Over-expression of Fungal Cytochrome P450nor Gene in Sf9 Cells by Using Bac-to-Bac Baculovirus Expression System

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Abstract: The Bac-to-Bac baculovirus expression system is a novel gene expression system that allows the rapid and efficient generation of recombinant baculovirus DNA by site-specific transposition in *Escherichia coli*, rather than homologous recombination in insect cells. The recombinant vector, pFast-P450nor, was constructed by inserting the cytochrome P450nor gene from *Cylindrocarpon tonkinense* into the *Bam*HⅠ/*Xba*Ⅰ sites of the transposing vector pFastBac1 in the correct orientation with respect to the polyhedrin promoter. After transformation, pFast-P450nor was introduced into the competent cells (*E. coli* DH10Bac) containing a shuttle vector, Bacmid. Recombinant bacmid pAc-P450nor was constructed by transposing a mini-Tn7 element from the donor plasmid, pFast-P450nor, to the mini-attTn7 attachment site on the bacmid, where the Tn7 transposition functions were provided in trans by a helper plasmid. The recombinant bacmid DNA was isolated and transfected into the insect cells (*Spodoptera frugiperda*, Sf9) to produce the recombinant virus, rAc-P450nor. Fresh insect Sf9 cells were infected with the recombinant virus containing cytochrome P450nor to express the target protein. Result showed that the recombinant bacmid expression vector, pAc-P450nor, was constructed successfully and the cytochrome P450nor gene was highly expressed in Sf9 insect cells. A specific band (about 43kD) was detected by SDS-PAGE and confirmed by Western blot analysis. Availability of this expression system should facilitate characterization of the role of cytochrome P450nor in the metabolism of NO.

Key words: Cytochrome P450nor, Bac-to-Bac system, Over-expression

Cytochrome P450s constitute a supergene family of heme-containing proteins that are involved in the metabolism of a variety of substrates and widespread in both eukaryotes and prokaryotes[1]. The databases currently list more than 2500 cytochrome P450 sequences belonging to 285 gene families including 70 genefamilies from animals, 52 genefamilies from plants, 86 genefamilies from lower eukaryotes and 77 genefamilies from bacteria (http://dmelson.utmen.edu/cytochrome P450.html)[2,3]. Cytochrome P450 enzymes usually function as monooxygenases, but some may catalyze other reactions such as reduction, isomerization, and dehydration[1]. For example, nitric oxide reductase (Nor) cytochrome P450nor (P450nor) is involved in fungal denitrification[4]. Denitrification is a biological process that plays an important role in the global nitrogen cycle, as it completes the nitrogen cycle performing the reverse reaction of nitrogen fixation. More attention is now being paid to N_2O, an intermediate of biological denitrification, as it exhibits a potent greenhouse effect and its concentration in the atmosphere is increasing rapidly. Denitrification is thought to be one of the main sources of N_2O that is emitted into the atmosphere[5]. On the other hand, biological denitrification is at present the most effective process to remove fixed nitrogen pollutants from aqueous ecosystems, in which they cause eutrophication. Therefore, increased knowledge about denitrification is important not only for biochemical research in this field but also for taking preventive measures against damage in the global environment[6]. P450nor is the most functionally diverse among known cytochrome P450 enzymes as it catalyzes the reaction shown in Reaction 1 without a redox partner such as cytochrome P450 reductase and thus receives electrons directly from NADH or NADPH. The catalytic turnover rate against NO is over 30000 min^{-1} at 30℃[7].

$$2NO + NAD(P)H + H^+ \rightarrow N_2O + NAD(P)^+ + H_2O \quad \text{(Reaction 1)}$$

Four isozymes of cytochrome P450nor, FoP450nor (CYP55A1) from *Fusarium oxysporum*, CtP450nor1 and CtP450nor2 (CYP55A2 and CYP55A3) from *Cylindrocarpon tonkinense*, and TcP450nor (CYP55A4) from basidiomycotina yeast *Trichosporon cutaneum*, were isolated and

