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Isolation of autophagocytosis mutants of *Saccharomyces cerevisiae*

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Abstract

Protein degradation in the vacuole (lysosome) is an important event in cellular regulation. In yeast, as in mammalian cells, a major route of protein uptake for degradation into the vacuole (lysosome) has been found to be autophagocytosis. The discovery of this process in yeast enables the elucidation of its mechanisms via genetic and molecular biological investigations. Here we report the isolation of yeast mutants defective in autophagocytosis (*aut* mutants), using a rapid colony screening procedure.

Key words: Yeast; Vacuole; Autophagocytosis; Screening procedure

1. Introduction

The vacuole (lysosome) is a center of proteolysis in eukaryotic cells [1–3]. In the yeast *S. cerevisiae* during logarithmic growth about 40% of the proteolytic capacity depends on the two major vacuolar endoproteinases yscA and yscB. Starvation for nitrogen increases the amount of vacuolar proteolysis considerably: up to 85% of the total proteolysis rate is due to vacuolar enzymes under these conditions. After 24 h of starvation, 45% of all cellular proteins are degraded within the vacuole [4].

Prior to proteolytic breakdown, cytosolic proteins must enter the vacuole. In higher eukaryotes, two pathways for protein import into the lysosome (vacuole) have been described: (i) a non-selective uptake of proteins into the lysosome due to autophagocytosis; [5]; (ii) a selective uptake of proteins induced by a KFERQ-related import sequence [6].

In yeast, the glucose-induced catabolite inactivation of fructose-1,6-bisphosphatase was proposed to be an example of selective vacuolar uptake [7]. However, recent experiments indicate that this process is dependent on the proteasome [8].

The existence of an non-specific autophagocytic pathway for protein uptake into the vacuole has been established in *S. cerevisiae*. The non-specific nature of this process was illustrated by following the concomitant uptake of several cytosolic proteins into the vacuole under starvation conditions [9]. Furthermore, under these conditions vesicles could be directly visualized inside vacuoles of vacuolar proteinase-deficient stains during starvation [10,11]. A detailed analysis identified these vesicles to be autophagocytic in nature and showed that the absence of proteinase yscB activity in vacuoles was sufficient for vesicles to accumulate in these organelles [11].

Here we describe the isolation of mutants of *S. cerevisiae* which are defective in the autophagocytic process. Using a colony screening procedure, mutants were selected which are unable to accumulate autophagocytic vesicles inside the vacuole during starvation.

2. Materials and methods

2.1. Chemicals

Nitrocellulose sheets were purchased from Schleicher & Schuell, Dassel, Germany; PMSF from Serva, Heidelberg, Germany; Zymolyase from Seikagaku, Tokyo, Japan; and galactose from Merck, Darmstadt, Germany. Goat anti-rabbit antibodies labeled with peroxidase or fluorescein isothiocyanate were from Medac, Hamburg, Germany; and fluorescein diacetate and propidium iodide from Sigma, Deisenhofen, Germany. All other chemicals were from Merck, Sigma or Roth, Karlsruhe, Germany, and all were of analytical grade. Antibodies directed against fatty acid synthase were described elsewhere [4,9].

2.2. Media

YPD medium (2% glucose, 1% yeast extract, 2% peptone) was prepared according to Ausubel et al. [12]. YPGal medium contained 2% filter-sterilized galactose instead of glucose. Acetate medium contained only 1% potassium acetate.

2.3. Strains

Yeast strains used were: BF 89.4-36 (*MATa fas1Δ::HIS3 ura3 trp1-289*) (H.-J. Schüller, Erlangen), YMS1 (*MATα fas1Δ::LEU2 his3-11,15 leu2-3,112 ura3*), YMS3 (*MATα fas1Δ::LEU2 pra1Δ::HIS3 prb1Δ his3-11,15 leu2-3,112 ura3*), WCG4α (*MATα his3-11,15 leu2-3,112 ura3*), YMTA (*MATα pra1Δ::HIS3 his3-11,15 leu2-3,112 ura3*), YMTAB (*MATα pra1Δ::HIS3 prb1Δ his3-11,15 leu2-3,112 ura3*).

