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Construction of a Genomic Library and Cloning of Glucoamylase Gene from *Aspergillus niger*

Luo Jinxian Zhang Tianyuan Li Wenqing Tan Yuhui Wang Yiliang

(Department of Biology and Biotechnology Research Center)

Abstract A complete genomic library of *Aspergillus niger* was successfully constructed using lambda EMBL3 DNA as vector. The donor *A. niger* DNA was partially cleaved with EcoRI, 15-20kb fragments were recovered from sucrose gradient and ligated to the vector lambda EMBL3 DNA with T4-DNA ligase at a proper ratio and the ligated mixture was packaged *in vitro* with the packaging extract prepared from E. coli SMR10 single strain system. The packaged phages were titered using *E.coli* 359 as host and 2.5×10^5 recombinant phages/ug DNA was obtained, which is 10x more than the number of recombinants required for an entire A.niger genome according to Clarke and Carbon. The glucoamylase gene was screened by *in situ* hybridization. The recombinant plaques were transferred to nitrocellulose filter and hybridize to ^{32}p labelled DNA probe, 2 positive plaques were obtained from 10^5 recombinant phages. After rescreening, DNA was extracted from the positive phage for further analysis. The glucoamylase gene was localized on a 2.5kb EcoRI -EcoRV fragment by Southern blotting. The 2.5kb fragment was subcloned in EcoRI site of -EcoRV pBR322.

Keywords Genomic library, Aspergillus niger, glucoamylase gene, cloning, subcloning, E.coli

1 Introduction

Aspergillus niger is an useful industrial strain for its easy growth and its several products with important commercial value like glucoamylase, gluconic acid and citri acid[1~4]. It has also been proven to be valuable in studying eukaryotic gene expression and regulation. Although A.niger has been extensively studied in the fermentation literature, little is known at the molecular level concerning the gene structure and expression. With the development of recombinant DNA technology, people become more and more interested in applying this technique to industrial fungi. Many eukaryotic gene products require post-tr-

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anslational modification before maturation while prokaryotic cells usually lack this mechanism. Fungal expression system has great potential for mammalian gene expession beeause of its similarity with mammals in pos t-translational processing[5]. Many filamentous fungi can secrete extracellular proteins and their genes may provide strong promoters and secretion signals having wide range of hosts, which can be used for constructing secretive expression vector. For instance, Cullen[6] has constructed an expression vector using the 5′ controlling region of *A.niger* glucoamylase gene to express bovine rennin gene in A. nidulans. The construction of genomic ibrary is useful to study the genomic structure by chromosome walking method[7], to analyse the sequence alignment on chromosome and to clone genes from the chromosome. The cloning of glucoamylase gene may improve the industrial strain[8] and would be of great value for the construction of starch-fermentable Saccharomyces cerevisiae by introducing the cloned gene into it[9] and for the construction of fungal expression system, we report here the construction of genomic library and the cloning of glucoamylase gene from *A.niger*.

2 Materials and methods

2.1 Strains, phages and plasmids

A.niger 3758 is from the Food and Fermentation Institute of Guangdong. *E. coli* Q358, *E. coli* Q359 and phage λEMBL3 are provided by Friendship Hospital, Beijing. *E. coli* SMR10 is from Guangdong Institute of Microbiology. Plasmid p4EX is a gift of Dr. Amsden.

2.2 Media

Zeiss medium is used for the culture of *A.niger*. L-broth is for bacterial growth and transformation. M9 medium is for phage infection.

2.3 Enzymes and Buffers

EcoRI, EcoRV, HindIII, DNA polymerase, DNase, RNase, Proteinase K and dNTPs were purchased from Boehringer Mannheim or SABC. Alpha-^{32}P-dCTP was from Fukrui Biotechnology Company, Beijing.

The buffers TE, STE, SM, TM, 50×TAE, 20×SSC, 20×SSPE, 50×Denhardt solution, prehybridization solution were prepared according to the formulas in reference(10).

Cocktail solution: 50%DMSO, 7.5mmol/L ATP pH 7.0.

