A modified end sequencing method for identifying the gene fragment (>40 k base pairs) inserted in fosmid

Jianfei Luo[#], Guoliang Tian[#] and Weitie Lin[*]

College of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, China
* Corresponding author. E-mail: wtlin@21cn.com

Abstract: End sequencing was usually applied to the identification of the gene fragment (>40 k base pairs) inserted in fosmid. However, the obstacles of low plasmid copy and high plasmid DNA purity requirement usually led to the difficulty and cost of end sequencing. To overcome these problems, we proposed a mortified end sequencing method. The protocol of this method consists of three steps: 1) fragmentation of fosmid circular DNA; 2) temperature gradient PCR by using fosmid specific primer coupling with random primer; 3) DNA band isolation and sequenced by fosmid specific primer.
[Jianfei Luo, Guoliang Tian and Weitie Lin. **A modified end sequencing method for identifying the gene fragment (>40 k base pairs) inserted in fosmid**. *Life Sci J* 2013; 10(3): 717-720]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 105 (# These are co-first author)

Keywords: Metagenomic; End sequencing; Fosmid; Random primer

1. Introduction

After the first time "metagenome" was used to describe the whole genomes of soil microbes in 1998 (7), metagenomic techniques basing on cosmid, fosmid or BAC plasmids were widely used to study the microbial ecology in natural and manmade environments (4, 5, 9), screen functional genes (8, 10), and isolate novel biocatalysts and bioactive molecules (1-3). After a metagenomic library is constructed, the clones are screened by phylogenetic markers (sequence-driven approach), such as 16S rRNA gene or functional enzyme genes (1, 2, 6). Once some interest clones with the expression of bioactive traits are screened or phylogenetic markers are not detected in clones, end sequencing is available for identifying the gene inserted in plasmid. However, the obstacles of: 1) cost of sequencing, 2) low plasmid copy and low DNA yields, 3) labor intensive and difficult to extract high purified plasmid DNA, and 4) formation of secondary DNA structure are often restricting the application of end sequencing. To overcome these problems, we proposed a modified end sequencing method for the identification of the gene fragment that inserted in fosmid.

2. Material and Methods

2.1. Fosmid library construction

A water sample was collected from a depth of 1 m in a shrimp aquafarm located in Zhongshan city. Environmental genomic DNA was extracted by a method previously described by Luo et al. (11). The total DNA was sheared by 200 µl pipette tip, and the DNA fragments approximately 40 kb were isolated and purified by LMP agarose gel electrophoresis. A fosmid library was constructed according to the CopyControl™ HTP fosmid library production kit (Epicenter Technologies) recommended procedure. Colonies growing on LB media that contained chloramphenicol were picked out and sorted into 96 well microtitre plates, and stored at -80 °C.

2.2. Fosmid DNA extraction

Fosmid clones were randomly picked, and inoculated into LB culture medium with chloramphenicol as selection stress, and cultivated overnight at 37 °C. The plasmids were extracted by using alkaline lysis minipreps, and purified by PEG8000 and LiCl precipitation method (12).

2.3. Protocol of the modified end sequencing method

As shown in Figure 1, the protocol consists of three steps. In step one, fosmid circular DNA were fragmented by 200 µl pipette tip after 100 passages; in step two, fragmented DNA were used as PCR templates for the temperature gradient PCR by using pCC2™ Forward Primer (5' -GTACAACGACACCTAGAC -3') coupling with random primer (5'-TGGCGCAGTG-3') as primer pair, or pCC2™ Reverse Primer (5' -CAGGAAACAGCCTAGGAA -3') coupling with random primer as primer pair. The PCR reaction mixture (20 µl) contained 1 µl template DNA, 10 µl PCR premix (Takara, Dalian, China), 0.5 µl each primer (20 µM) and 8.5 µl ddH_2O. The thermal cycle involved an initial denaturation at 94 ℃ for 5 min, followed by 30 cycles of 94 ℃ (45 sec), annealing (45 sec) and 72 ℃ (1 min); the annealing temperature range from 36 ℃ to 62 ℃. Amplification was completed by a final extension step at 72 ℃ for 10 min. All of the PCR products were examined in 1 % (w/v) agarose electrophoresis in 1×Tris-acetate EDTA (TAE) buffer and stained with ethidium bromide (0.5 µg ml^{-1}). In step three, DNA bands on agarose were isolated and purified by Agarose Gel

DNA Purification Kit Ver.2.0 (TaKaRa, Dalian, China). The purified DNA bands were sequenced by pEpiFOS™ Forward Primer or pEpiFOS™ Reverse Primer as sequencing primer by Beijing Genomics Institute (BGI, Shenzhen, China).

