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Sequencing and heterologous expression in *Saccharomyces cerevisiae* of a *Cryptococcus neoformans* cDNA encoding a plasma membrane H^+-ATPase

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Abstract

A cDNA containing an open reading frame encoding a putative plasma membrane H^+-ATPase in the human pathogenic basidiomycetous yeast *Cryptococcus neoformans* was cloned and sequenced by means of PCR and cDNA library hybridization. The cloned cDNA is 3475 bp in length, containing a 2994 bp open reading frame encoding a polypeptide of 997 amino acids. As in the case of another basidiomycetous fungus (*Uromyces fabae*), the deduced amino acid sequence of *CnPMA1* was found to be more homologous to those of P-type H^+-ATPases from higher plants than to those from ascomycetous fungi. In order to prove the sequenced cDNA corresponds to a H^+-ATPase, it was expressed in *Saccharomyces cerevisiae* and found to functionally replace its own H^+-ATPase. Kinetic studies of CnPMA1 compared to ScPMA1 show differences in V_{max} values and H^+-pumping in reconstituted vesicles. The pH optimum and K_m values are similar in both enzymes.

Keywords: P-type H^+-ATPase; cDNA sequence; Human pathogenic yeast; Heterologous expression; *Cryptococcus neoformans*

1. Introduction

The encapsulated yeast *Cryptococcus neoformans* causes serious and often fatal meningitis in patients with AIDS and in other immunocompromised patients [1]. The treatment options for cryptococcal infections are quite limited and the development of fungicidal antimicrobial agents for cryptococcosis is urgently needed. Unlike the better known ascomycetous human pathogenic fungi, *C. neoformans* is a basidiomycete. This group comprises a wide range of fungi, including mushrooms and many plant pathogens and symbionts.

The plasma membrane H^+-ATPase of *Saccharomyces cerevisiae* is an integral membrane protein belonging to the P-type class of ion-translocating ATPases. It is an electrogenic proton pump that regulates intracellular pH, maintains ion balance and generates the electrochemical proton gradient necessary for nutrient uptake [2]. This enzyme is the major protein in the plasma membrane, regulating the rate of cell growth

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[3], and it can be a new target for antifungal drug design [4]. The corresponding gene, *PMA1*, has been sequenced in 10 fungi and it is essential to yeast viability [5]. A second plasma membrane H^+-ATPase encoded by the gene *PMA2* was described in *S. cerevisiae* and is dispensable in the presence of a functional copy of *PMA1* [6]. In *Schizosaccharomyces pombe*, there are *PMA1* and *PMA2* genes with properties similar to those of their *S. cerevisiae* homonyms [7].

In plants, the proton pump of the plasma membrane is encoded by a large family of genes and they are expressed as well as regulated in a different way [8]. The heterologous expression of these genes in *S. cerevisiae* was initiated with the *AHA1*, *AHA2* and *AHA3* isoforms from *Arabidopsis thaliana*. Among them, only *AHA2* was able to sustain yeast growth at low level when expression of the yeast H^+-ATPase was turned off [9]. However, *pma2* and *pma4*, two isoforms of *Nicotiana plumbaginifolia*, were expressed in *S. cerevisiae* and successfully replaced the yeast H^+-ATPase gene, allowing cell growth [10–12].

When the biotrophic rust fungus *Uromyces fabae* cDNA encoding a putative P-type H^+-ATPase was expressed in *S. cerevisiae* [13], both the wild-type enzyme and a mutant derivative deleted for the 76 C-terminal amino acids were able to support yeast growth too.

To study the role of plasma membrane H^+-ATPase in *C. neoformans* and to enhance our knowledge of gene structure and function as related to membrane transport in this organism, we have cloned and characterized the gene *CnPMA1*. A cDNA of *C. neoformans* was isolated and sequenced, and the deduced amino acid sequence was compared with H^+-ATPases from other organisms. We have analyzed the expression of this gene in a *S. cerevisiae* strain deleted of its own two H^+-ATPase genes (*PMA1* and *PMA2*) to prevent their recombination with the cryptococcal *PMA1*. CnPMA1 is able to sustain *S. cerevisiae* growth, and after that we have biochemically characterized this enzyme.

