectively recovered using FACS and used in either cultivation or genetic experiments to characterize the microbial ecology of environmental samples more thoroughly than is possible without the innovative use of FACS described here. Preliminary results obtained by the authors using fluorescent dyes that have affinity for proteins or carbohydrates yielded similar results. Specifically, sub-populations of cells obtained using FACS yielded reproducible DGGE patterns, indicating that the composition of these sub-populations differs from that of the corresponding untreated environmental samples (data not shown). Research is currently underway to identify fluorescent dyes, staining conditions, and FACS procedures that will allow the selective enrichment of targeted phylogenetic groups of bacteria and archaea.

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