Microbes Environ. Vol. 26, No. 3, 261–265, 2011
http://wwwsoc.nii.ac.jp/jsme2/ doi:10.1264/jsme2.ME11111

MICROBES AND ENVIRONMENTS

Short Communication

16S rRNA Gene Microarray Analysis of Microbial Communities in Ethanol-Stimulated Subsurface Sediment

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(Received February 8, 2011—Accepted April 1, 2011—Published online May 11, 2011)

A high-density 16S rRNA gene microarray was used to analyze microbial communities in a slurry of ethanol-amended, uranium-contaminated subsurface sediment. Of specific interest was the extent to which the microarray could detect temporal patterns in the relative abundance of major metabolic groups (nitrate-reducing, metal-reducing, sulfate-reducing, and methanogenic taxa) that were stimulated by ethanol addition. The results show that the microarray, when used in conjunction with geochemical data and knowledge of the physiological properties of relevant taxa, provided accurate assessment of the response of key functional groups to biostimulation.

Key words: microbial community, 16S rRNA gene, DNA microarray, terminal electron accepting process, uranium reduction

DNA microarrays are widely used to monitor and characterize microbial communities in environmental samples (16, 19, 26, 31, 35, 38, 45). Microarray technology permits simultaneous interrogation of PCR amplicons or genomic DNA across a large number of probe sequences (6, 9, 10, 21, 42). Differences as small as one nucleotide base pair can be distinguished with an oligonucleotide-based microarray, although this degree of specificity is dependent on the sequence context (*e.g.* local melting temperature), hybridization conditions, and detection chemistry. Under optimal conditions, DNA microarrays can be used to efficiently screen a complex mixture of different sequences (42, 43).

During *in situ* bioremediation of uranium, mobile U(VI) is microbiologically (enzymatically) precipitated and immobilized as the insoluble U(IV) mineral uraninite (UO_2) (1, 22–25). A diverse range of microorganisms can reduce U(VI) to U(IV) under anaerobic conditions (39). Information on the status of microbial communities involved in U(VI) reduction and other electron-accepting pathways is important to understand the spatial/temporal dynamics and overall efficacy of in situ uranium bioremediation (1). Although 16S rRNA clone libraries and/or microarrays have been applied to various U(VI) reduction systems (2, 4, 7, 17, 28, 29, 40, 41, 44), knowledge remains scant of the quantitative coverage of these techniques, particularly in situations where multiple groups of organisms are active.

We report here on the use of a high-density 16S rRNA gene microarray (4) for analysis of microbial communities in a slurry of ethanol-amended, uranium-contaminated subsurface sediment from Oak Ridge National Laboratory. The samples analyzed came from an experiment designed to document patterns of microbial redox metabolism and community structure in response to the addition of an electron donor (ethanol) to stimulate U(VI) reduction activity (29). As part of that study, six conventional 16S rRNA gene clone libraries (90–134 clones each) were constructed from reverse-transcribed 16S rRNA extracted from subsamples obtained at different time points during the experiment. While these libraries provided important insights into the connection between microbial metabolism and community structure in the experiment, their construction and analysis were time consuming, and likely provided only limited coverage of the overall microbial community in the sediment slurry. For this reason, we decided to apply the 16S rRNA gene microarray to the same reverse transcribed 16S rRNA extracts analyzed by conventional cloning and sequencing. The goal was to evaluate whether the 16S rRNA microarray, the results of which were obtained and analyzed within *ca.* one week, compared to the 3–5 months required to construct, sequence, and analyze the six clone libraries, could detect specific changes in microbial community composition related to shifts in major redox metabolic pathways. Although 16S microarrays have seen broad use in environmental microbiology, to date their ability to reveal changes in microbial community structure during redox transitions in space or time has not been established. A prior experiment (29) provided an ideal context for evaluating this question, as it was a controlled system for which both good supporting geochemical data on redox metabolism, and independent information on microbial community composition (*i.e.* clone libraries), were available.

The microarray (PhyloChip G2) is a high-density oligonucleotide array fabricated for the Affymetrix platform that contains over 500,000-bp fluorescent probes, with approximately 300,000 of these targeting 16S rRNA gene sequences; the array is capable of simultaneously detecting almost 9,000 bacterial and archaeal taxa. Details on the design and use of the 16S microarray are available elsewhere (4) and for the sake of brevity are not repeated here. It is important to note, however, that rigorous procedures for quantitative detection

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and array response normalization were employed, and that background fluorescence (defined by the mean fluorescence intensity of probes producing the lowest 2% of all intensities) was subtracted from the fluorescence intensity of all probes.

