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Original article

Vacuolar morphology and cell cycle distribution are modified by leucine limitation in auxotrophic *Saccharomyces cerevisiae*

Z. Petek Çakar[a], Uwe Sauer[a], James E. Bailey[a], Martin Müller[b], Martin Stolz[c,d], Theo Wallimann[c], Uwe Schlattner[c]*

[a] *Institute of Biotechnology, ETH Zürich, 8093 Zürich, Switzerland*

[b] *Laboratory of Electron Microscopy I, ETH Zürich, 8092 Zürich, Switzerland*

[c] *Institute of Cell Biology, Swiss Federal Institute of Technology Zürich, Hönggerberg HPM, ETH Zürich, 8093 Zürich, Switzerland*

[d] *Present address: BSD–SRK AG, PCR Diagnostics, Murtenstrasse 133, 3001 Bern, Switzerland*

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Yeast vacuoles are highly dynamic and flexible organelles. In a previous paper, we have shown that subtle, often unrecognised amino acid limitations lead to much lower final cell densities in cultures of different commonly used auxotrophic *Saccharomyces cerevisiae* strains (Çakar et al., Biotechnol. Lett. 21 (1999) 611). Here, we demonstrate for two of these strains, CEN.PK 113.6B and CBS7752, that such subtle leucine limitations also affect the number and morphology of vacuoles, and that these changes are correlated with the cell cycle in batch cultures in a similar way as is known from synchronized cultures. Morphological aspects were studied by electron microscopy, using advanced high pressure freezing/freeze-substitution techniques for sample preparation that so far have been barely successful in yeast. Cells of leucine-limited cultures had single, large vacuoles with a hexagonal tonoplast pattern and were partially arrested in G1 phase. To relieve leucine-limitation, additional leucine was supplied extracellularly via the medium or intracellularly via enhanced leucine biosynthesis due to plasmid-based expression of a leucine marker gene. Such cultures reached more than two-fold higher final optical densities in stationary phase. Cells in later growth phase were characterized by fragmented vacuoles lacking any tonoplast pattern and by a smaller proportion of cells in G1 phase. These drastic effects of subtle leucine limitation on cell physiology, vacuolar morphology and cell cycle distribution present a note of caution for morphological and cell cycle studies in yeast. © 2000 Éditions scientifiques et médicales Elsevier SAS

auxotrophy / electron microscopy / leucine / *Saccharomyces cerevisiae* / vacuole

1. INTRODUCTION

The vacuole of *Saccharomyces cerevisiae* is central to the physiology of this organism and is involved in pH- and osmo-regulation, protein degradation and storage of various compounds (reviewed in Jones et al., 1997). Vacuoles are also highly dynamic and flexible organelles that are sensitive to many factors, including the genetic background of a particular strain, as well as growth conditions (Weisman et al., 1987; Raymond et al., 1990). The yeast vacuole may change size and

* *Correspondence and reprints:* fax: +55 21 562 6483.
E-mail address: schlattn@cell.biol.ethz.ch (U. Schlattner).

shape, alter its tonoplast morphology (Moeller and Thomson, 1979a, b), or fragment into multiple small vacuoles and vice versa (Wiemken et al., 1970; Bryant and Stevens, 1998). Most frequently, yeast cells have a single, large spherical vacuole (Wiemken et al., 1970). The tonoplast of these vacuoles may contain intramembrane particles that aggregate to form geometric, mostly hexagonal patterns. This was observed in batch cultures of *S. cerevisiae* A364A at the transition from exponential to stationary phase (Moeller and Thomson, 1979a, b) and in a temperature-sensitive secretory mutant of *S. cerevisiae* HMSF1 at restrictive temperature (Necas and Svoboda, 1986), but not in different other strains (Willison and Johnson, 1985). In synchronized cultures, the single, large vacuole that is present during G1 phase fragments into several smaller vacuoles prior to bud formation in S phase (Wiemken et al., 1970; Severs et al., 1976). Some small vacuoles migrate into the bud and, after completion of mitosis and cytokinesis, fuse again into a single, large vacuole. Here, fragmentation and fusion of yeast vacuoles are integral parts of vacuolar inheritance (reviewed in Bryant and Stevens, 1998). In asynchronous batch cultures of various *S. cerevisiae* strains, however, no correlation between vacuole fragmentation and cell cycle could be detected (Weisman et al., 1987), except for an adenine-supplemented *ade*$^-$ mutant strain. These authors concluded vacuolar morphology to be cell cycle independent. Thus, it is currently not known whether these changes in number and morphology of yeast vacuoles are triggered by general, well-defined molecular or physiological events or whether they simply depend on the yeast strain or the experimental procedure (Weisman et al., 1987; Willison and Johnson, 1985).

