

METAGENOMIC LIBRARY OF MICROBIAL GENETIC RESOURCES IN LIVESTOCK FARM

R. B. Liaw^{1*}, M. P. Cheng², T. H. Hsiao³, M. C. Wu¹, and C. Y. Lee⁴

¹ Breeding and Genetics Division, COA-LRI, Hsinhua, Tainan 71246, Taiwan

² Preparatory Office of Agricultural Biotechnology Park, COA, Changjhih, Pintung 90846, Taiwan

³ Management Division, COA-LRI, Hsinhua, Tainan 71246, Taiwan

⁴ Department of Agricultural Chemistry, National Taiwan University, Taipei 10617, Taiwan

* Corresponding author, e-mail: liawrb@mail.tlri.gov.tw

ABSTRACT

In this study, the livestock environmental sample of activated sludge was assessed for bacterial diversity. According to 16S rDNA analysis, there were different microbial phyla including *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Genera_incertae_sedis_OP10*, *Cyanobacteria*, and unclassified Bacteria in activated sludge and most of the 16S rDNA clones were closest to uncultured bacteria. Therefore, the bacterial diversity was rather abundant in such semi-tame samples and we can stand a good chance of discovering novel enzymes in such a sample. Furthermore, the metagenomic library derived from activated sludge was constructed. A lipase gene in the library was found by screening with tributyrin agar plate. The putative lipase gene consisting of 308 amino acids showed novel only 35.6% identity with a known enzyme AAS77233. The putative lipase gene has been subcloned into an expression vector for further enzymatic characterization. Thus, it is a potential route to discover novel enzymes through metagenomic approach.

KEY WORDS: Bacterial diversity, Lipase, Metagenomic library.

INTRODUCTION

It is estimated that there are at least 10^6 - 10^7 kinds of microbial species in the world (Cowan, 2000), but the vast majority can not be classified by artificial culturing. Microbes play key roles in ecological balancing on earth and go through environmental changes for a long time, so they have abundant genetic diversity. In the same way, there are many kinds of microbes in the livestock environment such as activated sludge. The microbial culturability in activated sludge ranged from 1% to 15% (Amann *et al.* 1995). So we stand a good chance of gathering novel, available and valuable genes to improve livestock production and related beneficial industry.

Due to the improvement of DNA extraction directly or indirectly from complicated samples such as soils, compost, and sediments, etc., we could easily isolate and acquire pure and enough DNA from such complicated samples and provide for metagenomic library construction. Recently, some novel genes or bioactive products were discovered through metagenomic approach, the genes or bioactive products included amylases (Yun *et al.*, 2005), lipases (Henne *et al.*, 2000; Lee *et al.*, 2004; Kim *et al.*, 2005; Ranjan *et al.* 2005; Rhee *et al.*, 2005), proteases (Gupta *et al.*, 2002), dehydrogenase (Wexler *et al.*, 2005), diol dehydratases

(Knietsch *et al.*, 2003), reductases (Eschenfeldt *et al.*, 2001), antibiotics (Gillespie *et al.*, 2002; Lim *et al.*, 2005). Among the enzymes listing above, lipases are important biocatalysts in some industries such as detergent, pharmaceutical, feed and foods, chemical synthesis, cosmetics and flavours, and so on. In this study, we assessed the bacterial diversity of activated sludge by 16S rDNA and discovered a novel lipase gene through metagenomic approach.

MATERIALS AND METHODS

Livestock environmental samples Activated sludge samples were collected from swine wastewater plant of Livestock Research Institute on May 6, June 8, and Aug. 4 of 2005, respectively. The pHs of the three samples were 7.59 (0526), 7.70 (0608) and 7.84 (0804), respectively. The temperatures of the samples belonged to be mesophilic (30 °C).

DNA extraction and purification: Activated sludge DNA was extracted using a commercial kit (PowerSoil DNA isolation kit, Mo Bio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Then the DNA was to test for quality by 0.8 % agarose gel electrophoresis.