A clearly defined temporal pattern of TEAPs developed in the ethanol-amended slurries, with NO_3^- reduction, Fe(III) reduction (Fe(II) production), SO_4^{2-} reduction, and CH_4 production proceeding in sequence until all of the added electron donors were consumed (Fig. S1A). Acetate accumulated during ethanol metabolism (Fig. S1B), and was converted to CO_2 and CH_4 during the methanogenic phase of the experiment. Approximately 60% of $NaHCO_3$-extractable U(VI) was reduced during the Fe(III) reduction phase between 4 and 12 d in the ethanol-amended slurries (Fig. S1B).

Analysis of the 16S microarray data was confined to the top 100 subfamilies (out of a total of 1608) that exhibited the greatest variation in array intensity among the different time point samples. Hierarchical cluster analysis (4, 11) was used to detect correlations between subfamilies. Overall, seven major groups were detected (Fig. 1A). A complete list of array intensities for the top 100 subfamilies and their group assignments is given in Table S1. The nitrate- and metal-reducing taxa that responded (based on the 16S rRNA clone libraries) most strongly to ethanol stimulation were grouped together (cluster 2, Fig. 1B) in cluster analysis, *i.e.* the pattern of variability in array intensity over time resulted in the clustering of these taxa into one group. Likewise, the methanogenic *Archaea* that proliferated toward the end of the experiment also formed a distinct cluster (cluster 4, Fig. 1B). Other clusters had no specific relationship to patterns of redox metabolism, although the large initial decline in *Clostrideaceae* sequences observed in the clone libraries was reflected in the pattern of array intensity for clusters 1, 3, and 7 which included these taxa. The relatively large apparent change in *Clostrideaceae* abundance may be attributed to oxidative stress triggered by exposure of the anoxic, nitrate-depleted sediment inoculum to mM levels of nitrate. The relative intensity of the overall group signals did not change markedly in relation to one another (maximum four-fold, Fig. 1B). Correlation analysis between array intensity for taxa detected by the 16S microarray and various geochemical parameters (*e.g.* ethanol, acetate, nitrate, or Fe(II) concentration; total electrons consumed; computed system redox potential) was performed within the R statistical programming environment (http://www.R-project.org) using the package 'multitest' (http://www.bepress.com/ucbbiostat/paper164) with correction for multiple observations performed using the Benjamini-Hochberg (BH) False Discovery Rate correction (3). This revealed a wealth of apparent significant correlations (R values >0.95) (Table S2); however, none of

