MATERIALS & METHODS
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1) Organism, media and growth conditions:

*C. albicans* wild type strains, ATCC 10261, ATCC 307 (D.H. Howard, UCLA, USA), and a clinical isolate 286 from All India Institute of Medical Sciences, New Delhi were used to isolate mutants. Wild type *S. cerevisiae* E1278b and mutant MG 2129 (*Pma1*) were obtained from Prof. A. Goffeau, Belgium. All these strains were maintained on YEPD agar plates (Appendix-I) at 4°C and were recultured monthly after single colony isolation. The orthovanadate resistant mutants (isolated in this study) were maintained on SD minimal medium (Appendix-II) containing 15 mM sodium orthovanadate at 4°C. Rich medium used were YEPD (Appendix-I), minimal medium SGM, SD and SDA were used (Appendix-II) for different experiments.

For experimental purposes all the cultures were grown at 30°C in rotary shaker up to stationary phase and then were inoculated to fresh medium at 30°C unless otherwise mentioned. The growth of all cells was monitored turbidometrically at 595 nm on Shimadzu UV-150 or UV-1201 spectrophotometer. Cells were harvested by centrifugation (1000xg for 5 min) and washed twice with distilled water before using them for various studies.

2) Chemicals:

Ampicillin, Antimycin-A, ATP, BSA, Coomassie Brilliant Blue, chloramphenicol, Cycloheximide, DMSO, DES, Erythromycin, Ethidium bromide, Glycyl-glycine, Glucose,6-P- dehydrogenase, Hygromycin-B, Hexokinase, MES, Miconazole, Nystatin, NADP+, NAD+, NADH, NTG, PMSF, Propionic acid, PPO, POPOP, Protamine sulfate, Periodic acid, Sucrose, Sorbitol, Sodium Azide, Sodium orthovanadate, Triton X-100, Trizma- HCl, Trizma-Base, Tetracycline, were from Sigma Chemical Co., St. Louis, USA.

X-bag (N,N’-(P-Xylylidene)-bis-amino guanidine-2 HCl) was a kind gift from the Department of Synthesis of Drugs of the Institute of Immunology.
and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland. Sodium 2 [14-C]-Propionate was procured from Amersham-Buchler, Germany. [14-C]-Inulin was from NEN Research Products, Dupont, USA. Uniformly labelled [14-C] amino acids (L-glu, L-lys, L-pro, gly) and [3H]-H2O were obtained from Bhabha Atomic Research Centre, Bombay, India. Other chemicals were procured locally and were of analytical grade.

In all the molecular biology experiment the chemicals were generously gifted by Prof. J. Gorman, Squibb Institute, New Jersey, USA.

METHODS

i) Preparation and storage of sodium orthovanadate solution:

A stock solution of 100 mM sodium orthovanadate was prepared in water, filter sterilized, and stored frozen at -20°C in equal portions. The pH of the solution was not adjusted for usual experiments. Occasionally the orthovanadate solution was boiled, cooled and used for experiments. For NADH oxidation experiments polyvanadate was used (kindly provided by Prof. T. Ramasarma, IISc, Bangalore).

ii) Mutagenesis:

Mutagenesis was done as described by Jenkinson and Shepherd (Jenkinson and Shepherd, 1987). An exponentially growing culture (50 ml) containing 10⁷ - 10⁸ cells/ml of each strain in YEPD medium at 28°C was centrifuged (1000xg for 5 min) and the cells were suspended at a density of about 10⁸ cells/ml in 200 mM of sodium acetate buffer, pH 7.0, containing NTG (0.45 mg/ml). The culture was incubated with gentle shaking at 28°C for 1 hr. The cells were then collected by centrifugation (1000xg for 5 min.) in 50 ml of YEPD after two washing by the same medium. These cells were then incubated for further 2 hrs with vigorous shaking. After the treatment, samples were taken, appropriately diluted and spread onto YEPD agar to determine viable counts. The mutagen treatment resulted in 96-97% killing of the cells.