Strain YMTA was obtained by gene disruption of the chromosomal *PRA1* gene in strain WCG4α using a *Sac*I–*Apa*I fragment of the integrative plasmid KS-PRA1Δ-HIS3 [13,14]. YMTAB is a segregant from a cross between strain YHH19 (*MATa prb1Δ his3-11,15 leu2-3,112 canR ura3Δ5*) [13] with strain YMTA.

Disruption of the chromosomal *FAS1* gene in strains WCG4α and YMTAB with a *Eco*RI fragment of the integrative plasmid pBF5 (H.-J. Schüller, Erlangen) yielded strains YMS1 and YMS3, respectively.

All strains disrupted in the chromosomal *FAS1* gene were transformed with plasmid pMT2 (see below) to allow growth on YPGal.

2.4. Molecular biological techniques and construction of plasmid pMT2

Standard cloning protocols were applied [15].

The vector pBM150, carrying the *GAL1-GAL10* promotor fragment between the *Eco*RI and *Bam*HI sites of YCp50 [16] was first converted

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into a plasmid containing the *LEU2* gene. *URA3* was deleted by cutting with *Sal*I–*Sma*I and the *LEU2* gene from plasmid YEp13 was inserted as a *Sal*I–(*Xho*I blunt-ended) fragment yielding plasmid pMT1. The *FAS1* structural gene, excised as a *Mro*I–*Sph*I fragment from plasmid pJS229 (H.-J. Schüller, Erlangen), was blunt-end ligated into the *Bam*HI site of plasmid pMT1. The resulting plasmid pMT2 carries the *FAS1* gene under the control of the *GAL1* promotor.

2.5. Mutagenesis with EMS

Mutagenesis was done according to [17] using 300 μl EMS for a 10 ml culture and an incubation time of 1 h at 30°C.

2.6. Screening procedure

Cells were grown on YPGal medium, replica plated onto nitrocellulose sheets, placed on YPGal plates, and grown for 2 days at 30°C. Thereafter, filter sheets were placed for 2 h on acetate plates, for 4 h on YPD plates, and for 3 (BF89.4-36,pMT2) to 9 (YMS1,pMT2) days on acetate plates. Cells were lysed by incubating the filters for 1 h on filter papers soaked with lysis solution. Lysis solution consists of 100 μl β-mercaptoethanol, 400 μl 10 N NaOH and 200 μl 10% SDS in 20 ml water. Lysed cells were removed from the filter sheets with water and the filter sheets were coated and stained with rabbit antibodies directed against fatty acid synthase, with subsequent binding of goat anti-rabbit antibodies coupled with peroxidase as described elsewhere [9,18].

2.7. Survival rates

10 μl of liquid culture were mixed with 90 μl YPD medium and 1 μl fluorescein diacetate (20 mg/ml in dimethylsulfoxide) and 1 μl propidium iodide (2 mg/ml in water). After a few minutes cells were washed with water and visualized with a Zeiss axioskop fluorescence microscope. Red cells were considered dead and green cells alive.

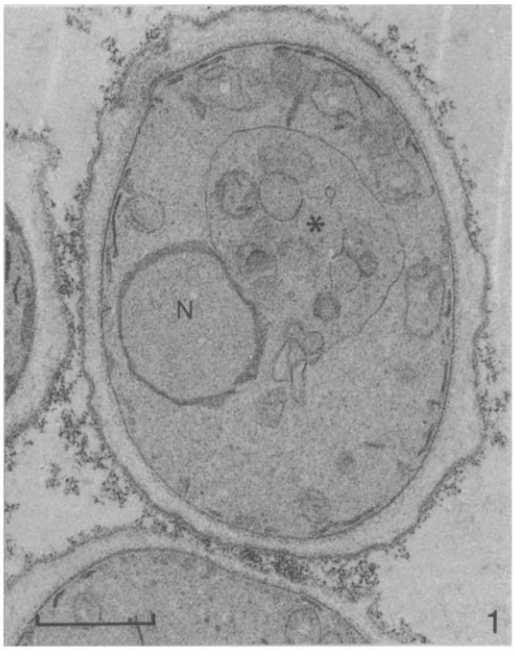

Fig. 1. Appearance of whole mitochondria in vacuoles of cells starved for nitrogen. Logarithmically growing cells of a proteinase wild-type strain (YMS1,pMT2) were starved for 4 h in the presence of 1 mM PMSF and prepared for electron microscopy according to [30].

