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Cloning of HAC1 gene of Candida parapsilosis using TA cloning method

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Abstract

C. parapsilosis exists as human commensal in the gastrointestinal and genitourinary tract of healthy individuals. However, it is notorious and infamous as it is the fourth leading cause of the bloodborne candidiasis in Europe. In India, several studies have reportedC. parapsilosis in the intensive care unit (ICU). Among the several virulence factors, the HAC1 gene came across as one of the major regulators of virulence in fungal pathogenesis. However, its role in C. parapsilosis pathogenies is not clear. To study the role of HAC1 in fungal pathogenesis, we have cloned this gene. It was also cloned to further study in genetic complementation and antifungal susceptibility assay.Cloning was achieved by the TA cloning method. TA cloning is a restriction endonuclease independent cloning method and based on the on T-overhangs at the cloning site. The efficiency of ligation of PCR products improves owing to the presence of A-overhangs.Here we report thatthe HAC1 gene is 1.15kb, which includes both translated as well as untranslated regions (introns). Further, the gene was characterized based onhomologyand DNA sequencing.

Introduction

Fungi, which are normal commensals of the human body, are mostly harmless. However, in people with low immunity, it becomes pathogenic and causes serious life-threating diseases. *C.parapsilosis* is infamous, as it can grow in total parenteral nutrition, its ability to form biofilms on catheters, medically implanted devices; hence, the patients who have gone invasive medical interventions are more prone to get an infection. Thenosocomial (Hospital-acquired) spread can also occur through hand carriage, as well as its persistence in the hospital environment [1].The isolated *C. parapsilosis* were classified into three groups I, II, and III. The group I *C. parapsilosis*, grouped II named *C. orthopsilosis* and grouped III named *C. metapsilosis*, respectively [2].

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Most of the rough endoplasmic reticulum (ER) associated proteins are co-translated in the ER lumen and folded by ER-resident chaperones. However, this mechanism often fails due to excessive protein load due to several stress conditions. The activation reassures this stress condition of the unfolded protein response (UPR). In some human fungal species, unfolded protein response (UPR) is involved in fungal pathogenesis. Activation of the UPR pathway gave an added advantage to these pathogens in coping with high temperature, nutrient acquisition, biofilm formation, resistance to cell wall disruptors, and antifungal agents. The two key molecules of these pathways are Ire1p and Hac1p [3]. In this study, we have cloned, *HAC1* gene of *C. parapsilosis* using a mighty TA cloning kit.

Material and Methods

Media components, Primers, and Strains

The media components used in the study are listed below in table 1. The components were procured form HIMEDIA®.The media components were all prepared in double distilled water, and an appropriate pH was adjusted whenever required. The media was autoclaved at 121°C at 15psi pressure for 15-20 minutes.

Table 1: Growth Media used in the study

Name	Media composition
1. YEPD/ YPD (broth)	1% yeast extract, 2% peptone, and 2% dextrose
2. YEPD / YPD(agar)	1% yeast extract, 2% peptone, 2% dextrose, and 3% agar
3.CHROMagar	15g peptone, 4g Yeast extract,1g Dipotassium hydrogen phosphate, 7.22g chromogenic mixture, 0.5g chloramphenicol, 15g Agar
4. LB Broth	10g Casein enzymic hydrolysate, 5g yeast extract, 10g Sodium chloride.
5. LB Agar	10g Casein enzymic hydrolysate, 5g yeast extract, 10g Sodium chloride, 15g Agar.

Table 2: List of primersused in the study

Primer Name	Sequence (5′-3′)	Remarks
M39-HAC1, C.p.F	5′ATTggatccATGGACGTAGATACTACTA C3′□	CPAR2_103720, HAC1 of *C.parapsilosis*,

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		Position 1-20 w.r.t to +1 ATG
M39-HAC1, C.p.R	5′CAGaagcttTCATACAGAAACGGCCAGAC3′	1138-1159 *w.r.t to +1 ATG*
M13 Forward	5′GTAAAACGACGGCCAGT3′	Universal primer (17 bases length)
M13 Reverse	5′AACAGCTATGACCATG3′	16 bases length
ITS1 Forward	5′TCCGTAGGTGAACCTGCGG3′	19 bases length
ITS4 Reverse	5′TCCTCCGCTTATTGATATGC3′	20 bases length

 The small letter depicts restriction endonuclease site BamHI and HindIII in forward and reverse primers, respectively.