The NTG treated yeast cells were harvested by centrifugation and suspended in 4 ml of SGM in a 90 mm sterile plastic petri dish and
irradiated for 40 sec with a 30 W germicidal UV light to induce mitotic segregation and to favour the formation of a mutant strain homozygous for the mutated allele. The UV treated cells were then added to warm (28°C) YEPD medium (20 ml), incubated with shaking at 28°C for further 2 hrs. Portions (0.1ml) were then diluted and plated onto SD agar containing sodium orthovanadate (15 mM) and incubated for 7 days at 30°C and resistant colonies were picked up (30% sterile glycerol was added to the remainder of the mutagenized cells, and was stored at -20°C for upto 7 days, during which time further aliquots were used to screen the mutants). The orthovanadate resistant clones were subcultured on YEPD rich medium 15-20 times and rechecked for orthovanadate resistance.

### iii) Measurement of growth rate of the mutants:

The wild types and orthovanadate resistant mutants were grown for 24 hrs at 30°C in minimal medium (or rich medium) and 10^6 cells/ml inoculated in fresh medium and for checking orthovanadate resistance the cells were grown for 8 hrs in minimal medium and different concentration of orthovanadate was added. Growth was followed for further 24 hrs and measured turbidometrically with the help of a spectrophotometer at 595 nm. Generation time (GT) and specific growth rate (SGR) was measured as following: Generation time was calculated using the formula

\[
g = \frac{0.301 (t - t_0)}{\log 10 B_t - \log 10 B_0}
\]

Where:
- \( g \) = Generation time
- \( B_0 \) = Reading of optical density at time 0
- \( B_t \) = Reading of optical density at time \( t \)
- \( t_0 \) = 0 time
- \( t \) = Anytime after \( t_0 \).

Specific growth rate was calculated according to Vallejo and Serrano, 1989, using the formula:

\[
\ln 2 / d.t. \quad \text{where,} \ d.t. = \text{doubling time}
\]
iv) **Scanning electron microscopy (SEM):**

Cells were grown up to stationary phase (24 hrs, as described earlier). 500 µl of cells were taken in eppendorf tubes and centrifuge for 5 min at 1000xg, the cell pellet was rinsed with 200 mM potassium phosphate buffer (pH 7.5). 25% of the glutaraldehyde (pH 7.5) was added to double the volume of the cells and kept for 2 hrs at 4°C. Cells were centrifuged and supernatant was discarded. The pellet was rinsed twice with phosphate buffer (200 mM, pH 7.5) and resuspended in same buffer. 1% of osmium tetroxide (OSO₄) was added (prepared in phosphate buffer) to the pellet (double the volume of the cell pellet), again kept for 2 hrs at 4°C. The pellet was rinsed once with phosphate buffer and then with glass distilled water (to remove phosphate crystals) and resuspended in distilled water. One drop of cell suspension was dropped on grid (previously marked and coated with silver emulsion stain and fixed on the smooth surface of the mirror glass), left for overnight to air dry. Cells were coated again (on the grids) with silver emulsion and put on the SEM microscope (Model 501B, Philips) for scanning. Scanning was done and was photographed at 10,000 x magnification.

v) **Crude membrane isolation:**

Crude membranes were isolated according to Tuduri et al. (Tuduri et al., 1985). From various mutant and wild type strains, cells were grown up to mid log phase in YEPD (inoculated with 10⁶ cells/ml) at 30°C, harvested and washed twice with distilled water and homogenized as follows: 450 mg wet weight of the cells were suspended in 1 ml of grinding medium containing 250 mM sucrose, 10 mM Tris-HCl, pH 7.5 and 1 mM PMSF. To 1 ml of this suspension 1 g of glass beads (0.45 µm size) were added and the mixture was agitated 9 times during 10 sec with an interval of 3 sec using a CO₂ refrigerated MSK Braun homogenizer with a micro adapter. The homogenate was centrifuged at 1000xg for 5 min and the pellet was washed at 1000xg for 5 min with 500 µl of grinding medium. The combined supernatants were centrifuged again for 5 min at 1000xg. The resulting supernatant was centrifuged for 40 min at 15000xg. The pellet was suspended in 300 µl of 250 mM sucrose, 10 mM Tris-HCl, pH 7.5, called as 'crude membrane fraction'. For routine assay this fraction of crude plasma membrane was used.
vi) **PM H⁺-ATPase Assay:**