2. Materials and methods

2.1. Media

C. neoformans cells were grown in the rich medium YPD (1% yeast extract, 2% peptone, 2% glucose). *S. cerevisiae* cells were grown in a medium with 2% of either glucose or galactose, 0.7% Yeast Nitrogen Base without amino acids (USBiologicals, Swampscott, MA, USA) and the appropriate requirements. Solid media contained, in addition, 2% agar. When indicated, medium was buffered with 50 mM Mes adjusted to pH 6.5 with Tris, or with 50 mM succinic acid adjusted to pH 3.0. The 5-fluoroorotic acid (5-FOA) medium was prepared as described previously [14].

2.2. Strains

C. neoformans B-3501 (ATCC 34873) [15] is a serotype D strain used for the cloning of *CnPMA1*. *S. cerevisiae* strain YAK2 [10] with the genomic markers: *MAT*α, *ade2-101, leu2*Δ*1, his3-*Δ*200*, *ura3-52, trp1*Δ*63, lys2-801, pma1-*Δ*::HIS3, pma2-*Δ*::TRP1* and carrying the plasmid *cp(GAL1)PMA1 (p(GAL1)::PMA1, CEN6, ARSH4, URA3)* was used for heterologous expression of *CnPMA1*.

Yeast cells were transformed according to Ito et al. [16].

The *Escherichia coli* strain XL1Blue (Stratagene) was used for plasmid transformation and amplification.

2.3. Plasmid constructions

The pRS316·p(*PMA1*)·t(*ADC1*) *Bgl*I fragment containing *URA3, ARSH4, CEN6* and part of *amp*^{r} was replaced with the corresponding *Bgl*I fragment of cp(*PMA1*)*pma2* [10] (derived from pRS315) containing *LEU2, ARSH4, CEN6* and the same part of *amp*^{r} yielding pRS315·p(*PMA1*)·t(*ADC1*) (centromeric, *LEU2*).

The *Sac*I–*Apa*I (blunted by T4 DNA polymerase) fragment (1400 bp) of the pRS315·p(*PMA1*)·t(*ADC1*) plasmid containing the promoter region of the *S. cerevisiae PMA1* gene and the terminator region of the *S. cerevisiae ADC1* was inserted between the *Sac*I site and the *Pst*I site (blunted by T4 DNA polymerase) of the Yeplac181 plasmid [17] yielding 2 μp(*PMA1*)·t(*ADC1*) (multicopy, *LEU2*).

The *PMA1* cDNA from *C. neoformans* B-3501 was cloned as a 3475 bp *Eco*RI–*Xho*I fragment in the pBluescriptSK(−) starting 187 bp upstream from

the initiation ATG of the *PMA* open reading frame and extending 294 bp downstream the stop codon. In order to avoid any interference of the 5′ and 3′ untranslated regions with the expression of *CnPMA1* in *S. cerevisiae*, these were eliminated by polymerase chain reaction (PCR). The oligonucleotides BPC5 (5′-CGCGGATCCAATACAACCATGTCTG-3′) and BPC3 (5′-TGCACTGCAGCTCTTTCAACGCGAACG-3′) were used. The sequence of BPC5, flanked by a *Bam*HI site, corresponds to nucleotides −12 to +7 of the *CnPMA1* translation initiation codon. BPC3 is complementary to nucleotides +3003 to +3019 downstream of the stop codon and contains a *Pst*I site. The 3 kb *Bam*HI–*Pst*I PCR fragment was cloned in pUC18 (Pharmacia) and sequenced to confirm the correct sequence of *CnPMA1* cDNA. The PCR was performed with Expand™ Long Template PCR System (Boehringer Mannheim). This fragment was introduced into the pRS315·p(*PMA1*)·t(*ADC1*) plasmid and into the 2 μp(*PMA1*)·t(*ADC1*) plasmid.

pSB32 (centromeric, *LEU2* plasmid) [18] and pSB32·*ScPMA1* (centromeric, *LEU2* plasmid bearing *ScPMA1* cDNA under the control of *ScPMA1* promoter) were used as controls for the transformation and complementation of YAK2.

2.4. Preparation of genomic DNA from C. neoformans

Genomic DNA from *C. neoformans* B-3501 was isolated essentially as described by Varma and Kwon-Chung [19]. Standard molecular biology procedures were performed according to Sambrook et al. [20].