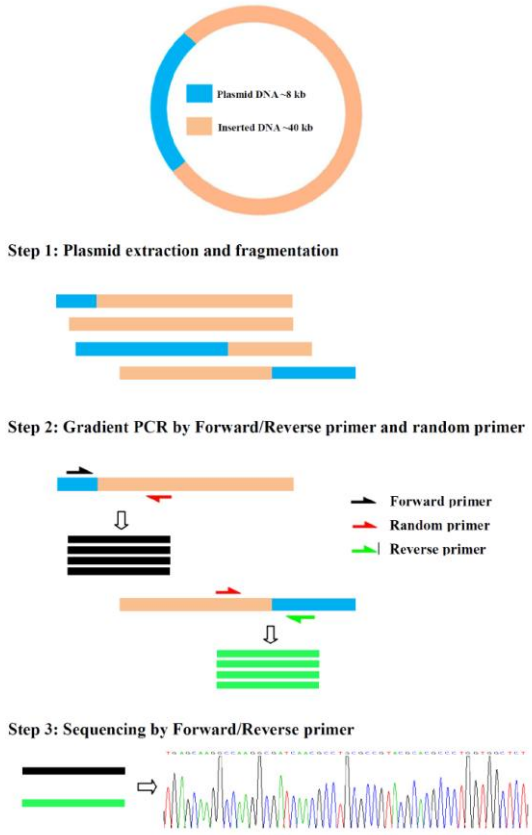

Figure 1. Protocol of the modified end sequencing method

The 16S rDNA inserted in fosmid was detected and amplified with primer pair 341F/1492R (341F: 5'-CCT ACG GGA GGC AGC AG-3'; 1492R: 5'-TAC CTT GTT ACG ACT T-3'), and sequenced by primer 341F.

2.4. Nucleotide sequence analysis

Nucleotide sequences were analyzed by using NCBI blast database. The sequences reported in this paper have been deposited in the GenBank database with the accession numbers: KC573032 to KC573035 for pair-end inserted gene; KC573036 for 16S rRNA gene.

3. Results and discussion

PCR product with three DNA bands was obtained by using primer pair pCC2™ Forward Primer and random primer (Figure 2A). DNA band F/1-1(~1700 bp) and F/1-2 (~1300 bp) were isolated and sequenced by using pCC2™ Forward Primer. The Blast analysis suggested F/1-1 (GenBank: KC573032) and F/1-2 (GenBank: KC573033) DNA sequences were closely related to *Acinetobacter calcoaceticus* PHEA-2 (CP002177.1) with a similarity of 97 % (Figure 2C). PCR product with four DNA bands was obtained by using primer pair pCC2™ Reverse Primer and random primer (Figure 2B). DNA band R/1-2 (~1400 bp) was isolated and sequenced by using pCC2™ Reverse Primer. The Blast analysis suggested R/1-2 (GenBank: KC573034) DNA sequence was also closely related to *Acinetobacter calcoaceticus* PHEA-2 with a similarity of 93 % (Figure 2C). In addition, we detected a 16S rDNA (GenBank: KC573036) sequence from the inserted DNA sequence, and identified it was also closely related to *Acinetobacter calcoaceticus* PHEA-2 with a similarity of 100 % (Figure 2C). All of those results indicated the mortified end sequencing method was available, and the inserted DNA sequence was identified to be from a strain belonging to the genus *Acinetobacter*. Moreover, the other three inserted DNA sequences from randomly picked clones were identified to be closely related to *Shewanella pealeana* ATCC 700345 genome (CP000851.1) with a similarity of 97 %, or uncultured bacterium sequence (JX649870.1) from an activated wastewater fosmid library.

