EXPRESSION, PURIFICATION AND *IN SILICO* CHARACTERIZATION OF A 100kDa PROTEIN FROM *Histoplasma capsulatum*

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INTRODUCTION

Histoplasmosis is a systemic and endemic mycosis widely distributed in the Americas caused by the dimorphic fungus *Histoplasma capsulatum*. The infection is usually asymptomatic in immunocompetent individuals. However, immunocompromised patients may contract the disseminated form of the disease, which has a bad prognosis and requires rapid diagnosis and treatment. The definitive diagnosis involves the isolation of *H. capsulatum* by culture from clinical specimens, which may take up to 4 weeks. In addition, molecular methods are expensive and have low sensitivity and immunoassays present many false-positive results.

Thus, the aim of this work is to express Hc100, a specific protein of 100kDa of *H. capsulatum*, to develop a novel direct immunoassay and to perform characterization studies of this protein as a first approach for the potential development of novel therapeutic strategies.

RESULTS

FIGURE 1. *Pichia pastoris* transformation. The gene that encodes for Hc100 was optimized for expression in *P. pastoris* and constructed with a secretory signal (α-MF) and a polyhistidine-tag. This construction was under the control of the methanol inducible promoter *aox* in the plasmid pPICZα A. *Pichia pastoris* X-33 strain was then transformed by electroporation and several colonies were obtained.

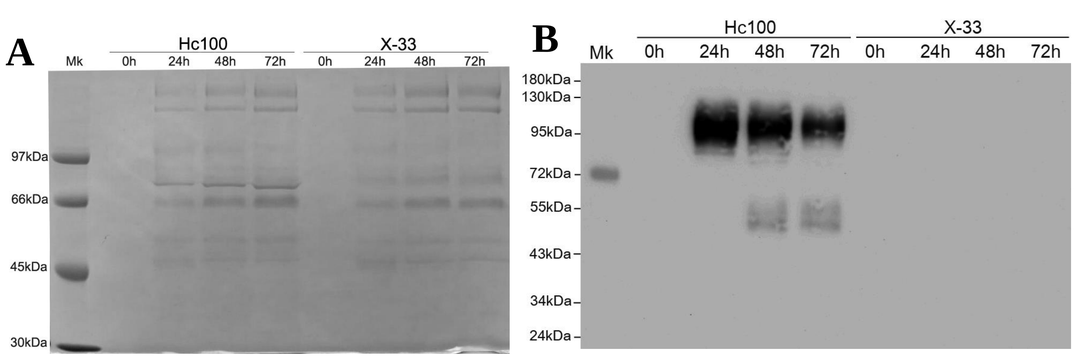

FIGURE 3. Kinetics of Hc100 expression in the cell culture supernatants. *P. pastoris* X-33 strain and clone A1 were grown in BMMY for 72h with pulse of 0.5% methanol every 24h to maintain induction. Cell culture supernatants collected at different time points of induction were analyzed by 8% SDS-PAGE following *Coomasie-blue* R250 staining (**A**) and Western blot (**B**) with anti-histidine antibodies. A band of the expected size was observed in the supernatants at 24, 48 and 72h of methanol induction with the highest expression level at 24h of induction. Also, a lower molecular weight band was observed at 48 and 72h of induction, probably due to degradation processes.

FIGURE 5. *In silico* characterization of Hc100. Hc100 is a 890-amino-acid protein with a theoretical molecular weight of 98.6kDa. Four Staphylococcal nuclease(SNase)-like domains of ~150 amino acids each, a shorter SNase like domain of ~100 amino acids and one Tudor domain were predicted using the Pfam 31.0 server (EMBL-EBI). The SN-like domains consists of a subdomain A, which contains an oligonucleotide/oligosaccharide-binding-fold, and a subdomain B containing two alpha helices. However, these SNase-like domains lack the active site residues of nuclease A. The tudor domain is a domain of unknown function that it has been described in several RNA-binding proteins.

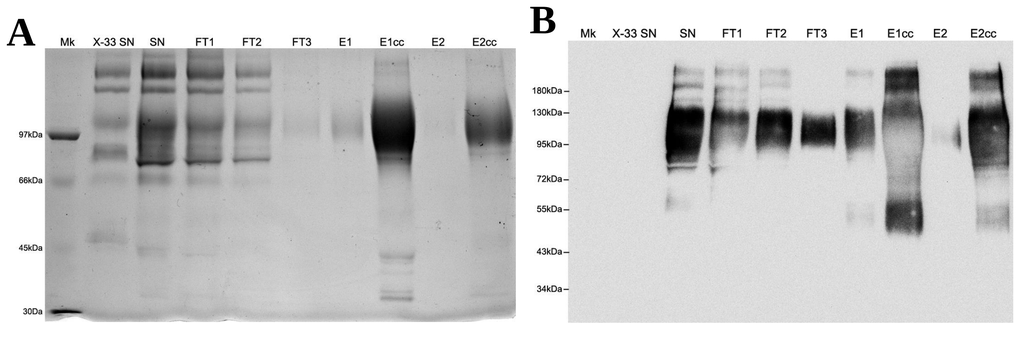

FIGURE 7. Purification of Hc100. Purification of Hc100 from a 24h cell culture supernatant (SN) was carried out using a Ni-NTA affinity chromatography column. Flow-throws from washes (FT1-3) and eluates (E1: 100mM Imidazole; E2: 250mM Imidazole) were analyzed by 8% SDS-PAGE following *Coomasie-blue* R250 staining (**A**) and Western blot (**B**). Purity of Hc100 in E1 was ~90%.

