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研究报告

Increasing transformation efficiency by topoisomerase II and its application for cloning of a γ-hexachlorocyclohexane dehydrochlorinase gene *linA-like* from a bacterial community

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Abstract: A simple modification was developed for construction of genomic/metagenomic libraries by T-vectors. Topoisomerase II was employed to introduce superhelical turns into relaxed circular T-A recombinant molecules, which greatly increased the transformation efficiency. By using this technique, a metagenomic library was constructed and a γ-hexachlorocyclohexane dehydrochlorinase gene *linA-like* was screened from it.
Keywords: Topoisomerase II, T-A recombinant molecules, *linA-like*

利用拓扑异构酶 II 提高转化效率及其在从细菌群落中克隆六六六 γ 异构体脱氯化氢酶基因中的应用

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摘 要: 一个简单的技术上的调整被应用于利用 T 载体构建的基因组或宏基因组文库策略中。该技术利用拓扑异构酶 II 将超螺旋引入松散的 T-A 环状重组分子, 从而提高转化效率。利用该技术构建一个宏基因组文库, 并从中克隆到一个六六六 γ 异构体脱氯化氢酶基因(*linA-like*)。

关键词: 拓扑异构酶 II, T-A 重组分子, 脱氯化氢酶基因

In functional genomics/metagenomics studies, cloning of desired genes is dependent upon the quality of libraries. The most important limiting factor in this case is the transformation efficiency of recombinant plasmids. Several modified strategies can increase the transformation efficiency, for instance, preparation of ultra competent cells instead of general competent cells, using electroporation instead of chemical trans-

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formation, or using T-vectors instead of dephosphorylated plasmids to construct libraries[1−2]. However, all these strategies ignore change of the configuration of recombinant plasmids. In these methods, recombinant plasmids are relaxed and have much lower transformation efficiency than superhelical plasmids. If superhelical turns can be introduced into recombinant plasmids, the transformation efficiency will be further increased greatly[2]. However in general construction strategies, dephosphorylated plasmids are employed to ligation with DNA fragments to form nicked recombinant molecules, which can relax superhelical DNA[2]. Fortunately in another library construction strategy by T-vectors, the recombinant plasmids have no nicks[1]. So in this study, we further developed this strategy by introducing superhelical turns into the relaxed circular recombinant plasmids by topoisomerase II[3]. As proof of principle, we constructed several microorganism genomic libraries by using T-vectors with or without topoisomerase II treatment and compared the transformation efficiency of both strategies. The modification method increased transformation efficiency obviously. Here, we also showed using this modification method successfully for cloning γ-hexachlorocyclohexane (γ-HCH) dehydrochlorinase gene *linA-like* from a metagenomic library.

Gram-negative bacterium *Pseudomonas putida* KT2440 and gram-positive bacterium *Bacillus halodurans* C-125 were grown at 30 °C in Luria Broth (LB) medium[4−5]. Cyanobacterium *Microcystis aeruginosa* NIES-843 was grown at 23 °C in BG11 medium under continuous light provided by Osram Universal White fluorescent tubes [30 μE/(m^2·s)][6]. Yeast *Saccharomyces cerevisiae* was grown at 28 °C in yeast peptone dextrose (YPD) medium[7]. Genomic DNA from *P. putida* KT2440, *B. halodurans* C-125 and *M. aeruginosa* NIES-843 strains were isolated by the method of high salt concentration precipitation[8]. Genomic DNA from *S. cerevisiae* was isolated by the Yeast DNA Extraction Kit [TaKaRa Biotechnology (Dalian) Co., Ltd.]. 2 μg of each genomic DNA was digested with *Sau*3A I at 37 °C and fragments (> 4 kb and <20 kb) were recovered by using TaKaRa Gel Extraction Kit. The DNA fragments were treated with *Tag* DNA ploymearse for filling 3 ends and creating the adenine (A) overhangs at the 3 ends. This reaction was performed as described previously by Kawata et al[1]. After phenol extraction and ethanol precipitation, 50 ng of DNA fragments were subjected to ligation with 50 ng of pMD18-T (Ampicillin^r) T-vectors [TaKaRa Biotechnology (Dalian) Co., Ltd] at 16 °C for 8 h. Next was the supercoiling step: After phenol extraction and ethanol precipitation, the ligation products was incubated with topoisomerase II (New England Biolabs Co., Beijing, China) for introducing superhelical turns into the relaxed recombinant plasmids DNA in a standard buffer (35 mmol/L Tris-HCl pH 7.5, 24 mmol/L KCl, 4 mmol/L $MgCl_2$, 2 mmol/L DTT, 1.75 mmol/L ATP, 5 mmol/L Spermidine, 0.1 mg/L BSA and 6.5% Glycerin) at 37 °C. After an hour, we electrophoresed the DNA and stained it with ethidium bromide (EB). The results showed in Fig. 1. In the absence of topoisomerase II (lane 2), low-mobility relaxed circular form of recombinant plasmids were observed. On the other hand, as adding