Foundation item: National Natural Science Foundation of China(30170011)
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Received date: 06-06-2003

cloned[8~11]. These isoforms differ in terms of intracellular localization (mitochondria and cytosol)[8]. As cytochrome P450nor genes have never been highly expressed in the eukaryotic cells, the Bac-to-Bac Baculovirus expression system, one of the most powerful eukaryotic systems for the expression of foreign genes, was utilized for gene expression. It is superior to the prokaryotic expression system for the modification of post-transcription and post-translation products. This paper reports the construction and expression of recombinant bacmid pAc-P450nor, which contained CtP450nor2 cDNA cloned from *Cylindrocarpon tonkinense*. This research will facilitate the analysis of the structure and the function of the cytochrome P450nor gene.

1 Materials and Methods

1.1 Plasmids, cells and biochemical regents

The pFastBac1 plasmid and *E. coli* DH10Bac cells containing Bacmid and the helper plasmid, were gifts from Professor QI Yi-Peng (College of Life Science of Wuhan University, China). The recombinant plasmid, pGEM-P450nor, cDNA was constructed as described previously[12]. *Spodoptera frugiperda* (Sf9) cell line is conserved in our laboratory. Mono-layer cultures of Sf9 cells and those infected with the recombinant viruses were maintained in Grace's medium supplemented with 5% (V/V) fetal bovine serum (FBS, GIBCO/BRL, USA). SSMC-7721 cell line (cancer cell line) was gift by Professor YANG Xu (College of Life Science of Central China Normal University, China), which was cultured in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% (V/V) FBS (GIBCO/BRL). Restriction endonucleases, T4 DNA ligase and *Taq* DNA polymerase were purchased from Promega Co. X-gal, IPTG, and antibiotics come from Hua-mei Co. Nitric oxide assay kit was purchased from Naijing Jiancheng Biotechnology Co.

1.2 Construction of recombinant plasmid pFast-P450nor

A DNA fragment containing P450nor was excised from the plasmid pGEM-P450nor by double digestion with *Bam*HⅠ/*Xba*Ⅰ, and subcloned into the transposing vector, pFastBac1, that contains the polyhedrin gene promoter. After the transformation and selection, the recombinant plasmid, pFast-P450nor, was obtained, in which P450nor cDNA was in the correct orientation with respect to the polyhedrin promoter.

1.3 Transposition between recombinant-pFast and bacmid

The recombinant pFastbac1 donor plasmid, containing P450nor gene, was transformed into competent DH10Bac cells for transposition into the bacmid. The transformation mixture was plated onto Luria agar plates containing kanamycin (500μg/mL), gentamycin (10μg/mL), tetracycline (10μg/mL), X-Gal (100μg/mL) and IPTG (40μg/mL) and incubated at 37℃ for 48 h for selection of the recombinant bacmid.

1.4 Identification of the recombinant bacmid

The recombinant bacmid was verified to be correct for insertion of the gene of interest by PCR with the pUC/M13 amplification primers and the recombinant Bacmid DNA as template. Forward primer P1: 5′-GTAAAACGACGGCCAGT-3′ and reverse primer P2: 5′-AACAGCTATGACCATG-3′ were purchased from Genecore Company (Shanghai, China). During PCR amplification, the profile was one cycle of 93℃ for 3 min and 30 cycles of 94℃ for 45 s, 55℃ for 45 s, 72℃ for 4 min. Finally it was extended at 72℃ for 10 min. An aliquot of PCR reactions were analysed on 1% (W/V) agarose gels.

1.5 Transfection of Sf9 cells with recombinant bacmid DNA

High molecular weight DNA of recombinant bacmid was prepared from overnight cultures of selected *E. coli* colonies and used to transfect fresh insect (Sf9) cells[13]. Following incubation for 96 h at 28℃, the supernatant containing P450nor was harvested from the transfected cells, and then used to infect fresh insect cells.

1.6 Preparation of recombinant virus DNA

The recombinant virus DNA was prepared from virus particles as previously described[14].

