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Construction and Analysis of Rumen Bacterial Artificial Chromosome Library from a Cashmere Goat Rumen Microflora

[1, 2]Mei-Li Zhang and [2]Yu-Lin Chen
[1]College of Animal Science, Yangtze University, 434025 Jingzhou, Hubei, China
[2]College of Animal Science and Technology,
Northwest A&F University, 712100 Yangling, Shaanxi, China

Abstract: A Bacterial Artificial Chromosome Library of rumen microorganism from Shanbei Cashmere goat was constructed to study the potential of the ruminant's ecosystem and the library storage capacity, stability and the functionality was also analyzed. The high molecular weight DNA was extracted and purified directly from rumen samples in the study by using embedding technology and pulsed field gel electrophoresis approaches. After digestion with HindIII, DNA fragments ranging from 50-150 kb was collected and ligated to pCC1BAC vector. The ligation mixture was transformed into *E. coli* TransforMax EPI300. The rumen microorganism BAC library contains about 14,400 clones and the average insert size of BAC clones was estimated to be 74.1 kb, mostly ranging from 50-90 kb. Based on the data mentioned above, the capacity of the BAC library was about 1,067 Mb, the frequency of clones without inserts was <2%. The BAC clones show good stability after cultured for 100 generations. RFLP analysis showed a great diversity of the insert fragments. Several BAC clones with activity of amylase had been screened from the BAC library. Eight clones with amylase activity were acquired. It was suggested that the constructed BAC library had good storage capacity, stability and functionality and could be used for further research on exploration of enzyme resource and rumen microflora of Cashmere goat.

Key words: Rumen microorganism, bacterial artificial chromosome library, Cashmere goat, screening, clone

INTRODUCTION

Rumen is an extremely complex symbiotic ecosystem, characterized by its highmicrobial population density, high diversity and complexityof interactions. Rumen microorganisms play an important role in ruminant digestion, nutrition and health (Bryant and Burkey, 1953; Miron *et al*., 2001; Delgado, 2005). However, due to the high complexity of rumen microbial communities as well as very strong symbiotic dependence symbiosis between species, it is very difficult to analysis and research of rumenecological and microbial resources by traditional culture-dependent techniques (Handelsman *et al*., 1998; Rappe and Giovannoni, 2003). The current research showed that only 10-20% of rumen microbes could been cultivated (Cowan *et al*., 2004; Edwards *et al*., 2004; An *et al*., 2005). This indicated >80% of the microbial diversity unexploited. The application of metagenomics approach in rumen microbial communities has expanded the scope of the research, accumulated a large number of gene sequence information, understood the biological function of rumen microbial diversity and provided the basis for the use of rumen microbial genetic resources (Singh *et al*., 2008; Huson *et al*., 2009).

Large insert metagenomic library (using Fosmid or BAC vectors, insert >40 kb) enabled us to gainbigger more microbial DNA fragments (Beja *et al*., 2000; Entcheva *et al*., 2001). This meanss researchers are able to isolation of genes and gene clusters to explore the complex metabolic pathways encoding by large DNA fragments to develop a more complete study on the sources, relationships and synergy of microbial genomes.

In the rumen metagenomic library research, New Zealand rumen microbial phage library with an average insert size of 5.5 kb was constructed and screened to a variety of cellulase and xylanase genes (Ferrer *et al*., 2005). A buffalo rumen metagenomic library was built and screen isolated 14 kinds of a cellulase gene (Duan *et al*., 2009). In another study, two glycosyl hydrolase genes were identified through activity screening of a bovine rumen metagenomic library (Shedova *et al*., 2009).

Bacterial Artificial Chromosome (BAC) of rumen microorganisms is necessary for rumen microbial ecology due to its large capacity, high clone stability and low DNA chimerism (Shizuya *et al*., 1992; Osoegawa *et al*., 2001; Venter *et al*., 2004). In this study, a BAC library from

Corresponding Author: Yu-Lin Chen, College of Animal Science and Technology, Northwest A&F University, 712100 Yangling, Shaanxi, P.R. China

Shanbei Cashmere goat rumen microflora was constructed and analyzed its storage capacity, stability and functionality.

