3. MATERIALS AND METHODS

3.1. Chemicals

The following chemicals were obtained from Sigma Chemical Company: Adenine sulfate, Ampicillin (sodium salt), L-Arginine-HCl, d-Biotin, L-Canavanine sulfate, Cesium chloride (Grade I), Chloramphenicol (Chloromycetin; D(-)threo-2,2-Dichloro-N[β-hydroxy-α-(hydroxymethyl)-p-nitrophenethyl] acetamide), CM-Sepharose, DL-Dithiothreitol, Erythromycin (Grade I), Ethidium bromide, Glass beads (Type V, size: 450-500 μm), L-Histidine-HCl, L-Leucine, Lysozyme (Grade III), D-Mannitol, 2-Mercaptoethanol, Oligomycin, Pencillin-G (Benzy1-pencillin, sodium salt), Polyethylene glycol (PEG)-4000, Ribonuclease A (RNase A, Type 1-A), Sodium azide, Sodium dodecyl sulfate (SDS; Lauryl sulfate, sodium salt), D-Sorbitol, Tetracycline-HCl, Thiamine-HCl, L-Threonine, Tris(Tris[hydroxymethyl]aminomethane; reagent grade), L-Tryptophan and Uracil.

Bacto-agar, Bacto-peptone, Bacto-tryptone, Bacto-yeast extract and Yeast nitrogen base w/o amino acids were procured from Difco Laboratories. Agarose
(SeaKem, LE grade) was from Marine Colloids Division of FMC Corporation. Bisbenzimide (Hoechst No.33258) was from Serva. Glusulase was from Endolaboratories. Polyethylene glycol (PEG)-6000 was from SISCO Research Laboratories. Restriction endonucleases were from Boehringer Mannheim. Sarkosyl (NL97) was from ICN Pharmaceuticals Inc.

DAPI(4',6-Diamidino-2-phenylindole) was a gift from Prof. O. Dann. λDNA was purified in our laboratory from λlysogen ci857tsSam7 by standard methods (Maniatis et al., 1982). Other chemicals used were of the highest grades available from BDH (Glaxo Laboratories, India), E. Merck (India) and Sarabhai M. Chemicals. Double distilled water was used for the preparation of all solutions and media.

3.2. Organisms

Plasmid YEpl3 (Broach et al., 1979; Fig.4) was maintained in E.coli strain C600 (F-,thi-1, thr-1, leuB6, lacY1, tonA21, supF44, λ-) or HB101 (F-,hsdS20 [r-, m-], recA13, ara-14, proA2, lacY1, galK2, rpsL20 [SmR], xyl-5, mtl-1, supF44, λ-). The S.cerevisiae strains are listed in Table 2.
Table 2. *Saccharomyces cerevisiae* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nuclear markers</th>
<th>Mitochondrial markers</th>
<th>Reference/Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>A22</td>
<td>a his1</td>
<td>rho^+omega^C^321_E^354</td>
<td>Sec. 3.8 and 4.3.5.</td>
</tr>
<tr>
<td>AN^R OR12D*</td>
<td>a his4 leu2 thr4</td>
<td>rho^+omega^+</td>
<td>(Gunge and Sakaguchi, 1979)</td>
</tr>
<tr>
<td>BO60AF-1</td>
<td>a ade2 arg4 leu2</td>
<td>rho^o</td>
<td>Nagley and Linnane, 1978</td>
</tr>
<tr>
<td></td>
<td>trp</td>
<td></td>
<td>Broach et al. 1979</td>
</tr>
<tr>
<td>BT1-9</td>
<td>a his4-15 ade2-1</td>
<td>rho^o</td>
<td>Sec. 4.3.2.</td>
</tr>
<tr>
<td>DC5</td>
<td>a leu2-3 leu2-112</td>
<td>rho^+</td>
<td>(Dujon et al. 1976)</td>
</tr>
<tr>
<td>DC5-0</td>
<td>a leu2-3 leu2-112</td>
<td>rho^o</td>
<td>(Netter et al. 1974)</td>
</tr>
<tr>
<td>IL16-10B</td>
<td>a his1</td>
<td>rho^+omega^C^321_E^354</td>
<td>(Dujon et al. 1976)</td>
</tr>
<tr>
<td>IL187-7A</td>
<td>a his1</td>
<td>rho^+omega^C^321_E^354</td>
<td>(Dujon et al. 1976)</td>
</tr>
<tr>
<td>IL249-8A</td>
<td>a trpl ural</td>
<td>rho^+omega^E^354</td>
<td>(Netter et al. 1974)</td>
</tr>
<tr>
<td>IL290-5D</td>
<td>a trpl ural</td>
<td>rho^+omega^E^354</td>
<td>(Netter et al. 1974)</td>
</tr>
<tr>
<td>IL8-5C/</td>
<td>a his1 ural</td>
<td>rho^+omega^C^321_E^354</td>
<td>Michel et al. 1979</td>
</tr>
<tr>
<td>H11A2</td>
<td></td>
<td></td>
<td>Dujon et al. 1976</td>
</tr>
<tr>
<td>IL8-8D</td>
<td>a ural</td>
<td>rho^+omega^C^321_E^514</td>
<td>Michel et al. 1979</td>
</tr>
<tr>
<td>IL841-6B</td>
<td>a ural</td>
<td>rho^+omega^E^354</td>
<td>(Netter et al. 1974)</td>
</tr>
<tr>
<td>KM55-2D</td>
<td>a ural</td>
<td>rho^+omega^E^354</td>
<td>(Netter et al. 1974)</td>
</tr>
<tr>
<td>5-8</td>
<td>a leu2-3 leu2-112</td>
<td>rho^+omega^C^321_E^514</td>
<td>Michel et al. 1979</td>
</tr>
<tr>
<td>5-841</td>
<td>a leu2-3 leu2-112</td>
<td>rho^+omega^E^354</td>
<td>(Netter et al. 1974)</td>
</tr>
<tr>
<td>9-8</td>
<td>a his4-15 ade2-1</td>
<td>rho^+omega^C^321_E^514</td>
<td>(Netter et al. 1974)</td>
</tr>
<tr>
<td>9-841</td>
<td>a his4-15 ade2-1</td>
<td>rho^+omega^E^354</td>
<td>(Netter et al. 1974)</td>
</tr>
</tbody>
</table>

