RESEARCH ARTICLE

Adenine auxotrophy – be aware: some effects of adenine auxotrophy in *Saccharomyces cerevisiae* strain W303-1A

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Abstract

Adenine auxotrophy is a commonly used genetic marker in haploid yeast strains. Strain W303-1A, which carries the *ade2-1* mutation, is widely used in physiological and genetic research. Yeast extract-based rich medium contains a low level of adenine, so that adenine is often depleted before glucose. This could affect the cell physiology of adenine auxotrophs grown in rich medium. The aim of our study was to assess the effects of adenine auxotrophy on cell morphology and stress physiology. Our results show that adenine depletion halts cell division, but that culture optical density continues to increase due to cell swelling. Accumulation of trehalose and a coincident 10-fold increase in desiccation stress tolerance is observed in adenine auxotrophs after adenine depletion, when compared to prototrophs. Under adenine starvation, long-term survival of W303-1A is lower than during carbon starvation, but higher than during leucine starvation. We observed drastic adenine-dependent changes in cell stress physiology, suggesting that results may be biased when adenine auxotrophs are grown in rich media without adenine supplementation.

Introduction

Like all micro-organisms, baker's yeast cells respond to environmental changes and adapt their growth and proliferation accordingly. A drop in nutrient availability is a signal for the onset of nutrient limitation and impeding starvation. Even a slight drop in the concentration of a critical nutrient is sufficient to induce alterations in cell physiology such as the initiation of a 'preconditioning programme', which prepares the cell for harsh conditions (Smets *et al.*, 2010).

Two types of limitations or starvations, dependent on the nutrient, can occur: 'natural limitations', which sets in when basic nutrients (carbon, phosphorous, sulphur and nitrogen) are scarce, and 'artificial limitations', which sets in when particular metabolites or metabolic intermediates are insufficient (Saldanha *et al.*, 2004). Additionally, depending on nutrient supply mode, distinction is made between starvation and limitation. Starvation for certain nutrient is defined if it is absent, whereas limitation occurs when certain nutrient is added in scarce amounts and thus limits the growth. Starvation is a typical phenomenon of batch cultivations, and limitation is usually attributed to chemostat cultivations. Auxotrophy is a typical example of an artificial limitation. Many common laboratory yeast strains (W303, S288C, CEN.PK and FY series) contain one or several auxotrophic markers. Histidine, leucine, uracil, adenine and tryptophan (his, leu, ura, ade and trp) are the most common auxotrophic markers of *Saccharomyces cerevisiae* strains used in physiology studies (Pronk, 2002; Da Silva & Srikrishnan, 2011). Insufficient concentration of an auxotrophic agent leads to artificial limitation. Depending on the type of limitation, the yeast cells exhibit different responses. Cell cycle arrest and subsequent quiescent state constitute a typical response to natural limitations (Boer *et al.*, 2008). However, cell cycle arrest and quiescence do not occur for an artificial limitation with either leucine or uracil. On the other hand, inability to complete cell division and to halt subsequent cell cycle leads to a decrease in viability in addition to an observable 'glucose wasting' phenomenon (Boer *et al.*, 2008).

The adenine auxotrophic marker, *ade2-1*, is common to the *S. cerevisiae* strain W303-1A and its derivatives. These strains are well known for the fact that they acquire red colouration during culture growth. This red pigment is the oxidised form of ribosylaminoimidazole,

an intermediate of the adenine *de novo* synthesis pathway. The adenine auxotrophy-dependent red colouration is used in white red mutant screens (Weng & Nickoloff, 1997) and synthetic lethality assays (Barbour & Xiao, 2006). In addition to *ade2*, *ade1* is also used in white red mutant screens, and *ade8*, another adenine *de novo* synthesis pathway gene, has been used as an integration site yielding moderate expression levels of heterologous genes (Sadowski *et al.*, 2007).

External adenine supplement in synthetic media is needed to promote proliferation of adenine auxotrophs. However, availability of adenine is sometimes ignored in rich media (e.g. yeast extract-based media) because it is assumed that all nutrients are present in sufficient levels. Several researchers point out that in rich media, adenine levels vary from batch to batch and the adenine is often depleted before exhaustion of the carbon source (VanDusen *et al.*, 1997; Zhang *et al.*, 2003).

Besides hampering proliferation, adenine auxotrophy might have other adverse effects on yeast physiology. Thus far, adenine auxotrophy has been associated with a decrease in heterologous protein expression. Interestingly, this has been observed for both low and high external adenine levels (VanDusen *et al.*, 1997; Zhang *et al.*, 2003).

W303-1A and its derivatives have been exploited in basic physiology research for 30 years (Carlson & Botstein, 1982; Ralser *et al.*, 2012). However, to our knowledge, no research on the effects of adenine auxotrophy on physiology of this particular strain has been performed. Many physiological studies are performed in batch mode using rich media where the effects of adenine limitation can become pronounced, obfuscating the physiological phenomenon of interest. The findings from the studies herein can help to minimize these undesired effects. In the present work, we report some basic culture physiology and cell morphology studies using W303-1A batch cultivation. We find that adenine depletion has a direct impact on the cell size, trehalose content and subsequent desiccation stress tolerance. Taken together, our results serve as a basis for new interpretations of some previous results regarding yeast stress physiology as well as a warning against assuming that adenine auxotrophy provides a neutral background for physiology studies.

Materials and methods

Strains

Laboratory strains used in this study are shown in Table 1. CEN.PK *ade8* disruption was created by homologous recombination using a *URA* cassette and screening for *ura*$^-$ mutants on 5-FOA as described in Sadowski *et al.* (2007).