Plasma membrane H⁺-ATPase activities in the presence and absence of inhibitors were determined at 30°C. in a reaction mixture containing 10 mM ATP, 10 mM MgCl₂, 25 mM MES-KOH pH 6.6, 10 mM NaN₃ and 30-40 μ of protein (crude membrane fraction) in 0.5 ml (Anand, 1989). The reaction was initiated with the addition of ATP and after 8 min the reaction was stopped by the addition of 3 volumes of 0.5% SDS, 2% H₂SO₄ and 0.5% ammonium molybdate. Inorganic phosphate was measured using 10% Ascorbic acid (20 μl) after 15 min of incubation (at 30°C) at 750 nm.

vii) **Protein Estimation:**

Protein was estimated by the Bradford method (Bradford, 1976). For estimations in whole yeasts, cells were boiled in 10% TCA for 30 min to release the tightly bound proteins. After cooling, cells were centrifuged and the pellet was dissolved in 0.1N NaOH. To a suitable aliquot (0.1 ml) 5 ml of working solution (W.S.) (Appendix-III) was added. For protein estimations in "Crude membrane fractions", 5 ml of W.S. was added to 10 μl of the fraction. After 2-10 min, the colour intensity was read at 595 nm using bovine serum albumin (BSA) as standard.

viii) **Measurement of H⁺ efflux:**

Glucose induced H⁺ efflux was measured by the addition of 10 mM Glucose in a water jacketed vessel in a total volume of 5 ml of yeast cells (20-40 mg dry wt.) at 30°C (Serrano, 1980), the medium contained 100 mM KCl and 10 mM glycyl-glycine (pH 5.8). For calibration pulses of 1 umoles of HCl were used. pH of the suspension was recorded using a Radiometer (Copenhagen Rec-80 Servograph). Full scale of the recorder was adjusted to 2.8 pH units and the proton ejection by the cells was started by the addition of glucose.

ix) **Germ tube induction:**

The parental wild type and mutants were grown on SDA for 24-72 hrs. The cultures were incubated at room temperature, fresh cultures (single
colonies) were inoculated in tube containing 0.1 ml of pooled human and rabbit serum with an appropriate control. The tubes were incubated at 37°C for 2 hrs. A loopfull of the cells were removed from each tube, placed on a glass slide and examined microscopically. The percentage of cells with germ tubes was determined by counting 100 cells (Odds, 1988).

**x) Phenotypic characterization of the mutants:**

pH sensitivity, osmotic pressure sensitivity, sensitivity to high ionic strength, and for drug resistance determination; rich YEPD medium was used according to McCusker et al., 1987. Single colonies were isolated and streaked on YEPD plates, grown for 24 hrs at 30°C as patches and then replica plated on respective test medium using sterile velveteen cloth. Plates were incubated at 30°C for 2-4 days, during which resistant cells grew as confluent patches whereas, less resistant cells failed to grow after 48 hrs. at 30°C but often produced papillae after 3-4 days, probably arising by spontaneous mutation. For pH sensitivity testing 0.05 M acetic acid was added to YEPD and pH was adjusted to pH 4.5 or 5.0 with NaOH or KOH before autoclaving. Osmotic pressure / high ionic strength sensitive growth was determined by using YEPD supplemented with KCl (2.5 M), NaCl (2.2 M) or glycerol (3.5 M). Temperature sensitivity of the cells were tested at 25°C, 37°C, 40°C using YEPD plates in incubator oven. Different drugs/inhibitors sensitivity/resistance was checked using different concentration of drugs/inhibitors dissolved in water or ethanol or DMSO and added after autoclaving (filter sterilized) (Ulaszewski et al., 1986).

