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Full Length Article

Isolation, Cloning and Sequence Analysis of *β-tubulin* Gene from *Bolinea lutea* F23523, a Potential Strobilurin Producing Fungus

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Abstract

Bolinea lutea F23523 is an ascomycetes fungus and known producer of potential antifungal metabolites, the strobilurins. To facilitate genetic engineering studies for production of new analogues of strobilurins, the gene cluster isolation and sequence analysis are essential prerequisites. Degenerate primers-based polymerase chain reaction (PCR) is commonly utilized for amplification and isolation of unidentified genes in fungi. The objective of this study was to isolate and characterize the *β-tubulin* gene from *B. lutea* which plays a major role in conferring resistance to fungicides specifically benzimidazoles. A 600 bp fragment of the *β-tubulin* gene was isolated from the genomic DNA of *B. lutea* using degenerate primers through PCR. The PCR amplicon was purified by agarose gel electrophoresis and then ligated into PCR 2-TOPO vector. The ligation mixture was subsequently transformed into Tam-1 *E. coli* cells using the heat shock method and spread on LB agar plate containing antibiotic. The positive transformants were checked for the gene fragment and sequenced. Basic Local Alignment Search Tool **(**BLAST) analysis revealed 80-85% homology with *β-tubulin* genes of *Hericium erinaceum, Coprinopsis candidolanata* and other fungal species. © 2013 Friends Science Publishers

Keywords: *Bolinea lutea*; Genomic DNA; Degenerate primers; β-tubulin gene

Introduction

Strobilurins are a small group of natural products produced by many species of fungi specially basidiomycetes (Anke *et al*., 1977). *Bolinea lutea* is the only reported ascomycete, which produces strobilurins (Fredenhagen *et al*., 1990). These metabolites possess antifungal activity against various pathogenic fungi and inhibit respiration of the pathogens in the Qo site of the bc1 complex in the respiratory chain (Becker *et al*., 1981).

B. lutea genome remains unrevealed despite of worth to be characterized for future metabolic engineering studies to utilize for generation of more useful analogues of strobilurins. The whole genome DNA assay has long been utilized for microbial identification by genomic similarities and comparison between two species (Kurtzman, 2006). However, this approach is time consuming, costly and difficult strategy. In recent years, it is swapped to target gene amplification and sequencing technique (Petti, 2007). The tubulin gene family consists of six highly conserved sub-families including: alpha-; beta-; gamma-; delta-; epsilon- and zeta-tubulins (McKean *et al*., 2001). Alpha- and beta-tubulins are the most abundant proteins in eukaryotic cell and have been studied most extensively. The *β-tubulin* is fairly conserved, ranging from 60 to 90% homology within species (Juuti *et al*., 2005).

In this study the β-tubulin gene fragment was isolated and sequenced from the fungus *B. lutea* strain F23523. This is the first gene reported from this fungus. *β-tubulin* gene has enormous significance in fungi including: developing resistance to certain fungicides; establishing evolutionary relationship among fungal species and phylogenetic marker for fungal classification. Keeping in view its importance isolation and sequence analysis of *β-tubulin* gene from *B. lutea* would pave the way for characterization biosynthetic pathway genes to generate new bioactive analogues using metabolic engineering strategies.

Materials and Methods

Chemicals and Reagents

All chemicals used were of molecular biology grade. Aqueous solutions were autoclaved before using for sterilization. Aqueous solutions that could not be sterilized by autoclaving were filtered through disposable sterile filter (0.2 µm). Tris-EDTA buffer pH 7.5 (TE buffer), 1M Tris-HCl, 50 mM Tris-HCl pH 7.5 (elution buffers) were prepared as (Sambrook and Russell, 2001). Restriction enzymes, *Taq* DNA polymerase, Reddy Mix™ PCR reagent mixture, T4 DNA ligase, shrimp alkaline phosphatase and other modifying enzymes were purchased from New England Biolabs (NEB), Promega, ABgene or Fermentas. 50 × TAE stock was prepared (Sambrook and Russell,

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2001). 10**X** gel loading buffer was prepared with bromophenol blue (0.25%) and glycerol (10%) in water. Inoculation of media and aseptic procedures were carried out in a microbiological safety cabinet to prevent contamination.

