New Genomic Strategies and Technologies for Studying Complex Microbial Communities

Mick Noordewier

Diversa Corporation

Background and Significance

The soil environment harbors a vast number of uncharacterized microorganisms. It is estimated that only 1% or less of the microorganisms that are known to exist in the soil environment have been cultured (Amann *et al.*, 1995; Whitman *et al.*, 1998; Dunbar *et al.*, 2002; Torsvik *et al.*, 2002). One-third of the ~40 bacterial divisions recognized to date consist entirely of so-far uncultured bacteria which have been described solely by their 16S rRNA gene sequences (Hugenholtz *et al.*, 1998; Dojka *et al.*, 2000). In addition, only a small fraction of organisms that can be cultured have been at least partially characterized (Rosselló-Mora and Amann, 2001). A major challenge faced by microbiologists today is to develop new strategies and technologies that enable access to the uncultured fraction of microbes in nature. Expanded access to these microbes and their metabolic capabilities is crucial to further our understanding of the role that microbes play in our environment.

Many important organisms that may drive environmental processes are known to exist but cannot be cultivated with traditional methods (Giovannoni and Rappe, 2000; Beja *et al.*, 2002). Organisms relevant to remediation, biogeochemical cycles, climate changes, energy production and biotechnology are of considerable interest.

Classic and Molecular Methods: Merits and Drawbacks

The enrichment and cultivation techniques developed by Koch, Beijerinck, and Winogradsky facilitated new discoveries in microbiology for over 100 years. Traditional enrichment and isolation techniques have captured representatives from a diverse array of bacterial and archaeal phylogenetic groups that inhabit a wide range of niches. Cultivating such a variety of different microbial species in the laboratory has been paramount to our understanding of microbial physiology and our appreciation of the metabolic capabilities of the prokaryotic world. These methods were adequate until molecular technologies, particularly PCR amplification of small subunit ribosomal RNA genes directly from DNA extracted from a microbial community, revealed that traditional techniques were only "scratching the surface" when used to describe the diversity of microbes inhabiting a particular niche.

Table 1. Comparison of different approaches used to "access" microbial diversity.

Methods	Access biodiversity	Total community genome	Single organism genome	Single organism physiology	Total community physiology
Traditional cultivation	-	-	++	++	-
Community DNA extraction/PCR	+	-	-	-/+	-/+
FISH[1]	+	-	-	-/+	-/+
FISH-MAR[2]	+	-	-	-/+	-/+
BAC[3] sequencing	+	+	-/+	-	-/+
GMD-based platform[4]	+	+	++	++	++

[1] Fluorescence in situ hybridization
[2] Microautoradiography
[3] Bacterial artificial chromosome
[4] Gel microdroplet-based cultivation platform

To overcome the limitation of traditional culturing, a variety of cultivation independent approaches have been developed in the last 20 years to study microbial ecology (Table 1). In conjunction with the development of phylogenetic analysis based on 16S rRNA analysis, methods such as fluorescence *in situ* hybridization (FISH) have been developed to study the microbial composition in complex microbial habitats (Amann and Ludwig, 2000). This technology allows researchers to better determine the true composition of microbial communities since single cells can be detected directly within the environmental sample. However, correlating the composition and structure of a microbial community to its functions is still limited to a very few time-consuming techniques. Physio-chemical parameters in a sample can be measured at sub-millimeter scales by microsensors and linked to community composition with 16S phylogenetic FISH probes (Santegoeds *et al.*, 1999). The combination of FISH probes and microautoradiography (FISH-MAR) aims to link, at the single cell level, the uptake of radioactively labeled substrates to phylogenetic information (Lee *et al.*, 1999; Ito *et al.*, 2002; Nielsen *et al.*, 2002). However this method is restricted to very few substrates, which can be labeled radioactively with a low radiation such as tritium or ^{14}C.