TSP: 40mmol/L Tris-HCl, 10mmol/L spermidine, 10mmol/L putrecine pH7.8.

TEK: 10mmol/L Tris-HCl, 10mmol/L EDTA, 50mmol/L KCl, pH8.0.

2.4 Isolation and Fractionation of Donor DNA

A.niger 3758 was grown in 100ml Zeiss medium at 30℃ on a shaker for 3 days, filtrated, rinsed and stored at -70℃. 10g of mycelia together with 10g of quartz were ground in liquid nitrogen on a mortar for 2 hours. The powder was dissolved in TES for 30 minutes, and incubated at 65℃ for 15 minutes. The mixture was extracted with phenol and chloroform and precipitaed with isopropanol. After rinsed with 75% ethanol, the pellet was dissolved in TE.

The *A.niger* DNA was partially digested with EcoRI and fractionated dy 10%～40% sucrose gradient centrifugation at 40000rpm for 6 hours, 0.2ml fractions were collected, the 15～20kb fractions were taken as donor DNA.

2.5 Vector DNA Preparation

The vector λEMBL3 DNA was prepared according to Silhavy[11]. The phage DNA was cleaved with EcoRI and annealed to see the integrity of cos sites. *In vitro* packaging was carried on to determine the titer. The DNA was then digested with EcoRI and precipitated with enthanol.

2.6 Preparation of Packaging Extract

The *E. coli* SMR10 single strain system was used[12]. The bacteria were grown in L-broth+KCl at 33℃ until OD550=0.8, the culture was shaken in 65℃ water bath until the temperature increased to 44℃ and incubated for another 15 minutes. The cells were transferred to a 37℃ shaker for 90 minutes and harvested by spinning at 4℃, 4500rpm for 10 minutes. The pellet was suspended in TSP, cocktail solution was added. The extract was stored in liquid nitrogen.

2.7 DNA manipulation and Construction of Genomic Library

The 15～20kb *A.niger* DNA and λEMBL3 DNA were ligated with T4-DNA ligase at 16℃ in 60mmol/L Tris-HCl pH8.0, 10mmol/L $MgSO_4$, 10 mmol/L 2-mercaptoethanol and 0.1mmol/L neutralised ATP.

The *in vitro* packaging was done according to Rosenberg[12]. The frozen packaging extract was melted at 28℃ for 30 seconds and mixed with 10μl DNA sample and incubated at 28℃ for another 90 minutes. 0.5ml SM, 50μg/ml DNase and 5μl chloroform were added. The mixture was vortexed until a clear lysate formed. The pellet was spinned down. 10μl of supernatant was used to infect *E.coli*.

2.8 In situ hyridization and Southern blotting

The packaging mixture was mixed with *E.coli* 359 suspended in 0.1

ml $MgSO_4$ and kept at 37℃ for 20 minutes, 3 ml melted top agar was added, and poured on the M9 medium and incubated at 37℃ overnight.

The 456 bp DNA probe was removed from plasmid p4EX and labelled with α-^{32}p dCTP by nick translation.

The nitrocellulose filter was placed onto the top agar so that it comes in direct contac with recombinant plaques for about 60 seconds. The filtes was then immersed in denaturation solution for one minute, neutralezation solution for 5 minutes and 2×SSPE for 5 minutes, dried at room temperature and baked at 80℃ for 2.5 hours in vacuum oven.

The filter attached with DNA was wetted in 6×SSC for 5 minutes, transferred in 60ml prehybridization solution and placed on a shaker at 42℃ for 4 hours. The denatured probe was added and the hybridization was done at 42℃ overnight. After hybridization, the filter was rinsed with 2×SSC-0.2% SDS for 3～4 times, dried and autoradiographied on X-film. The positive plaques were picked up, suspended in SM and infected *E.coli* Q359 for rescreening.

DNA of positive phage was isolated and cleaved with EcoRI and EcoRV and electrophoresed on agarose gel. The gel was transferred to nitrocellulose filter by Southern blotting. The DNA on the filter was hybridized with ^{32}P-labelled probe.