1.7 Detection of the expressed target protein by SDS-PAGE and Western blotting analysis

100μL of viral supernatant, harvested from transfected cells, was used to infect fresh insect cells in 2mL of Grace's medium. After 24h, 48h, 72h, 96h post-infection, cells were harvested and lysed with 2% (W/V) SDS. The expressed recombinant P450nor products were identified by SDS-PAGE with the concentrations of 5% (W/V) of stacking gel and 12% (W/V) of separating gel[14]. After electrophoresis, the samples were then electrophoretically transferred onto polyvinylidene fluoride (PVDF, Millipore Co. USA) membranes. The primary antibody was anti-CtP450nor2 (rabbit) and the second antibody was anti-rabbit antibody conjungated with horseradish peroxidase. The blot was visualized with

ECL kit (Pierce, Rockford, IL, USA).

1.8 Detection of the content of Nitric Oxide

Confluent SSMC-7721 cells were washed twice with cold phosphate-buffered saline (PBS) and scraped into 1.2 mL of cold homogenization buffer containing 50 mmol/L Tris-HCl, 10 mmol/L CHAPS, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.1% (*V*/*V*) β-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L benzamidine, 0.25μg/mL leupeptin, and 0.5μg/mL aprotinin, pH 7.5. Cells were lysed by sonication. NO assays were initiated by adding 200μL of Sf9 cell extract to SSMC-7721 cells. The content of nitric oxide in SSMC-7721 cells was measured according to the manual of nitric oxide assay kit (Naijing Jiancheng Biotechnology, China).

2 Results

2.1 Construction of recombinant transposing plasmid and recombinant bacmid

Restriction endonuclease analysis was performed to verify the recombinant pFast carrying the gene of interest. The recombinant plasmid pFast-P450nor digested with *Bam*HⅠ/*Xba*Ⅰ generated a 1.3 kb fragment and a 4.7 kb fragment (Fig.1 Lane 3). This result demonstrated a successful construction of the recombinant transposing plamid pFast-p450nor in which the cytochrome P450nor cDNA was correctly inserted.

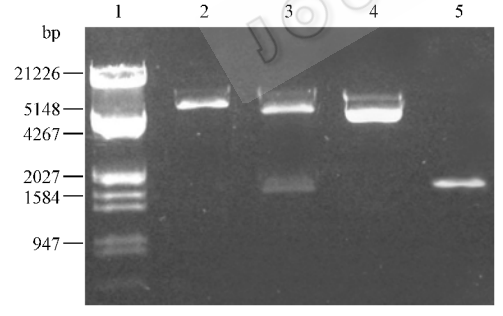

Fig.1 Identification of the recombinant transposing plamid with restriction endonulease digestion

1. Marker, λDNA/*Eco*RⅠ + *Hin*dⅢ; 2. Recombinant transposing plamid pFast-p450nor digested with *Bam*HⅠ; 3. Recombinant transposing plamid pFast-p450nor digested with *Bam*HⅠ and *Xba*Ⅰ; 4. pFastBac1 plasmid digested with *Bam*HⅠ; 5. P450nor gene recoverd from gel as control.

The recombinant plasmid pFast-P450nor was transformed into competent DH10Bac cells for transposition into the bacmid. The recombinant bacmids were constructed by transposing a mini Tn7 element from a pFastBac1 donor plasmid to mini-attTn7 attachment site on the bacmid when the Tn7 transposion functions are provided in trans by a helper plasmid. After transposition and selection, the recombinant bacmid pAc-P450nor was obtained. By transfection in Sf9 cells, the recombinant virus (rAc-P450nor) containing P450nor cDNA was generated.