Growth media

YPD [10 g L^{-1} of yeast extract (Biolife), 20 g L^{-1} of peptone (Biolife), 20 g L^{-1} of dextrose (Sigma)] was used for yeast cell physiology studies: growth dynamics, cell morphology, trehalose content and desiccation stress tolerance. Synthetic dextrose (SD) media [1.7 g L^{-1} of yeast nitrogen base w/o amino acids and ammonium sulphate (Difco), 5 g L^{-1} of $(NH_4)_2SO_4$, 20 g L^{-1} of dextrose] supplemented with leucine (260 mg L^{-1}), of tryptophan (80 mg L^{-1}), of uracil (100 mg L^{-1}), histidine (100 mg L^{-1}) and adenine (100 mg L^{-1}) was used for adenine titration experiments. For starvation experiments, SD media with either adenine, leucine or glucose omitted depending on starvation type investigated were used.

Cultivation

Yeasts were cultivated in shake flasks at 180 r.p.m. in 30 °C with broth volume not exceeding 20% of the flask volume.

Morphological measurements

Optical density (OD) was measured at 600 nm with Ultrospec 2100 pro (Amersham Biosciences), diluting cultures below 0.3 absorbance units.

The cells dry weight was determined by harvesting biomass from 20 to 40 mL of cultivation broth by centrifugation, washing twice with distilled water, and drying at

Table 1. Yeast strains used in this study

Strain name in text	Strain genotype	Source
W303 *ade2*	W303-1A *MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	Dr. Peter Richard
W303 *ADE2*	W303-1A *ADE2*	Dr. Arnold Kristjuhan
W303 prototroph	2832 – 1B *MATa can1*	Dr. Frederick R. Cross
CEN.PK prototroph	CEN.PK 113-1A	Dr. Peter Richard
CEN.PK *ADE8*	CEN.PK2 *MATa leu2-3/112 ura3-52 trp1-289 his3-1, MAL2-8c SUC2*	Dr. Peter Richard
CEN.PK *ade8*	CEN.PK2 *MATa leu2-3/112 ura3-52 trp1-289 his3-1, ade8, MAL2-8c SUC2*	This study

105 °C until reaching constant weight. For cell counts and morphology assessment, cells were fixed in 1% glutaraldehyde and then sonicated briefly. Cell number was counted by hemocytometer.

Cell size (for a sample size of $n > 300$) was determined by analysing micrographs with ImageJ software, approximating cell shape as an ellipse and calculating the area of the acquired ellipses (Jorgensen *et al.*, 2007). Buds, if they exceeded half the size of the mother cell, were defined as separate cells. Budding index, defined as the proportion of cells with buds, was determined for the population from a sample size of $n > 500$.

Metabolite measurements

Anthrone assay was used to determine trehalose and media glucose concentrations (Terevelyan & Harrison, 1956). For trehalose measurements, cells were washed with distilled water twice, disintegrated in 5% TCA with glass beads, and then the supernatant (diluted with water when necessary) mixed with anthrone (2 g L^{-1} in 75% H_2SO_4) in a 1 : 6 ratio. The mixture was heated at 100 °C for 10 min, and absorbance at 626 nm was measured. The same procedure was used for media glucose quantification.

The concentration of adenine in media was determined enzymatically following a modified protocol from Zhang *et al.* (2003). More specifically, the concentration of adenine in media was quantified fluorometrically by hypoxanthine oxidase (Sigma X4500)-coupled assay using horseraddish peroxidase (HRP; Sigma) and Amplex UltraRed dye (Molecular Probes®, ex/em 530/590 nm). The reaction mix contained 20 μL of sample, 2 mL 0.1 M, pH 7.5 sodium phosphate buffer, 0.02 U xanthine oxidase, and 2 U HRP. Reaction mixtures were incubated at 30 °C for 30 min at which point the emission at 590 nm (ex 530) was measured with a FluoroMax-3 (Yvon Horiba) spectrofluorometer.

Stress tolerance assessment

Desiccation tolerance was assayed by estimating CFU mL^{-1}, before and after dehydration. One millilitre of culture at $OD_{600} = 1$ was washed with distilled water twice, diluted serially and spotted on YPD plates. The remaining cell suspension was centrifugated, and the pellet was left to desiccate for 10 h at 30 °C in a desiccator and then rehydrated for 10 min in room temperature in distilled water. The suspension of rehydrated cells was serially diluted and spotted on YPD plates. The viability was calculated by dividing the number of CFU mL^{-1} before and after desiccation, as performed in Calahan *et al.* (2011).

To assess starvation stress tolerance, cells were grown in full SD media up to exponential phase, washed with distilled water, and re-suspended to $OD_{600} = 1$ in SD media lacking either sugar, leucine or adenine with all other broth components added in surplus. The yeasts were incubated for 10 days in a rotary shaker, and samples were taken upon inoculation (day 0) and on the 1st, 2nd, 4th, 7th and 10th day. Samples were diluted serially and plated on YPD plates to assess viability. Undiluted sample was fixed with glutaraldehyde and later used for budding index, cell size and count mL^{-1} measurements.

Statistical treatment of data

All the represented values are means from biological triplicates. Error bars and variation depict standard errors. Two-tailed, two-sample unequal variance Student's *t*-test or Wilcoxon rank-sum test (for cell size comparison) were used to compare means of physiological parameters. *P*-values < 0.05 were considered statistically significant.

Results

Growth characteristics and cell morphology

The effects of adenine auxotrophy on the *S. cerevisiae* strain W303-1A (from here on called W303 *ade2*) were explored during cell growth in YPD (glucose content 2%) media. A W303-1A-derived adenine prototroph (W303 *ADE2*) strain was used as a control. External adenine depletion was monitored by xanthine oxidase coupled to horseradish peroxidase assay. Also, red colouration of W303 *ade2* cells served as a signal for adenine depletion. The red pigment in yeast cells was observed after adenine became depleted in the media, as detected by xanthine oxidase assay. However, adenine auxotrophs tend to accumulate a vast amount of adenine and then use it upon depletion of the external adenine pool (VanDusen *et al.*, 1997). The adenine synthesis pathway and subsequent red pigment accumulation in adenine auxotrophs are induced after adenine *de novo* synthesis is started, which occurs when no free adenine remains inside the cell (Rebora *et al.*, 2001). Therefore, we assumed that pigment development is a physiologically more reliable signal for adenine starvation in the cell than the concentration of adenine in the media.