3 Results and Discussion

3.1 Analysis of donor and vector DNA

A.niger DNA was isolated by grinding with quartz under liquid nitrogen and extracting the lysate with phenol-chloroform. The isolated DNA was analysed at OD260 and OD280 to determine the amount and purity. OD260: OD280 = 1.9 was obtained. The DNA was also run on 0.8% agarose gel electrophoresis (Fig.1). The gel pattern shows a sharp band which is bigger than 50kb. These results indicate that DNA isolated by our protocol omitting the CsCl gradient centrifugation is also applicable for constructing the library.

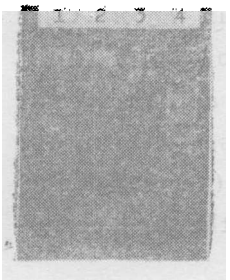

Fig.1 Gel electrophoresis of chromosomal DNA from *A.niger*
(1),(2) chromosomal DNA, (3)λEMBL3 DNA (49kb),
(4)λEMBL3 DNA/EcoRI

The vector λEMBL3 DNA was digested with EcoRI and the digested DNA was annealed at 42℃ for one hour. Fig.2 is the electrophoresis pattern, λEMBL3 DNA was cleaved into 3 bands of about 19kb, 13kb and 9kb with EcoRI, after annealing, the two arms (19kb and 9kb fragments) religated into a new band of about 28kb while the 19kb and 9kb fragments disappear. This indicates the integrity of the cos sites and the infectivity of the phage provided the phage DNA was packaged. 4×10^7 plaques/ugDNA was obtained when the isolated λEMBL3 DNA was in vitro packaged by our packaging extract with a titer of $10^7\sim10^8$ plaques/μg DNA.

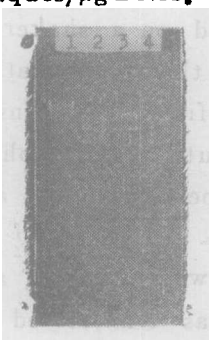

Fig.2 Gel electrophoresis of EMBL3 DNA and its restriction fragments
(1)λEMBL3 DNA (2)λEMBL3 DNA/EcoRI
(3)λEMBL3 DNA/EcoRI after annealing
(4)λcI857S7 DNA/HindIII

3.2 Construction of genomic library of *A.niger*

The annealed two arms of λEMBL3 DNA were ligated with 15-20kb A. niger DNA fragments at the ratios of 2:1 and 3:1 (w:w). The recombinant DNA was packaged and the hosts E.coli Q358 and Q359 were infected with the packaged phages. The result was shown in table 1.

Tab.1 Titration of packaged phages

Hosts *E.coli*	Titration(pfu/μg DNA)		
	Intact λEMBL3	EMBL3:*A.niger* DNA(μg:μg)	
		2:1	3:1
Q358	6×10^7	5.1×10^6	5×10^5
Q359	0	2.5×10^5	3.2×10^4

Tab.1 shows: (1) the intact λ EMBL3 cannot grow in *E.coli* Q359 (P_2 lysogen) because of its gam genotype while the recombunant λEMBL3 losing its gam due to insertion of foreign gene can grow and this eliminates the interference of packaging system on the results; (2) in our experimental conditons, the packaging efficiency of 2:1(vector: donor) ratio is 2.5×10^5pfu/ug donor DNA which is higer than the 3:1 combination.

According to Clarke and Carbon, 1.41×10^4pfu/μg DNA is required to represent an entire *A.niger* genomic library and our figure is 10× more than that.

3.3 Screening of glucoamylase gene

The packaging mixture containing about 3500 recombinant phages was used to infect *E.coli* Q359, the plaques formed were transferred to nitrocellulose filter and in situ hybridized with ^{32}p-labelled probe, 2 positive plaques were obtained from 10^5 recombinant phages (Fig.3) and was rescreened with the same probe(Fig.4).

