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# PRODUCTION AND CHARACTERIZATION OF A POLYCLONAL ANTIBODY AGAINST *BUR6* A SUBUNIT OF NC2 COMPLEX INVOLVED IN *CANDIDA ALBICANS* DRUG RESISTANCES

**Vipin Yadav and P. J. John**

Centre for Advanced Studies, Department of Zoology, University of Rajasthan, Jaipur, India.

**Corresponding Author: Dr. P. J. John**

Centre for Advanced Studies, Department of Zoology, University of Rajasthan, Jaipur, India.

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## ABSTRACT

*Candida albicans* is an opportunistic pathogen having clinical importance as it causing oral, vaginal or systemic infections in immuno-compromized patients, such as HIV+, cancer patients receiving chemotherapy and patients went through organ transplant. Cases of *Candida* infection dramatically increased due to emergence of drug resistance against antifungal drugs. Previously it was reported that *Ncb2* the subunit of *NC2* Complex is involved in drug resistance mechanism but the role of *Bur6* which is another subunit of *NC2* complex is still unknown. To identify the role of *Bur6* in *Candida* drug resistance, complete ORF of *Bur6* was cloned and expressed in pET28a vector. Purification of *Bur6* protein was performed and polyclonal anti sera against full length *Bur6* were raised in Mouse model and further experiments performed to characterize role of *Bur6* protein.

**KEYWORDS:** *Candida albicans*, drug resistance, transcriptional regulation.

## INTRODUCTION

Due to sudden increase in immunocompromised patients, cases of *Candida albicans* infection increased dramatically in last two decades.[22] *Candida albicans* is commensal and a constituent of the normal gut flora consisting microorganisms that live in the gastrointestinal tract and human mouth. *C. albicans* present in 80% of the human population without causing any negative health effects, although overgrowth of the fungus results in candidiasis.[19] Candidiasis is generally observed in immunocompromised individuals such as HIV-infected patients. Presence of candidiasis generally observed in the mucosal membranes of mouth or vagina, which is easily cured in people who are not immunocompromised.

*Candida albicans* is the most frequent species among the current fungal pathogens followed by other *Candida* species, non *Candida* species and molds[24] Azole belongs to a class of antifungal drugs that are widely used for the treatment of fungal diseases and especially those caused by *Candida albicans*. [17] Since azoles are fungi static drugs for *C. albicans*, cells repetitively exposed to these antifungal, adapt to the drug pressure and eventually become azole resistant.[8]

*CDR1* is a major efflux pump involved in drug resistance and it belongs to ABC class super family.[21] The regulation of *CDR1* is an intrinsic process.[25, 26] Various transcription factors regulate expression of *CDR1* in both positive and negative manner. *Tac1* is first identified transcription factorwhich regulates expression of *CDR1* in positive manner.[5] Recently it was shown that *Ncb2*, beta subunit of *NC2* complex also regulates expression of *CDR1*. [23] NC2 complex made up of two hetrodimeric subunit *Ncb2* and *Bur6*.[1] NC2 binds to TBP and by preventing preinitiation complex assembly it can repress transcription or stimulate activated transcription.[10] *Bur6* subunit is homologous to human NC2alpha.[18] Role of *Bur6* which is another subunit of *NC2* complex is not yet reported in drug resistance mechanism.[14] Therefore characterization, localization and other studies are required to understand the mechanistic role of *Bur6* in *Candida albicans* drug resistance. Purified antibodies have important role in diagnosis and treatment of various disease.[2,11] In this work, polyclonal antibody has been raised in mice against whole recombinant *Bur6* protein and localization of endogenous protein has been explored using raised antibody to establish role of *Bur6* in *Candida albicans* transcription and drug resistance mechanism.[9]

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

The *Candida albicans* strain used in this study is wild type laboratory strain SC5314. *C. albicans* strain was grown in YEPD (1% yeast extract, 2% peptone, and 2% dextrose) medium for routine purposes at 30°C or in synthetic defined (SD) dropout medium (0.67% yeast nitrogen base without amino acids and 2% glucose).