The cell cycle of yeast is controlled by the availability of nutrients. Deprivation of glucose, ammonia, sulphate, phosphate, biotin, or potassium leads to an arrest of cell division at the un-budded G1 phase of *S. cerevisiae* (Williamson and Scopes, 1962; Johnston et al., 1977). Auxotrophic starvation may cause yeast cells to arrest at various points in the cell cycle (Cooper et al., 1979). We have shown that even slight limitations in leucine supplementation of leucine-auxotrophic *S. cerevisiae* cause dramatic changes in growth physiology, i.e. a much lower final cell density in batch culture (Çakar et al., 1999). This raised the question whether an insufficient supply of auxotrophic amino acid(s) induces G1-arrest in these cultures. We also observed the frequent appearance of *trp*$^+$ revertants of strain CBS77752, denoted as CBS7752* (Çakar et al., 1999). When transformed with the yeast expression vector pAAH5 (carrying the *LEU2* gene), CBS7752* grew to much higher cell densities than wild type CBS7752 transformants, and this yield could not be further increased by leucine in the medium, in contrast to the wild type CBS7752 transformants. This indicated that the latter were leucine-limited, while CBS7752* transformants were not, essentially behaving as other auxotrophic yeast strains (Çakar et al., 1999).

Based on our previous report about leucine effects on growth physiology (Çakar et al., 1999), the aim of the present study was to evaluate whether subtle, often unrecognised amino acid limitations also affect yeast cell morphology, especially vacuole morphology. Further, we wanted to examine whether such changes in growth and cellular morphology are related to cell cycle in batch cultures. For this purpose, we have analysed the effect of a slight leucine limitation on both vacuolar morphology and cell cycle, using batch cultures of two leucine-auxotrophic *S. cerevisiae* strains. Leucine starvation was induced in two different ways: i) decreased leucine supplementation via the medium, and ii) use of wild type CBS7752 that grows only to suboptimal final cell densities if a leucine marker gene is supplied on a plasmid, together with the close descendant CBS7752* that grows normally if transformed with the same plasmid.

2. MATERIALS AND METHODS

2.1. Strains and plasmids, transformation protocol

S. cerevisiae strain CBS7752 (*MATa, ura3-52, leu2-3/112, trp1*) was from the '*Centraalbureau voor Schimmelcultures*' (Delft, the Netherlands), and CEN.PK strain 113-6B (*MATa, ura3-52, HIS3, leu2-3/112, trp1-289, MAL2-8*^{c}*, SUC2*) was provided by Dr. P. Kötter (Institute of Microbiology, Johann Wolfgang Goethe-University, Frankfurt, Germany). For auxotrophic complementation we used the constitutive yeast expression vectors pAAH5 (Ammerer, 1983) and YEP24 (Botstein et al., 1979). Strain CBS7752 was transformed by electroporation (Becker and Guarante, 1990), and CEN.PK by using the *S. cerevisiae* EasyComp™ transformation kit (Invitrogen BV). Transformants were selected by means of the *leu* or *ura* markers on the plasmids pAAH5 or YEP24, respectively.

2.2. Growth media and culture conditions

Yeast minimal medium (YMM) containing 6.7 g·L^{-1} yeast nitrogen base without amino acids (Difco) and glucose (0.5% w/v) as carbon source was used in all cultures. Filter-sterilized tryptophan and uracil were added where appropriate to a final concentration of 20 mg·L^{-1} each. The leucine concentration of the CEN.PK growth medium was either 80 or 240 mg·L^{-1}. Cells were grown until stationary phase using a rotary shaker at 30°C and 200 rpm, in 1-L baffled shake flasks containing 250 mL medium. Achievement of stationary

phase was verified by at least three consecutive optical density readings at 600 nm (OD_{600}).

2.3. FACS analysis

Sample preparation for fluorescence-activated cell sorting (FACS) analysis was done as described before (Lew et al., 1992; Deere et al., 1998) with some modifications. Liquid culture samples of 1 mL and 1×10^6 cells·mL^{-1} density were harvested and washed with ice-cold, sterile dH_2O. Absolute ethanol was then added to the cells to a final concentration of 70%. The cells were kept at 4°C overnight, for membrane permeabilization and fixation. They were then centrifuged and washed once with Na-citrate (50 mM, pH 7.0), and resuspended in 500 μL Na-citrate. RNase A was added to a final concentration of 1 mg·mL^{-1}, and the cells were incubated for 2 h at 37°C and subsequently transferred on ice. Cell densities were again adjusted to 1×10^6 cells·mL^{-1} by addition of cold, sterile PBS buffer (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and 3 μL of propidium iodide (1 mg·mL^{-1} in PBS buffer) was added. Samples were incubated on ice in the dark for up to 2 h before analysis. FACS analysis was performed using an EPICS ELITE Analyzer (Coulter, USA), and data were evaluated using the MultiCycle software.