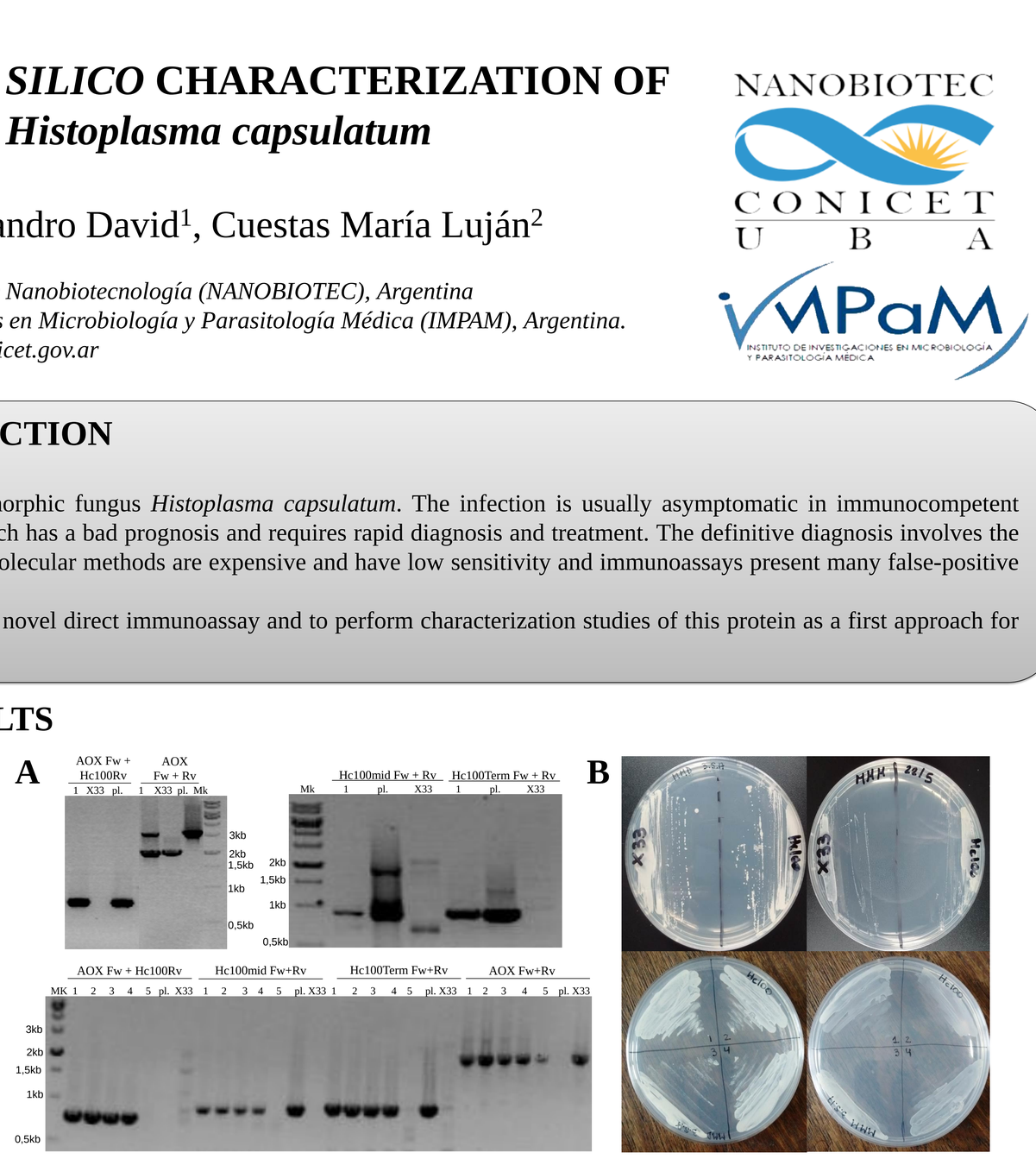

FIGURE 2. Genotype and phenotype of *P. pastoris* transformants. **A.** Genotype of five clones (1-5) was analyzed performing a *Colony* PCR using four sets of primers that amplified the initial (AOX Fw+Hc100Rc), middle(Hc100mid Fw+Rv) and terminal (Hc100Term Fw+Rv) region of the Hc100 sequence and the AOX and Hc100 complete genes (AOX Fw+Rv). Four out of five clones were positive for all amplification reactions. **B.** Phenotype was analyzed by growing the five clones and the X-33 strain in Minimal Dextrose Medium (MMD) and Minimal Methanol Medium (MMM) agar plates for 48h. All clones grow as the same rate as the X-33 strain, suggesting that all were phenotype Mut+ (Methanol utilization Plus).