* AN^R OR12D was originally CR II; it spontaneously became OS during maintenance on YPD slants.
3.3. Media

Media preparation was based on Coen et al. (1970), Sherman et al. (1981) and Maniatis et al. (1982). Percentage is on w/v basis unless otherwise mentioned. The compositions of the liquid media are given below and to prepare solid media, 2% agar was used.

YD : 1% yeast extract and 4% glucose
YPD : 1% yeast extract, 2% peptone and 2% glucose
YPDA : YPD with 20 μg/ml adenine sulfate
YPG : 1% yeast extract, 2% peptone and 3% (v/v) glycerol
SD : 0.67% yeast nitrogen base w/o amino acids and 2% glucose

Supplements are indicated by three letter abbreviations. SD-his-leu-can means SD with histidine, leucine and canavanine. Often the media are indicated as s his, $ leu, $ can etc. in the Tables. $ his means histidine is lacking; rest of the components of the complete medium (defined in respective Tables) are present. $ can means canavanine is present in addition to the components of the complete medium. The supplements were used at the concentrations mentioned in brackets: adenine sulfate (20 μg/ml); L-canavanine sulfate (20 μg/ml); L-histidine-HCl (20 μg/ml); L-leucine (30 μg/ml); L-threonine (200 μg/ml); L-tryptophan (20 μg/ml); uracil (20 μg/ml). L-threonine was autoclaved separately and mixed with the other components.
NO : 1% yeast extract, 1% peptone, 2% glucose and 50 mM Na-K-PO₄ (pH 6.25).

Na-K-PO₄ was prepared with Disodium hydrogen phosphate and Potassium dihydrogen orthophosphate.

NOEB40 : NO with 40 μg/ml ethidium bromide. Ethidium bromide was added to the autoclaved medium, at 55°C, from a sterile stock solution (10 mg/ml, prepared in water).

N2 : N3 with 0.1% glucose

N3 : 1% yeast extract, 1% peptone, 2% (v/v) glycerol, 2% (v/v) ethanol and 50 mM Na-K-PO₄ (pH 6.25). (Ethanol was added to the autoclaved medium, at 55°C, before pouring to the plates).

N3 with antibiotics : N3 with chloramphenicol (C) and/or erythromycin (E) and/or oligomycin (O). Chloramphenicol was used at 4 mg/ml (C4) or 2 mg/ml (C2) concentration. Erythromycin was used at 4 mg/ml (E4) or 2 mg/ml (E2) concentration. Oligomycin was used at 2 μg/ml concentration. They were added to the autoclaved media, at about 55°C, directly as solids or as ethanolic stocks. The ethanolic stocks were made at the following concentrations: Chloramphenicol: 200 mg/ml; erythromycin: 200 mg/ml; oligomycin: 1.8 mg/ml.

LB : 1% tryptone, 0.5% yeast extract and 1% NaCl (pH was adjusted to 7.5 with NaOH).
3.4. Purification of Lyticase

Lyticase was purified from *Oerskovia xanthineolytica* by a modified procedure of Scott and Schekman (1980). Enzyme induction was done with autoclaved yeast rather than with yeast glucan and CM-Sepharose was used instead of CM-cellulose.

Media: M63 contained per litre – 13.6 g KH₂PO₄, 2.0 g (NH₄)₂SO₄, 4.2 g KOH, 0.2 g MgSO₄·7H₂O, 0.001 g Fe₂(SO₄)₃·6H₂O, 1 mg thiamine and 1 mg biotin (thiamine and biotin were added to the autoclaved medium from sterile stock solutions). Carbon source was 20 g/litre autoclaved yeast (M63-Y) or 4 g/litre glucose (M63-G).

Preparation of autoclaved yeast: 5-841 was grown in 1 litre YPD to stationary-phase and harvested. The pellet was combined with an equal volume of distilled water and autoclaved. The autoclaved cells were washed with 100 ml distilled water, twice, and stored frozen at -20°C, until use.