2.4. Labelling of yeast vacuoles and light microscopy

Three mL of yeast culture in a light-protected tube were supplemented with 20 μL Cell Tracker™ Blue CMAC (10 mM stock solution, Molecular Probes, Eugene, OR, USA), a cell-permeable fluorescent marker that accumulates specifically in vacuoles. After incubation of the culture for 0.5–1 h at 30°C, yeast cells were harvested by centrifugation at 4°C, washed once with ice-cold SPM buffer (1.2 M sorbitol, 1 mM $MgCl_2$, 50 mM KH_2PO4, 0.1 mM EDTA, pH 7.3), resuspended in 100 μL SPM buffer and finally kept at 4°C in the dark. On a glass slide coated with Concanavalin-A and poly-L lysine, a drop of this suspension was immobilized under a coverslip and sealed with nail polish. Cells were observed using a Carl Zeiss Inc. Axiophot microscope with a 100× oil-immersion objective, equipped for differential interference optics (DIC), i.e. Nomarski optics, and epifluorescence. Fluorescence filters used were Carl Zeiss Inc. G365 (excitation), FT 395 (beam splitter), and LP420 (emission barrier). All DIC and fluorescent images were photographed using a Kappa CF8/1 FMCC video-camera.

2.5. Transmission electron microscopy

Cells were conventionally fixed (Luft, 1961) with 2.5% glutaraldehyde, followed by 1% OsO_4 and 1% K–ferrocyanide as described previously (van Tuinen and Riezman, 1987; Banta et al., 1988), rendering the cell walls permeable by treatment with 1% sodium metaperiodate. Thin sections were cut from Epon/Araldite blocks and poststained with 2% aqueous uranylacetate, followed by lead citrate (Reynolds, 1963). Micrographs were taken on a Jeol 200 transmission electron microscope at 80 kV. Cryopreparation of cells for thin section transmission electron microscopy (TEM) was performed as described in Hohenberg et al. (1994). The suspensions were sucked into porous cellulose capillary tubes and cryoimmobilized in their growth medium by high pressure freezing (Studer et al., 1989), using a Baltec HPM 010 instrument. Freeze substitution was performed in 2% OsO_4 in acetone (van Harreveld and Crowell, 1964). Embedding, sectioning, poststaining and microscopy were done as outlined above.

2.6. Cryo–scanning electron microscopy

Samples for cryo–scanning electron microscopy (cryo–SEM) were prepared and imaged as described elsewhere (Walther and Müller, 1997). A 100-mesh copper grid (diameter 3 mm) was dipped into the yeast suspension and mounted between two aluminium platelets. This sandwich was frozen in a high pressure freezing machine (HPM 010, Baltec, Balzers, Liechtenstein), and subsequently cryofractured in a freeze etching device (BAF 300, Baltec) by removing one aluminium platelet with the microtome knife (temperature 163 K, vacuum about 2×10^{-7} mbar). After 2 min of etching (sublimation of some water at the fracture face), the sample was coated by electron beam evaporation with 3 nm of platinum–carbon, from an angle of 45°, and 8 nm of carbon, perpendicularly. After coating, the samples were cryotransferred under liquid nitrogen into a cryo–SEM (Hitachi S-900 in-lens field emission) and analysed in the frozen–hydrated state on a cold stage (Gatan) at a temperature of 140 K. Images were recorded using the backscattered electron signal.

3. RESULTS

3.1. Leucine limitation dramatically affects growth, vacuolar fragmentation, and cell cycle distribution

CEN.PK 113-6B, a leucine-auxotrophic strain of the well-defined *S. cerevisiae* CEN.PK family (Brown, 1997), was grown in 0.5% glucose-containing minimal medium with different initial leucine concentrations. A concentration of 80 mg·L^{-1} leucine was insufficient for optimal growth, since cultures grown with 240 mg·L^{-1} leucine attained 50% higher cell densities (*figure 1*). Vacuole-specific fluorescence staining of cells grown at

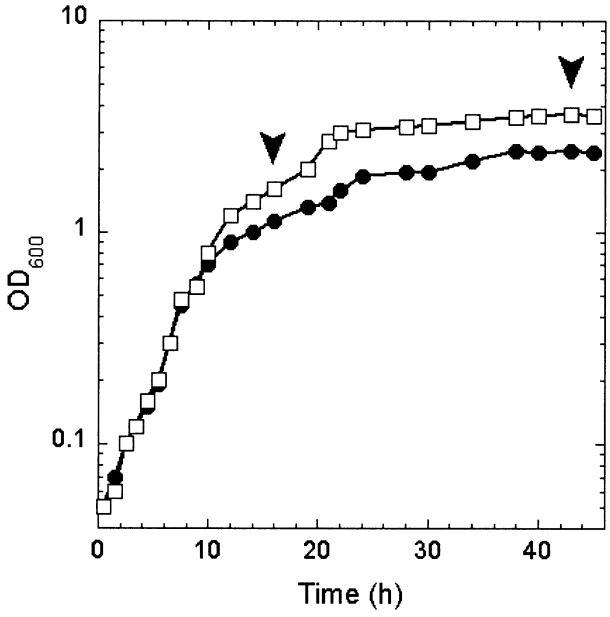

Figure 1. Growth behaviour of CEN.PK 113.6B on 0.5% glucose–YMM and at two different initial leucine concentrations, 80 mg·L^{-1} (filled circles) and 240 mg·L^{-1} (open squares). The arrows indicate the sampling time points for microscopy and FACS analysis.

