Replenishment and mobilization of intracellular nitrogen pools decouples wine yeast nitrogen uptake from growth

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Abstract

Wine yeast capacity to take up nitrogen from the environment and catabolize it to support ~~proliferation~~population growth, fermentation, and aroma production is critical to wine production. Under nitrogen restriction, yeast nitrogen uptake is believed to be intimately coupled to reproduction with nitrogen catabolite repression (NCR) suggested mediating this link. We provide a time- and strain-resolved view of nitrogen uptake, population growth, and NCR activity in wine yeasts. Nitrogen uptake was found to be decoupled from growth due to early assimilated nitrogen being used to replenish intracellular nitrogen pools rather than being channeled directly into reproduction. Internally accumulated nitrogen was later mobilized to support substantial population expansion after external nitrogen was depleted. On good nitrogen sources, the decoupling between nitrogen uptake and growth correlated well with relaxation of NCR repression, raising the potential that the latter may be triggered by intracellular build-up of nitrogen. No link between NCR activity and nitrogen assimilation or growth on poor nitrogen sources was found. The decoupling between nitrogen uptake and growth and its influence on NCR activity is of relevance for both wine production and our general understanding of nitrogen use.

Keywords

Wine yeast
Growth
Nitrogen metabolism
GAP1
Nitrogen catabolite repression

Electronic supplementary material

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Introduction

The capacity to take up nitrogen in its various forms from the environment and channel it into biomass and reproduction is a key determinant of fitness. The yeast *Saccharomyces cerevisiae*, a model

system for understanding nitrogen metabolism, assimilates a variety of nitrogen sources, including most amino acids, nucleotides and their derivatives, urea and allantoin, a plethora of di- and tri-peptides, and inorganic ammonium (Cooper 1982). Nitrogen source quality in terms of the capacity to support yeast population growth has traditionally been evaluated considering the yeast lab strains S288c or Σ1278b (Rytka 1975).

The molecular basis for why utilization of a particular nitrogen source results in a particular speed and efficiency of yeast population expansion is poorly understood. Times and costs of nitrogen conversion into next-generation biomass not only depend on reaction rates and costs of the individual reactions required for uptake, catabolism, and anabolism of a nitrogen source ~~(Rytka 1975).~~. It also depends on poorly understood, indirect effects of nitrogen sources and their reaction products on cell physiology and reproduction. For example, catabolism of aromatic and branched amino acids via the Ehrlich pathway produces keto-acids, aldehydes, and fusel alcohols. Many of these impede cell division (Hazelwood et al. 2008) or activate quorum sensing that coordinates cell behavior within populations (Chen and Fink 2006). Such effects also depend heavily on environmental factors that vary over time and space. Notably, ammonium is toxic in large concentrations but the level of toxicity varies over orders of magnitude depending on external potassium concentrations (Hess et al. 2006). A common perspective is that yeast tends to prioritize use of high over low-quality nitrogen sources (Jiranek et al. 1990). Indeed, many processes required for uptake of, and growth on, lower quality nitrogen sources are suppressed in the presence of higher quality nitrogen (Godard et al. 2007). Although question marks remain, nitrogen catabolite repression (NCR) is believed to be a key mechanism responsible for this quality-based prioritization of nitrogen use (Magasanik and Kaiser 2002). The mechanism is centered on the four GATA family zinc-finger transcription factors, Gln3, Gat1, Dal80, and Gzf3 and their regulator Ure2. In the presence of high concentrations of high-quality nitrogen, general amino acid transporters capable of transporting poor nitrogen sources, notably Gap1 and Agp1, are both transcriptionally repressed and post-translationally modified as a signal to degradation, by NCR (Hofman-Bang 1999; ter Schure et al. 2000).

In lab strains, NCR also suppresses transcription genes encoding of some amino acid specific permeases, e.g., the allantoin permease Dal4 and the proline permease Put4. When a preferred nitrogen source decreases in concentration, NCR is relaxed and its target nitrogen catabolic mechanisms are induced, facilitating a switch to use of non-preferred nitrogen (Beltran et al. 2004; Magasanik and Kaiser 2002).

Although nitrogen metabolism has been most thoroughly studied in lab yeasts growing on artificial medium, the capacity of wine yeasts to utilize nitrogen compounds present in grapes is of particular interest. Grape must is rich in carbon but poor in nitrogen. Consequently grape must nitrogen content tends to limit wine yeast growth, leaving sugar in the wine and reducing its ethanol content (Varela et al. 2004). Both the capacity of wine yeasts to convert different nitrogen sources into biomass (Gutierrez et al. 2013a) and the nitrogen content of grape musts (Bell and Henschke 2005) vary, with mismatches exacerbating the problem of stuck and sluggish fermentations (Bisson 1999). The resulting incomplete fermentations are not only a quality problem per se. The combination of glucose and nitrogen remaining in the grape must and ethanol being low allows microbes to invade the fermentations. These microorganisms convert the nitrogen remaining into foul-tasting secondary metabolites (Jiranek et al. 1995), spoiling the wine and causing substantial economic loss. The manner in which wine yeast uses nitrogen sources present in grape must also directly influences wine taste and aroma. Nitrogen metabolism is biochemically linked to the production of the major carbon metabolites glycerol, ethanol, and acetic acid (Beltran et al. 2005). These strongly influence wine flavor properties. Notably, glycerol content is often increased and ethanol content reduced, when inorganic rather than organic nitrogen is used (Albers et al. 1996). Second, organic nitrogen compounds are metabolic starting points for yeast production of more complex flavors that are positively associated with taste and aroma (Bell and Henschke 2005). Thus, from several perspectives, understanding and optimizing wine yeast utilization of the nitrogen sources present in the must is important to maximize wine quality, quantity, and production economics.