Preparation of substrate cells for assay: One ml of 5-841 inoculum (YPD stationary-phase culture) was transferred to 100 ml YPD. After 7 hr of growth, cells were harvested and washed with 50 mM potassium phosphate (pH 7.5) and 10 mM sodium azide, twice, using 50 ml at a time. Cells were resuspended in the above buffer and stored at 4°C.
Lyticase assay: The assay mixture contained 50 mM potassium phosphate (pH 7.5) and 10 mM sodium azide, sufficient substrate cells to give an absorbance of 0.8 to 0.9 at 800 nm and the enzyme in a total volume of 2.0 ml. The enzyme was added last, incubated at 30°C with shaking and after 30 min, absorbance was read at 800 nm. The decrease in absorbance compared to a no-enzyme control was calculated. Activity was determined from the slope between 20% and 70% reduction in absorbance, as provided by increasing enzyme concentration. One lytic unit is defined as 10% decrease in absorbance in 30 min.

Purification: *O. xanthineolytica* was grown in M63-G for 15 hr at 30°C with shaking. 650 ml of M63-Y was inoculated with 6.5 ml of M63-G culture and was incubated with shaking at 30°C. After 24 hr, the cells and residual substrate were pelleted (14,000 x g, 20 min, 4°C) and the supernatant was transferred to a processed dialysis tubing. It was concentrated overnight against solid PEG-6000 to 80 ml (this and the following steps were performed at 0°C to 4°C). The concentrate was dialysed against two changes of 10 mM sodium succinate (pH 5.0), 1 litre at a time.
CM-Sepharose (100 ml) was equilibrated with 100 mM sodium succinate (pH 5.0), overnight. It was packed in a column (4.5 x 15 cm) and washed with 300 ml of 10 mM sodium succinate (pH 5.0). The dialyzed concentrate of the culture supernatant was passed through the column at a flow rate of 120 ml per hour. The column was washed with 150 ml of 10 mM sodium succinate (pH 5.0) and eluted with 140 ml of 0.25 M sodium chloride and 10 mM sodium succinate (pH 5.0); 7 ml fractions were collected. 2 µl of the fractions was used in lytic assays. The fractions with lytic activity were pooled, transferred to a dialysis tubing, concentrated against PEG-6000 and dialysed, first against 1 litre of 50 mM potassium phosphate (pH 7.5) and 10 mM sodium azide and subsequently against 200 ml of above buffer with 20% (v/v) glycerol. The enzyme preparation was stored at 4°C. Protein concentration was determined by using the equation (Whitaker and Granum, 1980): Protein concentration (mg/ml) = (A_{235} - A_{280})/2.51.

3.5. Isolation of rho<sup>0</sup> Mutants

Rho<sup>0</sup> mutants were induced by growing the cells in a medium containing 40 µg/ml of ethidium bromide.
The method used was similar to that of Dujardin et al. (1980). Cells from a fresh slant were streaked on a NOEB40 plate and incubated in the dark at 30°C for two days. From this plate, the cells were streaked on a fresh NOEB40 plate and incubated as above. After two more such passages on NOEB40 plates, the cells were streaked on a YPDA plate. After growth, they were checked for the loss of mtDNA by DAPI staining.

3.6. DAPI Staining

The procedure used was based on that of Williamson and Fennell (1975). A small amount of cells was resuspended in 60% (v/v) ethanol. After 30 min incubation at room temperature, the cells were pelleted (Remi table-top centrifuge, 2,000 rpm, 5 min), washed once in distilled water and resuspended in 0.1 ml of 0.2 µg DAPI/ml water. After 30 min incubation, the cells were examined in a Leitz microscope with fluorescence attachment; filter block A (exciting filter: BP340-380; beam splitting mirror: RKP400; suppression filter: LP430) was used.
3.7. Cytoduction

Construction of 9-8: The procedure was based on that of Nagley and Linnane (1978). IL8-8D and BT1-9 were patched on a YPD plate and incubated overnight. They were crossed by mixing a small amount of cells in a drop of water on the same YPD plate. After 8 hr incubation, the mating mixture was resuspended in water and spread on a N3 plate. After one day, the cells from N3 plate were resuspended in water and streaked on a SD-his-ade-leu plate. The colonies obtained after three days of incubation were transferred to a YPD master plate along with the parental strains. After one day incubation, the master plate was replicated onto minimal and N3 plates. Growth response was scored after two days.

Construction of 9-841: IL841-6B was crossed with BT1-9. A procedure similar to that of 9-8 was used.

Construction of 5-8 and 5-841: DC5-O was crossed with a 9-8 or a 9-841 clone. The procedure used was similar to that of 9-8 except SD-his-leu- can plates were used in place of SD-his-ade-leu plates.
3.8. Isolation of \textit{ura}^+ Revertants from IL8-5C/H11A2

A small amount of cells from a fresh YPD slant was resuspended in a drop of water on a SD-his plate and spread. The plate was sealed with parafilm and incubated at 30°C. One big colony (about 1 mm in diameter) and a small colony (about 0.5 mm in diameter) were obtained after 14 days. The big colony, designated as A22, was used for further work.