Morphological Characters and Culture Condition for the Fungus *B. lutea*

B. lutea strain F23523 belongs to the order Boliniales and family boliniacea of the class ascomycetes, was obtained as gift from Novartis, Switzerland. The fungus was maintained on slants/agar plat of YGM medium containing yeast extract (0.4%), glucose (0.4%) and malt extract (1%) in water supplemented with 2% agar and oat meal (Fig. 1). The fungus was slow growing on agar plate culture and had a pluffy mycellial mass. The mycelia at the beginning of culture were whitish and turned to reddish with time. The fungus was identified from its morphology and confirmed for production of strobilurin A and B using High performance liquid chromatography (HPLC). Seed culture (25 mL) was prepared in 50 mL pre-sterilized tubes in circular wheel incubator to get homogeneous biomass. 100 mL of the pre-autoclaved glucose nutrient broth (GNB) media was inoculated with 5 mL of the seed culture and grown on a shaker at 25 °C and 200 rpm for 3-4 days to obtain fresh mycelia for DNA extraction.

Extraction of Fungal Genomic DNA

The fungal culture was collected and the mycelia were separated from the liquid medium by filtration under vacuum. The mycelia (1.5 g) were crushed with sterile pestle and mortar under liquid nitrogen to a fine powder. The gDNA was extracted by following the manufacture's protocol of the GenElute Plant Genomic DNA kit (Sigma) from 100 mg of the fungal tissues.

Polymerase Chain Reaction (PCR)

The extracted gDNA from *B. lutea* was used as a template in standard PCR reactions using β-tubulin degenerate primer pairs (5' ➔ 3') forward: GCTTCMRWGTCACCCACTCT and reverse: CTTGGGGTCGAACATCTGCTG. Using the primer pair specified, a part of gDNA was amplified by PCR.

For PCR 100 ng of the DNA template of *B. lutea* was mixed with REDDY MIX PCR components (ABgene) which contains Thermoprime Plus DNA polymerase, Tris HCl pH 8.8, $(NH_4)_2SO_4$, $MgCl_2$, Tween 20, dATP, dGTP, dTTP, dCTP and precipitant plus red dye for gel electrophoresis. The forward and reverse primers were added to a final concentration of 0.3 µM each, and the final reaction volume was made up to 25 µL with sterilized water. Samples were centrifuged and amplified in a programmable thermal controller. The following thermal cycling profile was run as standard: Initial denaturation at 94°C for 3 min. 10 cycles of denaturation at 94°C (15 sec), primer annealing at 55°C (30 sec), extension at 72°C (45 sec). Denaturation at 94°C (15 sec), primer annealing at 45-65°C (30 sec), extension at 72°C (48 sec for the first round and an additional 3 sec for every additional round, for a total of 20 rounds). Final extension at 72°C (6 min.), Cooling to 4°C. Control reactions without template and a positive control were also run for each set of reactions. Positive PCR products were identified by gel electrophoresis, using PCR reaction mixture (5-10 µL), on an agarose gel. Purification of PCR products were carried out using the NucleoSpin Extract II kit (Macherey-Nagel), according to the manufacturer's protocol.

Agarose Gel Electrophoresis

Analytical agarose gel (0.7%) was prepared and TAE buffering system (0.04 M Tris-acetate, 1 mM EDTA) was used to run the gel. Loading buffer (0.5 µL, 0.25% bromophenol blue, 10% glycerol in water) was added to the DNA sample, and an appropriate DNA marker was used simultaneously alongside. The gel containing DNA fractions were stained by immersion in an ethidium bromide solution for 5-10 min and DNA bands were visualized by UV light (312 nm), using a BioDoc-it (UVP) UV transilluminator and photographed with a Sony UP-895CE Video Graphic Printer on thermofilm (Sony).