The assumption of a strong association between the quality and quantity of nitrogen taken up from the environment, population growth, and NCR activity is attractive. However, whether this conjecture provides a sufficiently resolved and generalizable view of wine yeast behavior during wine fermentation is unclear. Assimilated nitrogen may not immediately be channeled into next-generation offspring, ~~and~~ NCR does not repress uptake and catabolism of all poor nitrogen sources, and mechanisms other than NCR- regulate~~d~~ nitrogen uptake and catabolism (Forsberg and Ljungdahl 2001; Ljungdahl 2009). Lab strains are also man-made chimeras that are poor representatives of the species (Warringer et al. 2011). It is therefore unclear to what extent findings can be extrapolated to wine yeasts. Here, we provide an exhaustive, time-resolved view of the quality and uptake of nitrogen sources present in grape must in wine yeasts. Simultaneously, we measure NCR activity in the form of transcriptional activation of the classical NCR target, the *GAP1* promoter.

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Materials and methods

Strains and medium

Four diploid commercial wine yeasts, the strains PDM, ARM, RVA, and TTA, were provided by Agrovin Company (Ciudad Real, Spain). A taxonomic description of these strains was carried out by the RFLPs of the ITS/5.8S region, as previously described (Guillamon et al. 1998). Strains are available for academic users at Agrovin (http://www.agrovin.com/) under these names. PDM, RVA, and TTA are pure *S. cerevisiae*, while ARM, also available under its alias EP2 (Maurivin, Australia), is a hybrid between *S. cerevisiae* and its relative *Saccharomyces kudriavzevii* (Dunn et al. 2012). All experiments were performed in synthetic grape must, prepared as previously described (Riou et al. 1997), but with 200 g/L of sugar (100 g/L glucose + 100 g/L fructose) and without anaerobic factors (Beltran et al. 2004). All media components were identical in all experiments, except for choice of nitrogen source. Each medium contained 140 mg N/L of a single nitrogen source. All low complexity nitrogen sources commonly detected in grape musts, as well as the key derivatives of such nitrogen

sources, were tested. This included adenine, ammonium, cytosine, GABA, allantoin, L-alanine, L-arginine, L-asparagine, L-aspartate, L-citrulline, L-glutamate, L-glutamine, L-isoleucine, L-leucine, L-methionine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-valine, and L-urea. A nitrogen complete synthetic wine must (SWM) with a mixture of ammonium and amino acids (40 % of ammonium + 60 % of amino acids), mimicking the nitrogen content of a typical grape must, was used as point of reference.

Inoculum and growth conditions

Strains extracted from glycerol stocks were grown at 30 °C on YPD plates (2 % glucose, 1 % yeast extract, 1 % peptone, and 2 % agar) and inoculated and pre-cultivated over 132 h at 30 °C in 10 mL of YNB medium (1.7 % YNB *w*/*w* nitrogen and 2 % glucose) with 0.5 % of ammonium chloride as sole nitrogen source. The extensive pre-culturing was necessary to deplete cells of nitrogen storages, to completely silence the *GAP1* and *DAL4* promoters (see below), and to completely degrade all GFP proteins expressed from these promoters during pre-cultivation growth. Pre-cultures were inoculated in SWM at an initial population size of 0.2 OD_{600nm} units. All fermentations were performed in triplicate in 50-mL Falcon tubes containing 40 mL SWM and took place at 28 °C with continuous orbital shaking (150 rpm). Thus, semi-anaerobic conditions were used, since Falcon tubes were capped with closures that enabled the carbon dioxide to escape and with limited aeration occurring during sampling. Cell growth was monitored by optical density (OD) changes at 600 nm, with an average measurement error (coefficient of variation) of 4 %. Following sampling at different time points and centrifugation, the supernatant was collected for nitrogen content measurements and stored at −20 °C. Dry yeast experiments were performed in biological triplicates. Dry yeast was first rehydrated for 30 min in sterile water and then used to inoculate experimental cultures, with no preceding pre-culture.

Nitrogen assimilation analysis

The concentration of free amino acid nitrogen remaining in the medium was determined by the σ-phtaldehyde/*N*-acetyl-L-cysteine spectrophotometric assay (NOPA) procedure (Dukes and Butzke 1998).