2.5. Identification of PMA sequences in C. neoformans

Two amino acid sequences conserved in all P-type H^+-ATPases, CSDKTGT (phosphorylation site) and TGDGVNDA (ATP-binding site) [21], were chosen to synthesize degenerate oligonucleotide primers, in which *Bam*HI restriction site was flanking the phosphorylation site as well as *Eco*RI restriction site was flanking the ATP-binding site. Using these primers and the genomic DNA from *C. neoformans* as template, a fragment of 800 bp was amplified by PCR. This fragment was purified with Prep-A-Gene DNA Purification System (Bio-Rad), digested with *Eco*RI and *Bam*HI and then inserted into the pGEM-7Zf(±) vectors (Promega). Nucleotide sequencing revealed an open reading frame encoding an amino acid sequence of 268 residues with an identity between 35 and 55% to known plasma membrane H^+-ATPases.

To obtain the entire coding sequence, the lambda Uni-Zap XR cDNA library of *C. neoformans* B-3501 (Stratagene) was screened under stringent conditions with the 800 bp PCR fragment as the hybridization probe. Several phages in the cDNA library contained sequences that hybridized with the PCR fragment. The plasmids containing hybridizing cDNA inserts were rescued according to the manufacturer's instructions. They were sequenced from both directions using fluorescent cycle sequence analysis on an ABI PRISM 377 sequencing apparatus (Perkin-Elmer) in conjunction with sequence analysis software.

2.6. Plasma membrane preparations and kinetic studies

S. cerevisiae plasma membranes were purified from glucose-metabolizing cells by differential and sucrose gradient centrifugation [22]. ATPase activity was assayed at 30°C in buffer containing 50 mM MES, 5 mM $SO_4Mg_2{\cdot}7H_2O$, 5 mM sodium azide to inhibit mitochondrial ATPase, 0.2 mM ammonium molybdate to inhibit acid phosphatase, and 50 mM potassium nitrate to inhibit vacuolar ATPase. This buffer was adjusted to pH 6.5 with Tris. Residual activity corresponded to specific ATP hydrolysis by the plasma membrane H^+-ATPase, as it was inhibited more than 80% by 0.2 mM diethylstilbestrol and more than 90% by 10 μM vanadate [2]. Protein concentration was determined by the method of Bradford [23], with the Bio-Rad protein assay reagent and bovine IgG as the standard.

2.7. Preparation of sealed plasma membranes

A 2 ml aliquot of a suspension of purified asolectin (75 mg/ml) was sonicated at room temperature in buffer R, which contained 25 mM K_2SO_4, 20% (w/v) glycerol and 10 mM BisTris adjusted to pH 7.5 with MES 0.5 mM, to form liposomes. Then, 1.5 ml of these was mixed with 500 μl of 8% (w/v) deoxy-