3. Results

Autophagocytosis is a non-selective process which leads to the import of cytosolic material into the vacuole. The imported material even contains whole mitochondria when vacuoles of yeast cells starved for nitrogen are analyzed (Fig. 1); [11]. Due to this non-selective import of cytosolic material into the vacuole, which is followed by non-specific breakdown carried out by the two vacuolar endoproteinases yscA and yscB, the steady-state level of cytosolic proteins should decrease in a wild-type strain during starvation (the conditions of heavy autophagocytosis) if synthesis of these cytosolic proteins remained unchanged or were decreased. In mutants defective in autophagocytosis, the mutationally induced inhibition of cytosolic protein import into the vacuole should abolish the decrease in the steady-state level of these proteins. Cytosolic proteins are then unable to enter the vacuole, thus escaping the vacuolar proteolytic machinery, and should therefore accmulate in the cytosol.

Fatty acid synthase is a cytoplasmic enzyme complex composed of two different subunits, α (expressed from the *FAS2* gene) and β (expressed from the *FAS1* gene), which assemble to the functional $\alpha_6\beta_6$ configuration [19,20]. The enzyme complex accumulates inside the vacuole during starvation of strains defective in the vacuolar endoproteinases yscA and yscB [9], and thus represents a potential candidate as a marker enzyme to trace the autophagocytic process. One would expect a drastic decrease in the enzyme level in wild-type strains due to rapid degradation after its autophagocytic uptake. However, in a proteinase wild-type strain, the expected significant decrease in the steady-state level of fatty acid synthase was not observed at different growth stages [9]. This is most likely due to the fact that degradation is paralleled by a continuing synthesis of fatty acid synthase during starvation.

Thus, use of the enzyme as a marker protein to trace defects in autophagocytosis required conditions which lead to a considerable decrease in the enzyme levels in the cell when autophagocytosis occurs. This could be achieved in the following way. Free, unassembled α-subunit of fatty acid synthase is subject to rapid cytosolic degradation via the proteasome [9]. Therefore, switching off only the synthesis of the β-subunit of fatty acid synthase leads to complete loss of fatty acid synthase antigenic material. Thus, a plasmid, pMT2, carrying the *FAS1* gene encoding the β-subunit under the control of the inducible and repressible *GAL1* promotor, was constructed and transformed into strains lacking the chromosomal *FAS1* gene. Over-expression of the β-subunit of fatty acid synthase leads to an increase in the steady-state level of the fatty acid synthase complex. Excess of free β-subunit is mainly degraded by vacuolar proteinases as is the fatty acid synthase complex under the appropriate conditions [9].

To achieve the appearance of easily visible autophagocytosis mutant phenotypes the following procedure was developed: cells were grown on nitrocellulose

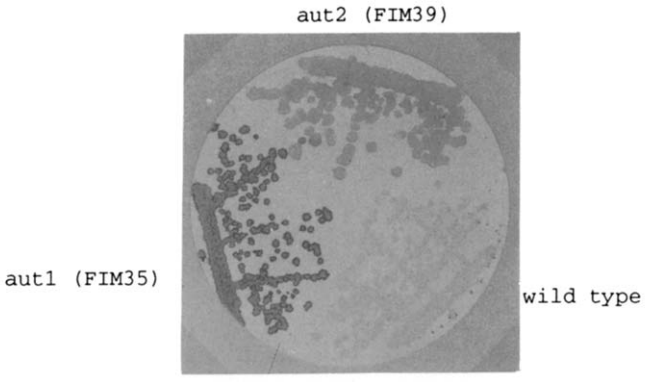

Fig. 2. Colony screen for the identification of autophagocytosis mutants. Cells were replica plated on nitrocellulose filters on YPGal plates and grown for 2 days at 30°C. Then the nitrocellulose filters were placed for 2 h on acetate, for 4 h on YPD, and finally for 3 days on acetate plates. Lysis and staining of the colonies was performed as outlined in section 2.

sheets on galactose-containing medium to induce the expression of the plasmid-encoded β-subunit of fatty acid synthase under control of the *GAL1* promotor. Thereafter, cells were shifted for 2 h into acetate medium to induce vacuolar proteinases [21–23]. A subsequent 4 h period on glucose-containing medium repressed further synthesis of the β-subunit of fatty acid synthase and allowed the cells to reduce the unphysiologically high fatty acid synthase concentrations. Finally, the nitrocellulose sheets were placed on acetate plates and starved for 3 (BF 89.4-36) to 9 days (YMS1) to follow the degradation of fatty acid synthase.