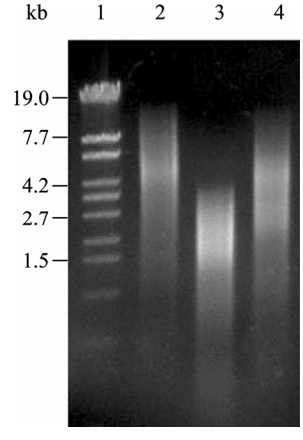

Fig. 1 Electrophoresis assay for recombinant products

图1 重组产物的电泳分析

Note: 1: λ-*Eco*T14 I digest; 2: T-A recombinant products; 3: T-A recombinant products incubated with topoisomerase II; 4: Supercoiled T-A recombinant products incubated with topoisomerase I.

topoisomerase II (lane 3), high-mobility form of recombinant plasmids with many superhelical turns were detected. The superhelical products were then subjected to electroporation with *Escherichia coli* DH5α competent cells [TaKaRa Biotechnology (Dalian) Co., Ltd] with the Gene Pulserr II (Bio-Rad). As a control, an equal amount of T-A ligation products without supercoiling was also transformed into *E. coli* DH5α competent cells by electroporation.

If supercoiling could cause increasing of the number of recombinant clones, relaxing of superhelical turns will cause transformation efficiency drops. In this study, we also investigated this. An equal amount of superhelical products were incubated with topoisomerase I (New England Biolabs Co., Beijing, China) for relaxing superhelical turns. This reaction was performed in a NEB buffer IV with BSA (20 mmol/L Tris-Ac pH 7.9, 50 mmol/L KAc, 10 mmol/L $Mg(Ac)_2$, 1 mmol/L DTT, 100 mg/L BSA) at 37 °C. After 30 minutes, we also electrophoresed the DNA and stained it with EB. The results showed that high-mobility form of recombinant plasmids disappeared and low-mobility form of recombinant plasmids were recovered (lane 4, Fig. 1), which indicated superhelical turns of circular DNA were relaxed successfully. These resulting products were also transformed into *E. coli* DH5α competent cells by electroporation. All transformants was grown on LB medium containing 50 mg/L of ampicillin and 40 mg/L of X-gal (Shanghai Sangon Biological Engineering Technology & Service Co., Ltd) at 37 °C. Recombinants were identified by blue/white selection.

The number of colonies and efficiency of each library were showed in Table 1. In each library, the probability of blue colonies was less than 0.01%. Plasmids DNA of 50 random selected white colonies from each library were isolated by the alkaline lysis method, and the size of inserted DNA fragments was more than 4 kb (Data not shown). Comparing with using T-A ligation products for transformation, much more recombinant colonies were obtained by using superhelical products. For instance, using latter, the genomic DNA library of *B. halodurans* C-125 contained 7.3×10^4 colonies. The efficiency of it was 1.46×10^6 colonies/μg vector DNA, which was nearly 10 times higher than control. However, after superhelical turns were relaxed by topoisomerase I, the number of colonies and efficiency dropped to the same with control (Table 1). The similar results also occurred in the other three microorganism genomic libraries. It demonstrated supercoiling indeed increased the transformation efficiency and improved the quality of libraries. Considering only using 50 ng of genomic DNA fragments in our study, this modified method was especially applied in construction libraries from small quantities of initial DNA.