We assessed growth of W303 *ade2* and its corresponding adenine prototroph W303 *ADE2* using yeast extract from only one producer due to known variance in adenine content across different commercial suppliers (VanDusen *et al.*, 1997; Zhang *et al.*, 2003). For all experiments, yeast extract from the same producer and single batch was used.

First, we compared growth dynamics of W303 adenine auxotroph and prototroph in YPD media to determine

whether adenine content in media influences general cell physiology.

OD measurements at 600 nm were used to monitor growth (Fig. 1a). Throughout the exponential growth phase (from 0 to 10 h), both strains, W303 adenine auxotroph and prototroph, grew similarly. The adenine content in the media, measured by the xanthine oxidase assay, was depleted at the same time for both strains (see Fig. 1). Moreover, specific glucose consumption rates and biomass yields were similar for both strains during the exponential growth phase, before exhaustion of external adenine.

Biomass yields per substrate consumed (Y, x/s, g g^{-1}) for W303 *ade2* and W303 *ADE2* were 0.097 and 0.099, respectively. Specific substrate uptake rates (q) were 0.98 and 0.95 g g^{-1} h^{-1} for W303 *ade2* and W303 *ADE2*, respectively.

There was still some sugar left in the growth medium after external adenine was exhausted (Fig. 1b). Surprisingly, adenine depletion did not interrupt an increase in OD for the W303 *ade2* strain, a metric that suggests uninterrupted growth. This observation conflicted with the prediction that adenine exhaustion would halt proliferation and an OD_{600} increase (Fig. 1a). When the ratio of dry weight to OD of the culture was analysed (Supporting Information, Fig. S1), both W303 strains, *ade2* and *ADE2*, maintained linear OD/dry weight ratios both before and after external adenine was depleted. As expected, W303 *ade2* formed red pigment as a response to external adenine depletion.

To resolve the discrepancy between the expected proliferation cessation and the clear increase in OD for W303 *ade2* in culture after adenine depletion, we compared optical densities with the corresponding cell counts mL^{-1} of both cultures (Fig. 2).

There was a notable difference in cell number mL^{-1} when comparing the adenine auxotroph and prototroph. While the cell number mL^{-1} grew steadily in adenine prototroph, its increase ceased for the auxotroph after adenine depletion. To show that this effect was not strain specific, but purely adenine dependent, we added extra adenine (100 mg L^{-1}) to W303 *ade2* strain in YPD media. As a result of not reaching adenine starvation through supplementation, the behaviour of W303 *ade2* cells followed the pattern of the W303 *ADE2* strain. Therefore, we concluded that an increase in OD_{600} after adenine depletion in adenine auxotrophic cultures is not caused by cell multiplication but most likely due to changes in the cells' optical properties.

Two hypotheses were proposed to explain the OD_{600} increase after adenine depletion: either pigment induced by adenine auxotrophy has absorption that overlaps 600 nm or increased light scattering occurs due to increase in cell size.

The red pigment does not have specific absorption around 600 nm (Smirnov *et al.*, 1967). Therefore, we concluded that red pigment had negligible, if any, impact on W303 *ade2* OD measurements at 600 nm. Beauvoit *et al.* (1993) have reported that yeast cell size affects culture suspension light scattering and attributed it to a special case of general light scattering Mie theory.

To determine whether cell size was contributing to the increase in OD_{600}, mean cell size in W303 *ade2* and W303 *ADE2* cultures was measured after 27.5 h of growth in YPD medium. Size was measured as an elliptical approximation of the cell's cross-section in microphotographs. The adenine auxotrophic cells were significantly ($P < 0.0001$) bigger than those of the prototrophs: 22 ± 14 vs. 16 ± 5 μm^2, respectively (Fig. 3).

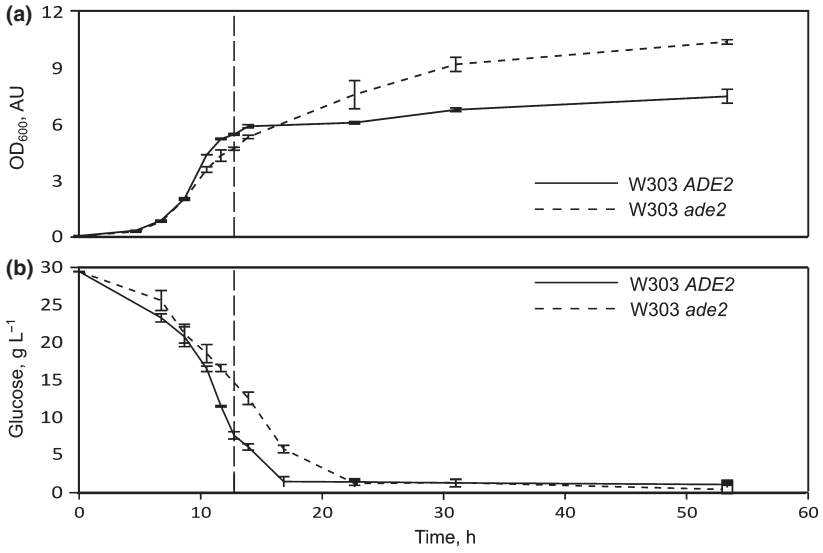

Fig. 1. W303 *ADE2* and W303 *ade2* culture growth (a) and glucose consumption (b) when cultivated in YPD media. Error bars represent standard deviation from three independent cultivations. Dashed vertical line indicates adenine depletion in the media.