*Escherichia coli* strains DH5α, DH10β, and BL21 (DE3) were used for routine cloning, sub cloning, and expression of cloned genes. All *E. coli* strains were maintained in LB medium. Ampicillin (100µg/ml) and kanamycin (50µg/ml) were added to the growth medium when required.

**Genomic DNA isolation from *Candida albicans***

The *C. albicans* cells were grown in 15 ml of YEPD media for 12-16 h, harvested at 3000 rpm for 5 min, resuspended in 1 ml of SOE (1 M sorbitol and 0.1 M EDTA) and pelleted down. SOE was then added to the cells to make final volume 500µl and 20µl of 10 mg/ml solution of zymolyase was added and the tubes incubated at 37°C. The tubes were then turned upside down gently after every 15 minutes. The contents of the tube were then centrifuged at 2000 rpm for 1 min until the cells were just pelleted enough. The supernatant was discarded and the cells were resuspended by vortexing thoroughly for 1 min in 500µl of 50 mM Tris, 20 mM EDTA mix. 50µl of 10% SDS was added to the above suspension and incubated at 65°C for 30 minutes. 200µl cold 5.0 M potassium acetate was added and the tubes were chilled on ice for 60 min after vortexing to mix the contents. After incubation on ice the cell suspension was centrifuged for 5 min at 4°C. The supernatant was recovered in fresh tubes following centrifugation and 1 volume of isopropanol was added. The tubes were inverted several times to mix and precipitate DNA and then centrifuged. The pellet obtained was air dried and resuspended in 150µl of TE. Subsequently, 1.5µl of RNase A solution (10 mg/ml) was added and the tubes were incubated at 37°C for 30 minutes. The tubes were chilled on ice and then centrifuged for 5 min at 4°C. The supernatant was transferred to fresh tubes and the DNA was precipitated with 2.5 volumes of ethanol in presence of 1/10 volume of 3.0 M sodium acetate (pH 5.2). The DNA pellet was washed with 70% ethanol, dried and finally dissolved in 100 µl of sterile TE buffer.[23]

**PCR amplification of *Bur6***

PCR was performed in 50µl reaction volume containing 50–100 ng templates of genomic DNA, 10 pmoles each of appropriate forward and reverse primer , 200 µM dNTPs and1.25 unit of Pfu 1X reaction buffer (20 mM TRIS-HCl (pH8.8), 10 mM (NH4)2SO4, 10 mM KCl, 0.1%(V/V) TritonX-100, 0.1 mg/ml BSA, 2 mM MgSO4 or 20 mM tris-HCl, 10 mM KCl, 10 mM (NH4)2SO4, 2 mM MgSO4, 0.1% (V/V) TritonX-100 (pH 8.8). Generally 30 cycles of following PCR steps were used for amplification of desired DNA templates. Denaturation at 94°C for 1 min, annealing at 50–55°C for 45 s, extension at 72°C for appropriate time and final extension step was for 7 min.

Primers used during *Bur6* cloning:

*Bur6* Forward Primers: CCATGGATGTCAAATTTACAGGATTTAATAAACCA

*Bur6* Reverse Primers: AAGCTTTGGTTTATTAAATCCTGTAAATTTGACAT

**Gel electrophoresis, digestion, and elution**

Amplified PCR products were subjected to agarose gel (1%) electrophoresis, and the desired band was cut out of the gel and purified using Qiagene gel elution kit. Purified DNA fragment was digested with the desired restriction enzymes, extracted, and finally precipitated with ethanol in presence of 0.3 M sodium acetate. Precipitated DNA was washed twice with 70% ethanol and finally dissolved in TE and stored at –20°C for further use. Desired plasmid DNA (vector) pET28a plasmid was digested with suitable restriction enzymes for 3 h at 37°C and 1 h treatment with CIP (calf intestine phosphatase, NEB) to remove 5′ phosphate groups from the linearized plasmid DNA. Dephosphorylated DNA fragment was subjected to agarose gel electrophoresis, and desired DNA fragment was cut out of the gel and purified using Qiagen gel elution kit according to the manufacturer's protocol.