Using this procedure, as expected, the fatty acid synthase level decreases significantly during starvation in a wild-type strain. Thus, autophagocytosis mutants could easily be visualized by growing EMS-mutagenized yeast cells as described above and thereafter lysing the colonies on the filters followed by immunostaining of the fatty acid synthase complex. Potential autophagocytosis mutants exhibited a dark blue colour on the filters, whereas wild-type colonies appeared nearly white (Fig. 2).

EMS mutagenesis was carried out in two different wild-type strains, YMS1 (*Matα*) and BF 89-4.36 (*Mata*), 25,000 colonies each were screened and 600 (YMS1 genetic background) or 800 (BF 89-4.36) putative mutant strains were identified. Mutants derived from strain YMS1 were named YBK, those from strain BF 89-4.36 were named FIM.

It should be stressed here that in the mutants, starvation-induced vacuolar degradation of fatty acid synthase might be affected not only by a mutationally induced block of the autophatocytic system, but also by a reduced activity of vacuolar proteinases resulting from other sites of mutation. Intact carboxypeptidase yscY is a good marker for an intact vacuolar proteolytic system, because for maturation of carboxypeptidase yscY to occur intact proteinase yscA inside the vacuole is required [24,25]. Therefore, all 600 putative mutants from the YMS1 genetic background were checked for activity of carboxypeptidase yscY using a well-established and simple overlay activity test [26,27]. Indeed, 14 mutants with reduced activity of carboxypeptidase yscY were

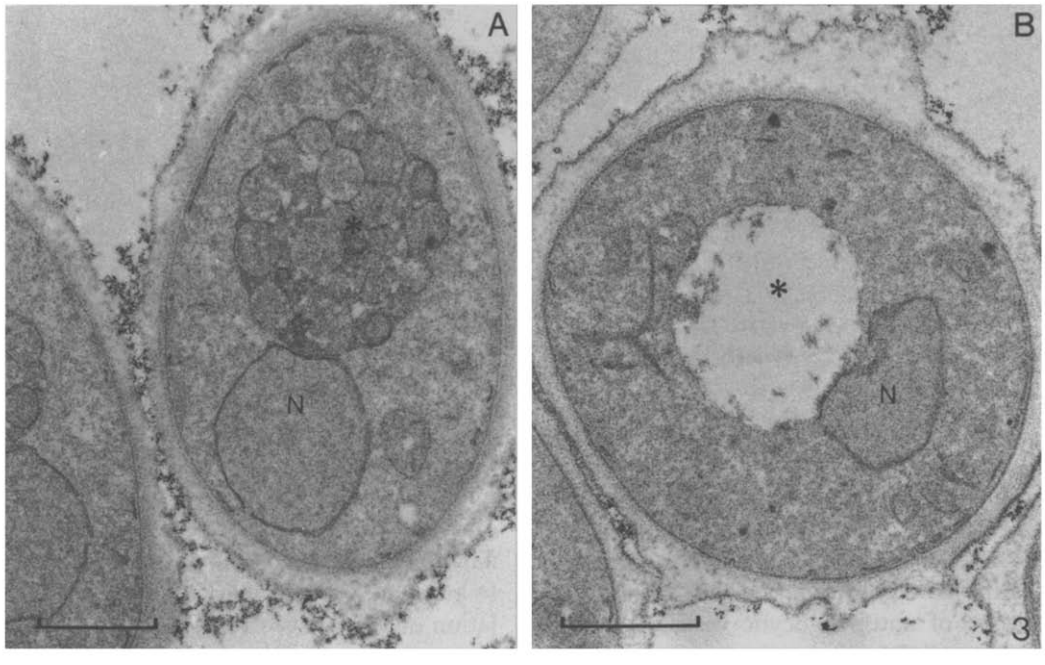

Fig. 3. Electron microscopic visualization of autophagocytic vesicles. (A) Autophagocytic vesicles accumulate in vacuoles of strain YMS3,pMT2, which is deficient in the proteinases yscA and yscB. Logarithmically growing cells of YMS3,pMT2 were starved for 4 h in the presence of PMSF and prepared for electron microscopy by fixation with potassium permanganate according to [30]. (B) Autophagocytosis mutants fail to accumulate autophagocytic vesicles inside the vacuole during starvation. Cells of the strain aut2 (FIM39,pMT2) were treated similarly to the cells shown in A. The marker represents 1 μm; N, nucleus; *, autophagocytic vacuole.

