Aluminum impairs morphogenic transition and stimulates $H^+$ transport mediated by the plasma membrane ATPase of Yarrowia lipolytica

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Abstract

The effect of aluminum on dimorphic fungi Yarrowia lipolytica was investigated. High aluminum (0.5–1.0 mM AlK(SO4)2) inhibits yeast–hypha transition. Both vanadate-sensitive $H^+$ transport and ATPase activities were increased in total membranes isolated from aluminum-treated cells, indicating that a plasma membrane $H^+$ pump was stimulated by aluminum. Furthermore, Al-treated cells showed a stronger $H^+$ efflux in solid medium. The present results suggest that alterations in the plasma membrane $H^+$ transport might underlie a pH signaling required for yeast/hyphal development. The data point to the cell surface pH as a determinant of morphogenesis of Y. lipolytica and the plasma membrane $H^+$-ATPase as a key factor of this process.

Introduction

Yeast has proven to be an excellent system for isolating and characterizing genes responsive to environmental signals including salt, oxidative and various metal stresses (Serrano, 1996; Avery, 2001; Gasch & Werner-Washburne, 2002 and references therein; de Freitas, 2003). Aluminum is the most abundant metal in the earth's crust, making up about 7% of its mass and has a ubiquitous and increasing presence in the human life, being widely used in modern industry. Therefore, the general environment is constantly exposed to relatively high levels of this metal that becomes mobilized and toxic for many plants in soils at pH lower than 5. Aluminum (Al(3+)) toxicity and its related nutrient deficiencies have been considered to be primary factors limiting crop production on the acid soils (Foy et al., 1978), which comprise ~40% of the world's arable land. In spite of intense investigations, the precise mechanism of Al actions remains unknown. Understanding the physiological basis for Al toxicity and the cellular mechanisms that some plants use to tolerate Al is essential in order to develop crop species that can be cultivated on the acid soils (Kochian et al., 2004).

Fungal and plant cells share similarity in ion homeostasis and bioenergetics and many cellular mechanisms involved in the homeostasis are common between these organisms. In the present study, the nonconventional yeast Yarrowia lipolytica were used to study aluminum toxicity. Yarrowia lipolytica is a dimorphic ascomycete, which, in recent years, has attracted growing interest due to its biotechnological value as well as a model organism to study fungal dimorphism, peroxisome and mitochondria functioning (Barth & Gaillardin, 1997; Kerscher et al., 2002). Yarrowia lipolytica grows at a wide pH range and can be found in different habitats including soil, thus being naturally exposed to Al. Yarrowia lipolytica grows as a mixture of yeast and...
filamentous cells. Different effectors involved in the di-morphic transition of Y. lipolytica have been described. Among them are the carbon and nitrogen source, serum and extracellular pH (Pérez-Campo & Domínguez, 2001; Ruiz-Herrera & Sentandreu, 2002; Szabo & Štofaniková, 2002).

The plasma membrane H\(^+\)-ATPase plays an essential role in fungal and plant cell physiology. It generates a large electrochemical gradient that drives the transport of amino acids, sugars and inorganic ions (Goffeau & Slayman, 1981). H\(^+\) extrusion catalyzed by this enzyme also contributes to the regulation of intracellular pH and surface pH of yeast. It is widely accepted that extracellular neutral or alkaline pH induces filamentation in Y. lipolytica and in Candida albicans, while extracellular acid pH favors the development of a yeast form (Ruiz-Herrera & Sentandreu, 2002; Szabo & Štofaniková, 2002). It was also reported that a transient increase in intracellular pH preceded the morphological transition of C. albicans (Kaur et al., 1988; Stewart et al., 1988).

It is reported here that Y. lipolytica growth is not affected by high Al concentrations, suggesting that Y. lipolytica exhibits more tolerance towards this toxic metal than plants. Yet, Al led to drastic changes in the morphological development of Y. lipolytica, inhibiting yeast-to-hypha transition. This was concomitant with an increase in the H\(^+\) transport mediated by P-type plasma membrane H\(^+\)-ATPase. The present data are discussed in relation to a possible Al-dependent disturbance of a pH signaling required for hyphal development.