Table 3: List of strainsused in the study

Strain name	**Type of strain**	**Received from**
Candida parapsilosis	Fungi	MTCC998
CLIB214 (WT strain)	Fungi	A gift from Dr. Butler Lab
HAC1Δ#1 (Disrupted strain)	Fungi	A gift from Dr. Butler Lab
E.coli BL21	Bacteria	MTCC1679

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Genomic DNA and Plasmid DNA Isolation

Genomic DNA: The genomic DNA was isolated from 3 mL overnight grown culture in YPD medium.The cells were harvested andresuspended in 600 µL of Lysis buffer (1% SDS, 100mM NaCl, 10mM Tris-Cl, and 1mM EDTA). To it, 100 µL of the autoclave glass bead and 5 µL of proteinase-K were added. The cells were lysed in vortex mixer 45 sec, in between cells were kept at the ice, this cycle was repeated for 6-8 cycles. After that, 250µL of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and centrifuged at 12000 r.p.m for 5 minutes. Aqueous layer aspirated and transferred into a new 1. mL microfuge tubes. To it, 200µL of Chloroform: Isoamyl alcohol (24:1) wasadded and again centrifuged at 12000 r.p.m for another 5 minutes. The DNA was precipitated by the addition of 50 µL of 7.5M ammonium acetate and 2.5 volumes of 100% ethanol. At last, the DNA sample was stored in

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50µL of 1X TE buffer and stored at -20°C. The purity of DNA was quantified by spectrophotometer and by running agarose gel electrophoresis.

Plasmid DNA: The plasmid DNA was isolated by the NucleoSpin Plasmid isolation kit as per the manufacturer's instruction. Alternatively,the pMD20 T vector was used from Mighty TA cloning KitMacherey-Nagel Germany (Takara Biosciences Pvt. Ltd.).

PCR and Ligation

*PCR:*The PCR reaction was set up for the isolated genomic DNA using gene-specific primers mentioned in table 2. For PCR amplification,rTaq DNA polymerase was used (Takara Biosciences Pvt. Ltd.). For confirmation and diagnostic PCR, alternatively, PCR supermix (Himedia) were also used.

*Ligation:*The ligation reaction was carried out by using the Mighty TA cloning kit (Takara Biosciences Pvt. Ltd.). The reaction was carried out in the vector: insert ratio of 1:3 at 10 µL reaction volume. The ligation mixture was incubated at 16°C for 30 minutes.

Transformation into bacteria and Blue-white screening

The *E. coli*DH5α chemical competent cells were used for transformation using the heat shock method. The cells were subjected to heat shock at 42°C for 45 seconds. The cells were outgrown in SOC medium for 1 hour at 37°C with shaking. After 1 hr incubation, 100µL of transformation mixture was plated on LB/ampicillin/IPTG/X-Gal plates. The plates were incubated overnight at 37°C. After overnight incubation at 37°C, the plates were kept at 4°C to enhance the visibility of the Blue and White colonies.

Results and Discussion

Characterization of *C. parapsilosis*

The strain of *C. parapsilosis* was obtained from the microbial type culture collection (MTCC998), Chandigarh, India. The strain was revived and grown in YPD mediumat 28°C. The cells appear as white creamy, round-shaped smooth colonies (Figure 1). To further characterize the strains, we have performed the PCR amplification of the ITS-I and ITS-II region. The PCR result suggests a band size of 550 bp (data not shown). It is similar to the observation previously reported [4].

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Identification of *HAC1* gene of *C. parapsilosis.*

The *C. parapsilosisHAC1* gene is a transcription factor that is involved in the unfolded protein response (UPR) pathway. The gene was identified initially by the BLAST search using the *HAC1* gene of *S. cerevisiae*as a query[5]. The gene is CPAR2_103720, according to the Candida genome database[6]. To identify whether it is present in our present strain, we have performed the PCR from the isolated genomic DNA. The genomic DNA was isolated using the glass beads method, as described in the methods section. Agarose gel electrophoresis suggests a band size above 10 Kb (Figure 2 A). The purity of DNA optical density (OD) A_{260nM}/A_{280nM} was 1.96. The PCR amplification was performed using gene-specific primers as enlisted in table 2. The conditions for PCR are mentioned in table 4 below.The result suggests the *HAC1* gene size is between 1-1.5 Kb (Figure 2 B).

