RESEARCH ARTICLE

Biochemical characterization of an ecto-ATP diphosphohydrolase activity in *Candida parapsilosis* and its possible role in adenosine acquisition and pathogenesis

Tina Kiffer-Moreira[1,2], Maria Ester Fernandes Sampaio[1,2], Daniela S. Alviano[3], Flavia Axelband[2,4], Gabriele Vargas Cesar[3], Daniela Cosentino-Gomes[1,2], Marcio L. Rodrigues[3], Leonardo Nimrichter[3], Adalberto Vieyra[2,4], Celuta S. Alviano[3] & José Roberto Meyer-Fernandes[1,2]

[1]Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; [2]Instituto Nacional de Ciência e Tecnologia de Biologia Estrutural e Bioimagem, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; [3]Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; and [4]Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Correspondence: José Roberto Meyer-Fernandes, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro (UFRJ), CCS, Bloco H, Cidade Universitária, Ilha do Fundão, 21541-590, Rio de Janeiro, Brazil. Tel.: +55 21 590 4548; fax: +55 21 270 8647; e-mail: meyer@bioqmed.ufrj.br

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Abstract

In this work, we describe the ability of intact cells of *Candida parapsilosis* to hydrolyze extracellular ATP. ATP hydrolysis was stimulated by $MgCl_2$ in a dose-dependent manner. The ecto-ATPase activity was increased in the presence of 5 mM $MgCl_2$, with values of V_{max} and apparent K_m for Mg-ATP^{2-} increasing to 33.80 ± 1.2 nmol Pi h^{-1} 10^{-8} cells and 0.6 ± 0.06 mM, respectively. Inhibitors of phosphatases, mitochondrial Mg^{2+}-ATPases and Na^+-ATPases had no effect on the *C. parapsilosis* Mg^{2+}-stimulated ATPase activity, but extracellular impermeant compounds, 4,4′-diisothiocyanatostilbene-2,2′disulfonic acid and suramin, reduced enzyme activity in yeast living cells by 83.1% and 81.9%, respectively. ARL 67156 (6-*N*,*N*′-diethyl-D-β-γ-dibromomethylene ATP), a nucleotide analogue, also inhibited the ecto-ATPase activity in a dose-dependent manner. ATP was the best substrate for the yeast Mg^{2+}-stimulated ecto-enzyme, but ADP, ITP, CTP, GTP and UTP were also hydrolyzed. A direct relationship between ecto-ATPase activity and adhesion to host cells was observed. In these assays, inhibition of enzyme activity resulted in decreased levels of yeast adhesion to epithelial cells. Based also on the differential expression of ecto-ATPase activities in the different isolates of *C. parapsilosis*, the possible role of this enzyme in fungal biology is discussed.

Introduction

Candida parapsilosis is a cause of serious nosocomial infections and is the second most common *Candida* sp. isolated from bloodstream infections in many regions worldwide (San Miguel *et al.*, 2005; Sarvikivi *et al.*, 2005; Almirante *et al.*, 2006; Trofa *et al.*, 2008). The mechanisms by which *C. parapsilosis* evades host defenses and colonizes host tissues are poorly understood. The search for new structures representing virulence factors that will enhance our understanding of the pathogenic steps of *Candida* infections is therefore extremely important.

Cell–cell recognition and adherence are central processes to many fundamental areas of biology. The cell wall composition is of primary importance during microbial adherence and infection establishment (Calderone *et al.*, 1994). Cell surfaces contain enzymes whose catalytic site faces the extracellular environment (Jesus *et al.*, 2002; Meyer-Fernandes, 2002; Gomes *et al.*, 2006; Pinheiro *et al.*, 2007; Peres-Sampaio *et al.*, 2008). The activities of these enzymes, referred to as ecto-enzymes, can be measured using living cells (Goding, 2000; Meyer-Fernandes, 2002; Amazonas *et al.*, 2009). This class of enzymes includes surface ATPases (ecto-ATPases), which are transmembrane glycoproteins that hydrolyze extracellular nucleoside tri- and/or diphosphates (Zimmermann, 2001; Meyer-Fernandes, 2002).

Ecto-ATPases, which are also known as E-type ATPases, are divalent cation-dependent enzymes that are insensitive

to inhibitors of P-type, F-type and V-type ATPases (Zimmermann, 2001; Meyer-Fernandes, 2002). E-type ATPases have been described on the surface of several microorganisms including protozoa (Meyer-Fernandes *et al.*, 1997; Barros *et al.*, 2000; De Jesus *et al.*, 2002; Sissons *et al.*, 2004; Leite *et al.*, 2007; Matin & Khan, 2008; Santos *et al.*, 2009), bacteria (MacFarlane *et al.*, 1994; Hopfe & Henrich, 2004; Sansom *et al.*, 2008a) and fungi, including *Saccharomyces cerevisiae* (Zhong & Guidotti, 1999), *Cryptococcus neoformans* (Junior *et al.*, 2005) and *Fonsecaea pedrosoi* (Collopy-Junior *et al.*, 2006). Several hypotheses have been suggested for the physiological role of these enzymes. They include: (1) protection from the cytolytic effects of extracellular ATP (Fillipini *et al.*, 1990; Steinberg & Di Virgilio, 1991); (2) termination of purinergic signaling (Weisman *et al.*, 1996; Westfall *et al.*, 1997); (3) involvement in signal transduction (Margolis *et al.*, 1990; Dubyak & El-Moatassim, 1993); and (4) involvement in cellular adhesion (Kirley, 1997; Bisaggio *et al.*, 2003; Pinheiro *et al.*, 2006; Santos *et al.*, 2009).

The characterization of the cell wall and other surface components apparently have an express impact in the development of new antifungal agents (Nimrichter *et al.*, 2005). In the present work, we characterized a surface ATPase activity in *C. parapsilosis* as an Mg^{2+}-stimulated ecto-enzyme. We found that *C. parapsilosis* isolates from the oral cavity and bloodstream express different levels of Mg^{2+}-stimulated ATPase activity at their surface. We also show that the inhibition of enzyme activity resulted in decreased levels of yeast adhesion to epithelial cells.

Materials and methods

Chemicals

All reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) or from E. Merck (Darmstadt, Germany). [γ-^{32}Pi]ATP was prepared as described by Glynn & Chappel (1964). Distilled water was deionized using a MilliQ system of resins (Millipore Corp., Bedford, MA) and was used in the preparation of all solutions.