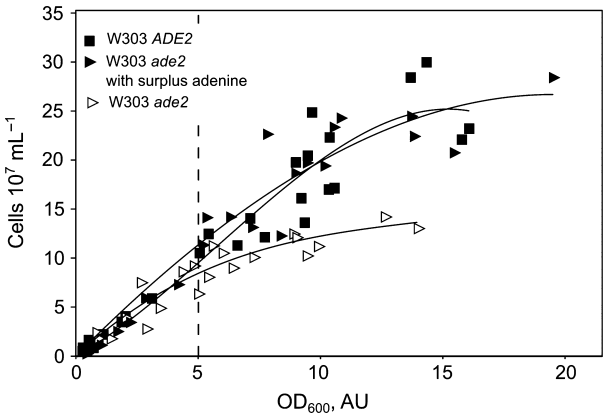

Fig. 2. W303 strain cell count mL^{-1} depending on OD_{600}. Yeast strains were grown in YPD media with (100 mg L^{-1}) and without extra adenine supplement. Cell number mL^{-1} was determined by hemocytometer. Vertical dashed line indicates adenine depletion during W303 *ade2* cultivation in YPD media without extra adenine supplement. Approximations of third-order polynomial functions were used for visualization of cell number mL^{-1} data. However, it does not fully account for relationship between the variables.

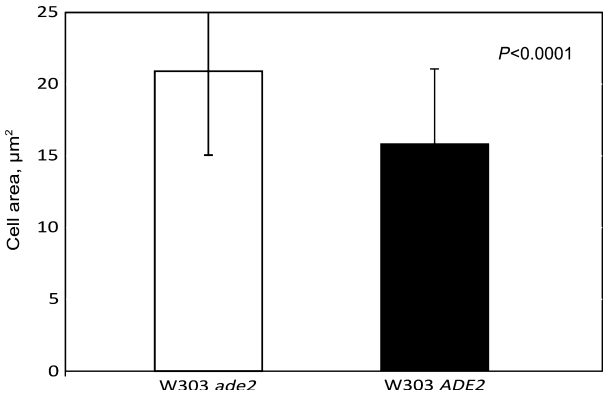

Fig. 3. W303 strain mean cell size. Cells were harvested after 27.5 h of cultivation, and their cross-section areas were measured from micrographs as described by Jorgensen *et al.* (2007). Cell number exceeded 300 for each strain. Means of both distributions were compared by Wilcoxon signed rank-sum test. Mean cell area of W303 *ade2* and W303 *ADE2* revealed to be significantly different ($P < 0.0001$).

Based on these data, we concluded that the increase in cell size has led to elevated turbidity of the cell suspension and, therefore, to the misleading impression of proliferation of adenine auxotroph cells after adenine depletion.

Our results indicate that growth of the W303 *ade2* strain in rich medium differs significantly from that of the adenine prototroph when adenine is depleted. VanDusen *et al.* (1997) reported on high variability of adenine content among yeast extracts of different vendors and different batches (ranging from 0.34 up to 1.91 mg g^{-1} yeast extract). The YPD media used in experiments described here contained 13 mg L^{-1} of adenine.

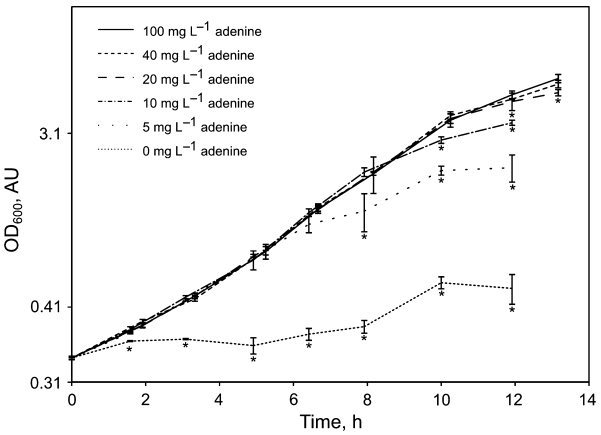

Fig. 4. Growth curves of W303 *ade2* strain in SD media with different adenine content (0, 5, 10, 20, 40 and 100 mg L^{-1}); all other nutrients were added in surplus. Each growth curve is mean of biological triplicates; error bars represent standard deviations. Statistical comparison between OD measurements during cultivation in medias of different adenine concentrations and 100 mg L^{-1} (positive control) was made. Asterisks below data point denote significant difference from positive control ($P < 0.05$).

Due to vast variability of adenine content among different manufacturers' yeast extracts, and obvious adenine insufficiency observed in our rich media, we decided to determine optimal adenine concentration for the W303 *ade2* strain cultivation. To ensure exact adenine concentrations, SD media were used. We were interested in changes in growth rate, which, besides red pigmentation, would indicate adenine exhaustion. We inoculated exponentially growing, freshly washed W303 *ade2* cells to flasks with media of different adenine content. We used an adenine concentration of 100 mg L^{-1} as a positive control (physiologically 'safe' after Pronk, 2002) against which growth curves of all other adenine concentrations (0, 5, 10, 20 and 40 mg L^{-1}) were compared (Fig. 4). Results are plotted on log axis to demonstrate growth rate changes. W303 *ade2* culture growth profiles when cultivated in media with 0, 5, 10 and 20 mg L^{-1} adenine diverged from 100 mg L^{-1} curve at different time points, and cells started to accumulate red pigment, thus indicating depletion of external adenine. Student *t*-test revealed significant differences for OD_{600} between 100 mg L^{-1} and each of these cultivations. At the time points when growth rate changed, glucose persisted in ample amounts (20, 15.4, 14.2, 8.68 g L^{-1} for flasks with 0, 5, 10, and 20 mg L^{-1} adenine supplement, respectively) further indicating adenine depletion. No statistically significant

($P > 0.05$) differences between culture OD_{600} were observed when comparing cultures containing 40 mg L^{-1} with cultures of 100 mg L^{-1} adenine supplement.