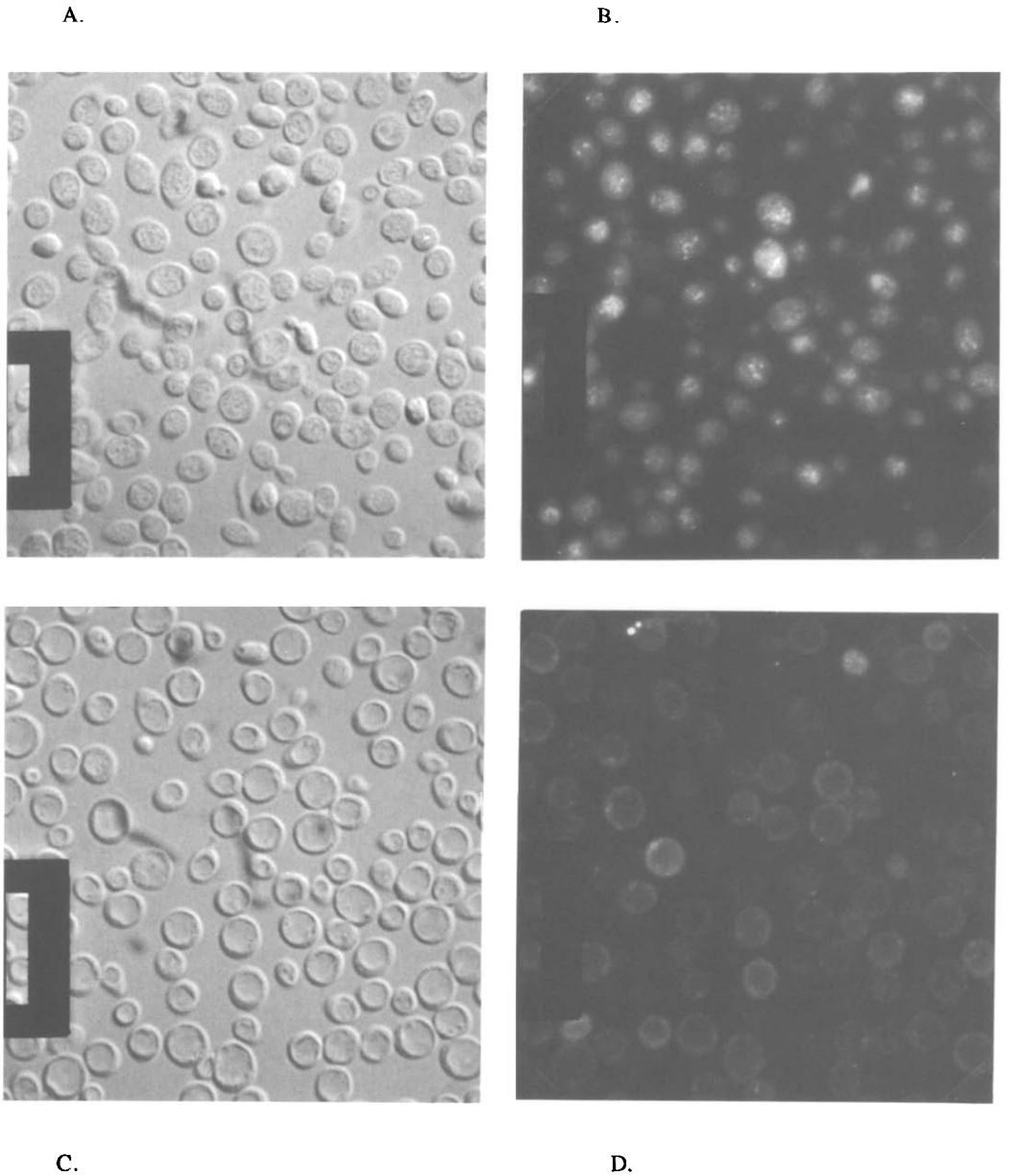

Fig. 4. *aut3* mutant cells fail to transport fatty acid synthase to the vacuole. Indirect immunofluorescence localization of fatty acid synthase in the proteinase yscA and yscB-deficient strain YMS3,pMT2 (B; (A, Nomarski); and a segregant of *aut3* deficient in proteinase yscA, from a cross of *aut3* (YBK26,pMT2) with the proteinase yscA-deficient strain YMTA (D; (C, Nomarski). Logarithmically growing cells were starved for 24 h in acetate medium and prepared for indirect immunofluorescence microscopy according to Pringle et al. [31].

found, thus confirming the vacuolar location of the degradation event of fatty acid synthase.