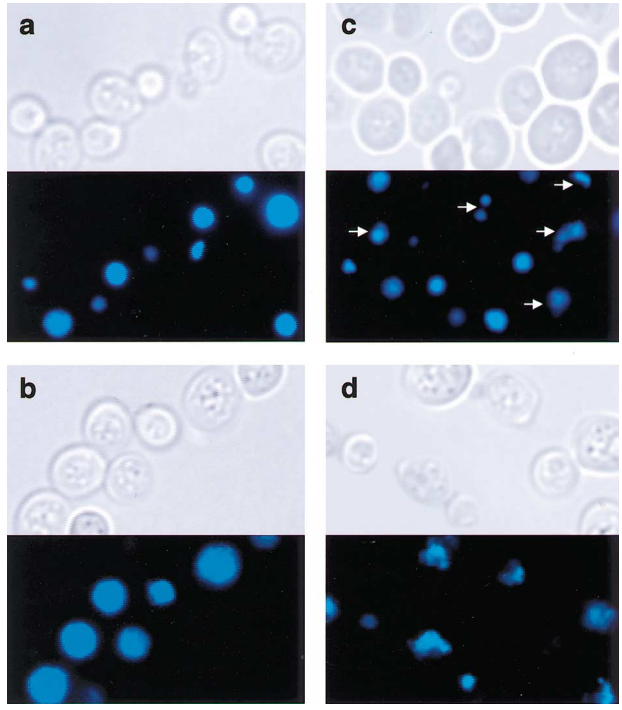

Figure 2. Vacuole fragmentation of CEN.PK 113.6B grown at 80 mg·L^{-1} (**a, b**) and 240 mg·L^{-1} (**c, d**) initial leucine concentration for 16 h (**a, c**) and 43 h (**b, d**). Cells were grown on 0.5% glucose–YMM and native stained with the vacuole-specific fluorescent marker dye Cell Tracker™ Blue CMAC. Images were taken with DIC (upper part of all paired panels) or fluorescence microscopy (lower part). Note that cells at 80 mg·L^{-1} leucine have single vacuoles after 16 h, which become even larger after 43 h (**a, b**), while vacuoles of cells at 240 mg·L^{-1} are smaller, 'diffuse', or partially fragmented after 16 h (**c**, see arrows for fragmented vacuoles) and almost completely fragmented after 43 h (**d**). Bar: 3 μm.

80 mg·L^{-1} leucine revealed a single, large vacuole throughout late exponential growth (16 h, *figure 2a*), increasing in size until stationary phase (43 h, *figure 2b*). In contrast, cultivation with 240 mg·L^{-1} leucine led to vacuolar fragmentation during exponential growth, clearly established after 16 h (*figure 2c*), and even more pronounced in stationary phase after 43 h (*figure 2d*). Thus, the appearance of vacuolar fragmentation correlated with increasing growth rates relative to the leucine-limited cultures, occurring after 10 h of growth (*figure 1*). In contrast, vacuole fragmentation was not related to the carbon source, since glucose was used up in both cultures when they approached stationary phase.

We then used FACS to investigate whether indeed slight leucine limitation in our batch cultures was correlated not only with vacuole morphology, but also with the cell cycle distribution, as was previously hypothesised for synchronized cultures (Wiemken et al., 1970; Severs et al., 1976). With an initial leucine concentration of 80 mg·L^{-1}, a higher percentage of the CEN.PK 113-6B cells was in G1 phase, and also in S phase, as compared to cells grown with an initial leucine concentration of 240 mg·L^{-1} (*figure 3*). This difference was most pronounced at later stages of growth (i.e. 16 h), supporting the hypothesis that insufficient supplementation of leucine prevents growth and cell division of yeast cells, thus leading to altered vacuolar morphology.

3.2. Insufficient leucine biosynthesis through marker gene expression affects growth and different aspects of vacuolar morphology

In many experimental systems, auxotrophic mutations are not countered by extracellular supplementation of the nutrient, but by transformation with plasmid-encoded auxotrophic marker genes. To investigate the effects of suboptimal leucine biosynthesis by strain-specific marker gene expression, we chose the two *S. cerevisiae* strains CBS7752, a wild-type strain, and CBS7752*, a previously described pleiotrophic mutant (Çakar et al., 1999). For CBS7752, the plasmid-based expression of a *leu2* marker gene is insufficient