Materials and methods

Yeast strain, media and cultivation conditions

The Y. lipolytica strain used in this work was JM12 MatB leu2-35 lys5-12 ura3-18. Cells were grown at 30 °C in YED medium (1% yeast extract, 1% glucose) adjusted to pH 4.5 with HCl. The effect of aluminum was tested at three different concentrations (0.1, 0.5 and 1 mM of AlK(SO\(_4\))\(_2\)× 12 H\(_2\)O). Liquid cultures were inoculated to an A\(_{600\text{nm}}\) of 0.01. Cell morphology was analyzed at 2-h intervals using a Zeiss Axioskop photomicroscope.

Membrane isolation

Yeast membranes were isolated and fractionated according to Okorokov & Lehle (1998). Briefly, the middle logarithmic phase cells grown in YED medium pH 4.5 for 18 h with or without 1.0 mM AlK(SO\(_4\))\(_2\) were transformed to the spheroplasts by incubation with hydrolytic enzymes from Tricho-derma and 30 mM of β-mercaptoethanol at 37 °C using 1.2 M sorbitol in 50 mM Tris HCl, pH 7.4. Spheroplasts were homogenated in lysis buffer (12.5% sucrose, 20 mM MOPS-Na, pH 7.4, 1 mM dithiothreitol, 1 mM benzamidin, 1 mM phenylmethanesulphonylfluoride and a cocktail of protease inhibitors) and total membranes were precipitated for 45 min at 120 000 × g. The total membranes were resuspended in lysis buffer and stored at −70 °C.

Biochemical assays

To measure H\(^+\) transport, 34 μg of total membrane vesicles were added in incubation medium containing 20 mM MOPS-KOH, pH 7.2, 2.5 mM MgCl\(_2\), 50 mM KCl, 12.5% sucrose and 1 μM 9-amino-6-chloro-2-methoxyacridine (ACMA). H\(^+\) transport was initiated by addition of 1 μM ATP, pH 7.2, and monitored by the fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (Okorokov & Lichko, 1983). Subsequent addition of 20 mM NH\(_4\)Cl was used to show recovery of the fluorescence that indicated a collapse of the preliminarily formed H\(^+\) gradient. F\(_{\text{max}}\) reflects steady-state amplitude of the ΔpH formation achieved after 10 min of H\(^+\) transport; it was calculated as ΔF/F and was expressed as a percentage. For determination of plasma membrane H\(^+\) transport, the membranes were preincubated with 0.2 mM Na\(_3\)VO\(_4\), a specific inhibitor of P-type ATPase, before addition of ATP. Addition of concanamycin had little effect on H\(^+\) transport.

ATPase activity was determined colorimetrically by measuring the release of Pi (Fiske & Subbarow, 1925; Okorokov & Lichko, 1983). The reaction media contained 30 mM MOPS-Tris, pH 6.5, 1 mM ATP, pH 7.2, 3.75 mM MgSO\(_4\) and 262 μM (NH\(_4\))\(_2\)Mo\(_4\), with or without 0.1 mM Na\(_3\)VO\(_4\). The reaction was started by addition of vesicle protein and stopped with ice-cold 5% (w/v) trichloracetic acid after 30 min of incubation at 30 °C.

In all the experiments, the H\(^+\)-ATPase activity was measured with and without vanadate, and the difference between these two activities was attributed to the plasma membrane H\(^+\)-ATPase.

Protein concentrations in membrane preparations were determined using the method of Bradford (1976).

Assessment of pH changes on agar plates

To visualize an alteration of the pH in solid medium, the same number of Y. lipolytica cells (≈50 per plate) were spread using glass beads on YED plates (adjusted to pH 4.5 with HCl) containing or not 1 mM AlK(SO\(_4\))\(_2\) and incubated at 30 °C for 3 days. After the appearance of colonies, a thin layer of 0.7% low-melting agarose containing 0.015% bromoresol purple was overlaid on yeast colonies. The initial pH of the agarose gel was also adjusted to 4.5. After 10–15 min, the alteration in color was recorded using a digital camera. The images were processed using Adobe Photoshop. The pH value was estimated using a colorimetric scale based on images of indicator gel at the indicated pH. Color change from yellow to purple reflected an
increase in the surface pH from acid (pH 4.5) to nearly neutral (pH 6.5).

