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RESEARCH ARTICLE

Construction of a Fosmid genomic library of *Streptomyces roseoflavus* Men-myco-93-63

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Abstract **To clone the antibiotic biosynthesis gene cluster of *Streptomyces roseoflavus* Men-myco-93-63, we constructed a Fosmid genomic library. The genomic DNA of the strain Men-myco-93-63 was isolated by the modified CTAB procedure, and the size of most genomic DNA fragments was larger than 150 kb. Then, a Fosmid genomic library containing more than 6000 clones was constructed. The average size of the inserted DNA in recombinant plasmids was 38.1 kb, and the probability of harboring any gene in the genome of the strain Men-myco-93-63 was 99.99%. The library coverage was at least a 10-fold genome equivalent. Therefore, the constructed Fosmid library meets the requirements as a standard genomic library**

Keywords *Streptomyces roseoflavus*, Fosmid, genomic library

Introduction

S. roseoflavus Men-myco-93-63 was isolated from the soil in which potato scab naturally declined and displayed an antagonistic activity against a wide variety of plant pathogenic fungi and bacteria (Liu, 1992; Sun, 2009). The fermentation liquid of the Men-myco-93-63 contains a large number of liposoluble antibiotics and has a strong antibiosis activity against cotton verticillium wilt and cucurbits powdery mildew in greenhouse and field (Meng et al., 2004). To clone the related antibiotic biosynthesis genes from this strain, a plasmid genomic library was constructed in which the average size of the inserted DNA was 1.7 kb (Di, 2006). Then, a cosmid genomic library was constructed, but the average size of the inserted DNA was approximate 20 kb (Liu, 2006). The inserted DNA fragments in both libraries were too small to screen the clone harboring the related antibiotic biosynthesis genes.

In recent years, Fosmid library has been widely used in gene map-based cloning, physical map construction, and comparative genomic studies. The average size of the inserted DNA was approximately 35 kb. To clone the antibiotic biosynthesis gene cluster, we constructed the Fosmid genomic library, and the results will be discussed in the following sections.

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Materials and methods

Materials

S. roseoflavus Men-myco-93-63 was cultured in TSB liquid media with a shaker at 200 rpm and at 28°C for 48 h. The CopyControl™ Fosmid Library Production Kit was purchased from Epicenter Biotechnologies Corp.

Isolation of genomic DNA

Adapted from the methods (Murrary et al., 1980; Tobias et al., 2000), isolation of genomic DNA was conducted by freezing mycelium with liquid media at −20°C overnight, followed by thawing and centrifuging 15-mL culture to harvest mycelium, which was washed with 15 mL TE25S, centrifuged at

10000×*g* at 4°C for 2 min, resuspended in 5 mL TE25S, and incubated at 37°C for 45min after adding 50 μL 100 mg/mL lysozyme solution and 10 μL 20 mg/mL RNaseA. Afterward, the supernatant solution was mixed with 140 μL 20 mg/mL proteinase K solution and 600 μL 10% SDS gently by inversion and incubated for 2 h at 55°C, which was then remixed with 1 mL 5 mol/L NaCl and 0.65 mL CTAB/NaCl gently by inversion, respectively, and incubated for10 min at 55°C.

The following step made was that when cooled to 37°C, it was gently remixed with 5 mL phenol/isoamyl alcohol (25∶1) and 5 mL chloroform by inversion for 15 min and 10 min, respectively, and centrifuged at 13500 × *g* at 4°C for 15 min. Finally, the supernatant was transferred to a fresh tube with 2.2 vol water-free ethanol. After cooling for 10 min, DNA was spooled onto a new tube, and the DNA was rinsed in 5 mL 70% ethanol, airdried, and dissolved in 0.3 mL TE buffer.

The size of genomic DNA fragments was examined by pulse field gel electrophoresis (PFGE) (Smith et al., 1987) with voltage and ramp times recommended by the manufacturer for separation of 50–500 kb DNA. The quality of genomic DNA was examined by UV spectrophotometer.