MFRPRLRREGDEVKFDISPGCPLHGFPVSGSSCPSAVLDGSLLTSYYFALTAQPFAFQSREFLRELLVGKVVKFQVDYTVPTRNGNTMIVKLHNNQENLAELCVAEGWVKVREDAG
KREESEDIVATVDKLRELENRARSESKGVWASTGGELETAYEVPDPKALIESEKGNQIAAVVERVLSGDRLIVRLLVAPHKHIQTLVVVAGIRAPATKRTNPDGTEQPGEPLGEQAQQF
VELRLLQRKVKISLLGVTPQNQLVAGVLHPNGNIAKFLLEAGLARCADHHSTMIGKDMTTLRQAENAAKEARKGLFMSHNAPKVGAGAAQADYVVTRVFSADTIFVRTKTGKDEKRIS
LSSVRHPRTSDPKQAPFILEAKEFMRKKLIGKHVKVKIDGKRPASEGYEEREVGTVMSGNTNIALALVQAGYASVIRHRRDDDDRSPEYDDLLRAEEAAQKEGKGMWESSKPPTVRA
PQDYSENVQKAKIQASVLQRQRKVPGVVDFVKSGSRFTILLPKDNAKLTLVLSGIRAPRSARNPGETGEPFGQEAHDFAYRRCMQRDVEIDVETIDKVGGFIGSLYINRESFSKILVEE
GLATVHAYSAEQGGHAAELFAAEKKAKEARKGLWHDWDPSKDLEEGETVATNGKNGAEAGADAPQQRKKDYRDVMVTNVDENGKLKIQQIGAGTTALTEMMSAFRAFHLNKAND
TALSGPPKAGDLVAARFTEDNEWYRAKIRRNDREAKKADVVYIDYGNSETVPWTRLRPLTQPQFSVQKIRPQATDTVLSFLQLPASPEYLRDAVGYLGERTLDRQLVANVDYTAPDG
TLHVTLMDPAESKSLEHSINADVISEGLAMVPRKLKEWERSSTETLAHLEKLENEAKEGRKGMWEYGATED

FIGURE 4. Mass spectrometry analysis. The band of 100kDa from the 24h induction cell culture supernatant was analyzed by HPLC-MS. A total of 205 peptides of high (light blue) and medium (grey) confidence were found to correspond to the Hc100 covering the 47% of the sequence, which confirmed its identity.

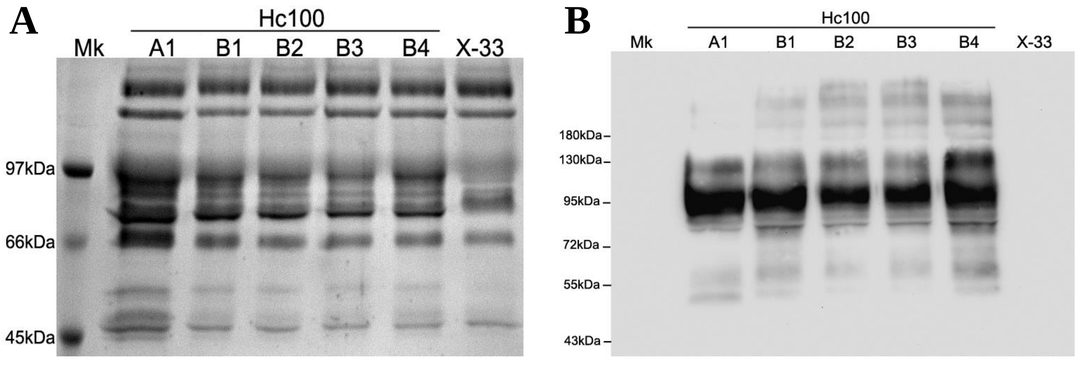

FIGURE 6. Hc100 expression in the different clones. Cell culture supernatants of 24h cultures of *P. pastoris* X-33 strain and Hc100 clones A1 and B1-4 were analyzed by 8% SDS-PAGE following *Coomasie-blue* R250 staining (**A**) and Western blot (**B**). A band of the expected size was observed in the supernatants of all clones. Also, higher and lower molecular weight bands were observed, probably corresponding to oligomeric and degradated forms of the protein, respectively.

CONCLUSIONS

- ✓ The Hc100 from *H. capsulatum* was successfully expressed in the cell culture supernatant of the methyilotrophic yeast *P. pastoris*.
- ✓ The identity of the Hc100 was confirmed by Western blot and mass spectrometry.
- ✓ The purification procces of Hc100 based on a Ni-NTA affinity chromatography reached a purity of ~90%.
- ✓ *P. pastoris* proved to be a valid biotechnological tool for the expression of the Hc100, thus encouraging the national production of novel fungal antigens for the potential development of new rapid diagnostic tests for this clinical relevant form of the histoplasmosis disease.