**Cloning of *Bur6***

Linearized vector DNA and processed amplicons were mixed in 1:3 molar ratios and ligated using "Quick ligase" ligation kit (NEB) or "Fast Link™ DNA ligation kit" (Epicenter Biotechnologies) according to the manufacturer's protocol. Ligated product was used for transformation of DH10β or DH5α competent E. coli cells. Transformed E. coli cells were platted on antibiotic containing LB agar plate.[13] Colonies were picked from selective plate for screening. Positive clones were selected by double digestion and PCR.

**Recombinant *Bur6* protein expression**

E. coli strains DH10β/DH5α were used for propagation of all types of E. coli cloning and expression vectors, while E. coli strain BL21 (DE3) was used for expression of plasmid having pET-28a vector back bone. When desired constructs were confirmed, freshly transformed E. coli colonies were inoculated into 5 ml of LB broth containing 100 µg/ml ampicillin, or 50 µg/ml kanamycin and grown overnight at 37°C for expression of recombinant protein. This primary culture was then used to inoculate 100 ml of fresh LB broth containing the same antibiotics. The culture was allowed to grow at 37°C till OD 600 reaches 0.6. At this stage, culture was induced with 1 mM isopropyl β -D-thiogalactoside (IPTG) for 3 h. Protein expression was checked by lysis of a small aliquot of pelleted cells subjected to SDS-PAGE.[7] Solubility of each protein was assessed in native lysate of desired bacterial cells followed by SDS-PAGE.

**Purification of recombinant Protein**

Overnight culture derived from a single E. coli BL21 (DE3) colony, harboring plasmid pET-528A (without tag) was used to inoculate 100 ml of LB, containing 50µg/ml kanamycin. Culture was allowed to grow at

37°C till OD/600 reaches 0.6. Thereafter, 1 mM IPTG was added to the media to induce the expression of recombinant *Bur6* and incubated further at 37°C for 3 h. Cells were then collected by centrifugation and washed with 50 pH-8.0, 150 mM NaCl, 1% Triton X-100, 10 mM βME, and 1 mM PMSF), sonicated and centrifuged at 10 K for 20 min in cold. Major portion of proteins were recovered in pellet as inclusion bodies. Protein was extracted from the inclusion body with 0.5% sarcosine containing lysis buffer. The extract was subjected to ammonium sulfate precipitation at different concentration followed by centrifugation at 10 K for 20 min at 4°C. Dialyzed ammonium sulfate fractionated protein was partially purified by ion exchanger chromatography in S-sepharose and **Q-sepharose column.**

**SDS-PAGE**

SDS-PAGE was carried with purified recombinant *Bur6* protein according to Laemmli's method.[16] A wide range molecular weight marker was used to identify and assess the molecular weights of protein bands observed in the CBB stained gel.

**Production of polyclonal antibodies against *Bur6***

The purified Bur6 was separated on 10% SDS-PAGE, visualized by incubating; the gel in chilled 0.1 M KCL solutions and the desired band was excised. The excised gel was pulverized mixed with Freund's complete adjuvant, emulsified and injected in to the mice. Overall three booster doses were administered at two week intervals. Blood was drawn from the immunized mice, two weeks after the third booster; serum was separated and stored in at - 20°C.[6]

**Western blot analysis**

Western blotting was performed by transfer of protein separated in SDS- PAGE on PVDF membrane (Sigma) according to standard protocol. SDS was included in the transfer buffer to increase efficiency of high-molecular weight protein. Five percent bloto (Genotech) dissolved in TBST were used to block the membrane. Respective antiserum was used to probe the transferred protein on membrane. Primary antibody bound membrane was incubated with 1: 10,000 dilution of HRP conjugated secondary antibody (Bio- Rad) and detected with enhanced chemiluminescence (ECL) method.

## RESULTS

**Cloning and expression of *Bur6* gene**

To characterize the role of *Bur6* in *Candida albicans* drug resistance the complete ORF was cloned in pET28a vector at NcoI and HindIII site. We kept stop codon intact so that there was no tag at C- terminal of *Bur6* protein. The clones were screened and confirmed by colony PCR and restriction digestion experiment.[3] After confirmation of positive clones the expression conditions of *Bur6* were optimized by using IPTG at concentrations of 1mM. After standardization of all optimal conditions we found good expression of *Bur6* protein upon induction (Figure 1).