Shearing and end-repairing the insert DNA

The DNA was sheared into approximately 40 kb fragments by pipetting up and down 40, 50, 60, and 70 times with a 200 μL small-bore pipette tip, and 2 μL sample was migrated on a 20-cm long 1% standard agarose gel at 35 V for 18 h using the 42 kb Fosmid Control DNA as a marker and λ-Mix markers, and thereafter, it was visualized and stained with ethidium bromide after the migration was completed.

According to the manual operation of CopyControl™ Fosmid Library Production Kit, the end-repair reaction was scaled as dictated by the amount of DNA available.

Recovery of the size-fractionated DNA

Sufficient end-repaired DNA was run by a 20-cm long 1% LMP agarose gel electrophoresis at 30 V for 18 h. The 42-kb Fosmid Control DNA was loaded into each of the outside lanes of the gel, followed by cutting off and staining the gel of the lanes containing the DNA size markers on both sides of the sample. On the basis of the markers, three gel slices below the position of the 42-kb Fosmid Control were excised successively.

The three size-fractionated DNAs with the Kit were separately recovered, with the size of DNA fragments examined by electrophoresis and the DNA concentration determined by spot tests on EB-stained agarose plates using dilutions of known amounts of λ-Mix markers as standard.

Ligation reaction and packaging the CopyControl Fosmid clones

We ligated 0.25 μg of about 40-kb insert DNA and about 0.009 pmol insert DNA to 1 μL CopyControl pCC1FOS according to the manual operation of the Kit.

We added 10 μL of the ligation reaction from the previous step to each 25 μL of the thawed MaxPlax Lambda Packaging extracts. At the end of the second incubation, Phage Dilution Buffer (PDB) was added to 0.5 mL final volume in each tube with 25 μL of chloroform added, mixed gently, and stored at 4°C.

Titering the packaged CopyControl Fosmid clones

Serial dilutions (10^{-1}, 10^{-2} and 10^{-3}) of 1 mL packaged phage particles from the prerious step into Phage Dilution Buffer were conducted, and 10 μL of each dilution was individually added to 100 μL prepared EPI300-T1^{R} host cells, with each tube incubated for 1 h at 37°C. The infected EPI300-T1^{R} cells were spread on an LB plate + 12.5 μg/mL chloramphenicol and incubated at 37°C overnight to select for the CopyControl Fosmid clones, with colonies counted and the titer of the packaged phage particles calculated.

Storage of the Fosmid library

Based on the titer of the packaged CopyControl Fosmid clones and the estimated number of clones required, the volume of the packaged Fosmid clones was calculated, which will be needed to prepare the CopyControl Fosmid library. The CopyControl Fosmid library was plated and selected by following the manual. For a longer term storage, we recommend the storage of the packaged DNA as a primary library or storage of the library in the EPI300-T1^{R} Phage T1-resistant *E. coli* plating strain.

Detecting the size of the insert DNA

The size of the insert DNA was detected by randomly picking up 19 clones into LB medium + 12.5 μg/mL chloramphenicol with the CopyControl Fosmid Autoinduction Solution, which was incubated overnight (18 h) at 37°C with shaking at 220 r/min, and then, the extracted plasmid was digested with *Sac*I.

Results and analysis

Genomic DNA

The size of most genomic DNA fragments ranged from 100 to 170 kb (Fig. 1). As (G + C)% of *Streptomyces* genomic DNA (*X*) was higher than the λ-Marker (*Y*), the size of the sample was corrected by the equation $Y = 0.96X - 18$ (Gravius et al., 1994; Sun et al., 2008). After correction, the size of most genomic DNA fragments was larger than 150 kb. The quality of genomic DNA was checked by UV spectrophotometer, and the DNA concentration was 141.2 μg / mL. $1.8 < OD_{260/280} =$

1.95 < 2.0 indicated no protein, RNA, or phenol contamination, and $OD_{260/230} = 2.23 > 2$ showed no salt contamination. Both the size of genomic DNA and the quality were fit for the construction of Fosmid genomic library.