Table 4: Conditions for PCR reaction

Stage	Temperature	Time
Initial Denaturation	95°C	2.5 min
Denaturation	95°C	20 sec
Annealing	54°C	30 sec
Extension	72°C	45 sec
Final extension	72°C	5 min
Hold	4°C	10 min

Our amplified product is as expected band size predicted from CGD. Based on this, we move ahead with the cloning of the gene.

Cloning of *HAC1* gene of C. *parapsilosis.*

The *HAC1* gene is cloned by the TA cloning method, as described in the methods section. This method uses a pMD20-T cloning vector. The amplified PCR product was purified by column purification method and then ligated in the vector. The ligated product was transformed into DH5α chemical competent cells. The experiment was carried out using LBampicillin plates onto which 40µL each of 20mg/mL X-Gal(5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside) and 100mM IPTG(Isopropyl β- D-1-thiogalactopyranoside) was spread plate with the help of glass spreader, and then plates were allowed to dry for some time. After that 100µL of the transformation,the mixture was spread plate,and the plates were kept at 37°C overnight. After overnight incubation, bothblue, as well as white colonies, were observed(Figure 3). The transformation efficiency of 0.05µg of transformed DNA was 7.09

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X10¹. The value is comparatively very less; we speculate this is due to bad competent cell preparation.

To confirm whether the white colonies were indeed our desired gene, we have randomly picked the white colonies and isolated the plasmid DNA. The plasmid DNA was double digested with BamHI&HindIII. After agarose gel electrophoresis,we found the expected size band (Figure 4A). The result suggests that the insert is present in the isolated plasmid DNA. We also observed other forms of DNA; we interpret it is due to the lack of complete digestion. To further confirm the insert DNA is indeed gene of the interest, we have performed the diagnostic PCR using the gene-specific primers. The result suggests out of the two cloned genes in the plasmid 1 and 2; plasmid 2 harbors the HAC1 gene as it is showing the desired 1.1 Kb band size. The experiment was further confirmed by DNA sequencing using M13 forward and reverse primers (data not shown).

Conclusions.

Based on the restriction digestion, diagnostic PCR, and sequencing experiments, we conclude that the *HAC1* gene of *C. parapsilosis* is around 1.1 Kb. The TA cloning method is a versatile gene cloning technique and gives a reliable result in a short time. It is free from the traditional approach of restriction digestion, ligation, and cloning. The blue-white screening techniques used in the study gave us an added advantage in the identification of the desired clone. Further studies are required to study the same gene is functional in genetic complementation assays.

Declaration of competinginterest

The authors have no conflict of interest to declare

Acknowledgments

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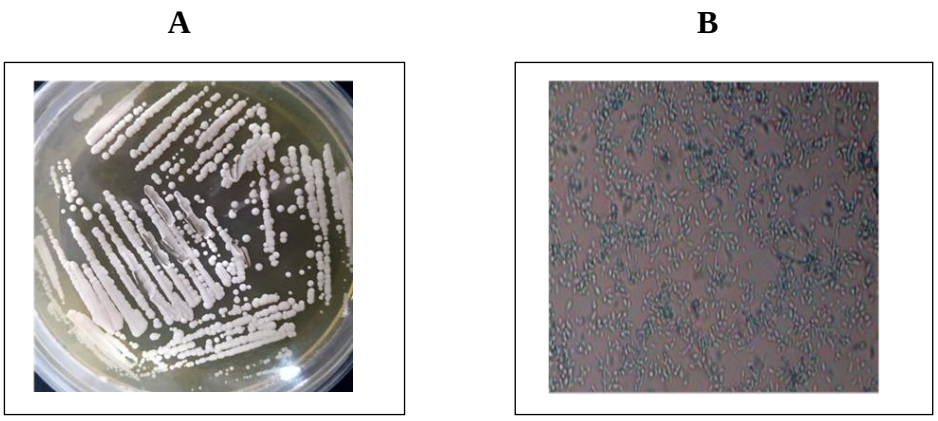

Figure 1:Morphology of *C. parapsilosis*. (A) *C. parapsilosis* strain was grown in YPD medium (B) Lacto-Phenol Cotton Blue stain of *C. parapsilosis*. The cells appeared as creamy white, roundto oval under the microscope.