Luria-Bertani Medium (LB medium)

Tryptone (10 g L^{-1}), yeast extract (5 g L^{-1}) and sodium chloride (10 g L^{-1}) were dissolved in water. The pH was adjusted to 7.0 and autoclaved. For LB-agar, Bacto agar (15 g L^{-1}) was added.

Soc (Post-Transformation Recovery)

Tryptone (20 g L^{-1}), yeast extract (5 g L^{-1}) and sodium chloride (0.5 g L^{-1}) were dissolved in water. Potassium chloride (10 ml L^{-1}, 250 mM) was added and the pH was adjusted to 7.0. The volume was then adjusted to the total with water before sterilization. Prior to use sterile magnesium chloride (5 mL, 2M) and glucose (20 mL, 20 mM) were added.

Bacterial Culture Conditions

Cultures of bacteria were grown in incubator at 37°C. Solid cultures were grown in oven incubator. Liquid cultures were cultivated in a rotary incubator at 250 rpm. Agar plate is used for short term maintenance where bacteria were streaked with the help of sterile loop onto a suitable medium containing an appropriate antibiotic selection. The plates were incubated at 37°C until formation of appropriate single colonies. The plate is then stored in a fridge at 4°C wrapped in parafilm viable for 1-2 months. Bacterial cells were kept in 2 mL cryovials made up to 20% glycerol (*w/v*) at -80°C for long term storage.

Fig. 1: *Bolinea lutea* strain F23523 grown on agar plate YGM nutrient medium supplemented with 2% oat meal

Cloning in *E. coli*

Ligation of Plasmid Vector and Insert DNA

Equi-molar amounts of insert and vector DNA were used. T4 DNA ligase (1 µL, NEB), 10 x ligase buffer (1 µL, NEB) were added and the final volume made up to 10 µL with nuclease-free water. The reaction was incubated at room temperature at least for one hr. The PCR product was ligated into the PCR 2-TOPO vector as per the manufacturer's protocol. The TOPO TA Cloning Kit (Invitrogen) was used to insert PCR products into pCR2-TOPO vector. The reaction products were cloned in Tam-1 *E. coli* cells using the heat shock method.

The heat shock method was used for *E. coli* transformation. Competent *E. coli* Tam-1 (Active Motif), Top 10 or DB3.1 (Invitrogen) were removed from storage at -80°C and allowed to thaw on ice. The DNA or the ligation mixture was added to the thawed cells up to 10% of the competent cell volume. The reaction was incubated on ice for 10 min and then heat shocked at 42°C for 30 sec. The mixture was incubated on ice again for 2 min and then SOC medium (5 × volume of the reaction) was added. SOC media was prepared as: 2 g Bacto Tryptone; 0.5 g Bacto Yeast extract; 0.2 mL of 5 N NaCl; 0.25 mL of 1 M KCl; 1 mL of 1 M $MgCl_2$; 1 mL of 1 M $MgSO_4$; 2 mL of 1M glucose dissolved in 100 mL distilled water and autoclaved. The mixture was incubated at 37 °C for not more than 1 hr, then aliquots of 50-200 µL were spread on pre-warmed LB solid medium plates supplemented with appropriate antibiotic(s), using an alcohol sterilized flamed glass spreader. The plates were incubated overnight at 37°C.

Plasmid DNA Extraction from *E. coli*

Plasmid Miniprep was made using the NucleoSpin plasmid DNA purification kit (Macherey-Nagel). Kit was used according to the manufacturer's protocol. DNA was stored at -20^{o}C.

Colony PCR

Cells from individual bacterial colonies were suspended in 25 µL lysis buffer (50 µg·mL^{-1} proteinase K in TE buffer) and incubated for 15 min at 55°C, then at 80°C for another 15 min. Cell debris was pelleted by centrifugation for 2 min, and 2.5 µL of the supernatant was used as DNA template in the analytical PCR reaction. Positive colonies from the transformation were identified by the presence of a PCR product of the predicted size by gel electrophoresis. Positive clones were then further tested by restriction digest analysis.