**Purification of recombinant *Bur6***

In order to purify the protein and eliminate the contaminating host protein, inclusion bodies were solubilized in 0.5% sarcosine containing buffer and fractionated by different concentration of ammonium sulfate (30%, 40%, 50%, and 70%). Maximum *Bur6* recovery was observed at 30% ammonium sulphate. Ammonium sulfate precipitated protein was subjected to dialyzed overnight against buffer and mixed with pre equilibrated S sepharose matrix and bound protein(s) was purified.[7] Profile of eluted purified protein was checked by SDS- PAGE and pure *Bur6* band was observed (Figure 2).

**Production of polyclonal antibody against recombinant *Bur6* in Mice**

Purified recombinant His tagged *Bur6* protein was injected into mice for antibody production.[15] By using total cell extract of *Candida* cells preimmune and immune serum was tested for specificity of *Bur6* by western blotting (Figure 3), and we found that it was specific for *Bur6*. There is no cross reactivity observed with *Candida* proteins as a single band appeared in western blot analysis, corresponding to *Bur6* protein. In this study we used anti *Bur6* antibody in western blots at a dilution of 1: 10,000/

***Bur6* is nuclear localized Protein**

By using anti *Bur6* antibody we identified that *Bur6* protein is localized in nucleus of *C. albicans* cells. The study confirmed the nuclear localization of *Bur6* using total cell extract, nuclear extract and membrane extract, and by performing western blot analysis we found that *Bur6* specifically localizes in nuclear compartment of cell (Figure 4). This result is in correlation with *in silico* analysis and previous report where it was predicted to be localized in nucleus.[15]

## DISCUSSION

*Candida albicans* exist in microbiome of immunocompromised as well as healthy individuals.[15] Continuous use of antifungal chemotherapy causes acquisition of drug resistance.[24] Major mechanism involved in drug resistance is over expression of efflux pumps.[20] *CDR1* is a major efflux pump involved in drug resistance of *Candida albicans*. In previous study[15] it was shown that beta subunit of NC2 complex which is also known as *Ncb2 is* involve in regulation of *CDR1*.[4] Here in this study, we attempt to characterize role of *Bur6* to identify its functional role in *Candida albicans* drug resistance mechanism.

*Bur6* is one of the major transcription factor involved in *Candida albicans* transcription. The characterization of *Bur6* protein would be helpful in the identifying the role of this protein in basal transcription, pathogenesis and drug resistance mechanism. In *Candida albicans* cells

*Bur6* interact with *Ncb2* protein and form NC2 Complex. In *Candida albicans* NC2 complex binds to TBP and it can suppress transcription by preventing preinitiation complex assembly or stimulate activated transcription.[15] To characterize role of *Bur6* first of all we generated full protein anti *Bur6* polyclonal antibody. The advantage of generation polyclonal antibody against full protein is that it is accessible to any part of protein and sensitivity of detection also increased due to availability of more epitopes.[11]

Polyclonal antibodies have various therapeutic role. polyclonal antisera is mainly used in replacement therapies where patients suffering from immune deficiency disease or neutralization of toxins and viruses. Availability of specific antibody help us to validate the function and fundamental process of particular protein.[2]

In the present study, we successfully cloned the *Bur6* gene of *Candida albicans* which is subunit of NC2 complex. NC2 complex is well known transcriptional regulator in eukaryotic system. Beta subunit of NC2 complex found to be involved in *Candida* drug resistance to identify the role of alpha subunit which is also known as *Bur6*.[23] Here we purified *Bur6* protein by using various techniques and for the first time generated antibody against *Bur6* a subunit of NC2 complex which is important component of transcriptional regulation. The antibody generated has high specificity which was confirmed by western blot analysis as shown in Figure 3. The titer value of antibody was identified by using various dilutions of anti *Bur6* antibody and titer of antibody was measured as 1:10,000 which is quite high. By using western blot analysis here we also identified the nuclear localization of *Bur6* protein. Through this study we were able to generate highly specific antibody against *Bur6* and we successfully identified localization of *Bur6* protein using this antibody. Further studies are necessary to understand the exact role of *Bur6* in *Candida albicans* drug resistance mechanism. In future recombinant *Bur6* protein may further open the doors to understand drug resistance mechanism and function in depth.