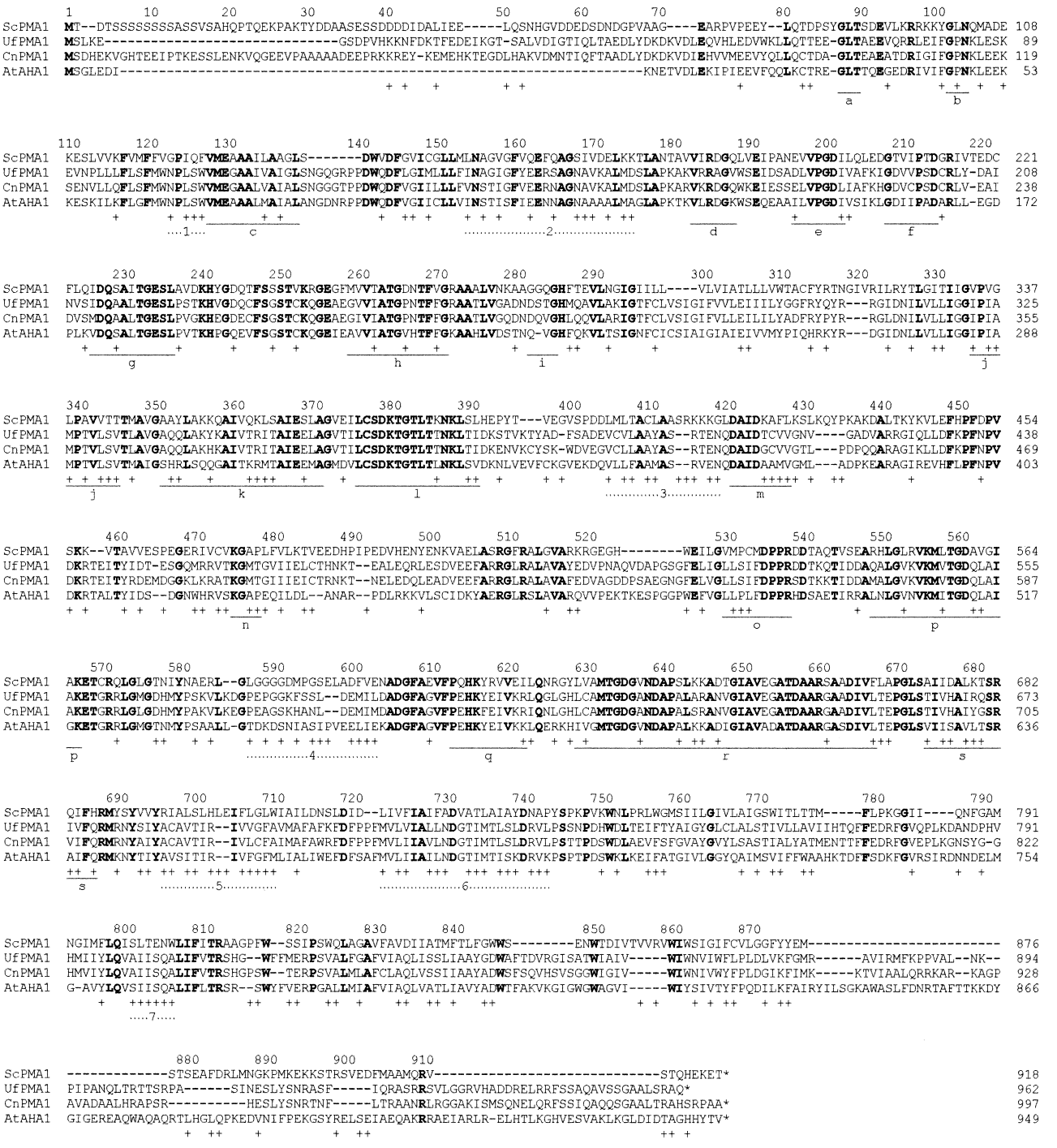

Fig. 1. Alignment of amino acid sequences of H^+-ATPases from an ascomycete (*S. cerevisiae*, ScPMA1), two basidiomycetes (*U. fabae*, UfPMA1, and *C. neoformans*, CnPMA1) and a plant (*A. thaliana*, AtAHA1). Previously reported sequences were obtained from the GenBank database. The alignment was initially performed by using the program Clustal V in MegAlign/Lasergene software (DNASTAR, Madison, WI, USA) [27] using the default parameters, and then refined manually. The numbers above the sequences are positions in ScPMA1; last digit of each number is aligned with the corresponding amino acid. Amino acid numbers of each sequence are to the right. Boldface indicates residues that are identical among the four ATPases. The 20 conserved regions (a–s) present in all P-type ATPases [21] are underlined. +, residues of ScPMA1 present at least in 10 sequences from other ascomycetes and not present in *Cryptococcus* and/or *Uromyces*. Dotted line designates regions (1–7) where these differences between ascomycetes and basidiomycetes are more pronounced. Gaps (−) were introduced to maximize the alignment. * indicates stop codon.

cholate in the buffer R, followed by 1 ml of the same buffer, and stored on ice [24]. Plasma membranes (2.5 mg protein) were diluted with buffer R and stored on ice. The mix of liposomes–deoxycholate was added to the plasma membrane suspension (2 ml) at 0°C and the mixture shaken manually every 15 s for 10 min. Then it was centrifuged at $100\,000 \times g$ for 1 h. The pellet was resuspended in 700 μl of buffer R.

2.8. Measurement of ATP-dependent proton transport

ATP-dependent proton translocation was measured by recording the fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA). A Perkin-Elmer fluorimeter (Luminescence Spectrometer LS50) with the excitation wavelength set at 410 nm and emission at 500 nm was used to measure the fluorescence. The assay mixture contained about 100 μg protein from *ScPMA1* cells or 375 μg from *CnPMA1* cells, and 10 μl of ACMA in a total volume of 3 ml with buffer R. A 100 μl aliquot of MgATP solution (MgATP in 10 mM MES, adjusted to pH 7.5 with Tris at final concentration 3 mM) was added after 3 min of incubation at 30°C to start the reaction. The amounts of ATP and Mg^{2+} to be added to the mixture at the pH indicated were calculated as described by Wach et al. [25] to obtain the desired concentration of MgATP and Mg^{2+}.