Restriction Analysis of Plasmid DNA

Plasmid DNA of the clones was obtained and restriction digests were performed to check the insertion of the PCR product into the vector. For analytical digestion, approximately 0.1- 0.2 µg plasmid DNA was used in a final volume of 10 µL and then incubated at 37°C for one hr. An agarose gel was used to visualize the digested DNA.

DNA Sequencing and Analysis

DNA sequencing was done by Cogenics. The sequence data was analyzed using the Vector NTi software package and NCBI-BLAST analysis from the database available on internet.

Results

Extraction of Genomic DNA from the Fungal Cells

The gDNA of *B. lutea* was prepared from the fungal cells using a miniprep kit (Qiagen) protocol. For good quality gDNA suitable for PCR amplifications, the fresh cells were extracted after 3-5 days of fermentations. The hyperladder1 marker was used for comparison the size and quality of the DNA template as shown in Fig. 2.

Isolation of β-tubulin Gene Fragment from *B. lutea* Genomic DNA

A set of β-tubulin degenerate primers; forward primer TubF: 5′-GGCTTCMRWGTCACCCACTCT-3′; and reverse primer TubR: 5′-CTTGGGGTCGAACATCTGCTG-3′, were designed and used in PCR amplification. The primers designed were expected to amplify the putative β-tubulin gene fragment of 600 bp from the genomic DNA template in PCR reactions. Thermoprime Plus DNA polymerase (ABgene) giving 3'-A overhung products was used. PCR products can be ligated directly into pGEM-T Easy (Promega) or PCR 2.1-TOPO (Invitrogen) cloning vectors.

The PCR reaction using *B. lutea* F23523 genomic DNA as a template with the TubF/R primer pair afforded a 550-600 bp product, which was the expected size.

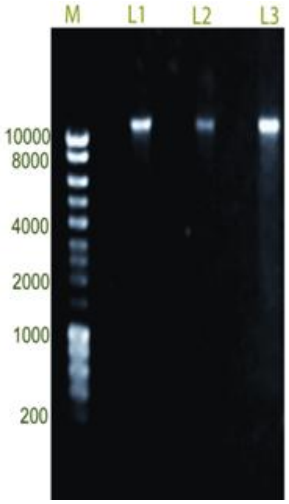

Fig. 2: Genomic DNA extracted from *B. lutea* F23523 by miniprep Kit method. (L1, L2 and L3 represent gDNA extracted after 3, 4 and 5 days of cultivation respectively, M represent the hyperladder-1)

The fragment of DNA was purified using 0.7% agarose electrophoresis gel and ligated into pCR®4-TOPO-TA cloning vector according to the manufacturer's instructions. The DNA sequence of the PCR product was obtained and translated into all possible amino acid sequences and used in BLAST (blastp) searches. The sequencing showed that the DNA sequence is a 600 bp-portion of a β-tubulin gene (Fig. 3a-c).

Discussion

β-tubulin is among the most conserved genes in fungi and can serve the purpose of checking the DNA quality for PCR reactions. Characterization of *β-tubulin* has been done for several different purposes. Davidson *et al*. (2006) studied the *β-tubulin* genes from sensitive and resistance fungi against the benzimidazole fungicides and reported a single mutation in *β-tubulin* genes of both the tolerant and the sensitive isolates. The *β-tubulin* can also be used as a phylogenetic marker for classification of *Saccharomyces sensu stricto* complex strains using direct sequencing (Huang *et al*., 2009). The *β-tubulin* gene isolated from the DNA library of *Aspergillus flavus* was utilized as a selectable marker for transformation as well (Seip *et al.*, 1990). It is also reported that intron positions within *β-tubulin* genes in fungi may provide some clues of evolutionary relationships among species (Ayliffe *et al*., 2001). Previously the *β-tubulin* gene has also been used to ensure the quality of fungal gDNA and conditions of PCR reaction (Dao *et al*., 2005).