Microorganisms and growth conditions

Candida parapsilosis strain CCT3834 (ATCC 22019) was kindly supplied by Dr Anibal Vercesi (Departamento de Patologia Clínica, Universidade Federal de Campinas, São Paulo, Brazil). Isolates RFO, obtained from the oral cavity of a human patient, and H297, from the bloodstream of an infected individual, were obtained from Faculdade de Odotonlogia and from Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Brazil, respectively. Stock cultures were maintained on solid brain–heart infusion at 37 °C. For measurements of enzyme activity, all the strains of *C. parapsilosis* were cultivated for 48 h in a complex medium containing glycerol (2% v/v), peptone (2% w/v; Bacto peptone; Becton Dickinson) and yeast extract (1% w/v) at room temperature with continuous shaking (Milani *et al.*, 2001). Yeast cells were obtained by centrifugation, and washed twice in a solution containing 116 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose and 50 mM HEPES-Tris buffer (pH 7.2). Cell growth was estimated by counting the number of yeast in a Neubauer chamber. Except for those assays using different isolates (H297 and RFO), experiments were performed using strain CCT3834. Cellular viability was assessed, before and after incubations, by Trypan blue dye exclusion. For Trypan staining, the cells were incubated in the presence of 0.01% Trypan blue for 10 min in the buffer used in each experiment (Kneipp *et al.*, 2004). The viability was not affected under the conditions used here.

Determination of ecto-ATPase activity

Intact cells (1.0×10^8) were incubated for 1 h at 30 °C in 0.5 mL of a mixture containing, unless otherwise specified, 116 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose, 50 mM HEPES-Tris buffer (pH 7.2) and 5 mM ATP in the absence or in the presence of 5 mM $MgCl_2$. The Mg^{2+}-stimulated ecto-ATPase activity was calculated from the total activity measured in the presence of 5 mM $MgCl_2$ minus the basal activity, which was measured in the absence of $MgCl_2$. The ATPase activity was determined by measuring the hydrolysis of [γ-^{32}P]ATP (10^4 Bq nmol^{-1} ATP) (Meyer-Fernandes *et al.*, 2000). The experiments were started by the addition of living cells and terminated by the addition of 1 mL of a cold mixture containing 25% charcoal in 1 M HCl. The tubes were then centrifuged at 1500 ***g*** for 10 min at 4 °C. Aliquots (0.5 mL) of the supernatants containing the released ^{32}Pi were transferred to scintillation vials containing 9 mL of scintillation fluid. The ATPase activity was calculated by subtracting the nonspecific ATP hydrolysis measured in the absence of cells from the total released ^{32}Pi. The hydrolysis of ATP was linear with time under the assay conditions used and was found to be proportional to the cell number. In the assays where other nucleotides were used, the hydrolytic activities were measured under the same experimental conditions described above, and Pi release was assayed spectrophotometrically by measuring the release of Pi from the nonradioactive nucleotides (Lowry & Lopes, 1946). The values obtained for ATPase activities measured using both methods (i.e. spectrophotometry and radioactivity) were the same. In the experiments where high concentrations of Mn^{2+}, Ca^{2+} and Sr^{2+} were tested, the possible formation of precipitates was checked as described previously (Meyer-Fernandes & Vieyra, 1988). In the reaction media containing 50 mM HEPES (pH 7.2), 116 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose and 5 mM ATP, no

phosphate precipitates were observed in the presence of these cations under the conditions used.

Reverse-phase HPLC analysis of ATP hydrolysis

The hydrolysis of ATP and the generation of ADP, AMP and adenosine was determined by incubating 1×10^8 cells mL^{-1} in a mixture containing 116 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose, 5 mM $MgCl_2$, 50 mM HEPES-Tris buffer (pH 7.2) and 100 μM ATP. After 5, 10, 15, 30, 45, 60 and 120 min, aliquots of 300 μL were taken and concentrated to dryness in a vacuum concentrator. The dried aliquots were resuspended in 0.1 mL of a solution containing 50 mM KH_2PO_4, 50 mM K_2HPO_4, 4 mM tetra-*n*-butylammonium bromide (TBAB), 10% methanol, pH 6.0, and immediately injected into a C-18 reverse-phase column (Rexcrom, 25 cm × 4.6 mm, Regis Technologies Inc., IL) coupled to an LC10AS-HPLC model (Shimadzu) through a 50-μL loop. The solvent system was the solution described above. The methodology used was modified from the original protocol described by Kawamoto *et al.* (1998). The nucleotides and adenosine were separated with a flow rate maintained at 1 mL min^{-1} (retention times, minimum: adenosine, 8.5 ± 0.1; AMP, 7.5 ± 0.07; ADP, 10.8 ± 0.2; ATP, 15.9 ± 0.2) and detected by UV spectroscopy at 254 nm. The amounts of remaining nucleotides and adenosine were calculated using the peak ratio area in the calibration graph constructed using standard concentrations of nucleotides and adenosine.

Interaction of *C. parapsilosis* with epithelial cells

Chinese hamster ovary (CHO) cells were purchased from the Rio de Janeiro cell culture collection (BCRJ, UFRJ, Brazil). The cells were grown in a 5% CO_2 (37 °C) in 25-cm^2 culture flasks containing Roswell Park Memorial Institute (RPMI) medium 1640 (GibcoBRL) supplemented with L-glutamine, 25 mM HEPES buffer and 10% fetal bovine serum (FBS). The initial inoculum was 5×10^4 cells mL^{-1}; this was subcultured every 2 days, and the cells were maintained in log-phase growth. For adhesion assays, animal cells were plated onto 24-well multidishes at a density of 10^5 cells per well. They were then incubated at 37 °C for 24 h in a culture medium supplemented with 10% FBS. Before interaction with animal cells, *C. parapsilosis* cells (10^6) (strain CCT3834) were incubated for 30 min at room temperature in 0.9% NaCl (control cells) or in the same solution containing 0.5 mM 4,4′-diisothiocyanostylbene-2, 2′-disulfonic acid (DIDS), 0.5 mM suramin and 0.5 mM ARL 67156. Yeast cells were then washed twice with 0.9% saline, and finally rinsed in RPMI. Fungal cells were suspended in the same medium and incubated with CHO monolayers. The CHO cell–yeast ratio used was 1 : 10. After the addition of fungi, the cells were incubated at 37 °C for 2 h, washed three times in phosphate-buffered saline to remove nonadherent yeast cells, fixed in Bouin's solution and stained with Giemsa. The index of association between *C. parapsilosis* and epithelial cells was determined using a microscope at a magnification of 1000 (Zeiss Axioplan 2, Germany). Representative images were taken at a magnification of 400. The index of association between *C. parapsilosis* and CHO cells was taken as the number of attached and internalized yeast cells per host cell (total number of host cell-associated yeast/total number of host cell). For each experiment, 400 animal cells were counted.