In recent years, shotgun, fosmid, or BAC cloning and sequencing of DNA extracted directly from environmental samples, or enrichments from samples, has been used to gain information on the possible or potential function of uncultivated microbes in the environment (Beja *et al.*, 2000; Quaiser *et al.*, 2002). Obviously, open reading frames that are linked to a 16S rRNA sequence can be immediately associated with an organism (Beja *et al.*, 2000). But difficulties remain in linking a particular cloned pathway to a specific organism (Rondon *et al.*, 2000). Assembly of complete (or even partial) genomes from a complex mixture of genomic clones of 100s to even 1000s of different organisms in an environmental sample has not yet been reported. Even with high throughput sequencing and advanced assembly programs, sequencing of cloned

community DNA is problematic because communities are uneven; for example, a given microbe playing a critical role in the environment may be present at 0.01% to 10% of the total population. This means that the great majority of sequencing is done on the few most dominant microbes, and sequence coverage of any non-dominant genome will be one to several orders of magnitude lower. If the dominant microorganisms (*i.e.*, those present at 10% of the total population) are sequenced to 10x depth, the great majority of the microbes will be sequences at 1x to 0.01x depth. Thus, with the exception of the few dominant microbes, community (BAC or shotgun) sequencing provides fragmentary genes and pathways, but results in little information on the physiology of the organisms as a whole. For this reason, methods to segregate specific microbes or groups of microbes prior to extraction of DNA are needed to avoid the unevenness problem and to reduce complexity.

The drawback of all these molecular methods is that they provide information on only a portion of the organism's biochemical capabilities or genetic potential. In this regard, conventional cultivation of microorganisms is preferable except that it is selective and biased for the growth of specific microorganisms (Ferguson *et al.*, 1984; Eilers *et al.*, 2000). The majority of cells obtained from nature and visualized by microscopy are viable, but they do not generally form visible colonies on plates (Xu *et al.*, 1982; Eilers *et al.*, 2000). Visible colonies on plate count medium require at least 105 cells and these media strongly select for microbes that are fast-growing, grow to high density, are resistant to high concentrations of nutrients, and are able to grow in isolation. Dilution to extinction liquid medium culturing requires 106 cells for turbidity-based spectroscopic detection of growth and is prone to the same selection biases. We argue that these culturing strategies are counter to the normal growth habit of many microbes, and are a major contributing factor for why most microorganisms have not been cultivated in pure culture. It has been shown recently that some previously uncultivable microorganisms can be grown in pure culture if care is taken to provide them with the chemical components of their natural environment (Connon and Giovannoni, 2002; Kaeberlein *et al.*, 2002; Rappé *et al.*, 2002). In addition, studies using modified media demonstrated the recovery of organisms not previously identified in culture by traditional cultivation methods (Bruns *et al.*, 2002; Janssen *et al.*, 2002; Bruns *et al.*, 2003). These studies highlight that attempts to closely simulate the microbes natural environment in the laboratory are bringing increasing success to cultivating previously uncultivated microorganisms.

Gel Microdroplet Cultivation as a Technology Platform to Study Uncultivated Microbes

In an effort to combine the merits of both cultivation and molecular analyses, and to gain greater access to uncultivated microorganisms and their genes, proteins, and biochemistries, Diversa has developed a technology based on encapsulation of single (or a small number) of cells within individual gel microdroplets (GMDs). The microbial community is then reconstituted by loading the GMDs into a column. The community is grown in the column under very low nutrient flux conditions using basal medium supplemented with low concentrations of nutrients extracted from the sampling site. Over time, each cell capable of growth under the conditions in the column forms a microcolony within its GMD. The high throughput nature of this technology is the use of a high-speed flow cytometer and cell sorter to identify GMDs containing microcolonies of >20 cells, and sort each positive GMD into a well of a microtiter plate for further analysis. Diversa has demonstrated that novel, previously uncultured organisms, can form microcolonies within the GMDs (Zengler *et al.*, 2002). Moreover, the GMDs provide a common technology platform for genomic, transcriptome, proteome, and metabolite studies of these novel, previously uncultured organisms.