Based on our results, we concluded that at least 40 mg L^{-1} supplement should satisfy uninterrupted W303 *ade2* mutant growth.

Desiccation and starvation responses

Reserve carbohydrate accumulation (glycogen and trehalose) is reported to occur in yeast under certain limitations (Lillie & Pringle, 1980; Klosinska *et al.*, 2011). Studies of different limitations in chemostats have revealed that trehalose accumulation is inversely related to the culture's growth rate, but independent of the nature of limitation, whether it be natural or artificial (Boer *et al.*, 2010). Due to the observed halt of proliferation after the onset of adenine depletion (Fig. 2), we decided to measure trehalose accumulation in a series of W303 strains (W303 *ade2*, W303 *ADE2*, and W303 prototroph) during cultivation in a YPD medium. A fully prototrophic strain was added to the analysis to assess possible pleoitropic effects from other auxotrophies present in W303 *ade2* and W303 *ADE2* cells. Cells were sampled at exponential growth phase, shortly before and after exhaustion of adenine, after exhaustion of glucose, and during stationary phase. Both (W303 *ADE2* and W303 prototroph) strains had consumed all the glucose after 13 h of growth. At the same time, we observed accumulation of red pigment and cessation of W303 *ade2* growth. Glucose measurements revealed that there was still 8 g L^{-1} glucose left in the media. Trehalose content in exponential phase cells, of all three strains, was close to zero. After adenine exhaustion, W303 *ade2* started to accumulate trehalose and its content increased with time. After glucose exhaustion, prototrophic strains began to accumulate trehalose as well, but at a slower rate. Interestingly, trehalose content continues to increase with time in a fully prototrophic strain but not in W303 *ADE2* (Fig. 5a). Trehalose content of adenine-starved W303 *ade2* cells differed significantly ($P < 0.05$) from W303 prototroph and W303 *ADE2*.

Traditionally, the increase in trehalose content has been linked to elevated tolerance to stress (e.g. desiccation). We assessed desiccation stress tolerance in cells sampled during various stages of culture growth, both adenine sufficient and adenine starved (Fig. 5b). All cell cultures sampled during exponential phase showed low desiccation stress tolerance, but desiccation tolerance of W303 *ade2* cells increased sharply after adenine depletion. Desiccation tolerance over time roughly corresponded to the pattern of trehalose accumulation, and for adenine-starved W303 *ade2* cells, desiccation tolerance differed significantly ($P < 0.05$) from W303 prototroph and W303 *ADE2*.

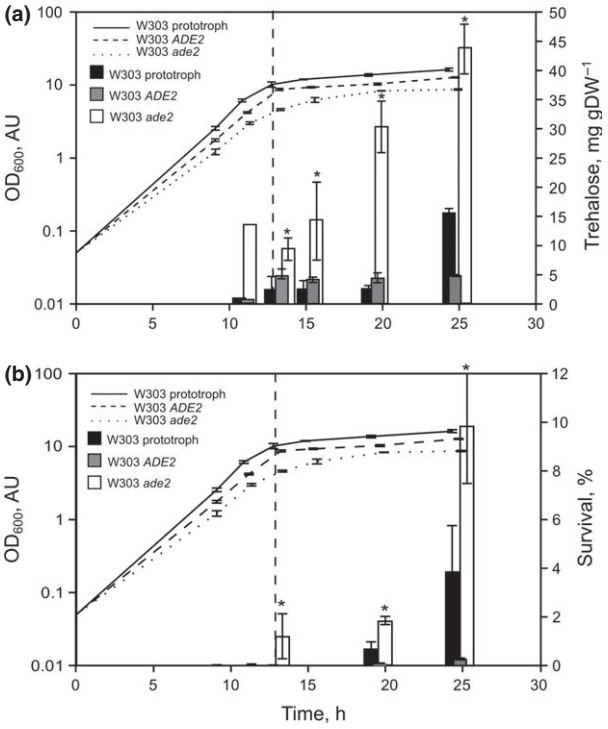

Fig. 5. Changes in accumulated trehalose (a) and desiccation tolerance (b) during W303 prototroph, W303 *ADE2* and W303 *ade2* cultivation in YPD media. Cells were desiccated in +30 °C for 10 h. Desiccation tolerance was quantified as in Calahan *et al.* (2011). In both (a) and (b), lines denote strain growth curves in logarithmic scale and bars trehalose (a) or survival (b). Error bars depict standard deviation from biological triplicates. Viability in first two time points (8 and 12 h) is close to zero (see b). Asterisks depict statistically significant difference ($P < 0.05$) between trehalose content of W303 *ade2* and both W303 prototroph and W303 *ADE2* strains (a). Also it depicts difference between W303 *ade2* and both W303 prototroph and W303 *ADE2* strains desiccation tolerance as statistically significant ($P < 0.05$) (b).

A distinction has previously been made between starvation for natural and artificial nutrients (Saldanha *et al.*, 2004). Cells starved for natural (C, P, N) nutrients survive for longer periods than cells under artificial starvations and do so by arresting the cell cycle. In contrast, auxotrophically starved cells have elevated glucose consumption rate and reduced survival and do not arrest their cell cycle (Brauer *et al.*, 2008). To place adenine starvation in the landscape of natural and artificial starvations, W303 *ade2* cells were incubated in synthetic media lacking either leucine, adenine or carbon source. While carbon-starved cells clearly showed high and stable survival rates when compared to both auxotrophies, survival of leucine and adenine differed significantly ($P < 0.05$) when measured at day 4, 7 and 10. Adenine starvation

led to higher culture viability than leucine starvation except for first 2 days where survival did not differ significantly between leucine and adenine starvation (Fig. 6a).