2.2 Analysis of recombinant bacmid

The verification of the insertion of the foreign gene is very difficult using classical restriction endonuclease digestion analysis because the recombinant bacmid DNA is too big (more than 135 kb). It is better to use PCR to confirm that the gene of interest has transposed to the bacmid. The pUC/M13 amplification primers are directed at sequences on either side of the mini-attTn7 site within the lacZα-complementation region of the bacmid. If transposition has occurred, the PCR product produced with these primers is 2300 bp plus the size of the insert. The size of bacmid transposed with recombinant pFast-P450nor was about 3.6 kb. With bacmid alone, the size of PCR product is 300bp as the primers could combine with mini-attTn7 attachment sites on the bacmid. Fig. 2 showed PCR analysis of recombinant bacmid and recombinant virus. PCR products from the empty Bacmid (lane 2) and from Bacmid in blue bacteria (lane 3) were 300bp; PCR product from non-p450nor recombinant Bacmid, transposed with only pFastBac1 (lane 4) was 2300 bp; PCR products from the recombinant Bacmid pAc-P450nor (lane 5) and from the recombinant virus rAc-P450nor (lane 6) were 3.6kb. These results indicated clearly that the recombinant bacmid and recombinant virus containing the P450nor cDNA were constructed successfully (Fig.2).

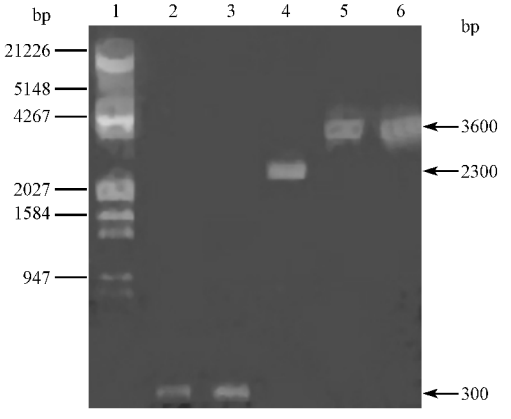

Fig.2 PCR analysis of recombinant bacmid and recombinant virus

1. Marker (λDNA/*Hin*dⅢ + *Eco*RⅠ); 2. Empty Bacmid as template; 3. Bacmid in blue bacteria as template; 4. Non-p450nor rBacmid as template; 5. rBacmid pAc-P450nor as template; 6. Recombinant virus rAc-P450nor as template.

2.3 The expression and biological activity of cytochrome P450nor

The Sf9 cells were infected with the recombinant bacmid pAc-P450nor and cultured for 24h, 48h, 72h, 96h at 28℃,

and harvested for SDS-PAGE and Western blotting analysis (Fig. 3). Figure 3 shows the result of the expressed target protein of cytochrome P450nor with a molecular weight of 43kD. The result proved a successful cloning and expression of cytochrome P450nor in Sf9 cells by using Bac-to-Bac system. It was found that the level of expression of the target protein was higher after 72h post-infection. The recombinant P450nor expression level was about 2.6% of total protein by gel scanning analysis. It can be seen that the expressed protein was increased from 24h to 72 h post-infection. The strongest band appeared after 72 h post-infection (Fig. 3, lane 4). After 96 h post-infection, the expression level was decreased rapidly. After addition of Sf9 cells extract to the culture of SSMC-7721 cells, cells were assessed for viability using the MTT assay[15]. The inhibition of these cell proliferation increases with concentrations of P450nor (not published). Table 1 show the concentration of NO of culture cells treated with or without P450nor.

Table 1 Metabolism of recombinant P450nor on nitric oxide ($\bar{x} \pm s$)

Samples	n	NO/(μmol/mL)
Control (extract from uninfected cell)	10	5.904 ± 0.14
P450nor (extract from infected cell) treated	10	$2.767 \pm 0.11^{*}$

* $P < 0.01$

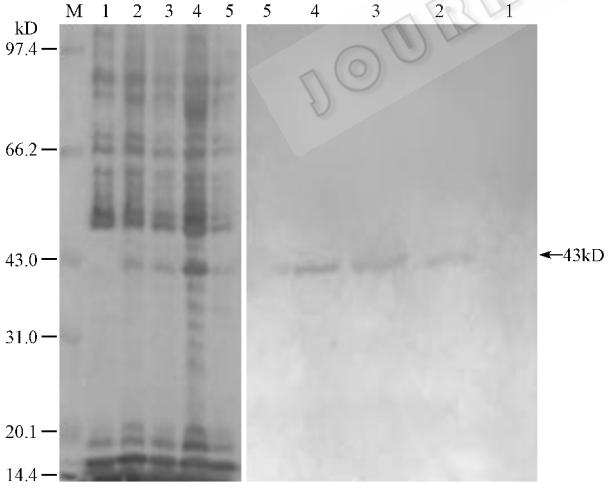

Fig. 3 SDS-PAGE (left) and Western blot (right) analysis of the recombinant p450nor expressed in Sf9 cells