The GMD cultivation technology can be thought of as a "high throughput" version and extension of Button's oligotrophic medium-limiting dilution approach using glass tubes (Button *et al.*, 1993). Several important advantages are realized by replacing glass tubes with microscopic "polymer cages" (*i.e.*, the GMD), and collectively these advantages result in improved cultivation and greater access to microbial diversity. These advantages are:

- The GMDs allow the reconstituted microbial community to be simultaneously cultivated 'together' and 'apart' because each "caged microcolony" can later be separated and analyzed. In contrast the limiting dilution approach physically isolates individual cells in glass tubes.
- The ability to reconstitute the community in the column of GMDs allows for diffusive cross-feeding of metabolites and other molecules (e.g. regulatory molecules) between members of the community. This feature also simulates the natural environment, and thus preserves some of the community interactions and other specific requirements that may be needed for successful cultivation.
- Microbes are grown in a flowing open system that simulates natural environments, where microbes are exposed continually to a very low concentration of nutrients. This is in contract to a closed batch system where microbes receive a high concentration of nutrients at one point in time and metabolic byproducts can build up to unnaturally high and inhibitory concentrations. The low concentration of nutrients also minimizes overgrowth of fast-growing organisms, thereby allowing propagation of microorganisms with extremely slow growth rates and/or that only grow to low cell densities.

It should also be noted that the GMD approach incorporates or can be made to incorporate any of the medium techniques recently used by others (Bruns *et al.*, 2002; Connon and Giovannoni, 2002; Janssen *et al.*, 2002; Kaeberlein *et al.*, 2002; Rappé *et al.*, 2002; Bruns *et al.*, 2003). The production and screening/sorting of GMDs is automated, high throughput, and easily and economically scaleable.

As stated above, microcolonies of previously uncultivated microbes can be readily interrogated with genomic, transcriptome, proteome, and metabolite studies. We are currently pursuing the development and application of methods to:

- Perform whole-genome amplification and sequencing from a GMD microcolony.
- Segregate GMD microcolonies of a given species by FISH identification and flow cytometry sorting.
- Conduct expression profiling on one or a small number of GMD microcolonies of a given species.

Anticipated Results and Benefits

The described work will allow us to apply this technology in combination with fluorescence *in situ* hybridization to target so far uncultivated microorganisms. By amplifying DNA directly from a microcolony, the microorganism will become accessible to whole-genome amplification. In combination with subsequent gene expression studies, this will not only enable us to get inside the genomes of so far uncultured microorganisms but also to study their physiological responses to environmental perturbations within a complex microbial community. Since every single organism is – within its own GMD – incubated in conjunction with the natural microbial community of the respective sample, environmental processes can be mimicked under any desired growth condition in vitro. This novel GMD microcolony platform could also be integrated into an ultra-high resolution proteomics platform and therefore allow the use of the GMD microcolony platform to provide proteome level information on novel previously uncultured microbes. We anticipate that this work will contribute to the understanding of metabolic capacities in complex microbial communities. Furthermore, it will enable us to predict community response and adaptation to certain

environmental changes. To identify the role of so far uncultured microorganims within environmental samples of interest might open new potential within areas of bioremediation, climate changes, energy production and biotechnology.