MATERIALS AND METHODS

DNA isolation from rumen sample: Fresh rumen contents (mixed solids and liquid) was collected from Shanbei Cashmere goats maintained on a grass/hay diet. The sample was prefiltered through gauze. The filtrate was centrifuged (12,000 g) for 10 min and the pellet suspended in phosphate-buffered saline so that the cell concentration was adjusted to 10^7~10^8 cells mL^{-1}. The cell suspension was mixed with an equal volume of molten 1.6% Low Melting Point agarose (LMP) and poured into plastic plug molds (Bio-Rad, USA). The agarose containing the embedded cells were lysed in 10 volumes of lysis buffer (10 mM Tris-HCl, 50 mM NaCl, 0.1 M EDTA, pH 8.0, 1% Sarkosy l, 0.2% sodium deoxycholate, 1 mg mL^{-1} lysozyme) at 37°C for 1 h in 5 volumes of ESP buffer (0.5 M EDTA, 1% Sarkosyl, 1 mg mL^{-1} proteinase K, pH 8.0) at 50°C for 24 h. The plugs were washed 3 times (1 h/wash) with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) plus 1 mM PMSF and then 3 times (1 h/wash) with TE at room temperature. The plugs containing the concentrated high molecular weight DNA (HMW DNA) were stored in sterile TE buffer at 4°C.

Partially digestion of genomic DNA: HMW DNA was partially digested by HindIII (New England Biolabs, USA) and then separated by PFGE on a 1% low-melting agarose gel in 0.5×TBE buffer, 6 V cm^{-1} with a 40 sec pulse for 16 h at 14°C using a CHEF-DR III apparatus (Bio-Rad, USA). Optimal sizes were generated with 0.15U of HindIII when plugs were incubated at 37°C for 4 h. The digestions were stopped with the addition of 50 mM EDTA. The partially digested DNA was subjected to PFGE. The agarose slices containing DNA in the size ranging from 50-150 kb were excised from the gel and subjected to PFGE for a second size selection to remove fragments <50 kb. The agarose slices with the targeted DNA sizes (50-150 kb) were dialysed with 0.5×TBE buffer.

BAC library construction: Approximately 100 ng partially-digested and size-selected genomic DNA fragments was ligated with 25 ng CopyControl pCC1BAC Cloning-ready Vector (HindIII-digested) (Epicentre Biotechnologies, USA) in a molar ratio of 1:6 using Fast-Link DNA Ligase (Epicentre Biotechnologies, USA). The ligation reaction was incubated for 12 h at 16°C. After desalting the ligation reaction, the ligation products was transformed *E. coli* Transfor MaxEPI300 (Epicentre Biotechnologies, USA) using an electroporation (Gene Pulser II, Bio-Rad, USA). The cells were grown in 1 mL SOC media (2% w/v bactotryptone, 0.5% w/v bacto yeast extract, 10 mM NaCl, 2.5 mM Kcl, 10 mM $MgCl_2$, 20 mM glucose, pH 7.0) at 37°C for 1 h with shaking at 220 rpm and then plated on to solid LB agar plates containing 12.5 μg mL^{-1} chloramphenicol, 40 μg mL^{-1} X-Gal, 0.4 mM IPTG incubated at 37°C for 24 h. The white recombinant BAC clones were picked to individual wells of a 96-well microtiter plate containing 100 μL LB freezing media (13.2 mM KH_2PO_4, 36 mM K_2HPO_4, 1.7 mM sodium citrate, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$, 4.4% v/v glycerol and 12.5 μg mL^{-1} chloramphenicol) and incubated overnight at 37°C. The whole library was stored -80°C.

Identification and evaluation of BAC library: In order to estimate the average insert size of the cloned DNA, 100 randomly selected BAC clones were incubated in 5 mL LB medium containing chloramphenicol 12.5 μg mL^{-1} at 37°C overnight. The BAC DNA was isolated using Standard Alkaline Lysis Methods (Sambrook *et al*., 1989). The purified BAC DNA was digested with Not I (New England Biolabs, USA) at 37°C for 4 h followed by PFGE in the presence of the Midrange PFGImarker (New England Biolabs, USA) and photographed.

Three randomly clones were picked from the library and incubated in 5 mL LB, respectively overnight at 37°C, primary cultures were considered as generation 0 then the cultures were diluted 10^6 fold in 5 mL LB and incubated overnight at 37°C. The process was repeated for 5 consecutive days representing 100 generations. The BAC DNA was isolated and taken for analysis by HindIII digestion at 37°C for 4 h followed by PFGE to analyze BAC clones subculture stability.

The 23 randomly selected BAC DNA were digested with HindIII and BamHI at 37°C for 2 h followed by PFGE to Restriction Fragment Length Polymorphism (RFLP) analysis of the insert fragments.