In this study the partial β-tubulin gene was isolated from the DNA template of *B. lutea* strain F23523 using PCR. The degenerate primers designed for this purpose from the conserved region of *β-tubulin* genes of other fungi was successfully amplified the target gene

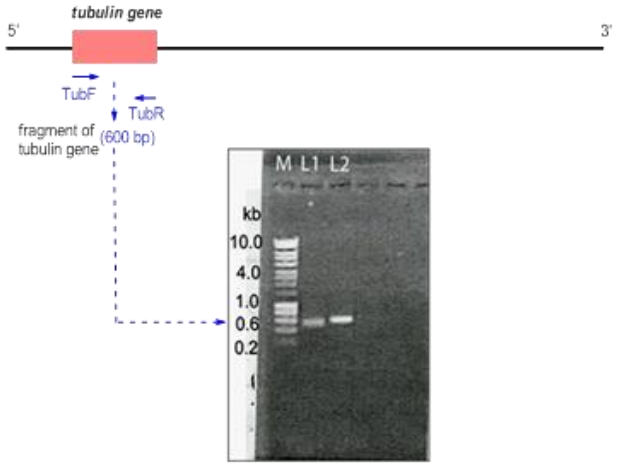

Fig. 3a: 0.7% agarose gel electrophoresis of the PCR product with TubF/R primers and *B. lutea* gDNA. M represent hyperladder-1 (0.2-10 kb) marker. Lane 1 and Lane 2 are 600 bp PCR amplified fragments of β-tubulin gene from gDNA of *B. lutea*

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1      ASVYPLSGWR YRCWYGYASH LEDS*RVPRP HDVHVLRRAQ   SQGLGYRCRG
51     TCSCIPGLRV VAKHISSSPT TPPSPSTSLS RTPTRPSVST   MRPCTTSASG
101    RSSCPPPPTA TSTTWSPSSC LVSRHACVSL VN*TPTCASL   PSTWVCVFPW
151    SPYSPLIIFT VPFPRLHFFM TGFAPLTARG SQQYRAVTVP   ELTQQMFDPK
201    K
```

Fig. 3b: Amino acid sequence of the PCR based product of primers TubF/R

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  1    GGCTTCAGTA TACCCACTCT CTGGGTGGAG GTACCGGTGC TGGTATGGGT
       ACGCTTCTCA TCTCGAAGAT TCGTGAAGAG TACCCCGACC GCATGATGTG
101    CACGTACTCC GTCGTGCCCA GTCCCAAGGT CTCGGATACC GTTGTCGAGG
       TACGTGTTCA TGTATCCCGG GACTACGCGT CGTGGCTAAA CATATCTCTT
201    CCAGCCCTAC AACGCCACCC TCTCCGTCCA CCAGCTTGTC GAGAACTCCG
       ACGAGACCTT CTGTATCGAC AATGAGGCCT TGTACGACAT CTGCTTCAGG
301    ACGCTCAAGC TGTCCACCCC CACCTACGGC GACCTCAACC ACTTGGTCTC
       CATCGTCATG TCTGGTATCA CGACATGCCT GCGTTTCCCT GGTCAATTAA
401    ACTCCGACCT GCGCAAGCTT GCCGTCAACA TGGGTTTGTG TTTTCCCTTG
       GTCGCCCTAC TCGCCGCTCA TCATCTTTAC AGTCCCCTTC CCCCGTCTCC
501    ACTTCTTCAT GACCGGCTTT GCCCCATTAA CCGCCCGCGG CAGCCAGCAG
       TACCGCGCCG TTACTGTTCC CGAACTGACC CAGCAGATGT TCGACCCCAA
601    GAAG
```

Fig. 3c: Nucleotide sequence of the amplified PCR product obtained from using *B. lutea* genomic DNA as a template and TubF/R primers. Primer position is highlighted with yellow and written in bold

fragment of 600 pb.

It is concluded that the degenerate primers can be used for isolation of new gene fragments from fungi. The tubulin primer pairs were used in PCR to obtain partial sequence of β-tubulin gene from the genomic DNA of *B. lutea* strain F23523.

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