References

1. Alfreider, A., Bogt, C., and Babel, W. (2003) Expression of chlorocatechol 1,2-dioxygenase and chlorocatechol 2,3-dioxygenase genes in chlorobenzene-contaminated subsurface samples. Appl Environ Microbiol 69:1372-1376.
2. Amann, R., and Ludwig, W. (2000) Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. FEMS Microbiol Rev 24: 555-565.
3. Amann, R.I., Ludwig, W., and Schleifer, K.-H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59: 143-169.
4. Beja, O., Suzuki, M.T., Heidelberg, J.F., Nelson, W.C., Preston, C.M., Hamada, T., Eisen, J.A., Fraser, C.M., and DeLong, E.F. (2002) Unsuspected diversity among marine aerobic anoxygenic phototrophs. Nature 415: 630-633.
5. Beliaev, A.S., and Saffarini, D.A. (1998) Shewanella putrefaciens mtrB encodes an outer membrane protein required for Fe(III) and Mn(IV) reduction. J Bacteriol 180: 6292-6297.
6. Beliaev, A.S., Saffarini, D.A., McLaughlin, J.L., and Hunnicutt, D. (2001) MtrC, an outer membrane decahaem c cytochrome required for metal reduction in Shewanella putrefaciens MR-1. Mol Microbiol 39: 722-730.
7. Blanco, L., Bernad, A., Lazaro, J.M., Martin, G., Garmendia, C., and Salas, M. (1989) Highly efficient DNA synthesis by the phage phi 29 DNA polymerase. Symmetrical mode of DNA replication. J Biol Chem 264: 8935-8940.
8. Bohuslavek, J., Payne, J.W., Liu, Y., Bolton Jr., H., and Xun, L. (2001) Cloning, sequencing, and characterization of a gene cluster involved in EDTA degradation from the bacterium BNC1. Appl Environ Microbiol 67: 688-695.
9. Bolton Jr., H., Li, S.W., Workman, D.J., and Girvin D.C. (1993) Biodegradation of synthetic chelates in subsurface sediments from the southeast coastal plain. J. Environ. Qual. 22:125-132.
10. Dunbar, J., Barns, S.M., Ticknor, L.O., and Kuske, C.R. (2002) Empirical and theoretical bacterial diversity in four Arizona soils. Appl Environ Microbiol 68: 3035-3045.
11. Eilers, H., Pernthaler, J., Glöckner, F.O., and Amann, R. (2000) Culturability and In situ abundance of pelagic bacteria from the North Sea. Appl Environ Microbiol 66: 3044-3051.
12. Ferguson, R.L., Buckley, E.N., and Palumbo, A.V. (1984) Response of marine bacterioplankton to differential filtration and confinement. Appl Environ Microbiol 47: 49-55.
13. Findlay, S. 1995. Importance of surface-subsurface exchange in stream ecosystems: the hyporheic zone. Limnol Oceanogr 40:159-164++
14. Fredrickson, J.K., Zachara, J.M., Brockman, F.J., Balkwill, D.L., Kennedy, D., Li, S.W., Kostandarithes, H.M., Daly, J.M., Romine, M.F., and Serne, R.J. (2003) Geomicrobiology of high level nuclear waste contaminated vadose sediments at the Hanford Site, Washington. Appl Environ Microbiol submitted.
15. Garmendia, C., Bernad, A., Esteban, J.A., Blanco, L., and Salas, M. (1992) The bacteriophage phi 29 DNA polymerase, a proofreading enzyme. J Biol Chem 267: 2594-2599.
16. Giovannoni, S.J., and Rappe, M. (2000) Evolution, diversity, and molecular ecology of marine prokaryotes. In Microbial Ecology of the Ocean. Kirchman, D.L. (ed): Wiley-Liss, Inc., pp. 47-84.
17. Harayama, S., Rekik, M., Wasserfallen, A., and Bairoch, A. (1987) Evolutionary relationships between catabolic pathways for aromatics: conservation of gene order and nucleotide sequences of catechol oxidation genes of pWW0 and NAH7 plasmids. Mol Gen Genet 210: 241-247.
18. Hendricks, S.P. 1993. Microbial ecology of the hyporheic zone: a perspective integrating hydrology and

biology. J. N. Am. Benthol. Soc. 12:70-78.