Interestingly, after aligning the inserted DNA sequences with the *Acinetobacter calcoaceticus* PHEA-2 complete genome sequence, we found the position of F/1 and R/1 sequences were at 2320424-2321095 bp and at 2007020-2007461 bp of *Acinetobacter calcoaceticus* PHEA-2 genome, respectively (Figure 2C). The DNA length between F/1 and R/1 was about 310 kb on *Acinetobacter calcoaceticus* PHEA-2 genome, which is significant different with our result that the inserted DNA in fosmid was only about 40 kb. In addition, the position of detected 16S rDNA was at 3321762-3322434 bp of *Acinetobacter calcoaceticus* PHEA-2 genome (Figure 2C), which is more far from the F/1-R/1 sequence range. Same as these results, the position of F/1, R/1 and 16S rDNA in the other two most closely related genomes were also different, but the F/1-R/1 length were all about 300 kb (Table 1). Comparing to the *Acinetobacter calcoaceticus* PHEA-2 genome, we found only one F/1 orR/1 similar sequence were presented (data not shown), which suggested there were no gene duplication in the related strain. The difference of sequence range might be the results of gene horizontal transfer or genomic recombination. As above, though we found the inserted DNA sequence was most similar with the sequence from *Acinetobacter* genome, especially the 16S rDNA (100 % similarity), the bacteria that the inserted DNA belongs might be a new *Acinetobacter* strain.

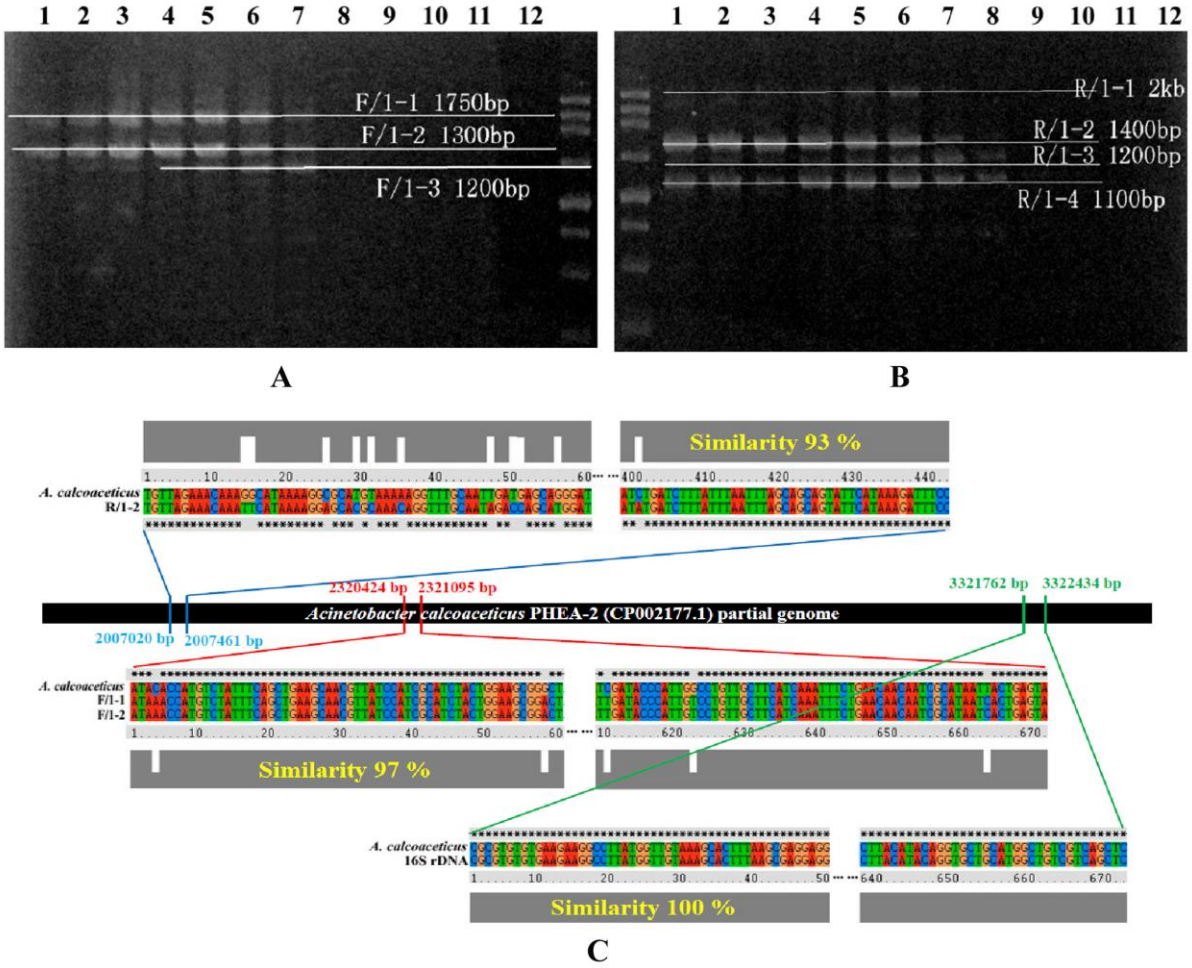

Figure 2. Gradient PCR product by using primer pair of pCC2™ Forward Primer and random primer (A), and primer pair of pCC2™ Reverse Primer and random primer (B), and alignments of inserted DNA sequences in fosmid plasmid with *Acinetobacter calcoaceticus* PHEA-2 (CP002177.1) partial genomic DNA. 1-12: temperature gradient range from 36 ℃ to 62 ℃.