Screening of BAC library for amylase activity clones: The libraries were replia plated onto LB agar supplemented with 1% soluble starch (Sigma, USA) and chloramphenicol (12.5 μg mL^{-1}) for amylase activity. Colonies were grown at 37°C for 20 h and then 5 mL of top agar (0.6%) containing D-cycloserine (60 μg mL^{-1}) was overlaid to allow detection of the intracellular enzymes. The plates were incubated for 1 day. Amylase activity was detected by flooding the plates with gram's iodine solution (1.0 g of I_2 and 2.0 g of KI in 300 mL of aqueous solution). Active colonies were detected as bright clear haloes upon fluorescent light illumination (Cho *et al*., 2000).

RESULTS

Construction of rumen microorganisms BAC library: Preparation of HMW genomic DNA is a key to construct a BAC library. A high molecular weight and high purity Metagenomic DNA were gained using the LMP agarose embedding. PFGE showed that the metagenomic DNA contained a substantial amount of HMW genomic DNA not degraded during preparation (Fig. 1).

The HMW DNA partial digestions were optimized by varying the concentration of restriction enzyme and time of digestion. In this study, HMW genomic DNA was optimally digested with HindIII. Partially digested DNA (50~100 kb) was ligated to BAC vector, ligations and transformations were performed to complete the construction of the rumen BAC library. A total of 14,400 clones were picked into 500, 96 well plates.

Identification and evaluation of BAC library: BAC DNA was extracted from 100 randomly selected clones, digested with NotI and submitted to PFGE to determine the insert sizes. The BAC library exogenous DNA insert size mostly ranged from 50-90 kb with an average of 74.1 kb (Fig. 2). The frequency of clones without inserts was <2%. The estimated total storage capacity of approximately BAC library was 1,067 Mb.

The stability of the BAC library was analyzed by continuously incubating three random clones for 6 days. It was demonstrated by restriction analysis with HindIII that the restriction fingerprinting of the 6 days were identical (Fig. 3). No loss or rearrangement of the inserted DNA fragments was found during the continuous subculture. The BAC library clones cultured for 100 generations still showed good stability.

RFLP analysis of the insert fragments was performed with 23 randomly picked clones, the restriction fingerprinting of each clone digested with HindIII and BamHI was different (Fig. 4). The result indicated that there was a great diversity in the insert fragments.

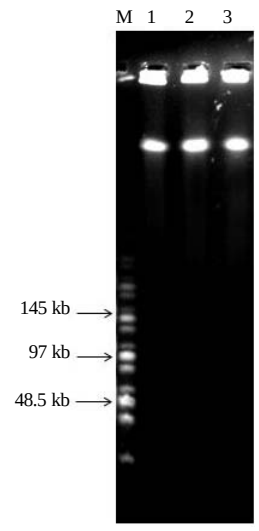

Fig. 1: The preparation of HMW DNA from rumen detected by PFGE; 1-3: HMW genomic DNA; M: MidRange PFGI marker

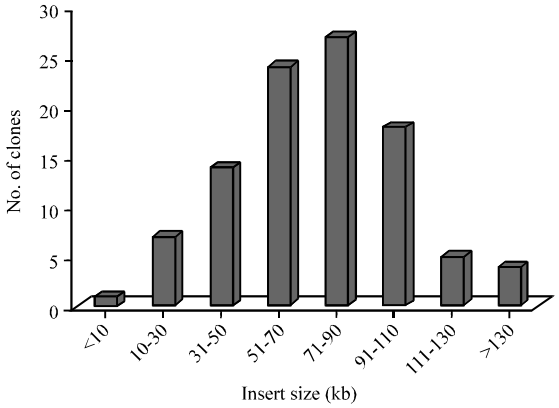

Fig. 2: Insert size distribution of the rumen BAC library clones

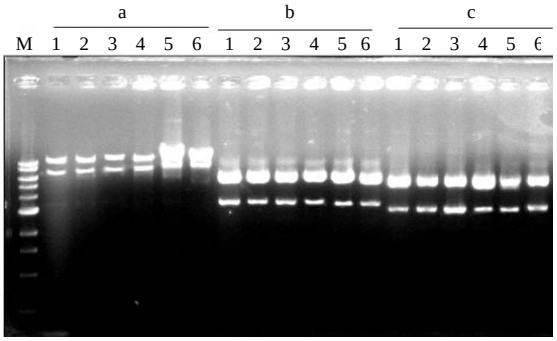

Fig. 3: The stability analysis of the rumen BAC library clones; M: 1 kb DNA marker; a-c: Clones digested with HindIII; 1-6: 5 time subculture