19. Hugenholtz, P., Goebel, B.M., and Pace, N.R. (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol 180: 4765-4774.
20. Ito, T., Nielsen, J.L., Okabe, S., Watanabe, Y., and Nielsen, P.H. (2002) Phylogenetic identification and substrate uptake patterns of sulfate- reducing bacteria inhabiting an oxic-anoxic sewer biofilm determined by combining microautoradiography and fluorescent in situ hybridization. Appl Environ Microbiol 68: 356-364.
21. Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M., and Sait, M. (2002) Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. Appl Environ Microbiol 68: 2391-2396.
22. Jastrow, J.D. (1987) Changes in soil aggregation associated with tallgrass prairie restoration. American Journal of Botany 74:1656-1664.
23. Jastrow, J.D., Boutton, T.W., and Miller, R.M. (1996) Carbon dynamics of aggregate-associated organic matter estimated by carbon-13 natural abundance. Soil Science Society of America Journal 60:801-807.
24. Jastrow, J.D., and Miller, R.M. (1997) Soil aggregate stabilization and carbon sequestration: Feedbacks through organomineral associations. In: (Eds), Soil Processes and the Carbon Cycle. Crc Press Inc, Boca Raton, pp. 207-223.
25. Kaeberlein, T., Lewis, K., and Epstein, S.S. (2002) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science 296: 1127-1129.
26. Killey, R.W.D., McHugh, J.O., Champ, D.R., Cooper, E.L., and Young, J.L. (1984) Subsurface cobalt-60 migration from a low level waste disposal site. Environ Sci Technol 18:148-157.
27. Lage, J.M., Leamon, J.H., Pejovic, T., Hamann, S., Lacey, M., Dillon, D., Segraves, R., Vossbrinck, B., Gonzalez, A., Pinkel, D., Albertson, D.G., Costa, J., and Lizardi, P.M. (2003) Whole genome analysis of genetic alterations in small DNA samples using hyperbranched strand displacement amplification and array-CGH. Genome Res 13: 294-307.
28. Lal, R., (1997) Residue management, conservation tillage and soil restoration for mitigating greenhouse effect by CO2-enrichment. Soil & Tillage Res 43:81-107.
29. Lee, N., Nielsen, P.H., Andreasen, K.H., Juretschko, S., Nielsen, J.L., Schleifer, K.-H., and Wagner, M. (1999) Combination of fluorescent in situ hybridization and microautoradiography - a new tool for structure-function analyses in microbial ecology. Appl Environ Microbiol 65: 1289-1297.
30. Rappé, M.S., Connon, S.A., Vergin, K.L., and Giovannoni, S.J. (2002) Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature 418: 630-633.
31. Riley, R.G., and Zachara J.M. (1992) Chemical Contaminants on DOE Lands and Selection of Contaminant Mixtures for Subsurface Science Research, DOE/ER-0547T. National Technical Information Service, U.S. Department of Commerce, Springfield, VA.
32. Rondon, M.R., August, P.R., Bettermann, A.D., Brady, S.F., Grossman, T.H., Liles, M.R., Loiacono, K. A., Lynch, B.A., MacNeil, I.A., Minor, C., Tiong, C.L., Gilman, M., Osburne, M.S., Clardy, J., Handelsman, J., and Goodman, R.M. (2000) Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl Environ Microbiol 66: 2541-2547.
33. Rosselló-Mora, R., and Amann, R. (2001) The species concept for prokaryotes. FEMS Microbiol Rev 25: 39-67.
34. Saffarini, D.A., Blumerman, S.L., and Mansoorabadi, K.J. (2002) Role of menaquinones in Fe(III) reduction by membrane fractions of Shewanella putrefaciens. J Bacteriol 184: 846-848.
35. Santegoeds, C.M., Damgaard, L.R., Hesselink, G., Zopfi, J., Lens, P., Muyzer, G., and de Beer, D. (1999) Distribution of sulfate-reducing and methanogenic bacteria in anaerobic aggregates determined by microsenor and molecular analyses. Appl Environ Microbiol 65: 4618-4629.
36. Schoolnik, G.K. (2002) Functional and comparative genomics of pathogenic bacteria. Curr Opin Microbiol 5:20-26.
37. Shutthanandan, J., Bailey, V. L., Bolton, H. J., Brockman, F. J. (2002). Shifts in Fungal and Bacterial Community Structure During Tallgrass Prairie Restoration. American Geophysical Union Fall Meeting, San Francisco, California.