In wild-type cells, the vacuolar endoproteinase yscB is involved in mediating the lysis of autophagic vesicles inside the vacuole. Therefore, addition of the proteinase yscB inhibitor PMSF to cells starving for nitrogen leads to the accumulation of autophagocytic vesicles within the vacuole [11]. Thus all the 600 putative mutants (YBK strains) isolated by the given screen for cytosolic accumulation of fatty acid synthase from the YMS1 genetic background were further examined in a second screen for defects in accumulation of autophagocytic vesicles inside the vacuole during starvation in the presence of PMSF using light microscopy ('vesicle test'). Also in this screen, mutants with defects in the activity of the vacuolar endoproteinases yscA and yscB would be discriminated, because mutations in these enzymes, in contrast to autophagocytosis mutants, would lead to an accumulation of vesicles in vacuoles.

A collection of 158 putative autophagocytosis mutants from the YMS1 genetic background which passed all screens described has been isolated. Currently three complementation groups have been identified. Complementation groups were named *aut1*, *aut2* and *aut3*, respec-

tively, for autophagocytosis mutants. A member of each complementation group was backcrossed three times with the wild-type strain WCG4α. Tetrad analysis using the vesicle test in the presence of PMSF showed a 2:2 segregation pattern, indicating that the corresponding mutant phenotype was due to a single gene mutation.

Heterozygous diploids from crosses of the *aut1, aut2* and *aut3* mutants with wild-type strain WCG4α showed a wild-type behaviour when using the vesicle test, demonstrating the recessive nature of the mutations. The inability of the *aut* mutants to accumulate autophagocytic vesicles inside the vacuole during starvation in the presence of PMSF was confirmed by electron microscopy (Fig. 3).

Strains defective in the vacuolar endoproteinase yscA almost completely lack their vacuolar proteolytic capacity [4,25]. In these strains cytoplasmic proteins entering the vacuole via the autophagocytic pathway escape proteolytic breakdown and can be visualized inside the vacuole by indirect immunofluorescence techniques [9]. In order to demonstrate that an *aut* mutant strain (*aut3*; YBK26), isolated by the screen applied, is not only defective in accumulation of autophagocytic vesicles and degradation of fatty acid synthase but also in transport of cytosolic proteins to the vacuole, strain YBK26 (*aut3*) was crossed with strain YMTA, defective in the vacuolar endoproteinase yscA. Segregants carrying the *aut3* mutation and an endoproteinase yscA deficiency were checked for the accumulation of fatty acid synthase inside the vacuole during starvation using indirect immunofluorescence microscopy (Fig. 4C,D). *aut3* mutant cells indeed failed to transport cytoplasmic fatty acid synthase into the vacuole. Strain YMS3, defective in the vacuolar endoproteinases yscA and yscB, exhibited the expected accumulation of autophagocytic vesicles in the vacuole (Fig. 4A). By indirect immunofluorescence microscopy cells showed a granulated structure of the vacuole (Fig. 4B). The cytoplasmic fatty acid synthase seemed to be located predominantly inside the autophagocytic vesicles.

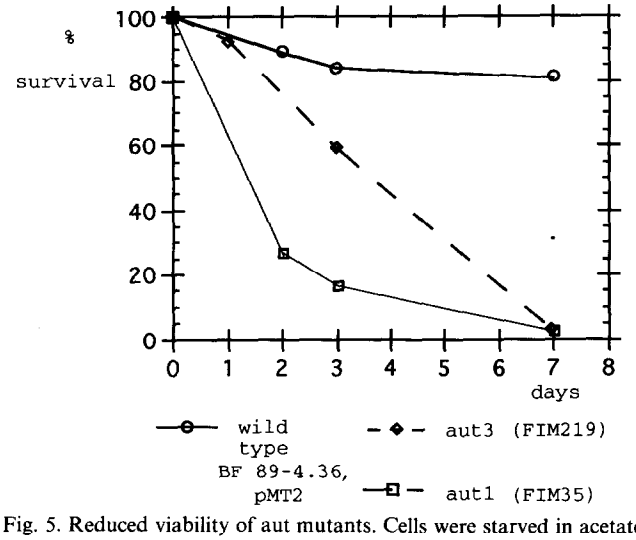

Fig. 5. Reduced viability of aut mutants. Cells were starved in acetate liquid medium. Survival rates were determined as described in section 2.