Generation of 16S rDNA clone library Bacterial-specific 16S rDNA was amplified by performing PCR in a thermocycler (9600, Applied Biosystems, Foster City, CA) with forward primer 27F (Edwards *et al.*, 1989) and reverse primer 1492R (Wilson *et al.*, 1990). The amplicons were ligated into the TA cloning vector (pOSI-T) and transformed into *Escherichia coli* competent cell with TA cloning kit (Genemark Technology Co, Ltd.). The clones were cultured and 1.5 µl of culture solution was picked up to perform PCR with primer set of T7 and SP6. Furthermore, the amplicons of every clone were purified using Micro-Elute DNA Clean/Extraction Kit (Genemark Technology Co, Ltd.). The purified amplicons were sequenced with primers of T7, SP6, 27F, 515F, 519R (Lane *et al.*, 1985), and 1492R by a BigDye Terminator v3.1 cycle sequencing kit and analyzed with a ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA). The DNA sequences of each fragment were assembled using a software VectorNTI (Invitrogen).

DNA sequence and phylogenetic analyses The 16S rDNA sequences were aligned to GenBank database with BLASTN program to search for the closest microbe. Phylogenetic analyses of the 16S rDNA sequences were performed with ClustalW 1.83. Phylogenetic trees were edited using TreeView.

Metagenomic library construction and lipase screening The extracted DNA was digested with *Hind*III and recovered the part of size range from 2 kb to 9 kb according to agarose gel electrophoresis. The digested DNA fragments were ligated to the same restriction enzyme treated vector pBluescript II KS (Stratagene). Then the ligated DNA was electroporated to competent cell *E. coli* EPI300 (EPICENTRE, Madison, WI). The transformants were grown on 1% tributyrin Luria–Bertani (LB) agar plate with ampicillin (50 µg/ml). After two or more days, there was a halo around the colony if the colony contained lipase activity.

RESULTS AND DISCUSSION

A total of 57 clones of near full length of 16S rDNA sequence were completed. After all of the sequences aligned with GenBank by BLASTN program, only two clones were belonged to

known microbes (*Nitrosomonas* sp. and *Hydrogenophaga* sp.) and two clones showed 90% to 93% identity with *Synechococcus* sp. and *Ectothiorhodospira shaposhnikovii*, respectively. The others were belonged to uncultured microbes (>96%). When all of the sequences were identified with Classifier program (Ribosomal Database Project II), there were different microbial phyla including *Proteobacteria* (including classes of *Alphaproteobacteria*, *Betaproteobacteria*, *Gamma*, *Deltaproteobacteria*, and *Epsilon*), *Bacteroidetes*, *Firmicutes*, *Genera_incertae_sedis_OP10*, *Cyanobacteria*, and unclassified Bacteria in activated sludge. The results were slightly different from that of Snaidr *et al.* (1997). It seemed that our activated sludge possessed more bacterial diversity than that of them.

The ligated DNA was electroporated to competent cell *E. coli* EPI300 and spread on 1% tributyrin agar plate. After two or more days of growth, a halo generated around a colony. We picked up them to fresh broth to cultivate and streaked on the fresh 1% tributyrin agar plate to confirm the lipolytic activity. The recombinant plasmid was extracted and digested with *HindIII* to be aware of the insert size about 4.7 kb. The insert was sequenced completely and had a putative open reading frame of lipase with 308 amino acids. When the deduced amino acid sequence was aligned by BLASTP program with GenBank, the results indicated that the putative lipase had only 35.6% identity with a known enzyme AAS77233 (Lee *et al.*, 2004). The AAS77233 lipase/esterase was discovered through metagenomic library of forest topsoil in Korea by fosmid vector system. We obtained a novel lipase gene by directly active screening without manipulating and maintaining huge numbers of clones.