1. The proteins extracted from uninfected Sf9 cell; 2. The proteins extracted from the cells post-infected for 24h; 3. The proteins extracted from the cells post-infected for 48h; 4. The proteins extracted from the cells post-infected for 72h; 5. The proteins extracted from the cells post-infected for 96h.

3 Discussion

Different from cytochrome P450 monooxygenases, cytochrome P450nor is a relatively new cytochrome P450. To further investigate its function, it is necessary to express cytochrome P450nor in eukaryotic expression system. This paper reports the successful cloning and expression of cytochrome P450nor in Sf9 cells by using Bac-to-Bac system. The expression level of the foreign protein was found to be higher compared to yeast expression system, which expressed P450nor at 1.4% of the soluble protein[10,12]. By SDS-PAGE analysis, it was found that the expression level was higher at 72h post-infection. Probably, if the time of infection is too short, there will be low yield of expressed protein, and if the time is too long, the infected cells will be damaged and most of the expressed protein will be secreted into the infection medium.

Although P450nor is involved in the denitrifying systems of fungi, it can not be a respiratory enzyme unlike nitrite or nitrate reductase, since P450nor received electrons directly from NADPH/NADH. P450nor should be a detoxifying enzyme. NO itself is a highly reactive radical, and known to cause defects in mitochondria. Furthermore, NO is well known to react with superoxide to form a more hazardous molecule, peroxynitrite. Peroxynitrite has higher reactivity than NO or superoxide against biological molecules and is known to inactivate the mitochondrial electron transport system[16]. From this work, we have learned that recombinant P450nor could reduce nitric oxide, since the proliferation of the cancer cell (SSMC-7721 cell line) was inhibited with P450nor. This result will redound to study the effects of P450nor on mice bearing tumor. Therefore, P450nor will be helpful to the diagnosis of a series of environmental and medical disorders and study on anaerobic respiration and NO metabolism in eukaryotic cells.

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利用杆状病毒 Bac-to-Bac 系统在 Sf9 细胞中表达真菌细胞色素 P450nor

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摘 要 利用 Bac-to-Bac 杆状病毒载体表达系统将真菌细胞色素 P450nor 基因克隆至转移载体 pFastBac1 中,得到重组质粒 pFastBac-P450nor,再将其转化进入含穿梭载体 Bacmid 的受体菌 DH10Bac 中发生转座作用,得到含 P450nor 基因的重组穿梭载体 rBacmid pAc-P450nor。分离提取重组 Bacmid DNA,并转染培养的昆虫细胞 Sf9,得到重组病毒 rAc-p450nor。经酶切和 PCR 鉴定,细胞色素 P450nor 基因正确地插入到病毒基因组的多角体蛋白基因启动子下,SDS-PAGE 分析证明:表达蛋白的分子量为 43kD 左右。Western blotting 分析结果表明:有一条特定的杂交带存在,且分子量相同(约 43kD)。进一步证明了含有真菌细胞色素 P450nor 基因的重组表达载体和重组病毒构建成功,并在昆虫细胞 Sf9 中实现了高效表达,经 MTT 法测定表达的细胞色素 P450nor 具有还原 NO 的生物学活性。

关键词:Bac-to-Bac 系统,细胞色素 P450nor,高效表达

中图分类号:Q753 **文献标识码**:A **文章编号**:0001-6209 (2004) 02-0177-05

基金项目:国家自然科学基金(30170011)

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收稿日期:2003-06-06,修回日期:2003-10-23