**Results**

**Al interferes with dimorphic transition**

To investigate the effect of aluminum on *Y. lipolytica*, yeast cells were grown in liquid medium in the presence of 0.1–1.0 mM AlK(SO₄)₂. In the absence of Al, the division of *Y. lipolytica* took place after a lag of ~6 h and the culture reached saturation after 26 h (Fig. 1a). The presence of 0.5 and 1 mM AlK(SO₄)₂ affected neither lag nor logarithmic phases and only slightly (10%) inhibited the growth at the stationary phase (Fig. 1a). Microscopic evaluation of cell morphology revealed that the yeast–hypha transition took place after 18 h in control culture. Consistent with this observation, the number of yeast cells at this time point reached a maximum, followed by a rapid decline reflecting the appearance of filamentous forms (Figs 1b and 2a). The cell numbers (Fig. 1b) were in agreement with the results of cell density until the transition point (Fig. 1a). Remarkably, when high Al amounts were added to the medium, changes in the cell morphology were detected. *Yarrowia lipolytica* culture exposed to 0.5 mM AlK(SO₄)₂ exhibited significantly less filamentous forms after 24 h than that of control (Figs 1b and 2b). Furthermore, at 1 mM AlK(SO₄)₂ the transition was delayed (24 h); many of the filamentous cells

![Fig. 1.](image1.png)  
Fig. 1. Effect of Al on JM12 strain. (a) *Yarrowia lipolytica* cell culture growth curve in YED medium, pH 4.5, in the absence (•) or presence of AlK(SO₄)₂ (▲, △). (b) The number of yeast cells at each time point was counted and the maximal value in control culture was considered to be 100%.

![Fig. 2.](image2.png)  
Fig. 2. Visualization of the Al-dependent effect on the morphogenic transition of *Yarrowia lipolytica* JM12 strain. Cell morphology was examined in cultures grown for 24 h in the absence (a) or presence of 0.5 mM AlK(SO₄)₂ and 1.0 mM AlK(SO₄)₂ (b and c, respectively).
were in the pseudohyphal rather than true hyphal form and the yeast form predominated even after 48 h (Figs 1b and 2c). Low Al concentrations (0.1 mM) neither suppressed the dimorphic switching nor interfered with growth (data not shown).

**Effect of Al on plasma membrane H\(^+\)-ATPase activity**

Next, the effect of Al on plasma membrane H\(^+\)-ATPase was analyzed. The P-type H\(^+\)-ATPase is modulated in plants by Al (Façanha & Okorokova-Façanha, 2002; Shen et al., 2005). Additionally, extracellular pH is among the key factors involved in the regulation of dimorphism in *Y. lipolytica* (Ruiz-Herrera & Sentandreu, 2002; Szabo & Štofaniková, 2002). To verify whether plasma membrane H\(^+\)-ATPase is a potential target of Al, the total membranes were isolated from cells exposed or not to Al. As shown in Fig. 3a, the ATP addition to membrane vesicles from unstressed cells promoted ACMA fluorescence quenching, which was prevented 85% by 0.1 mM vanadate, indicating that H\(^+\) transport in *Y. lipolytica* total membranes was mainly mediated by the plasma membrane P-type H\(^+\)-ATPase. The same percentage of vanadate inhibition was found in membrane vesicles isolated from Al-treated cells; however, the maximal amplitude of vanadate-sensitive H\(^+\)-transport was 1.8-fold higher than in untreated cells indicating that the P-type H\(^+\) pump was stimulated by Al (Fig. 3b, Table 1).

The hydrolytic activity of the plasma membrane H\(^+\)-ATPase was next examined. The comparison of control *Y. lipolytica* and Al-exposed cells revealed vanadate-sensitive ATPase activity (70% of total activity) that was slightly increased by Al treatment (1.2-fold, Table 1). Thus, Al promotes differential modulation of the H\(^+\) extrusion and ATP hydrolysis, suggesting an increase in the coupling of these two processes.

**Effect of Al on ambient pH changes in solid media**

To further confirm the data on the stimulation of plasma membrane H\(^+\)-ATPase by Al, the H\(^+\) extrusion from *Y. lipolytica* cells was assessed by visualization of the pH changes in solid acidic medium using bromocresol purple as a pH indicator. As Fig. 4a shows, *Y. lipolytica* colonies exhibited an increase in the external medium pH, indicated by the appearance of a purple area around the colonies. This alkalization was remarkably reduced when *Y. lipolytica* was grown in the presence of 1.0 mM AlK(SO\(_4\))\(_2\) (Fig. 4b), suggesting stronger H\(^+\) efflux from Al-grown cells. Additionally, the morphology of cells in solid medium was analyzed and the result corroborated the data obtained in liquid medium (Fig. 2), i.e. the dominance of yeast or filamentous forms on Al-containing and control plates, respectively (data not shown).