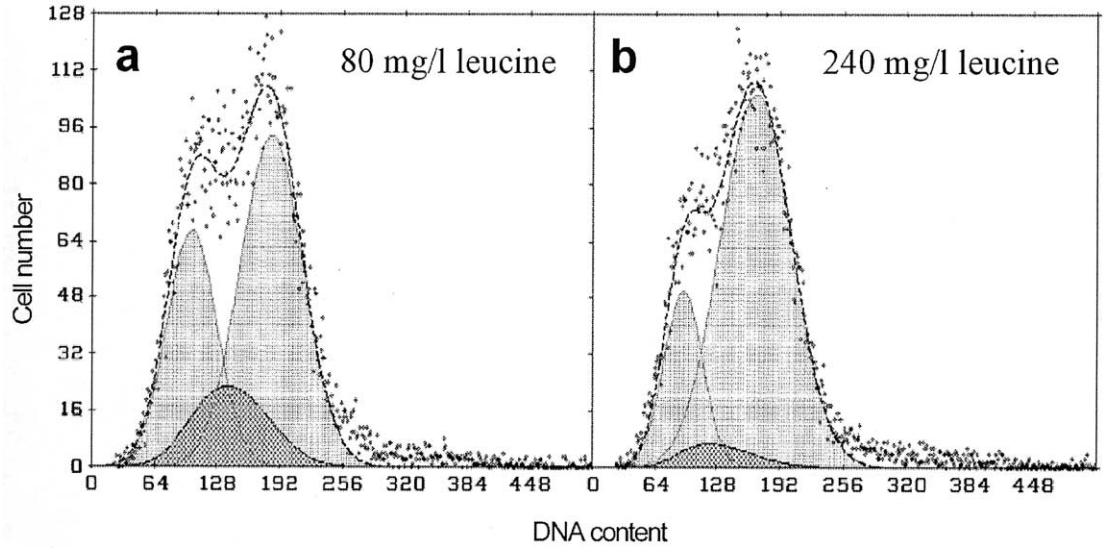

Figure 3. Cell cycle distribution of CEN.PK 113-6B in late exponential growth phase (16 h). Cells were grown on 0.5% glucose–YMM at two different initial leucine concentrations, 80 mg·L^{-1} (**a**) and 240 mg·L^{-1} (**b**). FACS was performed as described in materials and methods. The distribution of cell number versus propidium iodide fluorescence is recalculated as a distribution of cells in phase G1 (DNA content 1*n*), S and G2 (2*n*) (see gray peaks, from left to right). Note the higher proportion of cells in G1- and S phase at 80 mg·L^{-1} initial leucine concentration.

for optimal leucine supply, as evidenced by the higher final cell densities reached after addition of extracellular leucine. In contrast, transformants of CBS7752* reached maximal final cell densities without additional leucine supply, similar to other transformed leucine auxotrophic yeast strains, including CEN.PK (Çakar et al., 1999). This is confirmed by the growth curves of CBS7752 and CBS7752* in 0.5% glucose-containing minimal medium (*figure 4*). At stationary phase, transformants of CBS7752* reached at least two-fold higher final optical densities compared to transformants of CBS7752, consistent with cellular dry weight measurements and microscopic cell counts. Growth rates of both cultures started to diverge already after 2 h of growth. After another 6 h of growth, glucose was completely used up by CBS7752* cultures, while leucine-limited CBS7752 cultures retained about half of the initial glucose. At late stages of growth, however, before reaching stationary phase, glucose was entirely consumed in both cultures (data not shown). Furthermore, when grown in 2% glucose or 0.5% ethanol as a carbon source, CBS7752 and CBS7752* transformants showed very similar differences in growth rate and final cell density (Çakar et al., 1999, and unpublished data). Thus, the systematic differences in growth rate between transformants of CBS7752 and CBS7752* were not due to changes in carbon source.

The vacuolar morphology of CBS7752 and CBS7752* transformants was first analysed with the vacuole-specific fluorescent vital stain. Similar to leucine-limited CEN.PK cells (*figure 2a, b*), CBS7752 transformants generally had a single, large vacuole

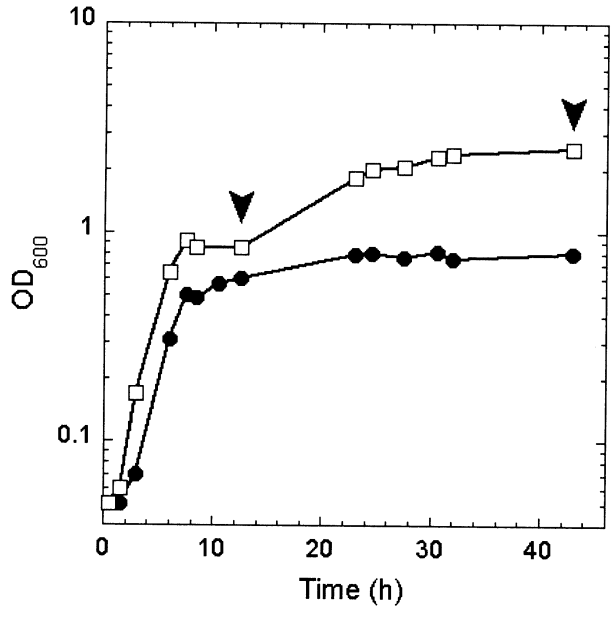

Figure 4. Growth behaviour of transformants of CBS7752 (filled circles) and revertant CBS7752* (open squares) on 0.5% glucose–YMM. The arrows indicate the sampling time points for fluorescence microscopy and EM analysis.