3.9. Replica-Cross Plating

The replica-cross technique of Deutsch et al. (1974) was used with minor modifications to check the purity of \textit{rho}^- cultures. It was also used to check the putative transformants obtained from strain DC5-0 in mtDNA mediated transformation experiments. The tester strains were grown on N3E2 plates before use. Cells of the tester strain were resuspended in 0.2 ml YPD and spread on a SD plate. The clones to be tested were replica plated on the tester lawn and incubated at 30°C. After three days, the diploid patches were replicated onto N3 and N3C2 or N3C4 plates. The growth response was scored after three or more days of incubation.
3.10. Standard Cross

S1 type standard cross (Coen et al., 1970): Prior to their use in crosses, the strains were grown on N3 plates with appropriate antibiotics. The strains to be mated were mixed in a few drops of water, spread on a SD plate and incubated at 30°C for three days. Fifty or more diploid colonies were resuspended in water, diluted and spread on SD plates. After three days, plates with appropriate number of colonies (50 to 200) were replicated onto N3C4E4, N3E4, N3C4 and N3 plates, in that order. Growth response was scored after five days of incubation.

S2 type standard cross (Michel et al., 1979): This method was used for crosses involving the rho- strain A22. Purity of A22 culture was first determined by replica-cross plating. Diploids obtained out of about 30 rho- A-type clones were pooled in water, diluted and spread on SD plates. After three days of incubation, the colonies obtained were replica plated onto N3C4E2, N3E2, N3C4, N3 and NO plates, in that order. Growth response was scored after five days of incubation.
3.11. Isolation of mtDNA

The procedure used was based on that of De La Salle et al. (1982). Cells from fifteen A22 A-type clones were transferred to 1.5 litres of YD medium. Growth was at 30°C with shaking for 24 hr. Cells were harvested (Sorvall GSA rotor, 2,000 rpm, 10 min, 4°C) and washed twice in distilled water, 150 ml at a time. The cells (25 ml packed cell volume) were resuspended in 150 ml of 1.0 M sorbitol, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA and 40 mM 2-mercaptoethanol. To the cell suspension, 5,000 units of lyticase was added and was incubated at 30°C for 1.5 hr.

Spheroplasts were pelleted (GSA rotor, 2,000 rpm, 15 min, 4°C) and were resuspended in 35 ml ice-cold 0.5 M sorbitol, 25 mM Tris-HCl (pH 8.0) and 5 mM EDTA (STE). The suspension was transferred to a precooled 0.5 litre bottle with acid-washed glass-beads (0.45 to 0.5 mm). The bottle was firmly stoppered and the spheroplasts were broken by the 'hand-shake' method (Lang et al., 1977). The bottle was shaken vertically, by half a metre distance, at the rate of 2 cycles per second, for 2 min. The broken spheroplast suspension was aspirated off the
glass-beads and the beads were washed four times with STE. The suspension and the washings were combined and spun in a GSA rotor at 3,000 rpm and 4°C for 15 min. The supernatant was spun in a SS34 rotor at 12,000 rpm and 4°C for 15 min which yielded a mitochondrial pellet.

The mitochondrial pellet was gently resuspended in 20 ml of 10 mM Tris-HCl (pH 7.6), 10 mM EDTA and 1.0 M NaCl. 1.2 ml of 35% (w/v) sarkosyl was added and gently mixed with the suspension. After 20 min incubation at room temperature, the lysate was extracted once with an equal volume of phenol: chloroform: isoamyl alcohol (50:49:1). The aqueous phase was dialyzed overnight against 1 litre of TE, pH 7.6 (10 mM Tris-HCl, pH 7.6, and 1 mM EDTA). It was adjusted to 0.3 M with respect to sodium acetate (pH 5.2) and the DNA was precipitated with 2 volumes of ethanol. The precipitate was pelleted (10,000 x g, 10 min, 4°C), washed with 70% (v/v) ethanol and briefly dried under vacuum. The pellet was dissolved in 4 ml of TE (pH 7.6). To the DNA solution, 10 µl of 35% (w/v) sarkosyl and 100 µl of 10 mg/ml bisbenzimide (prepared in water) were added. The
volume of the DNA solution was adjusted to 4.5 ml with TE (pH 7.6). 5.0 g solid cesium chloride was added to the above solution, dissolved and transferred to a single 13.5 ml Beckman polyallomer tube. The rest of the tube was filled with paraffin oil. The tube was capped and was centrifuged in Type 50 Ti rotor (45,000 rpm, 20°C, 24 hr).

After the run, the tube was observed under long-wave UV illumination. Three fluorescent bands could be seen. The top most band was just above the middle of the gradient. It was well resolved from the middle band. The third minor band was just below and partially resolved from the middle band. The top band was collected and the bisbenzimide was removed by repeated extractions with cesium chloride saturated isopropanol. The DNA solution was dialyzed overnight against 1 litre of TE (pH 7.6). It was adjusted to 0.3 M with respect to sodium acetate (pH 5.2) and the DNA was precipitated with two volumes of ethanol. The precipitate was pelleted, washed with 70% (v/v) ethanol, dried under vacuum and dissolved in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.
3.12. Transformation of *E. coli*

The method used was based on that of Mandel and Higa (1970). HB101 was used as the recipient strain. 25 ml of LB in a 500 ml conical flask was inoculated with 0.1 ml of an overnight culture and incubated at 37°C with shaking. Optical density was monitored periodically at 600 nm; when it was 0.1 (about 5 x 10^7 cells/ml), the culture was put on ice. The cells were harvested by centrifugation at 3000 x g for 5 min at 4°C. They were gently resuspended in 20 ml of ice-cold 0.1 M MgCl₂. The cells were spun down and gently resuspended in 10 ml of ice-cold 0.1 M CaCl₂. After 20 min incubation on ice, the cells were pelleted and resuspended in 1 ml of ice-cold 0.1 M CaCl₂. 0.2 ml of the bacterial suspension was mixed with 1 μg of YEpl3 plasmid DNA in 0.1 ml of 20 mM Tris-HCl (pH 8.0), 1 mM EDTA and 20 mM NaCl. The bacteria-DNA mixture was left on ice for 30 min. It was heat-shocked at 42°C for 2 min and again incubated on ice for 30 min. 2.7 ml of LB with 2 μg/ml tetracycline was added to the bacteria-DNA mixture
and incubated at 37°C for 1 hr. The cell suspension was spread on LB plates with 15 µg/ml tetracycline. Transformants were obtained after overnight incubation at 37°C.