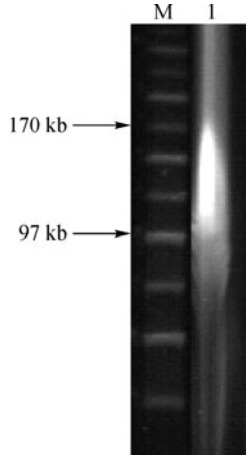

Figure 1 Detection of genomic DNA. M is MidRangeII PFG markers, and 1 is genomic DNA.

Sheared DNA

The DNA from the pipette tip was aspirated and expelled 40, 50, 60, and 70 times, respectively. Lanes 2 showed most of the genomic DNA migrated with the 42-kb Fosmid Control DNA when the genomic DNA was pipetted up and down 50 times, and the size of most sheared DNA fragments ranged from 36 to 48 kb (Fig. 2). The other lanes migrated slower (higher MW) than the 42-kb Fosmid Control DNA.

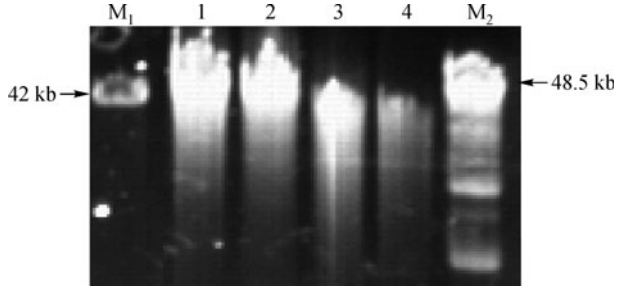

Figure 2 Sheared DNA. M_1 is 42-kb Fosmid Control DNA; M2 is λ-Mix Markers. 1–4 are genomic DNA aspirated and expelled 40, 50, 60, and 70 times, successively.

Size-fractionated DNA

On the basis of the markers, three gel slices were excised successively (Vertical lines, aclinic lines, and diagonal lines in Fig. 3A). The size of the above (vertical lines) aliquot DNA fragments was approximately 40 kb (Fig. 3B), which was fit for construction of a Fosmid genomic library at approximately 250 μg/mL (Fig. 3C).

Fosmid library

The number of Fosmid clones required to reasonably ensure any given DNA sequence in the library was determined by the following formula (Sambrook et al., 1989):

$$N = \ln(1-P)/\ln(1-f),$$

where P is the desired probability (expressed as a fraction); f is the proportion of the genome contained in a single clone; and N is the required number of Fosmid clones. To ensure 99.99% probability of a given DNA sequence of *Streptomyces* spp. (genome = 8.0 Mb) within a Fosmid library composed of 38.1 kb inserts, the following formula was used: $N = \ln(1 - 0.9999)/\ln[1 - (3.81 \times 10^4 \text{bases}/8.0 \times 10^6 \text{ bases})] = 1929$ clones.

Titering the packaged CopyControl Fosmid clones indicated that the Fosmid genomic library contained more than 6000 clones, of which about 2000 clones were stored for the amplified library and others for primary Fosmid library. Randomly detecting the size of the insert DNA of 19 clones showed that the average size of the inserted DNA in recombinant plasmids was 38.1 kb (Fig. 4). The probability of harboring any gene in the genome of the strain Men-myco-93-63 was 99.99%, and the library coverage was at least 10-fold genome equivalent.

Discussion

High-molecular-weight genomic DNA

Preparing high-molecular-weight genomic DNA from *S. roseoflavus* Men-myco-93-63 is necessary for the construction of a Fosmid genomic library. Although *Streptomyces* DNA can be isolated by many methods, and most methods provide DNA fragments of about 40 kb, preparing DNA fragments of more than 150 kb for Fosmid cloning can be quite a challenge (Hopwood et al., 1985; Pospiech et al., 1995). The concentration of the mycelium, optimal timing of the lysis step, and the care and skill exerted in the solvent extraction, phase separation, and precipitation steps may be more important than the choice of method. To isolate the desired genomic DNA, DNase action and mechanical shearing must be minimized. We took some effective measures for the CTAB procedure including freezing and thawing mycelium for centrifugation and lysis and washing of mycelium with TE25S to remove extracellular contamination (MacNeil et al., 1986). It was more convenient to add RNase together with lysozyme rather than treat DNA sample solely, with the lysozyme treatment for 45 min and phenol/chloroform/isoamyl mixed to denature and precipitate protein. At last, the genomic DNA at both the size and the quality fit for construction of Fosmid genomic library was isolated.

