Soil Metagenome to Explore Soil Microbial Resources

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Recent advances in molecular microbial ecology revealed that only small fraction of total microorganisms in nature were identified and characterized, because the majority of them are not culturable. A concept, metagenome, came from the idea that much more microbial resources could be tapped from the viable but non-culturable microbes compared to the small portion of culturable microbes.

Our long-term practical goal is to explore metagenomic library constructed from various soil environments and find out microbial products, which can serve for chemical biotechnology application. We have constructed soil metagenomic library in a fosmid using total microbial DNA isolated from four different soils. Screening to select metagenomic clones conferring copper tolerance on *Escherichia coli* generated 67 unique clones from 57,600-member library. Heavy-metal specific responses of the selected copper tolerant clones to several heavy metals suggested that our library cover the genome of diverse microorganisms. We have also selected 8 unique lipolytic active clones by screening 33,700-member metagenome library to obtain lipases, which will be used as biocatalysts for the chiral compound synthesis. Many useful genes would be exploited from our metagenomic library for chemistry applications.

Microbial Diversity

Recent studies on microbial ecology unveiled that earth ecosystem is dominated by microorganisms (Whitman et al., 1998). Over 99% of bacteria present in nature are not culturable (Amann et al., 1995; Hugenholtz and Pace 1996). Microbial community structure and microbial diversity have been revealed by culture-independent studies with the analysis of the complexity of 16S rRNA genes from various microbial communities. These studies indicated that microbial diversity is much more complicated than ever imagined (Liesack and Stackebrandt 1992; Borneman et al., 1996; Bintrim et al., 1997; Pace 1997). Recently, Torsvik et al (2002) summarized the reality of microbial diversity in various soil environments and sediments by comparing microbial counts with fluorescent microscopy and unique genome equivalent derived from community genome complexity. Microbial DNA diversity in forest soil and pasture soil appeared to carry more than 6,000 and 3,500 different microbial genomes, respectively.

Metagenome

Although the microbial diversity in nature has been estimated by culture-independent method, it is not certain if one could find a universal way to culture and characterize most of the bacteria present in natural ecosystem (Rondon et al., 1999). Many attempts to search valuable microbial products from microorganisms employ pure culture of microorganisms and culture-based activity screening. However, recently developed strategy adopts cloning total microbial genome, so called 'metagenome', directly isolated from natural environments in the culturable bacteria such as *Escherichia coli* (Rondon et al., 2000). By now, most of

microbial products for industrial applications were originated from cultured microbes and natural products from cultured bacteria often exhibit the simple rediscovery of already known product. However, exploiting the unculturable majority of bacteria is expected to reveal novel microbial world. The metagenomic approach is proven to be technically feasible to explore novel microbial resources by several recent studies (Rondon et al., 2000; Gillespie et al., 2002; Wang et al., 2000). Therefore, tapping metagenomic library provides us with a unique opportunity to search novel microbial resources.

Construction of Metagenomic Library

We constructed metagenomic library in a fosmid from four different soils such as forest topsoil, pine tree rhizosphere soil, pasture soil, and lime cave soil. We made metagenomic library mainly from forest soils because of the results from Torsvik et al. (2002) that the microbial diversity determined by DNA diversity is high in forest and pasture soils. High molecular weight metagenomic DNA were successfully isolated from the above soils with high humic acid contents. Subsequent purification of the DNA and end-repair made it possible to clone in a fosmid vector as blunt-end cloning. We have routinely obtained 50,000 clones per microgram of partially purified soil DNA. Restriction analysis of random selected fosmid clones indicated highly complex polymorphism with average insert DNA size ranged from 32 kb to 40 kb. Sequencing of insert DNA ends from randomly selected clones exhibited in about 61% of the sequences significant similarities to protein genes from the databases (e-value of $< e^{-10}$) in BLASTX searches) although many of them were similar to hypothetical proteins in database. We stored the library as pools containing 300-1000 clones until we used them for functional screening to select the novel microbial resources. We are currently depositing the libraries and the random fosmid end-sequencing data to the Microbial Genomics and Applications Center in Korea Research Institute of Bioscience and Biotechnology.

Metagenomic Clones

Exploiting metagenomic library to find useful resources is mostly based on the functional expression of the cloned environmental DNA in the cloning host (Rondon et al., 2000; Wang et al., 2001). However, it is also be possible to explore environmental microbial genome based on DNA homology dependent screening (Quaiser et al., 2002; Courtois et al., 2003). We choose the first strategy, which will screen the library based on functional expression of cloned metagenomic DNA.

Firstly, we selected metagenomic clones conferring copper tolerance on *E. coli* by taking the advantage of positive selection on the culture media with lethal concentration of copper (II). Copper is essential as a trace element but toxic at high concentration. Therefore, most microorganisms should have mechanism for intracellular copper homeostasis (Nies, 1999). Sixty-seven unique clones were selected from 56,700-member forest topsoil metagenomic library and heavy-metal specific responses were investigated from further selected 18 clones using cadmium, zinc, lead, and cobalt. Each clones showed different heavy-metal specific response and altered colony pigmentation. Interestingly, one clone (pEcp53) showed enhanced copper tolerance but was highly sensitive to other heavy metals tested compared to *E. coli* carrying an empty fosmid vector. Characterization of the clones may reveal the novel mechanism of heavy-metal tolerance in soil microorganisms. The copper tolerant clones selected in this study would represent the mechanisms of copper tolerance or homeostasis from the soil microbes. Furthermore, the analysis with the copper tolerant clones could be an indirect method to evaluate the coverage of soil microbial diversity in the constructed metagenomic library.

Our second interest from the soil metagenome is to find microbial resources for the applications in

chemical biotechnology. Lipases are the biocatalysts of our choice, frequently used for biotechnological application because of their regioselectivity, stereoselectivity, and chemoselectivity with stable activity in organic solvents (Jaeger and Eggert 2002). Screening lipolytic active clones from our metagenomic library was carried out on culture media supplemented with 1% tributyrin to select colonies showing clear zone (Fig. 1). Eight unique clones were selected from 33,700-member library constructed from forest topsoil. The responsible genes for lipolytic activity from the selected clones would be identified and potential usage of the lipases for biotechnology application will be tested.

(A) (B)

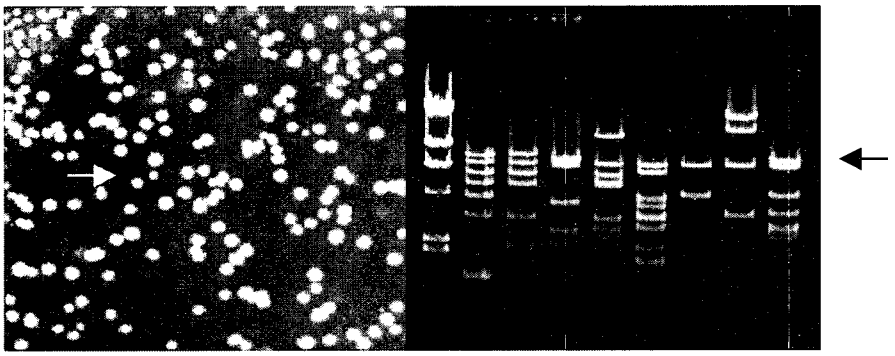

Fig. 1. Lipolytic active clones selected from forest topsoil metagenomic library.

(A) A lipolytic active clone (arrow) on LB with 1% tributyrin

(B) Restriction digest (*Bam*HI) of 8 lipolytic active clones separated by agarose gel electrophoresis. The first lane is the size standard λ-DNA digested with *Hind*III and the arrow indicates the fosmid vector.

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