Phylogenetic tree and sequence alignment

The phylogenetic tree was constructed with MEGA version 4.0 (Tamura *et al.*, 2007) using the neighbor-joining method. NTPDase protein sequences from *Mus musculus* (GenBank accession nos NP_033978; NP_033979; NP_848791; NP_080450; NP_001021385; NP_742115; NP_444333; NP_082369), *Homo sapiens* (GenBank accession nos NP_001767; NP_982293; NP_001239; NP_004892; NP_001240; NP_001238; EAW49862; NP_001028285) trypanosomatids, *Trypanosoma cruzi* (GenBank accession no. AAS75599); *Trypanosoma brucei* (GenBank accession no. AAZ13145); *Leishmania major* (GenBank accession no. DAA04950) and fungi, *Schizosaccharomyces pombe* (GenBank accession no. CAB57847); *S. cerevisiae* (GenBank accession no. EDN62971); *Aspergillus fumigatus* (GenBank accession no. XP_753547); and *Candida albicans* (GenBank accession no. XP_715624) were retrived from the NCBI protein database.

Protein sequence alignment and analysis were performed using the CLUSTAL X version 2 (Larkin *et al.*, 2007) and GENEDOC (Nicholas *et al.*, 1997) programs.

Statistical analysis

All experiments were performed in triplicate, with similar results obtained in at least three separate cell suspensions. The maximal velocity V_{max} and K_m for Mg-ATP^{2-} were calculated using a computerized nonlinear regression analysis of the data to the Michaelis–Menten equation (SIGMA PLOT 7.0; Jandel Scientific Software). Statistical significances were determined using Student's *t*-test. Values of $P < 0.05$ were considered as significant.

Results

Candida parapsilosis expresses an ecto-ATPase that is stimulated by divalent cations

Initially, we compared the enzyme activity in the presence or absence of divalent cations, potent modulators of ecto-ATPases (Meyer-Fernandes, 2002). In the absence of divalent metals, *C. parapsilosis* hydrolyzed ATP at a rate of 2.12 ± 0.07 nmol Pi h^{-1} 10^{-8} cells. Our results revealed that

addition of divalent metals also increased ecto-ATPase activity in *C. parapsilosis* (Fig. 1). $MgCl_2$ exerted the most prominent effect; however, other divalent metals, especially $MnCl_2$ and $CoCl_2$, also stimulated ecto-ATPase activity (Fig. 1a). Because Mg^{2+} cations were the most efficient activator and modulated the enzyme activity in a dose-dependent manner (Fig. 1b), all the following experiments were performed in the presence of 5 mM $MgCl_2$. The Mg^{2+}-stimulated ecto-ATPase activity (30.48 ± 1.41 nmol Pi h^{-1} 10^{-8} cells) represents the difference between enzyme activities measured in the presence of $MgCl_2$ (5 mM) minus the basal ecto-ATPase activity measured in the absence of added divalent metals.

The time course of ATP hydrolysis by the *C. parapsilosis* Mg^{2+}-stimulated ecto-ATPase was linear ($r^2 = 0.9740$) for at least 1 h (Fig. 2a). To certify that the observed ATP hydrolysis did not result from secreted enzymes, the cells were first incubated in a reaction mixture containing no ATP. Cells were then removed by centrifugation and the supernatant was assayed for ATPase activity. ATP hydrolysis was not detected, even in the presence of $MgCl_2$ (inset of Fig. 2a). This control excludes the possibility that the ATPase activity described here could be derived from lysed *C. parapsilosis* cells. Similarly, in assays to determine the influence of cell density, the Mg^{2+}-stimulated ecto-ATPase activity measured over 60 min was linear ($r^2 = 0.9941$) over a nearly 10-fold range of cell density (Fig. 2b).

Effect of pH and different inhibitors on the hydrolysis of ATP by yeast cells

The optimum pH for the Mg^{2+}-stimulated ecto-ATPase activity is within the alkaline range, while the ecto-phosphatase activity also present on the external surface of *C. parapsilosis* was inhibited in basic pH (Kiffer-Moreira *et al.*, 2007). In the pH range from 3.0 to 9.0, in which the cells were alive throughout the time course of the reaction, the Mg^{2+}-stimulated ATPase activity increased with the pH. At pH 9.0, the values of ATP hydrolysis were around sevenfold higher than those obtained at pH 3.0 (Fig. 3). Similar results were obtained for other microorganisms such as *Entamoeba histolytica* (Barros *et al.*, 2000), *Leishmania amazonensis* (Berrêdo-Pinho *et al.*, 2001) and *F. pedrosoi* (Collopy-Junior *et al.*, 2006). To confirm that ATP hydrolysis was not due to a phosphatase activity, described previously in this cell (Kiffer-Moreira *et al.*, 2007), experiments were performed in the presence of different inhibitors of phosphatases. Table 1 shows that classical inhibitors of different phosphatases, such as sodium fluoride and ammonium molybdate (acid phosphatases; Dutra *et al.*, 2001), levamizole (alkaline phosphatases; Van-Belle, 1976) and tartrate (secreted phosphatases; Dutra *et al.*, 2001), had no effect on the *C. parapsilosis* ATPase activity. Similar results were observed when the phosphatase substrates *p*-nitrophenylphosphate and β-glycerophosphate were tested as inhibitors. Ecto-ATPase activity was insensitive to 10 mM inorganic phosphate (Table 1), which inhibited 70% of ecto-phosphatase activity (Kiffer-Moreira *et al.*, 2007). Together, these data indicate that ecto-phosphatase activities also present in *C. parapsilosis* did not contribute to ATP hydrolysis.