To find out whether survival of adenine- and leucine-starved cultures can be explained by differences in the cells' ability to arrest their cell cycle, we estimated the budding index of the starved cultures. The number of cells with small buds was counted, and the relative fraction for all cells calculated. Presence of a small bud indicates that the given cell is in the beginning of S phase and is not arrested (Smets *et al.*, 2010). Typically, a large percentage of cells are in a budded state while starving for leucine or uracil (Brauer *et al.*, 2008). Adenine-starved W303 *ade2* cells showed a smaller percentage of budded cells. Differences between leucine and adenine starvations were established in the first 2 days of starvation and remained relatively unchanged through the remainder of the experiment (Fig. 6b). When comparing the viability and budding index results, we noticed that higher mortality during starvation corresponds to a higher percentage of budded cells. That confirmed the possible role of cell cycle arrest in viability during auxotrophic starvation.

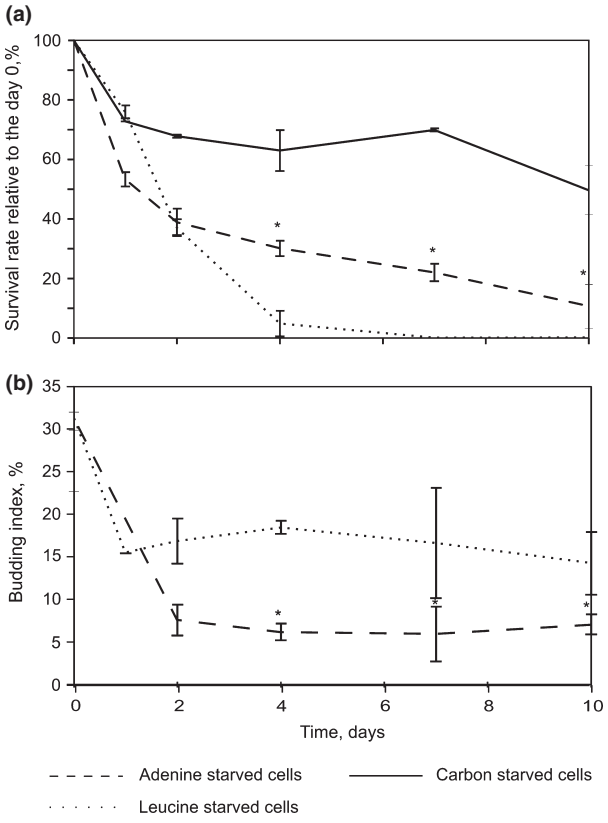

Fig. 6. Survival rate (a) and budding index (b) during prolonged W303 *ade2* auxotrophic (leucine or adenine) or carbon source starvation. Cells were grown in full SD media up to exponential phase, washed with distilled water and re-suspended to OD600 = 1 in SD media lacking sugar, leucine or adenine; all other broth components were added in surplus. Error bars represent standard deviation from three independent cultivations. Viability in (a) depicted as percentage of CFU from OD600 = 1 in the beginning of experiment. Budding index is calculated as ratio of cell number with small bud against total cell number. Asterisks depict statistically significant difference ($P < 0.05$) between adenine and leucine and carbon starvations.

Although exponentially growing, double-washed cells were used for SD adenine- and leucine-deficient media inocula, a two- to fourfold increase in OD of the culture was observed during the first days of cultivation (Fig. S2a). This might be explained either by cell multiplication due to accumulated resources within the cells or by cell size increase. We estimated cell number mL^{-1} during cultivation (Fig. S2b). Almost no increase in cell numbers was seen. When using the real cell density (cell number mL^{-1}) instead of OD_{600} measurements, viability curves showed no statistically significant ($P < 0.05$) difference in cell survival between adenine- and leucine-starved cultures, during the first 2 days of starvation (Fig. S2c).

Because differences in cell size and cell number per optical unit were noted during W303 *ade2* and W303 *ADE2* cultivation in YPD medium, we expected a similar effect during long-term adenine or leucine starvation. Mean cell size of the culture increased for both cultures, albeit to a different degree (Fig. 7). The increase in the mean cell size for leucine-starved cultures stopped after 2 days, whereas it continued to increase in adenine-starved culture. These differences are statistically significant for each day of cultivation ($P < 0.001$). When the distribution of the frequencies of cell size was plotted (Fig. S3), the distributions were shown to widen with each starvation day. While some of the starved cells slowly lost viability and retained their size, living cells became increasingly larger. It seems plausible that the increase in cell size is caused by metabolite (e.g. trehalose) accumulation.

On the basis of our results, we conclude that adenine auxotrophy is distinctively different from leucine auxotrophy. Although cell viability drops over time relative to carbon starvation, adenine starvation exhibits characteristics similar to those of other natural starvations: higher survival rates and ability to arrest cell cycle more efficiently when starved in comparison with artificial starvations such as leucine.