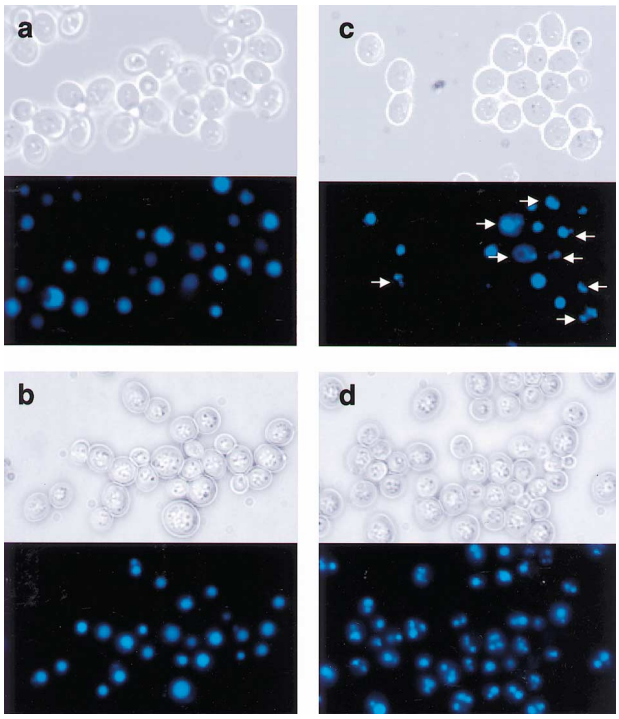

Figure 5. Vacuole fragmentation of transformants of CBS7752 (**a, b**) and revertant CBS7752* (**c, d**) for 12.5 h (**a, c**) and 43 h (**b, d**). Cells were grown on 0.5% glucose–YMM and native stained with the vacuole-specific fluorescent marker dye Cell Tracker™ Blue CMAC. Images were taken with DIC (upper part of all paired panels) or fluorescence microscopy (lower part). Note that each CBS7752 cell has a single, large vacuole throughout the entire growth phase (**a, b**), while CBS7752* cells show largely fragmented vacuoles at 12.5 h (**c**, see arrows), leading to multiple, small vacuoles in each cell after 43 h (**d**). Bars: 10 μm.

that persisted throughout their entire growth and stationary phase (*figure 5a, b*). In contrast, like the non-leucine limited CEN.PK cells (*figure 2c, d*), transformants of the mutant strain CBS7752* showed pronounced vacuolar fragmentation (*figure 5c, d*). Partial fragmentation was already observed at the end of a first growth phase after 12.5 h (*figure 5a*), where CBS7752* transformants showed a somewhat higher growth rate as compared to CBS7752 (*figure 4*). During a late, second growth phase, where growth rates between both strains differed even more (*figure 4*), vacuoles of CBS7752* transformants fragmented completely and cellular volume occupied by these vacuoles was significantly reduced (*figure 5d*). These changes in vacuolar morphology, occurring exclusively in CBS7752* transformants, were not related to carbon source, since glucose was exhausted in cultures of both strains at this time.

To analyse further aspects of vacuolar and cellular morphology that could possibly discriminate CBS7752 and CBS7752* transformants we have studied cells from late growth phase by electron microscopy. The applied techniques included conventional TEM (*figure 6a, d*), cryofixation-based (high pressure freezing–freeze substitution) procedures for TEM (*figure 6b, e*), and cryo–SEM of high pressure frozen and freeze-fractured samples (*figure 6c, f*) (Studer et al., 1989; reviewed in Kiss and Staehelin, 1995). The single, spherical vacuole of the CBS7752 transformants was found adjacent to the nucleus. The tonoplast showed a regular, geometric pattern of smooth hexagonal areas ($\phi \sim 200$ nm, *figure 6c*), which were depressed towards the organelle interior and separated by particle-rich rows. These rows corresponded to the edges of the scalloped pattern of the tonoplast seen in thin-sectioned cells, especially after high-pressure cryofixation (*figure 6b*). The typical CBS7752* transformant had multiple vacuoles distributed all over the cell, highly varying in size and a with a rather smooth tonoplast surface lacking geometric patterns (*figure 6f*). Numerous electron-microscopically isomorphous vesicles, not staining with the vacuole-specific dye (*figures 2* and *5*), most likely represented lipid droplets. They were present in both strains, but much more frequent in CBS7752* transformants (*figure 6d, f*).