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**DECLARATION OF INTEREST**
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

**Figures**

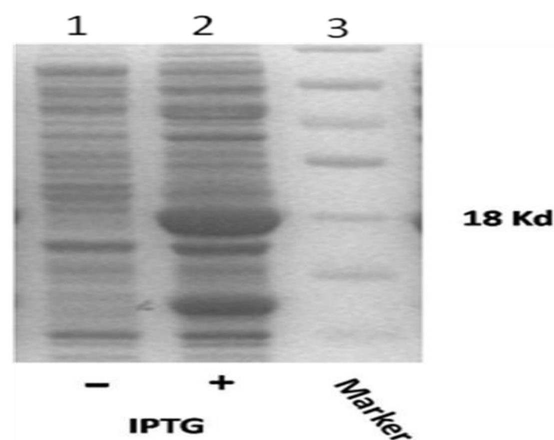


**Figure 1: Induction Profile of *Bur6* Protein:**

**Induction pattern of *Bur6* Protein :** Lane 1 : Bacterial Protein without Induction to IPTG, Lane 2: Bacterial Protein With 1mM IPTG Induction, Lane 3: Marker

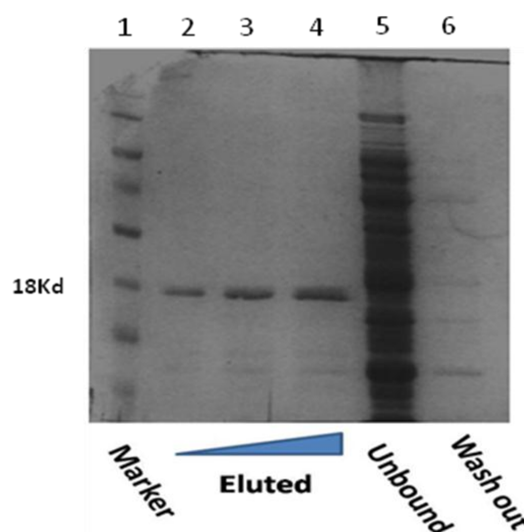


**Figure 2 : SDS-PAGE showing purification of recombinant *Bur6* by Affinity purification.**

**Affinity Purification of His tagged *Bur6* Protein:** Lane 1: Marker, Lane 2 ,3,4 : Eluted sample with incrasing amount of sample (5,10,15µl), Lane 5 : Unbound , Lane 6 : Wash Out

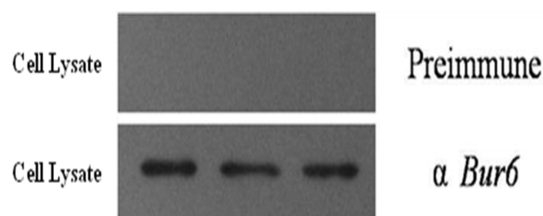


**Fig. 3: Western Blot analysis**

**Western Blot analysis of anti *Bur6* antibody.** Box 1: *Candida* cell lysate probed with pre immune serum, Box 2: *Candida* cell lysate probed with anti *Bur6* antibody.

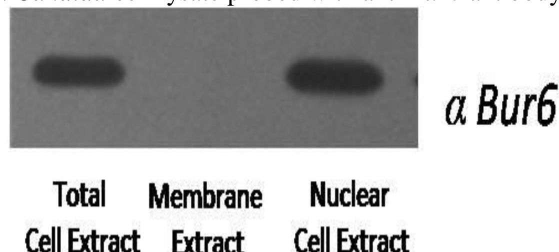


**Figure 4: Western blot analysis showing Nuclear localization of *Bur6***

**Cellular localization of *Bur6*:** Lane 1: total cell extract probed with *αBur6,* Lane 2: membrane extract probed with *αBur6,* Lane 3: Nuclear extract probed with *αBur6*

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