Discussion

Auxotrophy is a common property of haploid laboratory strains. Traditionally, amino acid and purine/pyrimidine auxotrophic markers are used in yeast strain genetic

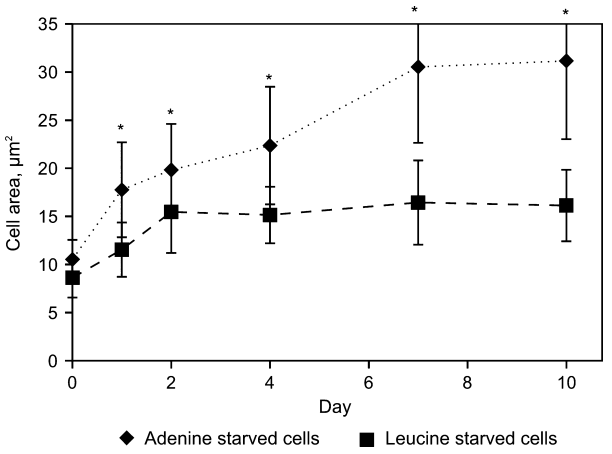

Fig. 7. W303 *ade2* culture mean cell size dynamics during prolonged adenine or leucine starvation. Cell cross-section areas were measured from micrographs as described by Jorgensen *et al.* (2007). Cell number exceeded 300 for each data point. Asterisks depict statistically significant difference ($P < 0.0001$) between cell sizes of adenine and leucine starvations, as determined by Wilcoxon rank-sum test.

engineering. Leucine, tryptophan, adenine, uracil, methionine, and histidine auxotrophies are typical markers and targets for complementation with plasmids or integration constructs (Pronk, 2002; Da Silva & Srikrishnan, 2011). However, questions have been raised regarding how this common strain property affects the general physiology of cells (Mülleder *et al.*, 2012; Liu *et al.*, 2013). Problems with auxotrophic strains might arise for two reasons: depletion of the auxotrophic agent in the growth medium may occur with great regularity if the concentration of the agent is insufficient and there may exist pleiotropic interactions between multiple auxotrophies in the same strain. Several protocols define different amounts of auxotrophic agents needed in synthetic media, and yeast cell physiologists have raised concerns that the amounts usually used might not be sufficient for uninterrupted cell growth (Pronk, 2002). However, rich media have been considered safe in respect to auxotrophies, and additional amino acids or nucleotides are seldom added.

Our results show that additional adenine (at least 40 mg L^{-1} or more), even to the rich media, should be added to avoid adenine starvation. Concerns are raised regarding growth attenuation effects of auxotrophic supplements, if added in excess (Mülleder *et al.*, 2012). Our results on synthetic media (Fig. 4) show no statistically significant differences in growth if 40 or 100 mg L^{-1} adenine is added. Furthermore, no difference between growth parameters of W303 prototroph and W303 *ade2* on YPD with extra 100 mg L^{-1} adenine supplement is seen ($\mu = 0.45\ h^{-1}$ for both strains). Similarly, VanDusen *et al.* (1997) and Zhang *et al.* (2003) reported on sufficient adenine concentration to be 50–80 mg L^{-1}.

Auxotrophic markers can have pleiotropic effects on *S. cerevisiae* physiology. There are many examples of the effects of tryptophan, methionine and histidine autotrophy on yeast physiology. For example, any gene deletion in the tryptophan biosynthesis pathway (*trp1-5* genes) leads to decreased growth in the presence of rapamycin, caffeine and SDS. Notably, wild-type characteristics are not regained after the respective gene complementation (González *et al.*, 2008). Also, depending on the length of the *HIS3* gene deletion (200 bp or 1 kbp) used to generate the histidine auxotrophy, various levels of respiration deficiency at 37 °C can be observed (Young & Court, 2008). Criticism has also been raised regarding the use of methionine auxotrophs because the need for methionine supplement masks the effects of other gene deletions as seen in *zwf1* strains (Thomas *et al.*, 1991; Pronk, 2002).

In contrast to the above auxotrophies exhibiting pleiotropic impact on physiology, our results on adenine auxotrophy show purely adenine-dependent effects. When ample amount of adenine is available in the media, adenine auxotrophs and prototrophs are physiologically indistinguishable: autotroph and prototroph cell size, OD and dry weight ratio, biomass yield, glucose consumption, and desiccation tolerance are similar. On the other hand, when adenine concentration in medium is limiting, cell morphology and physiology change significantly. Additionally, adenine-dependent effect is not strain or specifically *ADE2* gene dependent. We repeated desiccation tolerance experiments with strains of CEN.PK series: full CEN.PK prototroph, CEN.PK *ADE8* (CEN.PK2 *MATa leu2-3/112 ura3-52 trp1-289 his3-1*, *MAL2-8c SUC2*) and CEN.PK *ade8* deletion (made on CEN.PK *ADE8* background). Growth rate, trehalose accumulation and desiccation tolerance were measured. Results follow the same pattern as in W303 – the adenine auxotroph accumulates trehalose and shows significantly ($P < 0.05$) elevated desiccation tolerance when adenine becomes depleted (results are shown in Fig. S4). From this, we conclude that the observed phenomenon is not strain or *ade2* gene specific; instead, it relates to adenine auxotrophy generally.

When comparing fully prototrophic strains with their respective *his*, *leu*, *trp*, *ura* auxotrophs, a decrease in trehalose accumulation and desiccation tolerance can be seen; however, differences are not statistically significant ($P > 0.05$). Some or even all of these common auxotrophies could decrease desiccation resistance in prototrophs. To fully understand the phenotypical interplay between all auxotrophic markers, each auxotrophy alone and its combination with the others should be tested in otherwise prototrophic strains. Some interplay between adenine, histidine and tryptophan auxotrophies could take

place as they share some common elements in their biosynthesis pathway. Moreover, multiple stress-related phenotypic traits of tryptophan auxotrophs have been described before (González *et al.*, 2008). We did segregant analyses of crosses between $ade^{+}trp^{-}$ and $ade^{-}trp^{+}$ strains and found that increased desiccation stress tolerance and trehalose accumulation after adenine depletion occurs in ade^{-} segregants independently of *trp* gene functionality (data not shown). However, adenine auxotrophy is usually accompanied by other four auxotrophic markers in W303 series strains or through *ade8* disruption by an integration vector. Statistically significant differences among adenine auxotrophs and prototrophs that we observed in two strain backgrounds, W303 and CEN.PK, indicate that the observed effects are adenine auxotrophy specific and thus should be carefully considered when doing physiological studies with those strains.