4. DISCUSSION

In this paper, we demonstrate that slight leucine limitations in batch cultures of auxotrophic *S. cerevisiae* not only change growth rate (Çakar et al., 1999), but also have severe effects on vacuole fragmentation and morphology. In addition, these changes were related to cell cycle distribution, a correlation only found so far in synchronized yeast cultures (Wiemken et al., 1970; Severs et al., 1976). These data suggest that leucine limitation causes leucine-auxotrophic cells to accumulate at the G1 phase, thus reducing cell growth and division, as well as altering vacuolar morphology. Yeast cell morphology has been analysed with TEM using high pressure-frozen, freeze-substituted samples that preserve a maximum of their native structure. To date, this technique has hardly been successful with yeast due to the quite impermeable cell wall (Erk et al., 1998).

4.1. Vacuolar morphology and cell cycle distribution of leucine-auxotrophic *S. cerevisiae* depend on initial leucine supply

When comparing the cellular morphology of leucine-auxotrophic *S. cerevisiae* grown in batch culture under leucine-limited and leucine-sufficient conditions, we found the most striking differences in the number and the ultrastructure of the vacuole. Indeed, yeast vacuoles are a very flexible and dynamic compartment,

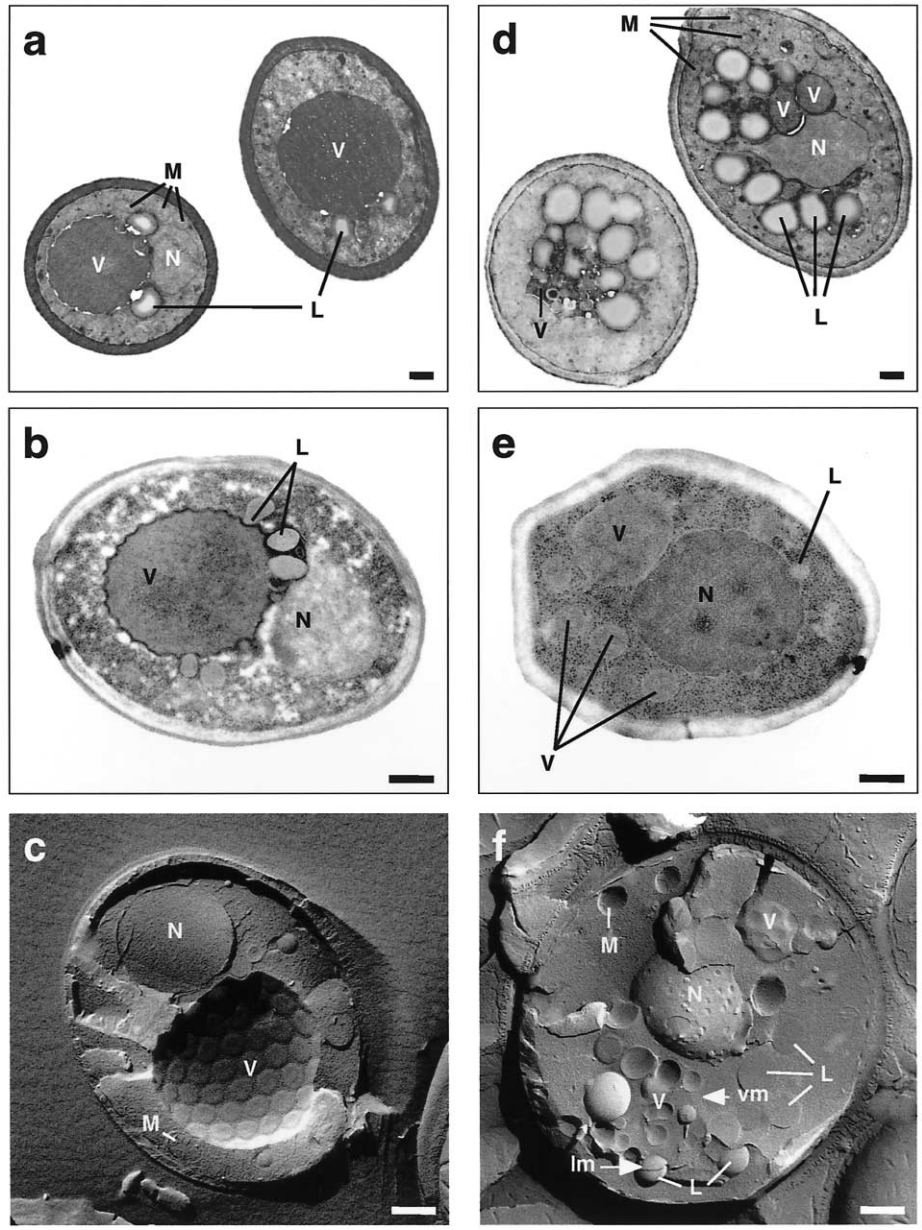

Figure 6. Cellular morphology of transformants of CBS7752 (**a**–**c**) and revertant CBS7752* (**d**–**f**) grown on 0.5% glucose–YMM. Micrographs were taken at late stages of growth (43 h) with TEM of chemically fixed, Epon-embedded cells (**a, d**) and high-pressure cryo–fixed cells (**b, e**) or with cryo–SEM of high-pressure frozen cells (**c, f**). Note the scalloped tonoplast membrane (**a, b**) corresponding to a hexagonal tonoplast pattern of aggregated intramembrane particles in leucine-limited CBS7752 (**c**), in contrast to the different vacuolar morphology and the accumulation of lipid droplets in CBS7752* (**d**–**f**). Abbreviations: N, nucleus; M, mitochondrion; L, lipid droplet; vm, vacuolar membrane; lm, membrane of lipid droplets. Bars: 0.5 μm.