**Discussion**

During the last decade, the budding yeast *Saccharomyces cerevisiae* has emerged as a model for studying the mechanisms of Al toxicity and tolerance. It has been shown that, as in the case with plants, Al caused *S. cerevisiae* growth inhibition (MacDiarmid & Gardner, 1996; Schott & Gardner, 1997; Ezaki et al., 1998).

In the present work, the effect of Al on dimorphic fungi *Y. lipolytica* is analyzed. It was found that *Y. lipolytica* growth was not impaired under Al stress, indicating that *Y. lipolytica* is more resistant towards Al than budding yeast. *Y. lipolytica* resistance in response to Al treatment could be related to organic acid exudation, a process described in plants.
Previous studies point to the plasma membrane H\(^+-\)ATPase formation takes place at pH above the neutral. in such a way that yeast form is favored at acidic pH while Y. lipolytica–hypha transition is controlled by environmental pH through regulatory cascades (Kadosh & Johnson, 2005). Factors that trigger the expression of hyphal-specific genes Dimorphic transition in inference with the transmission of hyphal-formation signal. Al-containing medium, indicating that there was an inter-genesis. The hyphal growth of induced in Al-treated Y. lipolytica cells.

Surprisingly, Al dramatically affects Y. lipolytica morphogenesis. The hyphal growth of Y. lipolytica was inhibited in an Al-containing medium, indicating that there was an interference with the transmission of hyphal-formation signal. Dimorphic transition in C. albicans is induced by different factors that trigger the expression of hyphal-specific genes through regulatory cascades (Kadosh & Johnson, 2005). Yeast–hypha transition is controlled by environmental pH in such a way that yeast form is favored at acidic pH while filament formation takes place at pH above the neutral. Previous studies point to the plasma membrane H\(^+-\)ATPase as an important factor of C. albicans morphogenesis (Kaur et al., 1988; Stewart et al., 1988). The involvement of the Y. lipolytica H\(^+\)-pump in morphogenesis and filamentous growth has not been examined previously. The search of the Y. lipolytica genome for the presence of P-type ATPases identified 13 ORFs encoding for P-type ATPases but only one, namely YALI0B2206/Q6CDQ7, exhibited high identity (78%) with the S. cerevisiae plasma membrane Pma1p (unpublished data).

It was found that Al stimulates the plasma membrane P-type H\(^+\)-ATPase of Y. lipolytica (Fig. 3, Table 1). In plants, Al can have both stimulatory and inhibitory effects on the H\(^+\)-ATPase activity depending on the concentration of Al used. The present findings are in agreement with data for plant enzyme where Al promoted H\(^+\)-ATPase stimulation at concentrations lower than the threshold of Al phytotoxicity (Façonha & Okorokova-Façonha, 2002; Shen et al., 2005). In case of Y. lipolytica, Al exerts a differential effect on its activity, being more effective in the enhancement of H\(^+\) pumping than ATP hydrolysis (Table 1). Thus, it is likely that the Al-dependent effect on pump involves posttranslational regulation that modifies the H\(^+\)/ATP coupling ratio of the enzyme. Indeed, posttranslational regulation involving phosphorylation was demonstrated for plant H\(^+\) pump (Shen et al., 2005).

The increased ATP-dependent H\(^+\) transport in membrane vesicles isolated from cells grown in the presence of Al reflected in vivo environmental pH changes as revealed by the pH indicator bromocresol purple. Previously, this dye was successfully used to detect rhizosphere pH changes (e.g. Ahn et al., 2002) and ambient pH in fungi colonies (St Leger et al., 1999). The changes in the pH of solid medium produced by Y. lipolytica cells under Al stress (Fig. 4) were consistent with Al-induced stimulation of H\(^+\) translocation in the membrane vesicles (Table 1) and reinforce the notion that Al influences the extracellular pH by activating the H\(^+\) pumping at the plasma membrane. The observed increase of external pH is due to ammonia production and release to the fungal colony surroundings (St Leger et al., 1999; Palková et al., 2002).