Previously two phenotypes were ascribed to mutants defective in the vacuolar endoproteinases yscA and yscB: (i) a drastically reduced sporulation frequency of homozygous diploids [4,28] and (ii) a reduced viability during starvation [4].

In homozygous diploids of *aut1, aut2* and *aut3* mutants from the YMS1 and BF89.4-36 strain background we indeed observed nearly no formation of asci. A reduced viability during starvation was found for *aut1* and *aut3* mutants (Fig. 5). *aut2* mutant strains showed no obviously reduced viability during starvation.

4. Discussion

We described a rapid colony screening procedure for the isolation of autophagocytosis mutants of yeast defective in vacuolar breakdown of a cytosolic protein, fatty acid synthase, during starvation. The isolated autophagocytosis (*aut*) mutants failed to accumulate autophagocytic vesicles in the vacuole when starved for nitrogen in the presence of PMSF.

In strains deficient in the vacuolar endoproteinase yscA, the capacity of vacuolar proteolysis is dramatically diminished in the degradation of proteins imported into the vacuole during starvation. This leads to accumulation of proteins and even whole mitochondria inside the vacuole. We checked the phenotypic appearance of vacuoles in the endoproteinase yscA and yscB-deficient strain YMS3 carrying the *FAS1* over-expressing plasmid pMT2. Vacuoles of this strain showed a granulated structure in immunofluorescence microscopy due to the accumulation of autophagocytic vesicles, and fatty acid synthase seemed to be located predominantly in these vesicles. This supports the idea that autophagocytic vesicles contain cytosolic material. Furthermore, using immunofluorescence microscopy, we demonstrated that a segregant from a mutant carrying the *aut3* allele and lacking the vacuolar endoproteinase yscA, accumulated neither fatty acid synthase nor autophagocytic vesicles in the vacuole under starvation conditions.

The block of vacuolar protein import in *aut* mutants should lead to a similar phenotype as inhibition of proteolysis inside the vacuole due to disruption of proteinases yscA and yscB. Indeed we found a drastically reduced sporulation frequency in homozygous *aut1, aut2* and *aut3* mutant diploids and a reduced viability of *aut1* and *aut3* mutants under nitrogen-deficient growth conditions. Strains carrying the *aut2* mutant allele exhibited no obviously reduced viability during starvation. Further studies must unravel if this is solely due to the mutation generated, or if this is a general feature of the *AUT2* gene.

Up to now only a minority of the isolated autophagocytosis mutants have been analyzed for their complementation behaviour. Among the mutants not analyzed in more detail yet, we expect to identify an additional number of complementation groups involved in the autophagocytic process.

Most recently another procedure for the isolation of autophagocytosis mutants, based on the rather pleiotropic phenotype of a reduced survival rate of mutant strains during starvation, was developed [29]. Using this procedure, the isolation of autophagocytosis mutants which survive starvation despite their defect would be impossible. In our hands only *aut1* showed a mutant phenotype using this colony screening procedure of a reduced survival rate; *aut2* and *aut3* showed wild-type behaviour, although *aut3* showed a somewhat reduced survival rate in liquid cultures. We expect the existence of additional mutants which are able to survive, because endoproteinase yscB-deficient strains, which show a defect in lysing autophagocytic vesicles inside the vacuole, are viable [4]. Our present screening procedure is also able to encompass such surviving mutants. However, all the mutants described by the group of Ohsumi [29] and all *aut* mutants described by us are obviously autophagocytosis mutants, because they all fail to accumulate autophagocytic vesicles inside the vacuole during starvation in the presence of PMSF. The identification and characterization of the genes affected in *aut* mutants will finally give insight in the mechanisms and function of autophagocytosis.

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