3.13. Minipreparation of Plasmid DNA

Presence of plasmid DNA in HB101 transformants was checked with minipreparations. The procedure is based on the alkaline lysis method of Birnboim and Doly (1979). All operations were carried out at room temperature unless otherwise mentioned. All centrifugations were done with an Eppendorf microfuge.

Bacteria were grown to saturation density in 5 ml of LB with 15 µg/ml tetracycline. Cells were harvested by 2 min centrifugation and resuspended in 200 µl of 25 mM Tris-HCl (pH 8.0), 50 mM glucose and 10 mM EDTA. They were lysed by the addition of 400 µl of freshly prepared 0.2 M NaOH and 1% SDS. After 5 min, 200 µl of 3 M sodium acetate (pH 4.8) was added, mixed and cooled on ice for 5 min. The precipitate was pelleted off by 5 min centrifugation. The supernatant was mixed with 0.6 volumes of iso-propanol and left on ice for 5 min. The precipitate obtained was collected after 5 min centrifugation, washed with 70% (v/v) ethanol, dried under vacuum
and dissolved in 100 μl of 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA and 200 μg/ml RNase A. After 30 min incubation at 37°C, the sample was extracted with phenol, phenol: chloroform: isoamyl alcohol (50:49:1), and chloroform successively. To the aqueous phase, two volumes of ethanol was added, mixed and incubated at -20°C for 30 min. The precipitate was collected by centrifugation (5 min), washed with 70% (v/v) ethanol, dried under vacuum and dissolved in 40 μl of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

3.14. Large Scale Preparation of Plasmid DNA

The procedure used was based on a modified procedure (Maniatis et al., 1982) of the alkaline-lysis method of Birnboim and Doly (1979). All solutions were prepared as described (Maniatis et al., 1982), except for 5 M potassium acetate which was prepared as follows: 0.3 moles of KOH was dissolved in about 40 ml of water; 0.5 moles of glacial acetic acid was slowly added to the above solution, keeping the temperature-rise within control; the volume was made upto 100 ml. The pH of the solution was found to be
When diluted 3x with water the pH came down to 5.1, and at 20x dilution the pH was 4.8.

The plasmid DNA was purified from a bacterial culture which was not amplified for the plasmid copy-number. An HB101 transformant with the YEpl3 plasmid was grown in 10 ml LB containing 15 µg/ml of tetracycline at 37°C with vigorous shaking for 12 hr. The entire 10 ml culture was transferred to 1 litre LB medium containing 15 µg/ml of tetracycline and was incubated as above for 12 hr. The cells were harvested by centrifugation (4,000 x g, 10 min, 4°C). The cell pellet was resuspended in 200 ml of ice-cold 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The cell suspension was distributed in two centrifuge tubes and was pelleted as above. Each pellet obtained was processed as follows.

The bacterial pellet was resuspended in 10 ml of 50 mM glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA containing 5 mg/ml lysozyme (freshly dissolved). After 5 min incubation at room temperature, 20 ml of freshly made 0.2 N NaOH and 1% (w/v) SDS was added, gently mixed and was incubated on ice for 10 min. To the above lysate, 15 ml of ice-cold 5 M potassium
acetate was added, mixed and was incubated on ice for 10 min. The white precipitate obtained was centrifuged off (30,000 x g, 20 min, 4°C) and the supernatant was combined with 0.6 volumes of isopropanol, mixed and incubated at room temperature for 15 min. The precipitate was pelleted (12,000 x g, 30 min, 20°C) and was washed with 70% (v/v) ethanol at room temperature. The pellet was briefly dried under mild vacuum and was dissolved in TE, pH 8.0 (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), in 4.5 ml.

To 9.0 ml of nucleic acid solution, 9.0 g of solid cesium chloride was added and dissolved; 0.9 ml ethidium bromide (10 mg/ml in water) was added, mixed well and distributed in two 13.5 ml Beckman polyallomer tubes (6.1 ml per tube). The remainder of the tubes was filled with paraffin oil. The tubes were capped and were centrifuged at 45,000 rpm in Type 50 Ti rotor for 24 hr at 20°C. Only one band could be seen, approximately at the middle of the gradient, under long-wave UV illumination. The band was collected and the ethidium bromide was removed by repeated extractions with water-saturated isoamyl alcohol. The DNA solution was dialyzed against 1 litre of TE (pH 8.0) for 12 hr.
It was made 0.3 M with respect to sodium acetate (pH 5.2) and the DNA was precipitated with 2 volumes of ethanol. The precipitate was pelleted, washed with 70% (v/v) ethanol, dried under vacuum and was dissolved in 80 μl of TE (pH 8.0).