### Table 1. Aluminum effect on Yarrowia lipolytica plasma membrane H\(^+\)-ATPase

<table>
<thead>
<tr>
<th>Condition</th>
<th>F(_{\text{max}}) (%)</th>
<th>ATP hydrolysis (nmol mg(^{-1}) min(^{-1}))</th>
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<tbody>
<tr>
<td>Control</td>
<td>687 ± 25</td>
<td>95 ± 10</td>
</tr>
<tr>
<td>1.0 mM AlK(SO(_4))(_2)</td>
<td>1258 ± 27</td>
<td>120 ± 18</td>
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</table>

Vanadate-sensitive H\(^+\) transport and ATP hydrolysis was assayed in total membrane vesicle preparations isolated from cells grown in the presence of 1 mM AlK(SO\(_4\))\(_2\) for 18 h. Determination of F\(_{\text{max}}\) was performed using data of Fig. 3 as described in "Material and methods". Values are the means ± SE of three experiments.

(Kochian et al., 2004; Shen et al., 2005). Yarrowia lipolytica is known for its capacity to produce organic acids (Barth & Gaillardin, 1997) and citrate efflux was suggested to be performed by a plasma membrane transport system rather than by vacuole (Kulakovskaya et al., 1993). It would be interesting to determine whether organic acid secretion is induced in Al-treated Y. lipolytica cells.

Fig. 4. Al-dependent effect on pH changes in solid media. Colonies of Yarrowia lipolytica were allowed to grow for 3 days in solid YED medium, pH 4.5, in the absence (a) or the presence of 1 mM AlK(SO\(_4\))\(_2\) (b) and the low-melting agarose containing bromocresol purple pH indicator (pH 4.5) was spread over the plate surface. Purple zones indicate alkalization.

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pH mediated by *S. cerevisiae* colonies was accompanied by a gradual decline in the expression of *PMA1* and *PMA2* genes encoding plasma membrane H\(^+\)-ATPase (Palková *et al.*, 2002).

The present results lead to the suggestion that alterations in the plasma membrane H\(^+\) transport might underlie a pH signaling required for hyphal development. The enhancement in H\(^+\) extrusion in response to the presence of Al can induce changes in membrane energization and surface pH, which became more acidic than in unstimpered cells. The present results, together with the data available in the literature on the importance of low pH as an effector of fungal dimorphism, suggest the existence of an extracellular pH sensor. It is tempting to speculate that surface pH plays a key role in the transduction of signal from pH sensor to pH-responsive factors, which elicit the transcriptional changes leading to morphological alterations.

The stimulation of *Y. lipolytica* PMA activity in response to Al treatment would result both in cell surface acidification and cytoplasm alkalization. A transient increase in intracellular pH was suggested to precede the morphological transition of *C. albicans* (Kaur *et al.*, 1988; Stewart *et al.*, 1988). The present results imply that extracellular pH rather than intracellular pH is a signal for morphogenesis. This is consistent with the idea that while plasma membrane ATPase-related cytoplasmic alkalization may be needed for the continued development of germ tubes and may aid in the polarization of morphogenesis in related biosynthetic processes in *C. albicans*, this phenomenon is unlikely to be the primary determinant in morphogenesis (Monk *et al.*, 1993).

The investigation of the molecular mechanisms of Al toxicity and tolerance demonstrated that Al has a multifactorial mode of action. In plant cells, where Al stress has been intensively studied for decades, it was reported that Al induces changes in the cytoskeleton, calcium homeostasis, ion fluxes, oxidative status and many other molecular targets that have been continuously revealed (for a review, see: Matsumoto, 2001). Similarly, it could be expected that Al affects other targets in addition to P-type H\(^+\)-ATPase in *Y. lipolytica*, which could also participate in the filamentation control. Clearly, further studies will be necessary to gain a deeper understanding of the role of the plasma membrane H\(^+\)-ATPase in this process as well as of other putative molecular targets involved in dimorphism of fungal cells.

In conclusion, the present data show the importance of the cell surface pH as a determinant of morphogenesis of *Y. lipolytica* and the plasma membrane H\(^+\)-ATPase as a key factor in this process. In addition, *Y. lipolytica* can be used to assess the role of plasma membrane H\(^+\)-ATPase in Al toxicity and tolerance. Finally, the present data provide a platform for future studies of the putative pH sensor(s) and associated signaling pathways.

### Acknowledgments

*Yarrowia lipolytica* JM12 strain was kindly provided by Prof. Angel Domínguez, Universidad de Salamanca. This work was supported by FAPERJ (Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro), CNPq (Conselho Nacional de Pesquisa e Desenvolvimento), IFS (International Foundation for Science) and UENF (Universidade Estadual do Norte Fluminense). A.L.O.F., A.R.F. and L.A.O. are research fellows of CNPq. This work is a part of the MSc thesis of F.A.L.

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