3. Results and discussion

3.1. Sequence analysis of an H^+-ATPase-encoding gene from C. neoformans

The nucleotide sequence data (about 3475 bp) of the longest cDNA clone obtained appear in the GenBank nucleotide sequence databases with the accession number AF077766. This sequence contained a putative open reading frame of 2994 bp in length starting from the ATG of the sequence ACAACCATGTC which fits the consensus sequence around the start codon for translation initiation by eukaryotic ribosomes as reported by Kozak [26]. This ATG is preceded by two in-frame TAA stop codons located 15 and 18 nucleotides upstream. Polyadenylation occurred at a site 276 bp after the stop codon. No canonical polyadenylation signal was found, as in the case of other sequenced *C. neoformans* genes [1]. The open reading frame encodes a protein of 997 amino acid residues with a calculated molecular mass of 108 692 and an isoelectric point of 5.3.

3.2. Comparison to other H^+-ATPase DNAs and protein sequences

Searching DNA and deduced amino acid sequence homology on the cDNA clone using the GenBank database revealed the highest overall sequence homology to P-type ion-transport ATPases, and more specifically, to H^+-ATPases. In addition, the primary structure contains most of the conserved sequences characteristic of this class of H^+ pumps (Fig. 1) [21]. Interestingly, *C. neoformans* H^+-ATPase is 50–54% identical to the P-type H^+-ATPase from higher plants, but only 27–32% identical to the corresponding proteins from 10 *PMA* genes of eight fungal species belonging or related to the *Ascomycota* (see legend of Fig. 2). So far, only one other P-type H^+-ATPase from a basidiomycetous fungus has been described; this is from the biotrophic rust fungus *U. fabae* [13]. As expected, the new sequence from *C. neoformans* is more homologous to this of *U. fabae* (70% of identity) than to those of ascomy-

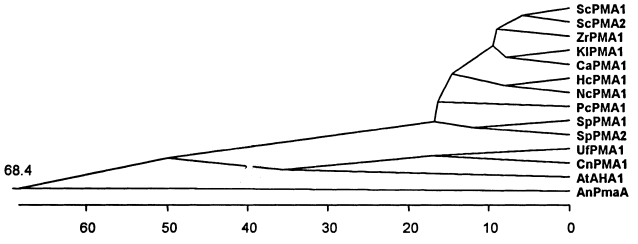

Fig. 2. Phylogenetic analysis of the plasma membrane H^+-ATPase based on the amino acid sequences from various fungi and one plant. The dendrogram was constructed from 13 different sequences found in the GenBank database plus the *C. neoformans* H^+-ATPase sequence reported here. The Clustal V method [27] was used for multiple alignment of the amino acid sequences. The length of each pair of branches represents the distance between sequence pairs. ScPMA1, *S. cerevisiae* 1; ScPMA2, *S. cerevisiae* 2; SpPMA1, *S. pombe* 1; SpPMA2, *S. pombe* 2; KlPMA1, *Kluyveromyces lactis*; ZrPMA1, *Zygosaccharomyces rouxii*; CaPMA1, *Candida albicans*; HcPMA1, *Histoplasma capsulatum*; NcPMA1, *Neurospora crassa*; PcPMA1, *Pneumocystis carinii*; UfPMA1, *U. fabae*; CnPMA1, *C. neoformans*; AtAHA1, *A. thaliana* 1; AnPmaA, *A. nidulans.*

cetous fungi (Fig. 2). Most striking are several residues non-conserved in these two basidiomycetes as compared to ascomycetous fungi (Fig. 1). According to the dendrogram of 14 P-type H^+-ATPase sequences from fungi and one representative plant shown in Fig. 2, H^+-ATPases can be divided into four groups: (1) all the ascomycetous fungi but *Aspergillus nidulans*, (2) basidiomycetous fungi, (3) plants and (4) *A. nidulans*. The PmaA H^+-ATPase of *A. nidulans* also displays lower levels of amino acid sequence identity with those from the other ascomycetes (Fig. 2). These differences suggest that structure–function relationships within the enzyme have not been so highly conserved as previously reported [21].