To rule out the possibility that ATP hydrolysis under the present experimental condition was promoted by intracellular ATPases, inhibitors of ATPases with ATP-binding sites facing the cytosol were tested (Table 1). Inhibitors of Na^+/K^+-ATPase (ouabain; Caruso-Neves *et al.*, 1998) and mitochondrial Mg^{2+}-ATPase (oligomycin and sodium azide; Meyer-Fernandes *et al.*, 1997) also had no effect on the Mg^{2+}-stimulated *C. parapsilosis* ecto-ATPase (Table 1). Ammonium molybdate, a potent inhibitor of 5′-nucleotidases (Gottlieb & Dwyer, 1983), was also inefficient as an inhibitor of the *C. parapsilosis* ecto-ATPase activity, as well

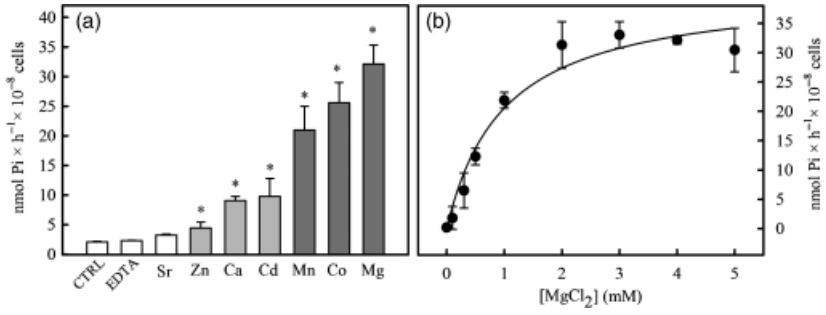

Fig. 1. Stimulation of the ecto-ATPase activity in *Candida parapsilosis* by divalent metals. (a) Influence of different divalent cations on the ecto-ATPase activity in intact cells of *C. parapsilosis*. Cells were incubated for 1 h at 30 °C in a reaction medium (control, described in Materials and methods section). Chloride salts of the ions described in the figure were added at a final concentration of 5 mM. Alternatively, the medium was supplemented with EDTA, in order to avoid cation interference. Asterisks denote significant differences ($P < 0.05$). (b) Mg^{2+}, the most effective stimulator, enhanced enzyme activity in a dose-dependent pattern, as determined after incubation of living yeast cells under the conditions described in Materials and methods section. The data are means ± SE of three determinations with different cell suspensions.

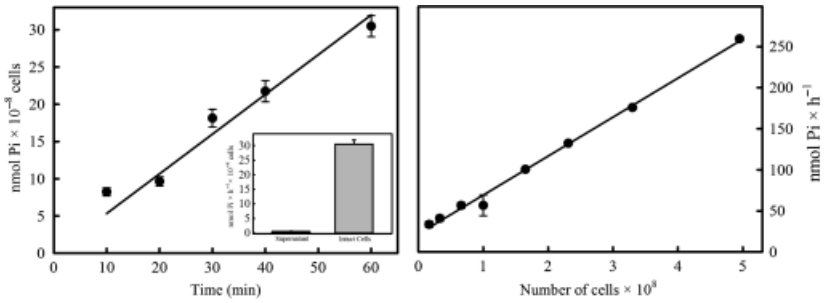

Fig. 2. Time course (a) and cell density dependence (b) on the Mg^{2+}-stimulated ecto-ATPase activity of *Candida parapsilosis*. Intact cells were incubated for different periods of time (a) or for 1 h (b) at 30 °C, in the reaction medium described in Materials and methods section. Inset: comparison between ATP hydrolysis measured in the supernatant and intact cells. The Mg^{2+}-stimulated ecto-ATPase activity was calculated from the total activity, measured in the presence of 5 mM $MgCl_2$, minus the basal activity, measured in the absence of $MgCl_2$. The data are means ± SE of three determinations with different cell suspensions.

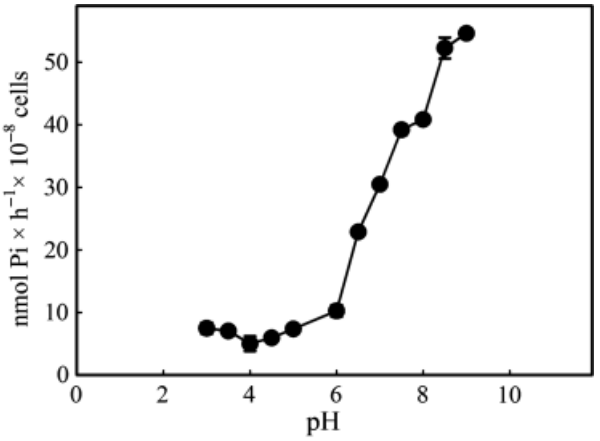

Fig. 3. Effect of pH on the Mg^{2+}-stimulated ecto-ATPase activity of intact cells of *Candida parapsilosis*. Intact cells were incubated with 50 mM MES-HEPES buffer adjusted to pH values between 3.0 and 9.0 with HCl and Tris, in the presence of all components of the reaction medium. In this pH range, cells were viable throughout the course of the reaction. The Mg^{2+}-stimulated ecto-ATPase activity was calculated from the total activity, measured in the presence of 5 mM $MgCl_2$, minus the basal activity, measured in the absence of $MgCl_2$. Data are means ± SE of three determinations with different cell suspensions.

Table 1. Influence of various agents on the ecto-ATPase activity of *Candida parapsilosis*

Compound added	Relative activity[†]
None (control)	100.0 ± 4.6
Levamizole (1.0 mM)	98.0 ± 10.8
Ammonium molybdate (1.0 mM)	90.8 ± 3.0
Sodium fluoride (10.0 mM)	87.5 ± 1.9
Sodium tartrate (10.0 mM)	118.1 ± 7.1
Ouabain (1 mM)	103.0 ± 5.4
Sodium azide (10.0 mM)	87.7 ± 8.0
Oligomycin (10.0 μg mL^{-1})	87.7 ± 8.0
5′-AMP (10.0 mM)	85.7 ± 7.5
p-NPP (10.0 mM)	94.4 ± 7.7
β-Glycerophosphate (10.0 mM)	88.3 ± 6.4
Pi (10.0 mM)	88.3 ± 6.4
DIDS (0.5 mM)	16.9 ± 0.1*
Suramin (0.5 mM)	18.2 ± 0.1*

[†]ATPase activity was measured at pH 7.2 using the standard assay as described in Materials and methods section. The ATPase activity is expressed as the percentage of that measured under control conditions, i.e., using the reaction medium in Materials and methods section, supplemented with 5 mM $MgCl_2$ (30.48 ± 1.41 nmol Pi h^{-1} 10^{-8} cells, 100%). The SEs were calculated from the absolute activity values of three experiments with different cell suspensions and were converted to a percentage of the control values. Under the conditions used, none of the externally added compounds reduced fungal viability.
*Significant differences ($P < 0.05$).

as the substrate for 5′-nucleotidases, 5′-AMP (Table 1). These data confirm that the enzyme detected in intact yeast cells of *C. parapsilosis* is a true ecto-ATPase.