38. Strunk, O., and Ludwig, W. (1998) ARB: a software environment for sequence data. In http://www.mikro. biologie.tu-muenchen.de: Department of Microbiology, Technische Universität München, Munich, Germany.
39. Taroncher-Oldenburg, G., Griner, E.M., Francis, C.A., and Ward, B.B. (2003) Oligonucleotide microarray for the study of functional gene diversity in the nitrogen cycle in the environment. Appl Environ Microbiol 69: 1159-1171.
40. Tiedje, J.M. (1975) Microbial degradation of ethylenediaminetetraacetic acid in soils and sediments. Appl. Environ. Microbiol. 30:327-329.
41. Tiedje, J.M. (1977) Influence of environmental parameters on EDTA biodegradation in soils and sediments. J. Environ. Qual. 6:21-26.
42. Torsvik, V., Ovreas, L., and Thingstad, T.F. (2002) Prokaryotic diversity--magnitude, dynamics, and controlling factors. Science 296: 1064-1066.
43. Vobis, G. (1992) The genus Actinoplanes and related genera. In The Prokaryotes. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds). New York: Springer-Verlag, pp. 1029-1060.
44. Wendisch, V.F., Zimmer, D.P., Khodursky, A., Peter, B., Cozzarelli, N., and Kustu, S. (2001) Isolation of Escherichia coli mRNA and comparison of expression using mRNA and total RNA on DNA microarrays. Anal Biochem 290: 205-213.
45. Whitman, W.B., Coleman, D.C., and Wiebe, W.J. (1998) Prokaryotes: the unseen majority. Proc Natl Acad Sci U S A 95: 6578-6583.
46. Willardson, B.M., Wilkins, J.F., Rand, T.A., Schupp, J.M., Hill, K.K., Keim, P., and Jackson, P.J. (1998) Development and testing of a bacterial biosensor for toluene-based environmental contaminants. Appl Environ Microbiol 64: 1006-10012.
47. Wise, A.A., and Kuske, C.R. (2000) Generation of novel bacterial regulatory proteins that detect priority pollutant phenols. Appl Environ Microbiol 66: 163-169.
48. Wu, L., Thompson, D.K., Li, G., Hurt, R.A., Tiedje, J.M., and Zhou, J. (2001) Development and evaluation of functional gene arrays for detection of selected genes in the environment. Appl Environ Microbiol 67: 5780-5790.
49. Xu, H.S., Roberts, N., Singleton, F.L., Attwell, R.W., Grimes, D.J., and Colwell, R.R. (1982) Survival and viability of nonculturable Escherichia coli and Vibrio cholerae in the estuarine and marine environment. Microb Ecol 8: 313-323.
50. Yang, L., Tran, D.K., and Wang, Z. (2001) BADGE, Beads aary for the detection of gene expression, a high throughput diagnostic bioassay. Genome Res 11: 1888-1898.
51. Zani, S., Mellon, M.T., Collier, J.L., and Zeher, J.P. (2000) Expression of nifH genes in natural microbial assemblages in Lake George, New York, detected by reverse transcriptase PCR. Appl Environ Microbiol 66:3119-3124.
52. Zengler, K., Toledo, G., Rappe, M., Elkins, J., Mathur, E.J., Short, J.M., and Keller, M. (2002) Cultivating the uncultured. Proc Natl Acad Sci U S A 99: 15681-15686.