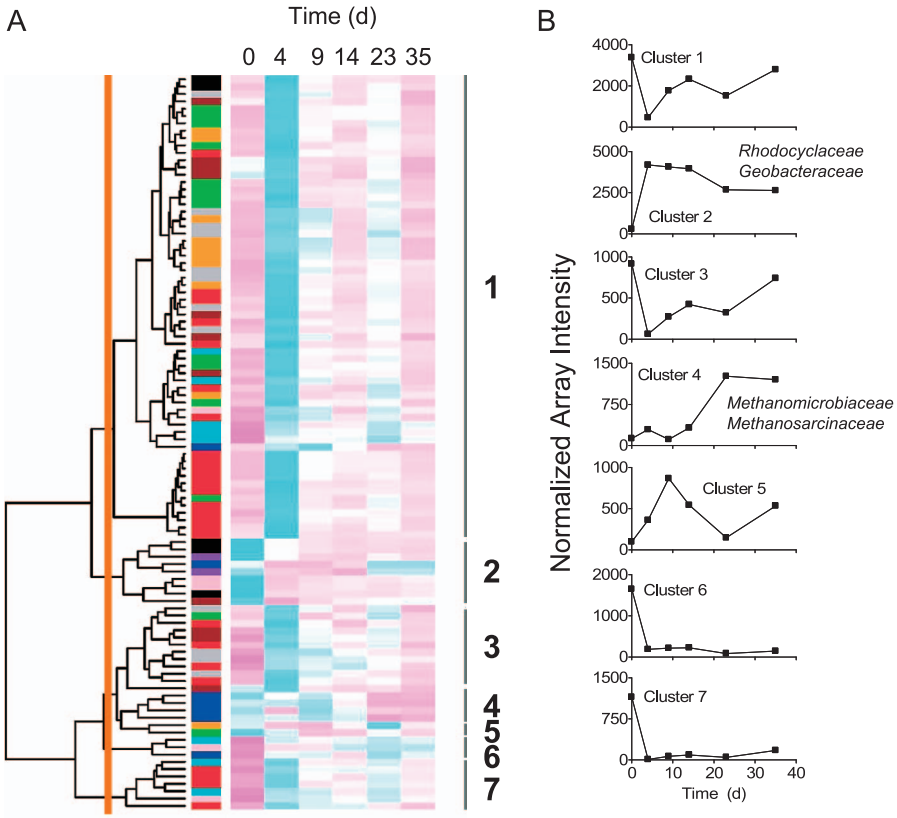

Fig. 1. (A): Heatmap and cluster analysis dendrograms showing the 16S microarray response of 100 subfamilies (shown on *y* axis) exhibiting the highest standard deviation among samples from different time points during slurry incubation (shown on *x* axis). Major taxa are indicated by colors in the left-most block (blue=*Archaea*; green=*Actinobacteria*; purple=*Bacteroidetes*; orange=*Bacillus*; red=*Clostridia*/*Desulfotomaculum*/*Symbiobacterales*; brown=*Alphaproteobacteria*; pink=*Betaproteobacteria*; black=*Deltaproteobacteria*; cyan=*Gammaproteobacteria*; grey=Others). The orange bar to the left of the color code block indicates the distance threshold at which cluster/groups were defined. The blue to purple color gradient in the heatmap for the different time points represents increasing array hybridization intensity. The lines and numbers to the right of the heatmap indicate taxa included in the seven major groups determined by cluster analysis. (B): Variation of mean array intensity for the major response groups detected by the cluster analysis over time.

the BH adjusted *P* values were significant due to the small number of geochemical data points relative to the large number of OTUs detected by the microarray.

Of specific interest was the extent to which the microarray detected temporal patterns in the relative abundance of major metabolic groups that might be expected to be stimulated by ethanol addition. We therefore examined average normalized array intensity for phylogenetic groups that include the major taxa that appeared (based on the chemical and clone library data) to be involved in ethanol metabolism. Analogous approaches have been used to assess the response of metal-reducing, sulfate-reducing, and methanogenic taxa during biostimulation for U(VI) and Cr(VI) immobilization (15, 34). Consistent with expectations, mean array intensities, binned at the family level, for *Geobacteraceae* (which includes *Geobacter* and related metal-reducing organisms), *Rhodocyclaceae* (which includes *Dechloromonas* and related nitrate-reducers) and *Oxalobacteriaceae* (which includes *Herbaspirillum* and related nitrate reducers) increased upon stimulation (Fig. 2).

The 16S microarray data revealed qualitatively similar temporal patterns in microbial community structure compared

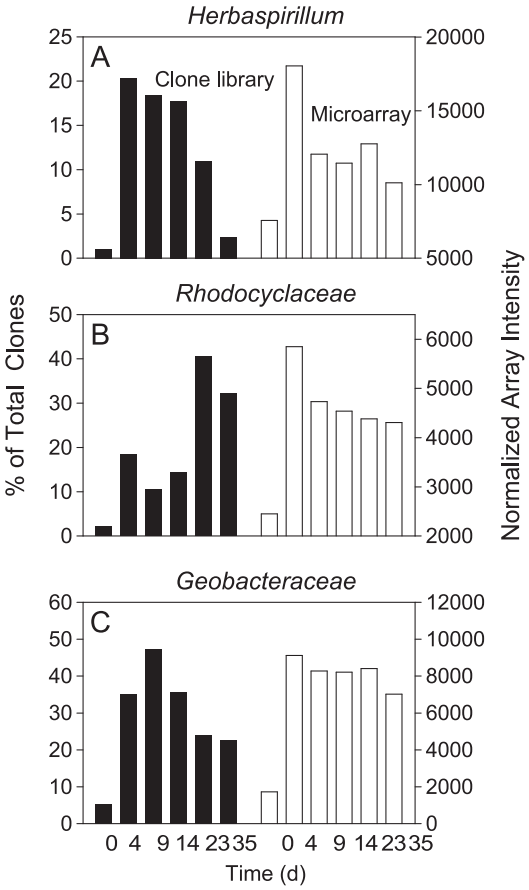

Fig. 2. Abundance of selected sequences, binned at the family level, from 16S rRNA clones libraries (filled bars; data from 29) and normalized array intensity for analogous taxa detected by the 16S microarray (open bars) over time during the sediment slurry incubation experiment. The Greengenes (greengenes.lbl.gov) suite of tools (12) was used to reclassify (relative to the classifications given in Table 1 in 29) the clone sequences using the same taxonomy (G2_chip) upon which the 16S microarray is based. A complete list of the clone library sequence taxonomic assignments is given in Table S3.

to conventional 16S rRNA gene clone library data reported previously (29) for the slurry incubation experiment (Fig. 2). Correlations (R values) between family-level percent abundance in the clone libraries and mean array intensity were 0.93 ($P<0.01$), 0.37 ($P=0.46$), and 0.61 ($P=0.19$) for *Oxalobacteriaceae*, *Rhodocyclaceae*, and *Geobacteraceae*, respectively. Array signals for *Rhodocyclaceae* and *Geobacteraceae* were not responsive when the relative abundance of these taxa in the clone libraries exceeded 15%; the reason for this lack of response is unknown. A recent 16S rRNA gene clone library-based study (36) of microbial community development in suspensions of ethanol-amended U(VI)-contaminated sediment (similar to the sediment used in this study) revealed the proliferation of *Deltaproteobacteria* and *Betaproteobacteria* taxa analogous to those detected in this study.