By definition, a true ecto-enzyme should be inhibited by nonpenetrating reagents (Meyer-Fernandes, 2002), which was addressed using DIDS (Meyer-Fernandes *et al.*, 1997) and suramin (Zighanshin *et al.*, 1995). Our results demonstrated that ATP hydrolysis in the presence of 0.5 mM DIDS and suramin was strongly inhibited (Table 1). We also tested the nucleotide analogue 6-*N*,*N*′-diethyl-β-γ-dibromomethylene ATP (ARL 67156), considered to be a selective inhibitor of ecto-ATPase activities from the CD39/NTPDase family (Crack *et al.*, 1995; Lévesque *et al.*, 2007). As shown in Fig. 4, the Mg^{2+}-stimulated *C. parapsilosis* ecto-ATPase was inhibited by ARL 67156 in a dose-dependent manner. The inhibition of the ecto-ATPase activity promoted by ARL 67156 was attenuated when the cells were incubated in the presence of a high concentration of ATP (Fig. 4, open circles), as also observed with other ecto-ATPase activities (Lévesque *et al.*, 2007). Under these conditions, no hydrolysis of ARL 67156 could be detected (data not shown).

Substrate affinity and reverse-phase HPLC analysis

The influence of different Mg-ATP^{2-} concentrations on the *C. parapsilosis* ecto-ATPase activity was determined, as demonstrated in Fig. 5. The values of V_{max} and apparent K_m for Mg-ATP^{2-} corresponded, respectively, to 33.8 ± 1.2 nmol Pi h^{-1} 10^{-8} cells and 0.6 ± 0.06 mM. The ability of *C. parapsilosis* to hydrolyze other nucleotides shows a selectivity pattern that follows (Table 2) ATP > ITP > ADP. The ecto-ATPase also hydrolyzes UTP at a relatively high rate, but GTP and CTP are less preferred substrates. Using HPLC, we obtained the ATP decay along time allowing the identification and quantification of the products sequentially generated from ATP as the result of the surface-located enzymes (Fig. 6). The disappearance of ATP, the biphasic time course of ADP formation and the accumulation of AMP and adenosine are clearly indicative of the sequential steps of hydrolysis ATP → ADP → AMP → adenosine.

Ecto-ATPase is expressed by different isolates of *C. parapsilosis* and interferes with adhesion to the host cell

The Mg^{2+}-stimulated ecto-ATPase activity of three different isolates of *C. parapsilosis* was analyzed in this study, including the laboratory-adapted strain (CCT3834) and two recently isolated strains (RFO and H297). The profiles observed in Fig. 7 show that the oral isolate RFO strain exhibited the highest levels of enzyme activity (86.09 $\pm$ 3.98 nmol Pi h^{-1} 10^{-8} cells), followed by the bloodstream isolate H297 (52.45 $\pm$ 2.36 nmol Pi h^{-1} 10^{-8} cells) and the CCT3834 strain (30.48 $\pm$ 1.41 nmol Pi h^{-1} 10^{-8} cells) (Fig. 7). Enzyme inhibition by DIDS and suramin (Table 1) and by ARL 67156 (Fig. 5) led us to compare the ability of *C. parapsilosis* to attach to epithelial cells when Mg^{2+}-stimulated ecto-ATPase activity was fully functional or inhibited. Yeast cells adhered to the cell surface rather than being internalized by host cells (data not shown). Pretreatment of fungi with the inhibitors DIDS, suramin and ARL 67156 caused a significant ($P < 0.05$) reduction in the

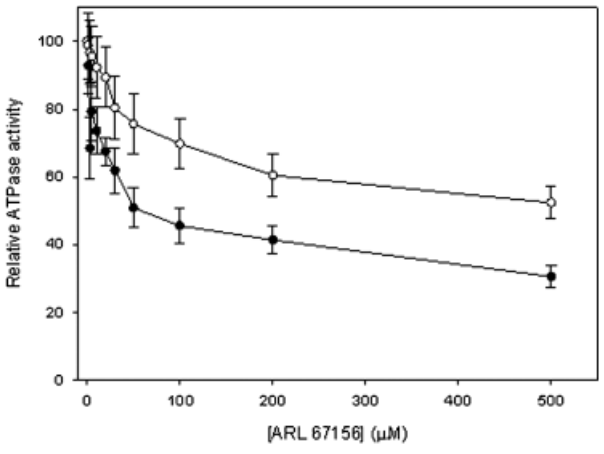

Fig. 4. Effects of increasing concentrations of ARL 67156 on the Mg^{2+}-dependent ecto-ATPase activity of intact cells of *Candida parapsilosis*. Cells were incubated for 15 min at 30 °C in a reaction medium described in Materials and methods section, in the presence of 0.5 mM ATP (●) or 5 mM ATP (○). Data are means ± SE of three determinations using different cell suspensions.

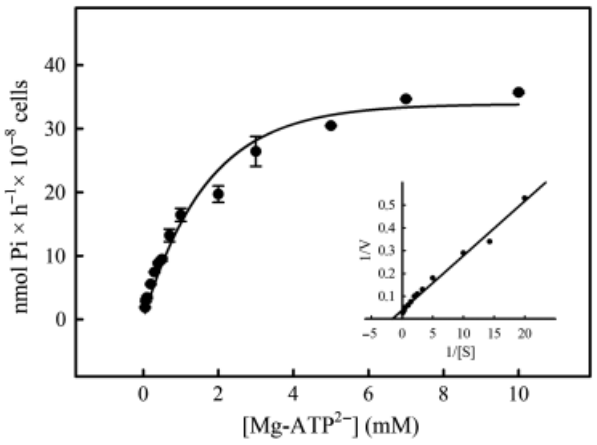

Fig. 5. Ecto-ATPase activity of *Candida parapsilosis* in the presence of varying concentrations of Mg-ATP^{2-}. The ATPase activity was measured in the same reaction medium as that described in the Materials and methods section, in the presence of increasing concentrations of Mg-ATP^{2-} as shown on the abscissa. The curve represents the fit of experimental data by nonlinear regression using the Michaelis–Menten equation as described under Materials and methods section. Inset: Lineweaver–Burk plot. Data are means ± SE of three determinations with different cell suspensions.