We explored this phenomenon further to determine the extent of biases in yeast physiology that can be produced due to insufficient adenine in the medium. Measurements of OD, a common indicator of cell growth used in microbiology (Madrid & Felice, 2005), can be misleading in adenine auxotrophs due to the swelling of cells – OD_{600} increases even after cell proliferation has stopped. One of the reasons why *ade* markers are widely used in genetic research is because the accumulation of red pigment is a convenient visual marker used when distinguishing segregants. The same pigment is autofluorescent and hinders cell visualization (Weisman *et al.*, 1987), so usually care is taken to use adenine-enriched media when growing cells for visualization studies. On the other hand, stress physiology research is quite often performed on adenine auxotrophs after exponential growth phase in rich media, without any additional supplementation (Carrasco *et al.*, 2001; Petrezselyova *et al.*, 2010). In our opinion, increased stress tolerance of stationary-phase adenine auxotrophs in rich media is due to adenine depletion and not because of other strain characteristics.

Desiccation is a multifactorial stress that challenges cells with hyperosmolarity, hyperoxidation, hyperionicity and protein misfolding/aggregation during dehydration and rehydration (Chakrabortee *et al.*, 2007; França *et al.*, 2007). Trehalose is a widely discussed storage carbohydrate, which accompanies various stress conditions (Crowe *et al.*, 1998). Still, whether trehalose is an important desiccation stress protector or just a metabolite that accumulates during slow growth remains unclear (Paalman *et al.*, 2003; Ratnakumar & Tunnacliffe, 2006). Although trehalose accumulation in W303 *ade2* coincides with the increase in desiccation tolerance, adenine prototroph strain does not show the same relationship between trehalose accumulation and desiccation tolerance. This indicates that trehalose accumulation could serve as a signal for elevated desiccation tolerance, but it might not be a prerequisite for it. Previously, rate of trehalose accumulation has been attributed to the growth rate (Paalman *et al.*, 2003). Adenine auxotroph cells indeed accumulate far greater amounts of trehalose after they cease to proliferate, and dynamic trehalose levels that change over time in arrested cells indicate that there may be additional regulatory mechanisms.

Trehalose levels in cells are determined as an outcome of dynamic equilibrium of trehalose synthesis and hydrolysis (Hohman & Mager, 2003). The activity of trehalase and trehalose synthase is regulated by the cAMP-PKA pathway, which is upregulated in the presence of glucose (Winderickx *et al.*, 1996). A great increase in trehalose levels in adenine-starved cells could indicate downregulation of cAMP-PKA pathway and consistent downregulation of trehalase activity and upregulation of trehalose synthase complex activity. Research shows that yeast cells, when starved for carbon, nitrogen or phosphorous, upregulate both sides of the trehalose metabolism – synthesis and hydrolysis. However, trehalose accumulates only in nitrogen-starved cells (Klosinska *et al.*, 2011). We have measured trehalase activity in *ade8* deletion strain in CEN.PK background, and it shows the same tendency – trehalose accumulation is accompanied by elevated trehalase activity during adenine starvation (data not shown). Additional research is needed to clarify dynamics of trehalose accumulation during adenine starvation to determine whether they are similar to those observed in nitrogen starvation.

The recent work of Welch *et al.* (2013) elucidates the role of TOR and RAS pathway in acquiring desiccation stress resistance. These two pathways regulate cell growth rate by monitoring external carbon and nitrogen supplies (Smets *et al.*, 2010). Here, we show that adenine depletion does stop cell division and increases cell viability after desiccation, indicating a possible role for these two signalling pathways in physiological changes observed during adenine starvation. The increase in desiccation tolerance in response to carbon, nitrogen and phosphorus starvation has already been reported (Welch *et al.*, 2013).

Although adenine and uracil are nucleotides, different physiological responses to starvation can be observed for them. It is possible that either yeast cells sense external adenine levels or, as adenine becomes limited, the cells sense limited or imbalanced adenylate levels intracellularly. Yet there are no reported transcriptional response mechanisms to disturbed nucleotide balance (Ljungdahl & Daignan-Fornier, 2012). Little is known about adenylate levels in cells undergoing adenine starvation, so further studies are required to elucidate possible cell response mechanisms. Existence of specific transcriptional response leading to a quiescent state for yeast is under debate (Klosinska *et al.*, 2011).

To summarize our findings, the physiological state of W303-1A changes dramatically after adenine is exhausted in YPD media. Cell proliferation ceases while cells increase in size and accumulate trehalose. Prominent increase in desiccation stress tolerance follows. Trehalose accumulation and elevated desiccation tolerance imply that adenine auxotrophs change their carbon flow and internal signalling after adenine depletion. In prolonged starvation experiments, adenine-deficient cells show increased ability to arrest cell cycle and are viable for longer period of time compared with cells starved for leucine. These facts distinguish adenine starvation from other auxotrophies described thus far. To avoid unwanted phenotypic changes due to adenine depletion in rich media, we suggest adding extra adenine for *ade*$^-$ if cell physiology will be studied after exponential growth phase, to avoid adenine exhaustion before glucose depletion.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. W303 adenine auxotroph and prototroph dry weight dependence on optical density measurements when cultivated in YPD medium.

Fig. S2. Optical density (a), cell count mL^{-1} (b) and relative survival rate (c) during W303 *ade2* adenine (diamonds) and leucine (squares) starvation.

Fig. S3. Cell size distribution during adenine (upper panel) and leucine starvation (lower panel).

Fig. S4. Changes in accumulated trehalose (a) and desiccation tolerance (b) during CEN.PK prototroph, CEN.PK *ADE2* and CEN.PK *ade2* cultivation in YPD media.