The *C. neoformans* ATPase is the larger PMA1 protein reported. Only the PMA2 proteins from *S. cerevisiae* (947 amino acids) and *S. pombe* (1010 amino acids), which represent a different class of fungal proton pumps, are more similar in size to the ATPases from basidiomycetes, although this similarity does not exist at the sequence level (Fig. 2). Hydropathy analysis of the *C. neoformans* H^+-ATPase presented 10 putative transmembrane helices (data not shown) which is consistent with the current structural model of fungal and higher plant H^+-ATPases [2].

3.3. Functional expression of CnPMA1 in S. cerevisiae

The *C. neoformans PMA1* cDNA was cloned into the *LEU2* centromeric expression vector pRS315·p(*PMA1*)·t(*ADC1*) and into the *LEU2* multicopy plasmid 2 μp(*PMA1*)·t(*ADC1*) under the control of the *S. cerevisiae PMA1* promoter. The pSB32 and pSB32·p(*PMA1*)·*ScPMA1* plasmids were used as controls in the transformation (see Section 2).

These four plasmids were transformed into the haploid *S. cerevisiae* strain YAK2. This strain was deleted from its chromosomal H^+-ATPase genes *PMA1* and *PMA2* but can survive with the yeast *PMA1* under the control of the *GAL1* promoter in the centromeric plasmid pRS316 [10–12]. Independent transformants obtained in MGal-His, Leu, Trp, Ura were plated on MGlu-His, Leu, Trp, Ura medium and they could grow, showing the *C. neoformans* H^+-ATPase is able to support *S. cerevisiae* growth. Then the loss of the cp(*GAL1*)*PMA1* plasmid was induced by plating the transformants on a Glu medium containing 5-FOA, and after that treatment the surviving colonies had lost the *URA3* plasmid. Fig. 3 shows the growth at different pH levels of *S. cerevisiae* cells expressing its own H^+-ATPase (from the *PMA1* gene in the pSB32 plasmid) which can grow at pH 3.0, pH 5.5 and pH 6.5, or expressing *CnPMA1*. Among the pH levels shown, CnPMA1 can only replace the *S. cerevisiae* H^+-ATPase at pH 5.5 and 6.5.

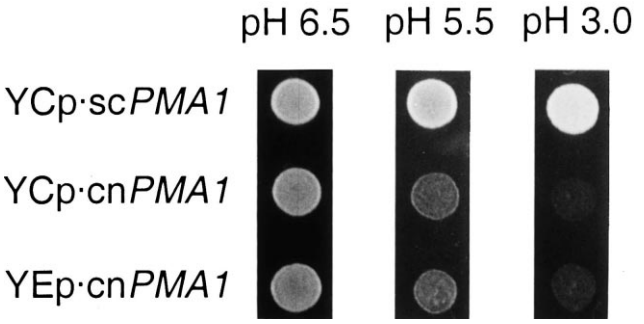

Fig. 3. The *C. neoformans* PMA1 supports *S. cerevisiae* growth. Strains YAK2+ YCp·sc*PMA1* (plasmid pSB32·Sc*PMA1*), YAK2+ YCp·cn*PMA1* (plasmid pRS315·p(*PMA1*)·t(*ADC1*)) and YAK2+ YEp·cn*PMA1* (plasmid 2 μp(*PMA1*)·t(*ADC1*)) were grown in MGlu-His, Leu, Trp at pH 6.5 containing 5-FOA. Colonies were plated on MGlu-His, Leu, Trp and then spotted onto solid media MGlu-His, Leu, Trp at different pH.

3.4. Enzymatic properties of C. neoformans PMA1

Before analyzing the kinetic properties of the CnPMA1 expressed in *S. cerevisiae*, it was of interest to determine whether or not the enzyme responds to activation by glucose. The ATPase activity of the enzyme from glucose-starved and glucose-fermenting cells was compared. We used *S. cerevisiae* cells grown in glucose medium, harvested in exponential phase and, before homogenization, incubated 7 min in 2% glucose. Glucose treatment largely released ATPase activity 7-fold in plasma membranes from yeast cells expressing *ScPMA1* whereas the CnPMA1 was defective in glucose-induced activation (data not shown).

The apparent K_m for ATP and V_{max} for ATP hydrolysis were determined on a purified plasma membrane fraction from glucose-fermenting cells (Table 1). The Eadie–Hofstee plots showed a higher V_{max}

for ScPMA1 compared to CnPMA1, whereas their K_m values were not significantly different, indicating a similar affinity for the substrate.

The pH optimum was found to be 6.5 for both *S. cerevisiae* and *C. neoformans* enzymes (Table 1).