Table 2. Substrate specificity of the *Candida parapsilosis* ectonucleotidase

Compound added	Relative activity*
ATP	100.0 ± 4.6
ITP	70.7 ± 2.1
UTP	43.3 ± 3.6
GTP	20.7 ± 3.9
CTP	15.9 ± 1.2
ADP	82.2 ± 1.6

*The ecto-nucleotidase activity was measured at 30 °C in a medium containing the nucleotides listed (5 mM), 50 mM HEPES, pH 7.2, 116 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose, 5.0 mM $MgCl_2$ and 1.0×10^8 cells. ATP hydrolysis (control) was taken as 100% (30.48 ± 1.41 nmol Pi h^{-1} 10^{-8} cells). The SEs were calculated from the absolute activity values of three experiments with different cell suspensions and were converted to a percentage of the control value. In these experiments, release of Pi from all nucleotides, including ATP, was measured using a spectrophotometrical assay as described in Materials and methods section.

adhesion of the *C. parapsilosis* to host cells (Fig. 8), indicating that Mg^{2+}-stimulated ecto-ATPase did indeed influence the interaction between yeast and epithelial cells.

In the *C. albicans* genome, the first sequenced *Candida* genome available (Jones *et al.*, 2004), we identified one gene for nucleoside diphosphatase (NDPase/NTPase) in the NCBI database. The gene CaO19.10432 encodes a hypothetical protein (GenBank accession no. XP_715624.1) with 695 amino acids and significant similarity to human ecto-nucleoside triphosphate diphosphohydrolase 4 (GenBank accession no. NP_004892) (29% of identity, 49% of similarity and an *E* value of 9e − 62). In *C. parapsilosis*, although the genome has been sequenced recently (Butler *et al.*, 2009) by the Wellcome Trust Sanger Institute Pathogen Genomics group (http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/), most of the genes have not been completely annotated yet. To verify the existence of nucleoside diphosphatase sequences in the *C. parapsilosis* genome, we used the gene sequence from *C. albicans* as a query, and then a BLASTN search was performed for related sequences in the *Candida* database on the Broad Institute server, for a comparative analysis of genes and genomes across the *Candida* clade (http://www.broadinstitute.org/annotation/genome/candida_group/MultiHome.html). We observed at the gene CPAG_04037 transcript product a conserved domain for CD39 family. The predicted *C. parapsilosis* protein sequence for nucleoside diphosphatase shares 62% and 31% identities and 74% and 49% similarities to the corresponding *C. albicans* and human sequences, respectively. The conserved amino acids are shown in the protein alignments (Fig. 9).

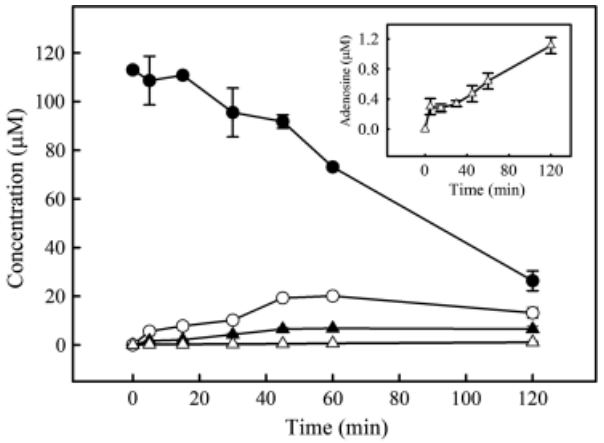

Fig. 6. Time course of ATP hydrolysis in the presence of intact *Candida parapsilosis* cells. The cells were incubated for each indicated period of time at 30 °C in the presence of 116 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose, 5 mM $MgCl_2$, 50 mM HEPES-Tris Buffer (pH 7.2) and 100 μM ATP. Filled circles: ATP concentration; open circles: ADP concentration, filled triangle: AMP concentration; open triangle: adenosine concentration. Inset: amplified view of adenosine generation. Data are means ± SE of three determinations with different cell suspensions.

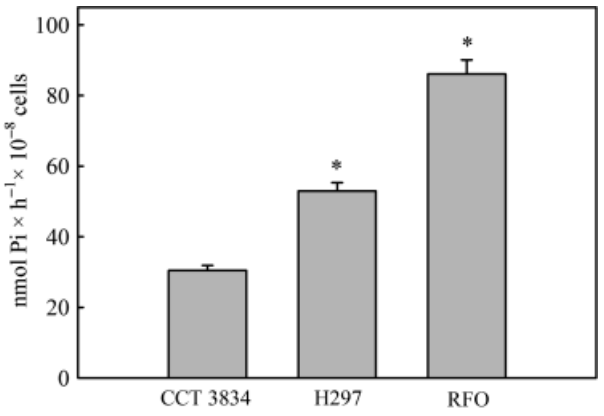

Fig. 7. Expression of Mg^{2+}-stimulated ecto-ATPase by different isolates of *Candida parapsilosis*. Equivalent cell suspensions were incubated for 1 h at 30 °C in the same reaction medium described in Materials and methods section. Enzyme activities in strains H297 and RFO were significantly higher ($^*P < 0.05$) than that observed in strain CCT3834. The Mg^{2+}-stimulated ecto-ATPase activity was calculated from the total activity, measured in the presence of 5 mM $MgCl_2$, minus the basal activity, measured in the absence of $MgCl_2$. Data are means ± SE of three determinations with different cell suspensions.

The phylogenetic relationship between NTPDase sequences from *Candida* sp., mammals, other fungi and parasites such as trypanosomatids can be seen in Fig. 10. The alignment of the eight well-known NTPDase groups of proteins from *H. sapiens* (Hs) and *M. musculus* (Mm) resulted in separate clades in the phylogenetic tree, whereas the orthologues sequences from *T. cruzi* (Tc), *T. brucei* (Tb) and *L. major* (Lm) formed a different clade. As expected, all fungi species, *S. pombe* (Sp), *S. cerevisiae* (Sc), *A. fumigatus* (Af), *C. albicans* and *C. parapsilosis* were also grouped in a unique clade, suggesting homologous functions between the proteins.