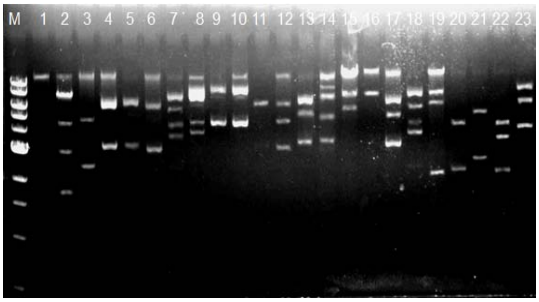

Fig. 4: PFGE of digested product of BAC clone; M: 1 kb DNA marker; 1~23: Randomly clones digested with HindIII and BamHI

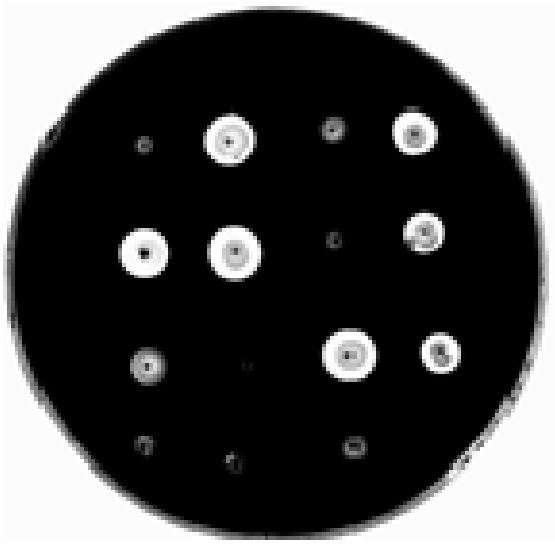

Fig. 5: Hydrolysis halos produced by clones with the activity of amylase on LB plate

Screening of BAC library for amylase activity: Amylase activity screening resulted in eightamylolytic clones on an LB agar plate containing soluble starch (Fig. 5). The different sizes of bright clear haloeson the screening plate of eight clones showed that there were different on amylase activity of clones.

DISCUSSION

HMW genomic DNA is a key to construct a BAC library. Purity of DNA fragment determines the quality of digestive enzyme there by affecting subsequent molecular manipulation. The vigorous extraction methods required for high yields of DNA often result in excessive DNA shearing. In this study, fractionating rumen contents using gel embedding technology, the bacterial cells were embedded in LMP agarose. The cell lysates and the gene isolation from the cells and purificationare carried out in the gelto protect the DNA fragment from degradation. HMW genomic DNA was obtained with high purity.

Assessing the quality of large insert libraries is the average insert size, the stability and integrity of inserts (Moullet *et al*., 1999). In the study, rumen microorganism BAC library contains about 14,400 clones. The average insert size of BAC clones was estimated to be 74.1 kb mostly ranging from 50-90 kb. Based on the data mentioned above, the capacity of this BAC library is about 1,067 Mb, the frequency of clones without inserts was lower than 2%. The insert sizes of the BAC library averaging 74.1 kb was apromising quality for the use of this library in large scale sequencing and gene-screening projects.

Eight clones were acquired with the activity amylase by activity screening. Restriction analysis of these clones and partial sequence analysis indicated that these clones contain different DNA sequences. This demonstrated the usefulness of the metagenomic approach to identify novel enzymes from rumen.

Rumen has been identified as inexhaustible sources of biotherapeutics, enzymes genes. Rumen metagenomic libraries were constructed from bovine (Ferrer *et al*., 2005; Beloqui *et al*., 2006; Palackal *et al*., 2007; Lammle *et al*., 2007), buffalo (Wang *et al*., 2009; Singh *et al*., 2012), yak (Bao *et al*., 2012), dairy cow (Zhao *et al*., 2010; Hess *et al*., 2011). In the study, rumen BAC library was constructed from Cashmere goat.

The ability to clone large fragments of metagenomic DNA is able to isolation of genes and gene clusters to explore the complex metabolic pathways encoding by large DNA fragments. This approach has been applied to the isolation of several multigenic pathways (Brady *et al*., 2001). Aiming at complex rumen environment, metagenomics BAC library was established in this study.

CONCLUSION

Through the identification and analysis of the BAC library it will be valuable for research of subsequent rumen microbial metabolic pathways, exploration of functional microflora, development of rumen microbial resources.

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