Although 16S rRNA sequences corresponding to sulfate-reducing bacteria were detected both on the 16S microarray and in the clone libraries, neither approach detected the proliferation of sulfate-reducing bacteria in conjunction with the brief period of sulfate consumption between days 10 and 14 of the experiment (see Fig. S1) (data not shown). Sequences related to other potentially important physiological groups, *e.g.* metal-reducing taxa, such as *Anaeromyxobacter* and *Shewanella*, which were not present in the 16S rRNA libraries, were detected by the microarray, although there was little change in the array intensity of these groups over time (data not shown). Practical considerations prevented conventional 16S rRNA gene clone library analysis of archaeal taxa in this study. The 16S microarray, however, readily detected a major increase in methanogenic taxa during the latter stage of the experiment (Fig. 1B) when methane production took place (Fig. S1B).

As observed in other recent studies (13), the microarray detected a much larger number of microbial groups than the clone libraries (Table S4). Clone libraries are known to underestimate microbial diversity due to sequencing of an insufficient number of clones (33) and/or to preferential amplification of specific sequences, leading to misrepresentation of sequence abundance within genomic DNA extracts (32, 37). The 16S microarray approach is also subject to such bias, since the DNA analyzed on the array was generated by PCR. Both the 27F-927R (used for the clone libraries) and 27F-1492R primers (used for the 16S microarray) target all known bacteria and have been widely used in previous microbial ecological studies (5, 14, 18, 20, 27, 30). Discrepancy in the assessment of bacterial diversity due to differences in the primer sets used was thus probably insignificant, especially given that all of the sequences identified in the clone libraries were detected by the array. The possibility that the high diversity detected by the array was due to nonspecific hybridization (leading to false positives) can be discounted because of the high specificity and reproducibility of hybridization between the probes, and 16S rDNA targets hybridization and reproducibility (4, 40). Rather, the much greater diversity detected by the microarray can be attributed simply to the very large number of probe sequences on the array compared to the relatively small number of 16S rRNA clones sequenced (13).

The advent of high-throughput pyrosequencing of 16

rRNA gene amplicons (*e.g.* 36) may provide an alternative for sequencing-based detection of both dominant and rare taxa. Cardenas *et al.* (8) recently employed 16S rRNA gene pyrosequencing to track the development of microbial communities during ethanol-driven in situ remediation of U(VI)-contaminated groundwater. As in this study, the sequence information was combined with knowledge of the geochemical conditions and the physiological and phylogenetic properties of key "indicator" taxa (*e.g. Deltaproteobacteria* analogous to those reported in this study) to holistically interpret the response to ethanol stimulation. This general approach provides a simple but powerful method to utilize definitive information on microbial community composition from 16S microarray or pyrosequencing analyses to interpret microbial responses to natural or engineered environmental perturbation.

In summary, 16S microarray characterization of microbial communities in the biostimulated sediment was consistent with the results of conventional 16S rRNA clone libraries, and also with the results of other recent sequence-based studies of subsurface sediment microbial community response to ethanol stimulation (*e.g.* 8 and 36). Targeted analysis of the array response for bacterial and archaeal taxa likely to respond to biostimulation (based on observed geochemical data) provided evidence of a response by these groups. The array detected a wide range of taxa not recovered in the clone libraries, although no obvious trends in the relative abundance of these taxa were evident. Collectively, the results agree with those of other recent studies where the 16S microarray was used to detect and monitor the microbial population response to biostimulation (4, 15, 34). Although it seems unlikely that the array alone can gauge the quantitative response of different taxa, when used in conjunction with general knowledge of the physiological properties of key taxa together with relevant geochemical data, the array can provide accurate assessment of the response of key functional groups (15, 34) much more rapidly and with equal or greater robustness than conventional cloning and sequencing.

Acknowledgements

The work was supported by grants DE-FG02-06ER64184 and ER64172-1027487-001191 from the Environmental Remediation Science Program, Office of Biological and Environmental Research, U.S. Department of Energy, Office of Science. Part of this work was supported by the U.S. Department of Energy under contract no. DE-AC02-05CH11231 with the Lawrence Berkeley National Laboratory.

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