Discussion

In the present study, we aimed at the biochemical characterization of an ecto-ATPase activity in intact cells of *C. parapsilosis*. This activity was stimulated by Mg^{2+} and Mn^{2+}, as demonstrated in *S. cerevisiae*, *C. neoformans* and *F. pedrosoi* (Zhong & Guidotti, 1999; Junior *et al.*, 2005; Collopy-Junior *et al.*, 2006). The surface localization of the enzyme was supported by its sensitivity to the impermeant reagents DIDS (Barbacci *et al.*, 1996; Meyer-Fernandes *et al.*, 1997; Berrêdo-Pinho *et al.*, 2001) and suramin. The Mg^{2+}-stimulated enzyme activity cannot be ascribed to ecto-phosphatases or 5′-nucleotidases, because it was not affected by inhibitors and substrates of these enzymes. The Mg^{2+}-stimulated ecto-ATPase hydrolyzes ITP, GTP, CTP and UTP (Table 2) with different selectivities indicating that the

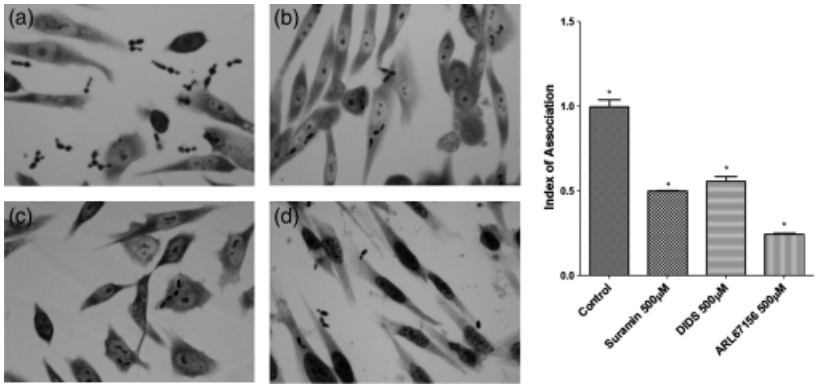

Fig. 8. Adhesion of *Candida parapsilosis* to epithelial cells is influenced by ecto-ATPase activity. Giemsa staining of *C. parapsilosis* strain CCT3834 pretreated with phosphate-buffered saline (a) or the ecto-ATPase inhibitors DIDS (b), suramin (0.5 mM) (c) and ARL67156 (0.5 mM) (d) and incubated with CHO cells resulted in decreased levels of association with host cells (e). Magnification: × 400. Data are means ± SE of three determinations with different cell suspensions. Asterisks denote a significant difference ($P < 0.05$) in relation to the control system.

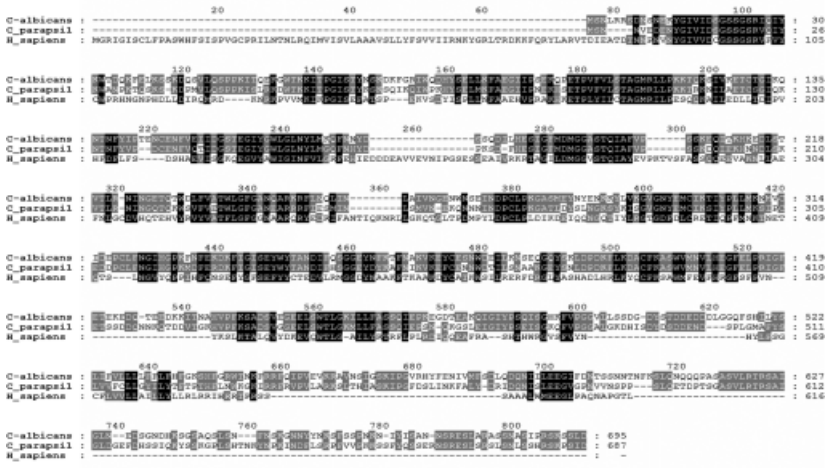

Fig. 9. Amino acid alignment of *Candida parapsilosis* predicted sequence for nucleoside diphosphatase. The predicted sequence of *C. parapsilosis* (CPAG_04037) for nucleoside diphosphatase was found to have homology to *Homo sapiens* ectonucleoside triphosphate diphosphohydrolase 4 (GenBank accession no. NP_004892) and *Candida albicans* hypothetical protein (GenBank accession no. XP_715624.1) for nucleoside diphosphatase. Conserved amino acids are shaded in black (100% conserved), dark gray (80% conserved) and light gray (60% conserved). Dashes indicate gaps. The alignments and analysis were constructed using the CLUSTAL X version 2 (Larkin *et al*., 2007) and GENEDOC (Nicholas *et al*., 1997) programs.

nucleoside moiety of the substrate is important for its fitting at the active site. ADP was also recognized as a substrate and the ATP : ADP hydrolysis ratio was 1.0 : 0.8, a ratio very similar to that observed with the E-NTPDase type 1, a plasma membrane-associated ecto-enzyme (Zimmermann, 2001). In addition, ARL 67156, a selective inhibitor of E-NTPDase type 1 (Lévesque *et al*., 2007), inhibited the ecto-ATPase activity in a dose-dependent manner (Fig. 4). Taken together, these results indicate that the currently characterized enzyme activity is an authentic ecto-ATPase as described in *C. neoformans* (Junior *et al*., 2005) and other organisms (Meyer-Fernandes, 2002; Lévesque *et al*., 2007; Sansom *et al*., 2008b).

The presence of surface-located ATPases has been reported in many microorganisms (Zhong & Guidotti, 1999; Bernardes *et al*., 2000; De Jesus *et al*., 2002; Hopfe & Henrich, 2004; Sissons *et al*., 2004; Junior *et al*., 2005; Collopy-Junior *et al*., 2006). In several of these microbial models, including *L. amazonensis* (Berrêdo-Pinho *et al*., 2001), *T. cruzi* (Meyer-Fernandes *et al*., 2004; Santos *et al*., 2009) and *Trypanosoma rangeli* (Fonseca *et al*., 2006), maximum values of ATP hydrolysis were obtained at alkaline pHs, such as that currently described here. The specific functions of these enzymes are not fully known, but it has been demonstrated that they participate in many relevant biological processes (Zimmermann, 2001; Meyer-Fernandes, 2002). For instance, Fillipini *et al*. (1990) have shown that ATP can kill various types of cells, with the exception of those that express a high level of ATP-breakdown activity on their surface. In *S. cerevisiae*, ecto-ATPase genes similar to those involved in the expression of the animal ecto-apyrase (CD39) have been described (Zhong & Guidotti, 1999). More direct evidence of the functions of ecto-ATPase in fungal cells has been provided recently by our group using *C. neoformans* as a model (Junior *et al*., 2005). In this pathogen, the addition of the ecto-ATPase natural substrate (ATP) to the culture medium rendered yeast cells significantly more resistant to the antifungal drug fluconazole. This