Fig.3 In situ hybridization of recombinant phages with ^{32}P labelled DNA probe

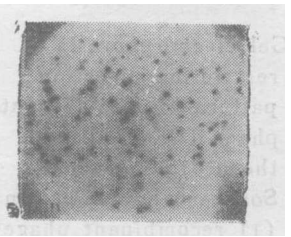

Fig.4 Autoradiogram of the resreening of glucoamylase positive phage

3.4 Analysis of the cloned fragment by Southern blotting

DNA was isolated from positive plaques and digested with EcoRI and EcoRV and run on agarose gel electrophoresis which is shown in fig. 5. The DN Abands were transferred to the filter by Southern blotting and hybridized with ^{32}p-labelled probe. Fig.5 is the restriction digestion pattern and the autoradiogram of Southern hybrid-izatfon.A 2.5kb fragment appeared to be the cloned glucoamylase gene.

3.5 Subcloning of the 2.5kb fragment into pBR322

The 2.5kb fragment was excised from the recombinant phage DNA and recovered from low melting temperature agarose, ligated to EcoRI -EcoRV digested pBR322. the recombinant plasmid was introduced to *E.coli* HB101.

The recombinant plasmid was isolated from the Amp^r and Te^s transformant and designated as pGAM2. Fig.6 is the EcoRI-EcoRV restriction digestion pattern of pGAM2, showing the 2.5kb cloned giucoamylase gene.

The restriction map of this clone pGAM2 is given in fig.6.

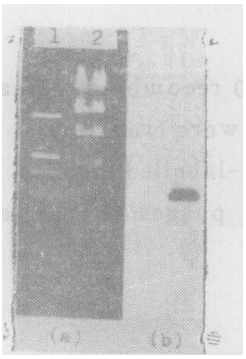

Fig.5 Gel electrophoresis of restriction digestion pattern of recombinant phage DNA (A) and theautoradiogram of Southern hybridization (B)
(1) recombinant phage DNA/EcoRI + EcoRV,
(2) λcI857S7 DNA/HindIII

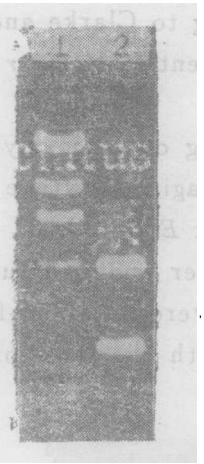

Fig.6 EcoRIEcoRV restriction digestion pattern of plasm id pGAM2
(1) λcI857S7 DNA/HindIII,
(2) pGAM2 /EcoRI + EcoR

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黑曲霉基因文库的构建及葡萄糖淀粉酶基因的克隆*

罗进贤** 张添元 李文清 谭宇惠 王良义

摘　要　用噬菌体λEMBL3 DNA为载体成功地构建了黑曲霉3758的完整的基因文库。供体黑曲霉染色体DNA经EcoRI部份酶切和蔗糖密度梯度离心，回收15—20kb片段，以一定的比例与经EcoRI处理的载体λEMBL3 DNA连接，连接产物用大肠杆菌SMR10单菌系统提取的包装蛋白进行体外包装，包装物感染宿主大肠杆菌Q359，获得2.5×10^5重组噬菌体/μgDNA的包装效率，比Clarke-Carbon公式对构建黑曲霉染色体基因文库要求的重组克隆数高10倍。以编码葡萄糖淀粉酶第329—481位氨基酸的DNA作为探针，用原位噬菌斑杂交的方法，从10^5个重组噬菌斑中筛选出2个杂交阳性噬菌斑。复筛以后，用快速法提取DNA，经EcoRI和EcoR V双酶切以后，走电泳，用Southern印迹法将DNA转移至硝酸纤维素膜上，与^{32}p-标记的探针杂交，证实葡萄淀粉酶基因位于2.5kb的EcoRI、EcoR V片段上，该片段已亚克隆至ρBR322。

关键词　基因文库，黑曲霉，葡萄糖淀粉酶基因，克隆，亚克隆，大肠杆菌

** 生物学系/生物工程研究中心