**xi) Measurement of phosphate uptake:**

Phosphate uptake was measured using the same protocol of amino acid uptake by Verma and Prasad 1983. For uptake, a 4% cell suspension of mid long phase cells in 100 mM Tris- Citrate buffer (pH 4.5) was incubated at 30°C for 10 min. The reaction was initiated by the addition of 600 μM of 32P-labelled phosphate (2.59 MBq/μ). After the indicated time interval 0.1 ml of aliquot was taken out and filtered rapidly through Millipore (Millipore, Bangalore, India) filter discs (0.45 μM), and washed twice with ice cold phosphate (KH₂PO₄ 600 μM) in 10 mM of Tris-citrate (pH 4.5). Filters were dried and the radioactivity retained on the filter paper discs was measured
in a Beckman Liquid Scintillation Counter (Model LS 1801) using toluene based scintillation fluid (Cocktail 'O') (Appendix- V). For pH optimum of phosphate uptake Tris-citrate buffer was adjusted to different pH's and uptake studies were done. For kinetic studies, 10-1000 μm of phosphate (³²P) was used at pH 4.5. For derepression of phosphate uptake studies the mid log phase grown cells (in high phosphate medium) were grown for further 3 hrs in low phosphate medium (SGM minus KH₂PO₄) and uptake was done as described.

**xii) Measurement of amino acid uptake:**

Transport of amino acids was performed in a reaction mixture, containing 5% cells (w/v). The cells were preincubated in 100 mM Tris-citrate pH 4.5, containing cycloheximide (200 μg/ml) for 10 minutes at 30°C to inhibit the protein synthesis. The reaction was initiated by the addition of ¹⁴C-labelled amino acids (37 KBq./umole, final concentration 100 μM of glycine; L-glutamic acid; L-lysine, and L-proline) to the reaction mixture. At indicated time intervals, a 0.1 ml of aliquot was removed by Eppendorf pipette, diluted 20-fold with chilled Tris-citrate buffer (10 mM, pH 4.5) containing 100 μM of respective cold amino acids, filtered rapidly through Millipore filter discs (0.45 μM) and washed thrice with the same buffer. The filters were dried and radioactivity retained on the filter discs was measured in a Beckman Liquid Scintillation Counter (Model LS 1801) using toluene base scintillation fluid (Appendix-IV). The rate of amino acid uptake was calculated from the time course of uptake upto 195 sec using linear regression analysis. For kinetic studies different concentration of radioactive amino acids (10 - 1000 μM) were used. Transport assay conditions were similar to those described above with the exception of single time point (1 min) was taken for uptake.

**xiii) Dry weight measurement:**

Small glass test tubes (1.5 ml size) empty weight was taken and 500 μl of diluted cell suspension (5-50% depending on the nature of the experiment(s) was taken (duplicate) with the help of a eppendorf pipette. Tubes were kept (in a stand) at 70-80°C inside a oven for 24 hrs. Tubes were taken out and cooled it to room temperature and sample weight was taken.
The sample weight minus empty weight of the tube gives the actual dry wt. value of the cells / 500 µl of cell suspension.

xiv) Measurement of membrane stimulated vanadate dependent NADH oxidation:

The NADH oxidation assay was carried out according to Ramasarma et al., 1981. The assay system consisted of NADH (0.1 mM), potassium phosphate buffer (50 mM pH 7.0) vanadate as polyvanadate (500 µM) and various concentrations (30-50 µg proteins) of crude membrane fractions in 1 ml. NADH oxidation activity was measured at 30°C by a decrease in absorbance at 340 nm using a UV-190 spectrophotometer attached to a Unicom linear recorder. The nmoles of NADH oxidized per minute was calculated from the recorded slope using the molar extinction coefficient of NADH (ε = 6.22 liters/mol/cm). Total NADH oxidation is the disappearance of NADH measured in the presence of both vanadate and protein. Nonenzymatic NADH oxidation is measured as the disappearance of NADH in the presence of vanadate alone. These activities were determined in separate assays. The vanadate stimulated crude membrane dependent activity is calculated as total NADH oxidation in presence of antimycin- A (to stop mitochondrial oxidation) minus nonenzymatic NADH oxidation.

xv) Measurement of intracellular volume:

Intracellular water space (volume) of the cell was measured according to Rottenberg using 3[H]H2O (Rottenberg, 1979). A 50% (w/v) cell suspension of mid-log phase grown cells was prepared. 400 µl cell suspension was preincubated for 10 min in a microfuge tube at 30°C and 50 µl of 3[H]H2O (37 MBq/ml) and 50 µl of 14C -Inulin (1.85 MBq/ml) was added. After 30 min of incubation at 30°C the whole eppendorf tube was centrifuged and the pellete and supernatant was separated. 1 ml of 1 M perchloric acid was added to pellet and 950 µl of 1 M perchloric acid was added to 50 µl of supernatant. 50 µl of each suspension was counted in Beckman Liquid Scintillation Counter (LS 1801) using 10 ml of cocktail 'T' (Appendix-V).
The intracellular volume was calculated using the formula:

\[ V_n = V_s \left( \frac{3[H]^P}{3[H]S} - \frac{14[C]^P}{14[C]S} \right) \]

where, \( V_n = \text{Volume (µl)} \), \( V_s = \text{Volume of aliquot} \), \( P = \text{Pellet} \), \( S = \text{Supernatant} \).

**xvi) Measurement of intracellular pH (pHi) using weak acid:**

pHi values were calculated by determining the steady state distribution of propionic acid across the plasma membrane (Cartwright *et al.*, 1986). A typical reaction mixture containing 100 mM Tris citrate buffer (of indicated pH) and cells (10-15 mg dry wt./ml) was incubated at 30°C. The distribution of propionic acid was initiated by the addition of 10 µM sodium 2-[\(^{14}\text{C}\)]-Propionate (1.85 kBq/ml). For actual pHi determinations, triplicate samples (0.3 ml) were removed after 4 minutes and rapidly filtered through Millipore filter discs (0.45 µM). Cells retained on the filters were washed four times with 1 ml of ice cold unlabelled 10 µM propionic acid. Radioactivity retained was counted using a toluene based scintillation fluid (Appendix- IV) in Beckman Liquid Scintillation Counter LS-1801. pHi values were calculated according to Waddell and Butler

\[ \text{pHi} = pKi + \log \left[ R \left( 10^{(pHe - pKe)} + 1 \right) - 1 \right] \]

where \( R = \frac{TAi Ve}{TAe Vi} \) and \( Ai \) and \( Ae \) are, respectively, the internal and external total amounts of propionic acid; \( Vi \) and \( Ve \) the intra and extracellular volumes; \( pKi \) and \( pKe \) the dissociation constants for propionic acid in the internal and external environments and pHi and pHe, the intra and extracellular pH. The intracellular concentrations of propionic acid was calculated by taking 2.0 µl (for wild types) and 2.4 µl (for mutants) volume of intracellular water mg/dry wt. (as described in earlier paragraph). The internal and external dissociation constants for propionic acid were calculated from the Davies’ simplified version of the Debye-Huckel equation, assuming that the ionic strength within the cells was in the region, 0.15 - 0.25 (Conway and Downey, 1950; Davies, 1962). Values for \( pKi \) and \( pKe \) were calculated to be 4.86 and 4.75, respectively.
Protein glycosylation using PAS staining:

Changing in protein glycosylation were assessed in crude membrane fractions from the polyacrylamide gel electrophoresis patterns following the Periodic Acid Schiff's (PAS) staining. Periodic acid reacts with vicinal hydroxyl group commonly found in sugar molecules to generate aldehydes which can be detected by their colour reaction with reduced Schiff's reagent (Fairbankes et al., 1971). 8% SDS-PAGE run gel (according to Gupta et al., 1991) was fixed with 25% isopropanol, 10% Acetic acid overnight and the gel was transferred to 10% isopropanol, 10% Acetic acid mixtures for 6-9 hrs. The gel was transfer to 10% acetic acid for overnight. Then the gel was treated with 10 volumes of 0.5-% periodic acid for 2 hrs in the dark at 4°C. Gel was transferred to 0.5% sodium arsenite, 5% acetic acid for 30-60 minutes, treated again with 0.1% sodium arsenite and 5% acetic acid for 20 minutes. Then the gel was transferred to 10 volumes of acetic acid for 20 minutes. 10 volumes of Schiff's reagent (Appendix-VI) was added and left overnight in the dark at 4°C. The glycoproteins were stained rose Pink in colour. To increase the contrast and retard fading, the gel was washed with 0.1% metabisulfdide in 10 mM HCl for several hrs until the rinse solutions fails to turn pink upon addition of formaldehyde.