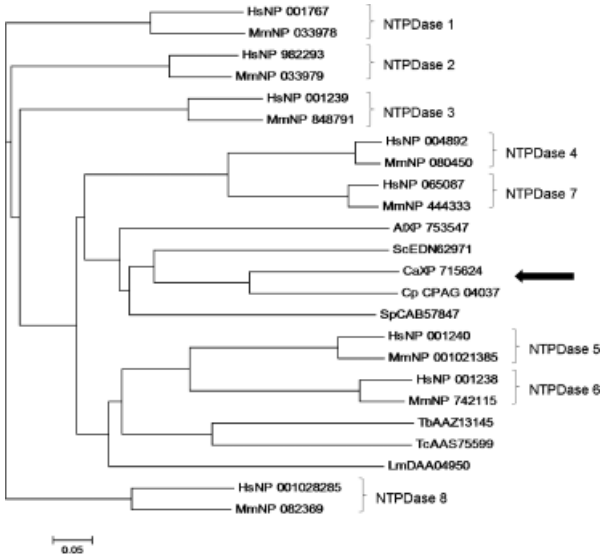

Fig. 10. Phylogenetic tree of the NTPDase family. The *Candida parapsilosis* (CPAG_04037) predicted sequence for nucleoside diphosphatase was shown to have a strong relationship with *Candida albicans* sequence (GenBank accession no. XP_715624.1). The deduced amino acid sequences were aligned using the CLUSTAL X version 2 (Larkin *et al.*, 2007) program and the phylogenetic tree was constructed using MEGA version 4.0 program (Tamura *et al.*, 2007). NTPDase sequences grouped consistently *Homo sapiens* (Hs), *Mus musculus* (Mm), *Trypanosoma cruzi* (Tc), *Trypanosoma brucei* (Tb), *Leishmania major* (Lm), *Schizosaccharomyces pombe* (Sp), *Saccharomyces cerevisiae* (Sc), *Aspergillus fumigates* (Af), *C. albicans* (Ca) and *C. parapsilosis* (Cp). The arrow indicates *Candida* sequences.

result may suggest that ecto-ATPase activity is a relevant factor regulating the transport of solutes, which may have an impact on fungal nutrition and/or drug resistance (Junior *et al.*, 2005).

In animal cells, several functions of ecto-ATPases have been proposed including the termination of purinergic signaling, cellular adhesion, vesicular transport and purinergic recycling (Zimmermann, 2001). Extracellular ATP and its degradation products ADP, AMP and adenosine are normal components of the extracellular mileu. Extracellular nucleotides do not cross the cell membrane, but rather mediate their biological actions through specific receptors on the cell surface, where they are locally metabolized by ecto-nucleotidases (El-Moatassim *et al.*, 1992; Dombrowski *et al.*, 1998; Zimmermann, 2001). The different enzymatic activities (ecto-ATPase, ecto-ADPase and ecto-5′ nucleotidase) present on the surface of *C. parapsilosis* might sequentially dephosphorylate ATP to adenosine (ATP → ADP → AMP → adenosine), as observed on the reverse-phase HPLC experiment, making adenosine available to *C. parapsilosis* from nucleotides. In our HPLC analysis (Fig. 6), we observed that only a part of the nucleotides consecutively generated by ATP hydrolysis accumulated in the extracellular medium. The possible hypothesis to explain the observed phenomenon could include uptake of adenosine by a nucleoside transport process as described in *C. albicans* and *Candida glabrata* (Fasoli & Keridge, 1990).

The function of the ecto-ATPase in *C. parapsilosis* is not completely elucidated. Besides its relevance in fungal physiology, this molecule could have an impact on fungal pathogenesis, due to its external location and probable exposition to the host cell during infection. In the present study, we observed that three isolates of *C. parapsilosis*, i.e. the laboratory-adapted strain (CCT3834), the bloodstream isolates (H297) and the oral cavity isolates (RFO), show different levels of ecto-ATPase activity. RFO isolates showed the highest enzyme activity, followed by H297 isolates and CCT3834. We showed previously that RFO isolates also presented a high capacity of adhesion to CHO cells, followed by the H297 and CCT3834 isolates (Kiffer-Moreira *et al.*, 2007). Variations in the production of enzymatic virulence factors, as secreted aspartic proteinases, have also been found by comparing systemic and superficial isolates, with a tendency towards a higher activity in superficial *C. parapsilosis* isolates (Dagdeviren *et al.*, 2005).

The characterization of an ecto-ATPase activity in *C. parapsilosis* and the detection of different levels of activity in recent isolates may represent important initial steps to understand the possible role of the enzyme as a pathogenic marker. Other surface enzymes (ecto-phosphatases) of *C. parapsilosis* have been demonstrated to modulate infection to epithelial cells (Kiffer-Moreira *et al.*, 2007), stimulating experiments on the role of ecto-ATPases during the interaction of different isolates with host epithelia. In order to evaluate the importance of ecto-ATPase activity in *C. parapsilosis* infectivity, we performed experiments with three known enzyme inhibitors of ecto-ATPases. DIDS, suramin and ARL 67156 inhibited ecto-ATPase activity in live yeasts (Table 1, Fig. 4). In parallel, we observed that pretreatment with these inhibitors also led to a decrease of *C. parapsilosis* adhesion to host cells (Fig. 8). It suggests that this enzyme, when active, participates in the *in vitro* infectious process. Similar results with the inhibitor suramin were also reported for the *in vitro* infectivity of *Acanthamoeba* isolates (Sissons *et al.*, 2004), *T. cruzi* (Bisaggio *et al.*, 2003; Santos *et al.*, 2009) and *Balamuthia mandrillaris* (Matin & Khan, 2008) and the *in vivo* virulence of *T. cruzi* (Santos *et al.*, 2009).

The involvement of ecto-ATPases during infections has been described for several microorganisms including protozoa (Bisaggio *et al.*, 2003; Sissons *et al.*, 2004; Pinheiro *et al.*, 2006; Matin & Khan, 2008; Santos *et al.*, 2009) and bacteria (Sansom *et al.*, 2008a). In recent years, the identification of an increasing number of human pathogens with plasma-membrane-associated ecto-ATPase activity has raised a number of intriguing questions regarding the role of these enzymes in interactions with the mammalian cells (Sansom *et al.*, 2008b). Genetic manipulation aiming the generation of mutants lacking the expression of ecto-ATPases will aid

the functional elucidation of these enzymes in *C. parapsilosis*, including its involvement in virulence.

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