3.15. Restriction Endonuclease Analysis

Restriction endonuclease digestion was performed as described in Maniatis et al. (1982). Low salt buffer was used for *Hpa* II; medium salt buffer for *Hind* III and *Bam* HI and high salt buffer for *Mbo* I and *Sal* I. Their composition is as follows: low salt buffer: 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂ and 1 mM dithiothreitol; medium salt buffer: 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂ and 1 mM dithiothreitol; high salt buffer: 100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂ and 1 mM dithiothreitol. These were prepared as 10x stocks. The volume of the reaction mixtures was 20 μl. One unit of restriction enzyme was used per μg of DNA. Incubations were done overnight at 37°C. For *Sal* I-*Hpa* II double digestion of mtDNA, first digestion was done with *Sal* I. Then the digest was phenol extracted, ethanol
precipitated, redissolved in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA and was used for Hpa II digestion.

To determine the size of restriction endonuclease fragments, Hind III digest of DNA was run in parallel in the agarose gels. Standard graphs were drawn as semi-log plots of the mobility of DNA fragments (in linear scale) against their known size (in log scale). The size of the unknown fragments was found by interpolation.

3.16. Agarose Gel Electrophoresis

For electrophoresis, samples were prepared by mixing five volumes of DNA with one volume of gel loading buffer (0.25% [w/v] bromophenol blue and 40% [w/v] sucrose in water).

The electrophoresis buffer was 40 mM Tris-acetate and 2 mM EDTA (pH 8.0). SeaKem LE agarose was used at 0.8% [w/v] concentration. The gel as well as the electrophoresis buffer contained 1 μg/ml ethidium bromide. The gel dimensions were 8.5 x 5.2 x 0.5 or 0.7 cm. A comb with 1 mm thick and 2.5 mm wide teeth was used to form the wells. Electrophoresis was done in a horizontal mini-gel apparatus (16 x 6 x 6 cm) at 100 V for 50 to 60 min.
3.17. MtDNA Mediated Transformation

The transformation procedure was based on that of Hinnen et al. (1978); the important modifications are discussed in sec. 4.3.8.

Solutions and Media
(Percentages are on w/v basis unless otherwise mentioned).

SMTM : 0.5 M sorbitol, 0.5 M mannitol, 10 mM Tris-HCl (pH 8.0) and 40 mM 2-mercaptoethanol. (2-Mercaptoethanol was added just before use).

SMTC : 0.5 M sorbitol, 0.5 M mannitol, 10 mM Tris-HCl (pH 7.6) and 10 mM CaCl₂
(CaCl₂ was autoclaved separately and was combined with the other components, after the solutions have cooled down to room temperature)

SMTC-YPD : SMTC with 0.67% yeast extract, 1.34% peptone and 1.34% glucose

PEG : 20% PEG-4000, 10 mM Tris-HCl (pH 7.6) and 10 mM CaCl₂
(Prepared fresh and filter sterilized)

Selective medium : 0.5 M sorbitol, 0.5 M mannitol, 0.67% yeast nitrogen base w/o amino acids, 2% glucose, 40 μg/ml L-histidine-HCl, 0.01% yeast extract, 0.02% peptone and 2% agar.
(Penicillin G was added, to a final concentration of 100 units/ml to the autoclaved medium at about 55°C)

Over- Selective medium with 1.5% agarose (LE lay grade, SeaKem) instead of agar; autoclaved agarose in 2.5 ml parts.
Procedure

The following protocol would yield sufficient spheroplasts for ten experiments. It was scaled down when necessary. All centrifugations were done in a swing-out rotor of a Remi table-top centrifuge at 2,500 rpm, for 5 min. All operations were performed at room temperature (\( \sim 22^\circ C \)) unless otherwise stated. A laminar flow bench was used to avoid contamination.

An inoculum culture of the strain to be transformed was prepared in YPD (24 hr growth at 30°C with shaking). It was diluted 100x in YPD or YPD4 and was incubated for 5 to 6 hr (1.5 to 2 \( \times 10^7 \) cells/ml). The culture was stored overnight at 4°C before use. For viable cell count, 100 \( \mu l \) of a \( 10^4 \)x dilution was spread on YPD.

Cells from 100 ml culture were pelleted, washed once in 10 ml water, once in 10 ml SMTM and resuspended in 10 ml SMTM. Lyticase (600 units) was added to the cell suspension, mixed and incubated at 30°C with occasional shaking, for 30 min. The spheroplasts were pelleted, washed once in 20 ml SMTC and were resuspended in SMTC in a total volume of 1.1 ml.
DNA samples were prepared by mixing 1 μg of YEpl3 DNA with appropriate amount of mitochondrial DNA. The volume of the DNA mixture was 14.5 μl for all experiments (the volumes were made up with 10 mM Tris- HCl, pH 8.0 and 1 mM EDTA). For each experiment, 100 μl of the spheroplast suspension was mixed with the DNA and incubated for 15 to 20 min. To this, 1 ml of PEG was added, mixed and incubated for 20 to 30 min. The spheroplasts were pelleted, resuspended in 150 μl SMTC-YPD and incubated at 30°C for 2 hr.