Table 1 The number of colonies and efficiency of each library
表 1 各文库库容及转化效率

		Pseudomonas putida KT2440	*Bacillus halodurans* C-125	*Microcystis aeruginosa* NIES-843	*Saccharomyces cerevisiae*
T-A ligation products (Control)	Number of colonies ($\times10^4$)	1.00	0.76	0.85	0.95
	Efficiency (colonies/μg vector DNA, $\times10^4$)	20.0	15.2	17.0	19.0
Recombinant plasmids (Supercoiling)	Number of colonies ($\times10^4$)	8.0	7.3	9.4	7.6
	Efficiency (colonies/μg vector DNA, $\times10^4$)	160	146	188	152
Recombinant plasmids (Superhelical turns were relaxed)	Number of colonies ($\times10^4$)	0.87	0.81	0.90	0.97
	Efficiency (colonies/μg vector DNA, $\times10^4$)	17.4	16.2	18.0	19.4

In previous study, we have obtained a bacterial community with γ-hexachlorocyclohexane (γ-HCH) degrading activity from a γ-HCH contaminated area but failed to isolate the γ-HCH degrading bacterium. Based on sequence homologies with previously conserved γ-HCH dehydrochlorinase gene *linA* sequence from *Sphingobium japonicum* UT26 and *linA1* and *linA2* sequences from *Sphingobium indicum* B90A[9−10], we designed the primers and tried to amplify *linA* gene by polymerase chain reaction (PCR). But only partial fragment of *linA* gene was cloned and complete *linA* gene was not amplified. It was deduced that 3 end or 5 end of *linA* gene from this community was not homology with previously published *linA* sequence. Then we attempted to construct the metagenomic library by T-vector to clone the *linA-like* gene. However, as quantities of extracted metagenomic DNA was very limited, we tried several times but only obtained a small number of recombinant colonies and failed to detect a positive clone with γ-HCH dehydrochlorinase activity. In this study, we reconstructed the metagenomic library by using the modified method. Only 100 ng of metagenomic DNA was extracted from the bacterial community as described by Porteous et al[11]. After partially digested with *Sau*3A I, the DNA fragments was modified of 3 ends and ligated with 50 ng of T-vectors. After supercoiling, the recombinant products were transformed into *E. coli* DH5α competent cells by electroporation and nearly 3×10^4 recombinant colonies were obtained. γ-HCH dehydrochlorinase activity was visually assayed by spraying γ-HCH solution (50 g/L in ethanol) on the transformants grown on the LB plates[9].

One candidate clone contained a 1.5 kb DNA fragment showed strong dehydrochlorinase activity which could make clear zone on the plate. Sequence analysis revealed that a gene with a size of 459 bp encoding 153 amino acids sequence was present in this DNA fragment. This gene was designated *linA-like* gene, which had a sequence similarity of 93% to the dehydrochlorinase gene *linA* from UT26 and of 94% to another dehydrochlorinase gene *linA1* from B90A. The DNA upstream sequence of *linA-like* gene shared 100% identical to *linA* and *linA1* gene region, while the downstream sequence differed from the two genes region, which caused amplification of *linA-like* gene failed previously. The few amino acid differences at the C-terminal end of LinA-like compared to those of LinA and LinA1 did not result in any obvious difference in dehydrochlorinase activity (Data not shown). The nucleotide sequence reported in this paper has registered with the GenBank nucleotide sequence databases under accession number HQ325842. As the probability of a positive clone occurred in this library was extremely low, we deduced *linA-like* gene was a rare gene and maybe the quantity of host bacteria was very low in this community.

In summary, the modified method presented here provides a more effective means of construction of genomic/metagenomic libraries. By using it, the probability of screening rare gene was greatly increased. It can be also utilized in increasing transformation efficiency of large plasmids as bacterial artificial chromosome (BAC vector) or other circular ligation products without nicks.

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