Vanadate, a specific inhibitor of cation-transporting ATPases forming a phosphorylated intermediate during their catalytic cycle, inhibited the ATPase activity of the *ScPMA1*-expressing strain, with a K_i of 1 μM. The CnPMA1 ATPase activity was also inhibited by vanadate, but its half maximal inhibition was obtained at 2.5 μM (Table 1).

We must consider these results as a first approach to study CnPMA1 because all these assays take into account the total amount of plasma membrane proteins applied. Gel electrophoresis of the plasma membrane fraction from *ScPMA1*- and *CnPMA1*-expressing strains revealed a lower expression level in *S. cerevisiae* of *CnPMA1* compared to *ScPMA1* (data not shown).

In order to provide a final proof that the *C. neoformans* PMA1 is indeed a proton pump, we studied the ATP-dependent proton translocation using purified plasma membranes [28] resealed by a lecithin/deoxycholate treatment [24]. The proton-pumping activity of *C. neoformans* PMA1, and *S. cerevisiae* PMA1 as control, illustrated in Fig. 4, was estimated by measuring the rate of ATP-dependent quenching of ACMA fluorescence. ACMA quenching was partially released by the proton-specific ionophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The initial rate of ATP-dependent proton-pumping was proportional to the ATPase activity measured at the same pH of the assay, pH 7.5, of ScPMA1- and CnPMA1-containing vesicles. The rate of quenching is much lower in CnPMA1-containing vesicles, but we must consider the ATPase activity is also lower than ScPMA1 ATPase activity, so it can be a problem of real quantity of enzyme in plasma membranes from *S. cerevisiae*.

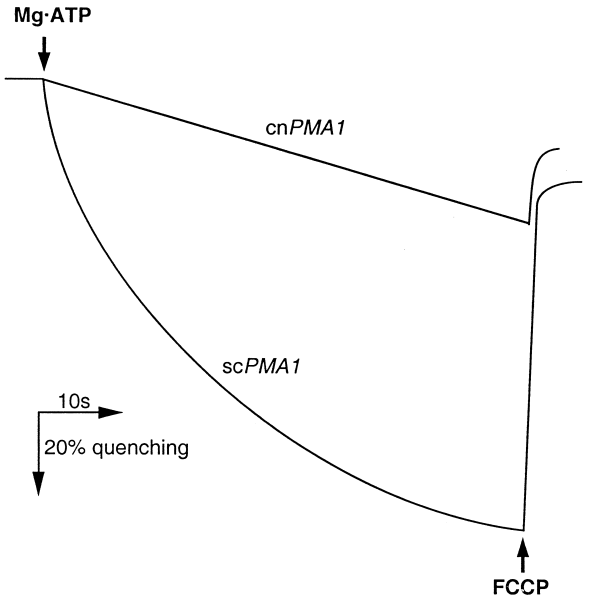

Fig. 4. ATP-dependent fluorescence quenching of ACMA in the presence of sealed plasma membrane vesicles from *S. cerevisiae* expressing only its own *PMA1* gene and *S. cerevisiae* expressing only *C. neoformans PMA1* gene.

In conclusion, this is the second plasma membrane H^+-ATPase gene from a basidiomycetous fungus that has been sequenced, confirming that these proteins form a new phylogenetic cluster more identical to the P-type H^+-ATPases from higher plants than from ascomycetous fungi. In spite of this, the cryptococcal H^+-ATPase can fully complement a *S. cerevisiae* mutant lacking *PMA1* and *PMA2*. In addition to enhance our knowledge base of *C. neoformans* genomic architecture, the structural and functional information about this human pathogen plasma membrane H^+-ATPase will be useful for elucidating if it can be a new target for antifungal development.

Table 1
Kinetics of *S. cerevisiae* PMA1 and *C. neoformans* PMA1 expressed in YAK2[a]

Expressed gene	K_m (mM)	V_{max} (μmol/min/mg protein)	K_i (μM)	Optimum pH
ScPMA1	1.60	1.40	1.00	6.50
CnPMA1	1.50	0.33	2.50	6.50

[a]Plasma membranes were purified from the strain YAK2 expressing only the *S. cerevisiae PMA1* and the same strain YAK2 expressing only the *C. neoformans PMA1* grown in media containing glucose buffered at pH 6.5 (SD6.5), and incubated in the presence of glucose. The conditions of the assays are described in Section 2.

Acknowledgements

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