In conclusion, the modified end sequencing method for identifying the gene fragment that inserted in fosmid was proved to be a available and simplified method. Comparing with the standard end sequencing, this identifying protocol no needs to extract an amount of high purified plasmid DNA, and no needs cost much of time and sequencing price. It will be a useful method to identify the gene inserted in interest plasmids with the expression of bioactive traits or phylogenetic markers are not detected.

Table 1 Position of F/1, R/1 and 16S rDNA in the closely related strain genomes

	A. calcoaceticus PHEA-2 (CP002177.1)	*A. baumannii* AB307-0294 (CP001172.1)	*A. baumannii* AB0057 (CP001182.1)
F/1	2,320,424-2,321,095 bp	837,076-837,747 bp	3,221,475-3,222,146 bp
R/1	2,007,020-2,007,461 bp	1,159,800-1,160,246 bp	2,884,661-2,885,107 bp
F/1-R/1	313,404 bp	323,170 bp	337,485 bp
16S rDNA	3,321,762-3,322,434 bp	18,734-19,405 bp	56,415-57,086 bp

Acknowledgements:

Authors acknowledge the supports by the National Natural Science Foundation of China (No. 21076090; No. 21276099) and the Specialized Research Found for the Doctoral Program of Higher Education of China (No. 20120172120045).

*Corresponding Author:
Prof. Weitie Lin
College of Bioscience and Bioengineering,

South China University of Technology,
Guangzhou 510006,
China.
E-mail: wtlin@21cn.com

References

[1] Allen HK, Moe LA, Rodbumrer J, Gaarder A, Handelsman J. Functional metagenomics reveals diverse β-lactamases in a remote Alaskan soil. ISME J 2009, 3: 243-251.

[2] Chen ZW, Liu YY, Wu JF, She Q, Jiang CY, Liu SJ. Novel bacterial sulfur oxygenase reductases from bioreactors treating gold-bearing concentrates. Appl Microbiol Biotechnol 2007, 74: 688-698.

[3] Daniel R. The soil metagenomic-a rich resource for the discovery of novel natural products. Curr Opin Biotechnol 2004, 15: 199-204.

[4] Debroas D, Humbert JF, Enault F, Bronner G, Faubladier M, Cornillot E. Metagenomic approach studying the taxonomic and functional diversity of the bacterial community in a mesotrophic lake (Lac du Bourget-France). Environ Microbiol 2009, 11: 2412-2424.

[5] DeLong EF. Microbial community genomics in the ocean. Nat Rev Microbiol 2006, 3: 459-469.

[6] Handelsman J. Metagenomics: application of genomics to uncultured microorganisms. Appl Environ Microbiol 2004, 68: 669-685.

[7] Handelsman J, Rondon MR, Brady SF, Clardy J, Goodman RM. Molecular biological access to the chemistry of unknown soil microbes: A new frontier for natural products. Chem Biol 1998, 5: R245-R249.

[8] Parsley LC, Consuegra EJ, Kakirde KS, Land AM, Harper WF, Liles MR. Identification of diverse antimicrobial resistance determinants carried on bacterial, plasmid, or viral metagenomic from an activated sludge microbial assemblage. Appl Environ Microbiol 2010, 79: 3753-3757.

[9] Riaz K, Elmerich C, Moreira D, Raffoux A, Dessaux Y, Faure D. A metagenomic analysis of soil bacteria extends the diversity of quorum-quenching lactonases. Environ Microbiol 2008, 10: 560-570.

[10] Suenage H, Ohnuki T, Miyazaki K. Functional screening of a metegenomic library for genes involved in microbial degradation of aromatic compounds. Environ Microbiol 2007, 9: 2289-2297.

[11] Luo JF, Lin WT, Guo Y. Method to detect only viable cells in microbial ecology. Appl Microbiol Biot 2010, 86: 377-384.

[12] Sambrook J, Russell DW. Molecular cloning - a laboratory manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 2001.

7/25/2013