sensitive to different physiological and genetic conditions (Weisman et al., 1987; Bryant and Stevens, 1998). Cells with limiting extracellular leucine supplementation or insufficient leucine biosynthesis based on plasmid-based marker gene expression had always single, large vacuoles. These are characteristic of G1 phase in the cell cycle of synchronized cultures (Wiemken et al., 1970; Severs et al., 1976) and of a transition to stationary phase (Moeller and Thomson, 1979a), eventually leading to G0-arrest (reviewed in Werner-Washburne et al., 1996). The ultrastructure of the vacuole was characterized in more detail for strain CBS7752. The tonoplast showed geometric, hexagonal patterns of intramembrane particles, similar to those seen in cells approaching stationary phase (Moeller and Thomson, 1979a,b) or in secretion mutant *sec1* (Necas and Svoboda, 1986). These patterns are due to the aggregation of intramembrane particles and were explained by phase separation of lipid molecules in the tonoplast membrane (Moeller et al., 1981). In contrast, rapidly growing, non-leucine-limited batch cultures of *S. cerevisiae* showed a fragmentation of the single, large vacuole into multiple, small vacuoles of variable size, having rather homogeneous tonoplast morphology.

Such a vacuole fragmentation in budding yeast has been described for strain A363A in synchronized cultures, undergoing cell division (Wiemken et al., 1970; Severs et al. 1976) and for several *vps* mutants (Bryant and Stevens, 1998). Consistently with these changes in yeast morphology, the portion of cells in G1 phase was much more prominent in leucine-auxotrophic cells as compared to cells grown with sufficient leucine supplementation. This indicates a 'partial' G1-arrest (Pringle and Hartwell, 1981) under leucine-limitation and a relationship between cell cycle and vacuolar morphology under batch growth conditions.

4.2. Vacuolar morphology and cell cycle are related under batch growth conditions

Earlier studies already indicated a close relation between vacuolar morphology, tonoplast ultrastructure and cell cycle in synchronized yeast cultures (Wiemken et al., 1970; Severs et al., 1976; Moeller and Thomson, 1979a). However, this conclusion has been questioned by more recent studies. Using asynchronous batch growth conditions, Weisman et al. (1987) found that vacuole fragmentation was cell cycle-independent in strain X2180-1A, and Willison and Johnson (1985) failed to observe geometric patterns at the tonoplast surface of strain AG1-7 during later growth phase. The authors concluded that changes in vacuolar morphology related to cell division were restricted to specific yeast strains or dependent on synchronized culture conditions. In contrast, our results demonstrate that asynchronous batch cultures, when grown under slight amino acid limitation, also show an alteration of vacuolar morphology and tonoplast ultrastructure that is related to cell cycle. Notably, when the *ade2* mutant of strain X2180-1A was grown with high levels of adenine, it also showed significant vacuole fragmentation (Weisman et al., 1987). This is probably also due to an auxotrophic limitation, as we have found for W303-1A, another *ade*$^-$ strain of *S. cerevisiae* (Çakar et al., 1999).

4.3. Cell cycle studies with auxotrophic *S. cerevisiae* can be misleading if the supply of auxotrophic substrate is limited

Our results clearly show that not only nitrogen, phosphate, or sulphate starvation (Williamson and Scopes, 1962; Johnston et al., 1977), but also subtle, often unrecognised amino acid limitations in different auxotrophic *S. cerevisiae* strains can drastically affect cell physiology, vacuolar morphology and cell cycle distribution. This presents a note of caution for cell cycle studies in yeast, in particular as 80–100 mg·L^{-1} of leucine is not generally recognised as limiting and even recommended by laboratory manuals (Adams et al., 1998). In fact, there are several published reports that are likely to be affected by this correlation. For example, the cell cycle behaviour of auxotrophic *S. cerevisiae* strains was studied by flow cytometry, using minimal media with an insufficient amino acid supplementation (Lew et al., 1992; Porro and Srienc, 1995). In addition, some of the constructs used in those studies were based on gene disruption via insertions of amino acid-encoding genes like *leu2* (Lew et al., 1992; Aon and Cortassa, 1998). It follows from our results that the comparisons made with the wild type could be misleading, since the amino acid supplied by a gene does not seem to have the same physiological effects as the externally supplied corresponding amino acid (see also Çakar et al., 1999). In order to minimize such complications, the amino acid concentrations that make auxotrophic strains comparable to their wild types have to be determined carefully for each particular strain, before using them in cell cycle, as well as physiological or morphological studies.

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