The entire transformation mixture or aliquots were embedded in overlay agarose and spread on selective plates. Before plating, the plates were preincubated at 37°C for 2 hr. The spheroplasts were added to 2.5 ml overlay agarose (which was liquified and kept in a 45°C water bath for about an hour before use), mixed immediately with a vortex mixer and quickly spread on a selective plate. For determining the extent of spheroplasting, 100 μl of \(10^2\) or \(10^4\) x dilution of the transformation mixture was spread on YPD plates.

The \textit{leu}^+ transformants of 5-841 could be counted after 3 days of incubation. When they were more than \(10^3\) per plate, an inverted microscope was
used to determine their number. On the fourth day, they were replicated onto N3C plates. The \textit{leu}^+ transformants of DC5-0 were counted after 6 days of incubation. Then, they were crossed with a \textit{rho}^+ tester strain by the replica-cross method (sec. 3.9).

3.18. Mitochondria Mediated Transformation

3.18.1. Osmotic Shock Method

The experiments were based on that of Tuppy and Wildner (1965).

Experiment 1

\textbf{Media:} All minimal media contained 0.67\% (w/v) yeast nitrogen base \textit{w/o} amino acids, 20 \textmu g/ml adenine sulfate, 20 \textmu g/ml L-arginine-HCl, 30 \textmu g/ml L-leucine, 20 \textmu g/ml L-tryptophan and 2\% (w/v) agar. In addition, one set of plates contained 1.1 M mannitol and 3\% (v/v) glycerol; the second set of plates contained 1.1 M mannitol and 2\% (w/v) glucose; the third set of plates contained 2\% (w/v) glucose.

\textbf{Isolation of Mitochondria:} All centrifugations were done in Sorvall SS34 rotor at 4^\circ\text{C}. AN^R OR12D was grown in YPG to late-log phase, harvested (1,000 x g, 5 min), and washed once in 40 ml ice-cold water. The packed cell volume was 0.5 ml. The cells
were washed once in 10 ml cold STE (0.25 M sucrose, 20 mM Tris-HCl and 5 mM EDTA, pH 7.4), and resuspended in 1.8 ml of the same buffer. The cell suspension was transferred to a precooled 100 ml measuring cylinder with 8 g acid-washed glass-beads (0.45 to 0.5 mm). The cylinder was stoppered firmly and was shaken vertically, by half a metre distance, at the rate of 2 cycles per second, for 3 min. The suspension was recovered from the beads and was spun at 4,600 x g for 5 min, twice. The pellet was discarded and the supernatant was centrifuged at 25,000 x g for 15 min. The mitochondrial pellet was resuspended in 1.5 ml STE and stored on ice until use (about 24 hr).

Isolation of Recipient Spheroplasts: All centrifugations were performed at room temperature, in a Remi table-top centrifuge with a swing-out rotor at 2,500 rpm, for 5 min. B060AF-1 cells were harvested from a late-log phase YPDA culture (10^8 cells/ml). Spheroplasts were isolated from 10^7 cells. Cells were washed and resuspended in 1 ml water. The cell suspension was made 2% (v/v) with respect to 2-mercaptoethanol and was incubated at 30°C for 20 min. The cells
were pelleted, washed and resuspended in 2 ml of 0.9 M mannitol, 20 mM Tris-HCl, 5 mM EDTA and 0.1% (v/v) 2-mercaptoethanol (pH 7.4). 20 μl Glusulase was added to the above suspension, mixed and incubated at 30°C for 1 hr with occasional shaking. Spheroplasts were pelleted, washed and resuspended in 0.9 ml of 0.9 M mannitol, 20 mM Tris-HCl and 5 mM EDTA (pH 7.4).

Transformation: 100 μl of spheroplast suspension was mixed with 10 μl of mitochondrial suspension. 110 μl of 1.3 M mannitol, 20 mM Tris-HCl and 5 mM EDTA (pH 7.4) was added and mixed with the spheroplast-mitochondria suspension and incubated at 4°C for 2 hr. The spheroplast-mitochondria mixture and the dilutions (made in the respective liquid media) were directly spread on the minimal plates. Plates were scored after 8 days of incubation.

Experiment 2

Media: All minimal media contained 0.67% (w/v) yeast nitrogen base w/o amino acids, 15 μg/ml adenine sulfate, 20 μg/ml L-arginine-HCl, 30 μg/ml L-leucine, 20 μg/ml L-tryptophan, 0.03% (w/v) yeast extract, 0.08% (w/v) peptone and 2% (w/v) agar. In addition, the M1 plates contained 6.8% (w/v) glycerol; the M2
plates contained 6% (w/v) glycerol and 2% (w/v) glucose; the M3 plates contained 2% (w/v) glucose; 0.45 M sorbitol and 0.45 M mannitol; the M4 plates contained 2% (w/v) glucose.

Isolation of Mitochondria: All centrifugations were done with a Sorvall SS34 rotor at 4°C. Ice-cold buffers were used. IL8-8D was grown in 100 ml YPD to late-log phase. The cells were harvested (1,000 x g, 5 min), washed twice in water (40 ml) and once in 20 ml of 0.35 M sucrose, 20 mM Tris-HCl (pH 7.4) and 5 mM EDTA. The pellet (1.5 ml packed cell volume) was resuspended in 2.5 ml of above buffer, transferred to a precooled test tube (2 x 20 cm) containing 8 g acid-washed glass-beads and vortexed at minimum speed for 3 min. The broken cell suspension was recovered from the beads and spun at 4,600 x g for 5 min, twice. The supernatant was spun at 20,000 x g for 15 min. The mitochondrial pellet was resuspended in 0.45 ml of 0.35 M sucrose, 20 mM Tris-HCl (pH 7.4) and 5 mM EDTA and was stored in ice until use (about 24 hr).