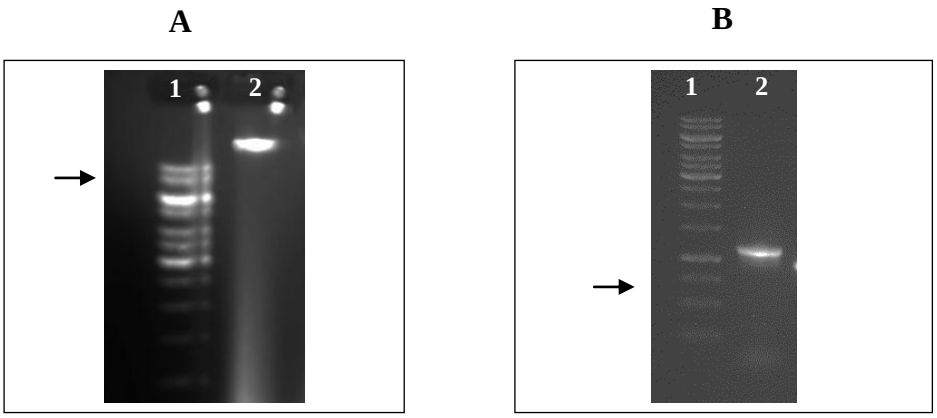

Figure 2:(A) Genomic DNA of *C. parapsilosis*was visualized on 1 % agarose gel. Lane 1, 1 Kb ladder and Lane 2, Genomic DNA. (B) PCR amplified product of the *HAC1* gene shown in lane 2. The arrow mark shows the genomic DNA and PCR amplified band, respectively.

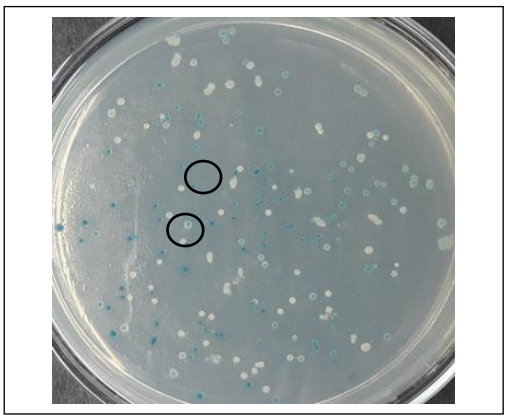

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Figure 3:Blue-White Screening of *HAC1*gene. The LB amp plates containing X-GAL and IPTG as an inducer**.** A plate is showing recombinants (positive clones) and non-recombinants (blue colonies).

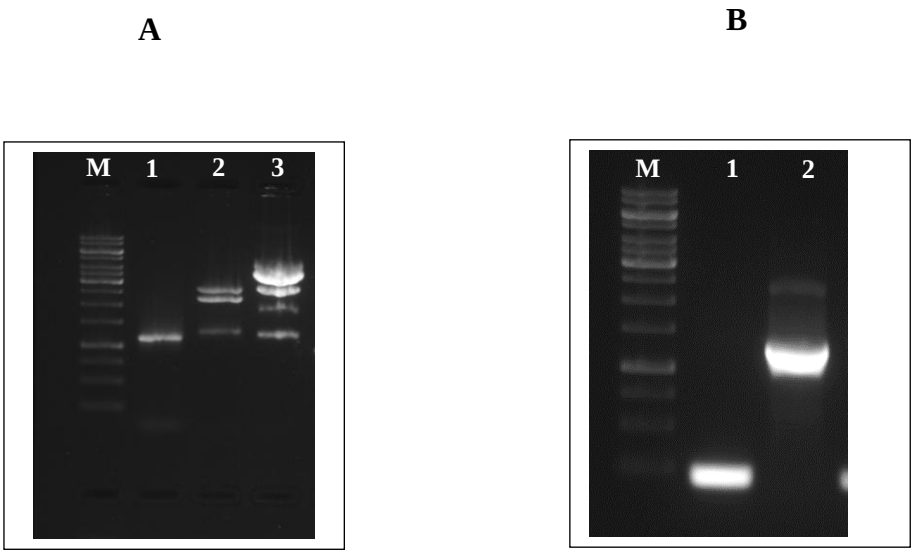

Figure 4:(A)Restriction digestion of the cloned*HAC1* gene. The M denotes 1 Kb ladder; Lane 1 denotes the insert used for the ligation; Lane 2 to 3 depicts double digested plasmid DNA. **(B) Diagnostic PCR of the *HAC1* gene.** The lane M denotes the 1 Kb ladder, Lane 1 false-positive and Lane 2 the desired product size.