It has been suggested that G + C content has an influence on the migration of DNA molecules in PFGE (Cantor et al.,

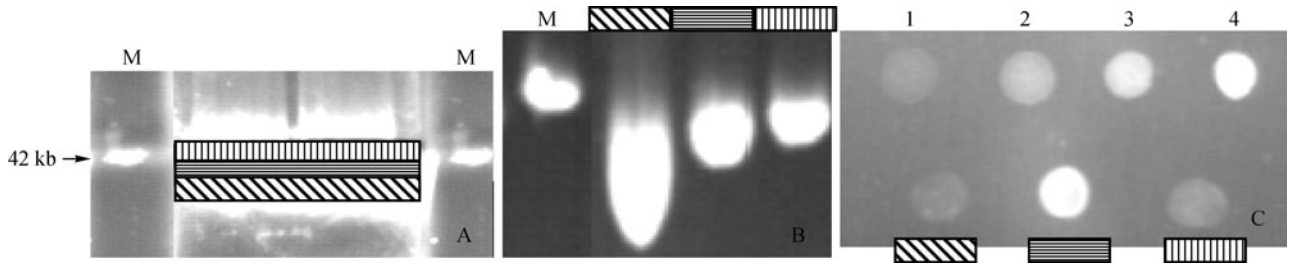

Figure 3 Size-fractionated DNA. Note: M is 42-kb Fosmid Control DNA. 1–4 are the concentration of λ-Mix markers at 200, 300, 400, and 500 μg/mL.

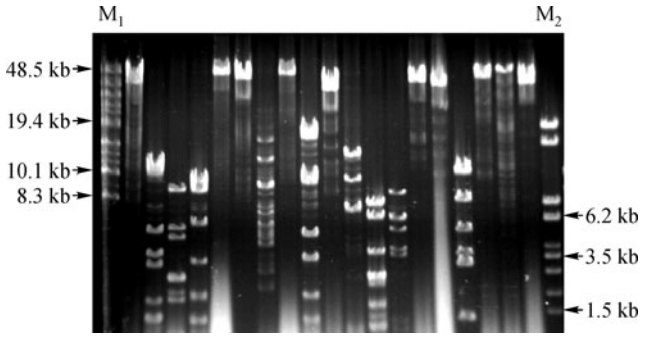

Figure 4 Detecting the size of the insert DNA. M_1 is λ-Mix markers. M_2 is λ-*Eco*T14 markers. Other lanes are 19 clones digested with *Sac*I.

1988; Maniloff, 1989), with 70%–74% (G + C) content of *Streptomyces* spp., which was probably the highest among prokaryotes (Hopwood et al., 1990). Gravius et al. (1994) suggested that the best fit linear regression line is $Y = 0.96X - 18$, where X is the size of *Streptomyces* spp. DNA, and Y is the size of Marker (Y). Thus, the size of the G + C-rich fragments is all underestimated; however, the relative error falls from over 30% (for a 60 kb fragment) to 7.5% (for a 500 kb fragment).

Unbiased primary Fosmid library

The Fosmid library utilized a novel strategy of cloning randomly sheared and end-repaired DNA. Shearing the DNA led to the generation of highly random DNA fragments in contrast to more biased libraries resulting from fragmenting the DNA by partial restriction digests (Hradecna et al., 1998; Wild et al., 2001; Wild et al., 2002).

Features of the Fosmid vector

Fosmid vector is now popular alternative cosmid vector for a large fragment library of the new vector. Compared with BAC library construction, the Fosmid library construction was easier and quicker. The other advantages are given as follows: (1) *E. coli* F factor-based partitioning and single-copy origin of replication make Fosmid vector stable in the host bacteria; (2) CopyControl Fosmid and PCR clones can be induced from single copy to about 50 copies per cell to improve DNA yields for sequencing, fingerprinting, subcloning, *in vitro* transcription, and other applications; and (3) good randomness guarantees every inserted DNA fragment in the library with equal frequency.

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