REFERENCE

- Amann, R. I., W. Ludwig, and K. H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev.* 59: 143-169.
- Cowan, D. A. 2000. Microbial genomes--the untapped resource. *Trends Biotechnol.* 18(1): 14-16.
- Edwards, U., T. Rogall, H. Blocker, M. Emde, and E. C. Bottger. 1989. Isolation and direct complete determination of entire genes. *Nucleic Acids Res.* 17:7843-7853.
- Eschenfeldt, W. H., L. Stols, H. Rosenbaum, Z. S. Khambatta, E. Quaiter-Randall, S. Wu, D. C. Kilgore, J. D. Trent, and M. I. Donnelly. 2001. DNA from uncultured organisms as a source of 2, 5-diketo-D gluconic acid reductases. *Appl. Environ. Microbiol.* 67: 4206-4214.
- Gillespie, D. E., S. F. Brady, A. D. Bettermann, N. P. Cianciotto, M. R. Liles, M. R. Rondon, J. Clardy, R. M. Goodman, and J. Handelsman. 2002. Isolation of antibiotics turbomycin A and B from a metagenomic library of soil microbial DNA. *Appl. Environ. Microbiol.* 68: 4301-4306.
- Henne, A., R. A. Schmitz, M. Bomeke, G. Gottschalk, and R. Daneil. 2000. Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on *Escherichia coli*. *Appl. Environ. Microbiol.* 66: 3113-3116.
- Kim, Y. J., G. S. Choi, S. B. Kim, G. S. Yoon, Y. S. Kim, and Y. W. Ryu. 2005. Screening and characterization of a novel esterase from a metagenomic library. *Protein Expr. Purif.* Online

- Knietsch, A., S. Bowien, G. Whited, G. Gottschalk, and R. Daniel. 2003. Identification and characterization of coenzyme B12-dependent glycerol dehydratase- and diol dehydratase-encoding genes from metagenomic DNA libraries derived from enrichment cultures. *Appl. Environ. Microbiol.* 69: 3048-3060.
- Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. *Proc. Natl. Acad. Sci. U.S.A.* 82: 6955-6959.
- Lee, S. W., K. Won, H. K. Lim, J. C. Kim, G. J. Choi, and K.Y. Cho. 2004. Screening for novel lipolytic enzymes from uncultured soil microorganisms. *Appl. Microbiol. Biotechnol.* 65: 720-726.
- Lim, H. K., C. E. J. Hung, J. C. Kim, G. J. Choi, K. S. Jang, Y. R. Chung, K. Y. Cho, and S. W. Lee. 2005. Characterization of a forest soil metagenome clone that confers indirubin and indigo production on *Escherichia coli*. *Appl. Environ. Microbiol.* 71: 7768-7777.
- Ranjan, R., A. Grover, R. K. Kapardar, and R. Sharma. 2005. Isolation of novel lipolytic genes from uncultured bacteria of pond water. *Biochem. Biophys. Res. Commun.* 335: 57-65.
- Rhee, J. K., D. G. Ahn, Y. G. Kim, and J. W. Oh. 2005. New thermophilic and thermostable esterase with sequence similarity to the hormone-sensitive lipase family, cloned from a metagenomic library. *Appl. Environ. Microbiol.* 71: 817-825.
- Snaidr, J, R. Amann, I. Huber, W. Ludwig, and K. H. Schleifer. 1997. Phylogenetic analysis and *in situ* identification of bacteria in activated sludge. *Appl. Environ. Microbiol.* 63: 2884-2896
- Wilson, K. H., R. B. Blichington, and R. C. Green. 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. *J. Clin. Microbiol.* 28: 1942-1946.
- Yun, J., S. Kang, S. Park, H. Yoon, M. J. Kim, S. Heu, and S. Ryu. 2004. Characterization of a novel amylolytic enzyme encoded by a gene from a soil-derived metagenomic library. *Appl. Environ. Microbiol.* 70: 7229-7235.