Isolation of Recipient Spheroplasts: All centrifugations were done as in experiment 1. BO60AF-l cells were harvested from a late-log phase (10^8 cells/ml)
YPDA culture. Spheroplasts were isolated from $10^7$ cells. The cells were washed in 1 ml water and resuspended in 0.9 M sorbitol. 2-Mercaptoethanol was added to 2% (v/v) concentration, mixed and incubated for 20 min. The cells were pelleted, washed with 1 ml of 0.9 M sorbitol, 10 mM citrate-phosphate (pH 5.9) and 0.1% (v/v) 2-mercaptoethanol, twice. They were resuspended in 1 ml of the above buffer and 10 µl Glusulase was added and mixed. After incubation for 90 min at 30°C with occasional shaking, the spheroplasts were pelleted and washed twice with 0.9 M sorbitol and 20 mM Tris-HCl (pH 7.4), 1 ml at a time.

Transformation: To the spheroplast pellet, 20 µl of mitochondrial suspension and 1 ml of 1.1 M mannitol, 20 mM Tris-HCl (pH 7.4) and 5 mM EDTA were added, mixed and incubated at 4°C for 3 hr. The transformation mixture or the dilutions were directly spread on plates. Plates were scored after 9 days of incubation.
3.18.2. PEG-Fusion Method

The procedure is partly based on that of Gunge and Sakaguchi (1979) and Yoshida (1979). The isolation of recipient spheroplasts and transformation are similar to that of DNA mediated transformation experiments.

Solutions and Media:
(Percentages are on w/v basis unless otherwise mentioned).

SMTM, SMTC, SMTC-YPD and PEG were prepared as described in sec. 3.17.

PEG ‡ : 20% PEG-4000, 10 mM Tris-HCl (pH 7.6),
osmotic a 10 mM CaCl₂, 0.4 M mannitol and 0.4 M
SMTE sorbitol (prepared fresh and filter
SMTEM sterilized).

SMTE : 0.5 M sorbitol, 0.5 M mannitol, 10 mM Tris-
SMT HCl (pH 8.0) and 10 mM EDTA.

MTE : SMTE with 40 mM 2-mercaptoethanol (added
Selective just before use).

Medium 0.5 M sorbitol, 0.5 M mannitol, 0.67% yeast
nitrogen base w/o amino acids, 40 µg/ml
L-histidine HCl, 60 µg/ml L-leucine, 3% (v/v)
glycerol, 0.1% glucose and 2% agar (Penicillin
G was added to the autoclaved medium, at about
55°C, to a final concentration of 100 units/ml).
Overlay: Selective medium with 1.5% agarose (LE agarose grade, SeaKem) instead of agar; autoclaved in 2.5 ml parts.

Isolation of Mitochondria: All centrifugations were performed at 4°C in a Sorvall SS34 rotor. AN30R12D was grown in 200 ml YPD to late-log phase (9 x 10^7 cells/ml). The cells were harvested (1,000 x g, 10 min), washed once in 100 ml water, once in 20 ml SMTEM and resuspended in 20 ml SMTEM. Lyticase (4000 units) was added, mixed and incubated at 30°C for 1 hr with occasional shaking. The spheroplasts were pelleted (1,000 x g, 10 min) and washed once in 20 ml ice-cold SMTE. The spheroplasts were resuspended in 2 volumes of ice-cold MTE. Sterile, pre-cooled glass-beads (0.45 to 0.5 mm) were added (3.5 g per ml of suspension). The spheroplast glass-bead mixture was vortexed for 2 min at the maximum speed. The suspension containing broken spheroplasts was aspirated off the beads and the beads were repeatedly washed with MTE. The suspension and the washings were combined and centrifuged at 3,000 x g for 10 min. The supernatant was again spun as above. The pellet was discarded and the supernatant was centrifuged at
at 12,000 x g for 20 min to obtain a mitochondrial pellet. The pellet was washed once in MTE and was stored on ice until use (for about 15 hr). Just before use, the pellet was resuspended in SMT in a total volume of 400 µl.

Isolation of Recipient Spheroplasts: The spheroplasts were isolated from DC5-0 cells (YPD culture) as described in sec. 3.17. They were stored as a suspension in SMTC, at 4°C, until use (for about 24 hr).

Transformation: Different amounts of mitochondria were combined with the spheroplasts. 0.1 ml of spheroplast suspension (in SMTC) was used in each experiment. Irrespective of the amount of mitochondria used, their volume was the same (80 µl) in all experiments which was adjusted with SMT. The spheroplast-mitochondria mixture was incubated at room temperature for 15 min. One ml of PEG or PEG osmotica was added, mixed and incubated at room temperature for 40 min. The spheroplast-mitochondria aggregates were pelleted (Remi table-top, 2,500 rpm, 5 min, room temperature), resuspended in 150 µl SMTC-YPD and incubated at 30°C for 2 hr. The transformation mixtures were embedded in overlay agarose (as described in sec. 3.17) and spread on selective plates.