Results were expressed as mg nitrogen (N)/L. Proline cannot be determined by the NOPA procedure and the proline content was therefore measured by HPLC. This was performed as for the determination of intracellular nitrogen (below). To determine intracellular nitrogen, free intracellular amino acids and ammonium were extracted by boiling cell pellets, corresponding to 10^7 cells, in 500 μL of sterile MilliQ water for 15 min. Free amino acids and ammonia in the extract were analyzed by diethyl ethoxymethylenemalonate (DEEMM) derivatization using the Agilent 1100 Series high-performance liquid chromatography (HPLC) (Agilent Technologies, Santa Clara, USA) (Gomez-Alonso et al. 2007). Separation was performed in an ACE HPLC column (C18-HL) with a particle size of 5 μm (250 × 4.6 mm) and thermostated at 20 °C. The concentration of each compound was calculated with the help of internal and external standards and the Agilent ChemStation software (Agilent Technologies). The average measurement error (coefficient of variation) over triplicates corresponded to 6.2 %.

NCR repression of *GAP1* and *DAL4* promoters by flow cytometry

In diploid strains, one of the *GAP1* or *DAL4* loci was altered such that native ORFs were replaced by that of green fluorescent protein (GFP), creating heterozygotic *GAP1*/*GAP1prGFP* and *DAL4*/*DAL4prGFP* strains (Gutierrez et al. 2013b). Heterozygotic constructions grew, fermented, and consumed nitrogen identically to the WT in nitrogen complete synthetic grape must (data not shown). Cells were harvested at different time points, rinsed with PBS, and analyzed in a Becton Dickinson FACSAria flow cytometer (BD Bioscience, San Diego, CA). The individual fluorescence of a total of 10,000 events (cells) was measured using the GFP-A filter and standard settings. Filtering out anomalous events, the median fluorescence of the remaining counted cells was taken as the GFP expression at an individual time point.

Results

Nitrogen source capacity to support growth is wine strain dependent

To provide an exhaustive view of the capacity of four widely used commercial wine yeast strains, PDM, ARM, RVA, and TTA, to utilize nitrogen compounds present in grape must, we cultivated strains individually in 40 mL of synthetic grape must (SWM), and followed population density change during semi-anaerobic growth (see "Materials and methods"). Growth was supported using 140 mg N/L, which is typical and adequate for grape must fermentations with the (200 g/L) sugar content used here (Gutierrez et al. 2013a; Martinez-Moreno et al. 2012). All nitrogen sources normally present in grape must and several of their most important catabolic derivatives were thus evaluated as sole nitrogen sources. To ensure that the growth observed reflected utilization of the nitrogen source supplied, internal nitrogen storages were first exhausted by cultivating pre-cultures well into stationary phase (>6 days). This resulted in a relatively long ensuing lag phase (<24 h). RVA had generally faster and TTA generally slower growth (Fig. 1a, Student's *t* test, one vs. all, $p < 0.005$ for at least one time point), as earlier reported (Gutierrez et al. 2013a). With some exceptions, the traditional, lab strain-based (Godard et al. 2007) classification of nitrogen compounds into "good" and "poor" agreed reasonably well with the growth achieved by the four wine yeasts in grape must (Fig. 1b, c). Wine strains were generally better than expected from the Godard et al. (2007) lab strain findings at converting urea, allantoin, GABA, and valine, classically considered intermediate nitrogen sources, into growth. They also showed better growth than expected from the Godard et al. lab strain data on leucine and isoleucine, which traditionally are classified as very poor nitrogen sources. In contrast, they performed worse than expected from lab strain findings on glutamate, aspartate, citrulline, and proline, the latter of which may be due to limited access to oxygen, which is required for proline growth. However, as epigenetic and environmental influences cannot be completely excluded, strict conclusions on a purely genetic origin of the differences to Godard et al. (2007) cannot be drawn. Complicating generalization, variations between wine strains were substantial with the largest variations on poor nitrogen sources, in line with what has been reported (Gutierrez et al. 2013a; Ibstedt et al. 2015). Compared to the other wine strains, RVA grew better on adenine, isoleucine, threonine, and proline, but not on other nitrogen

sources, whereas TTA performed better on cytosine and citrulline (Fig. 1c, Student's *t* test, one vs. all, $p < 0.0002$ for at least one time point). Large variations were also observed for the poor nitrogen sources methionine and tryptophan. The slow PDM growth on methionine was recently mapped to variation in *ARO8*; *ADE5*,*7*; and *VBA3* (Gutierrez et al. 2013a). Except for the prolonged recovery from pre-culture stationary phase, the reported data agreed well with what was recently reported using miniaturized cultivations in 1 % of the current volume (Gutierrez et al. 2013a). Relative growth on different nitrogen sources therefore appears reasonably independent of cultivation scale.

Fig. 1

Nitrogen source capacity to support growth is wine strain dependent. Four commercial wine yeasts were independently cultivated in 23 different nitrogen sources and synthetic wine must ($n = 3$). Changes in population cell density over time were followed by measurements of optical density. **a** Mean growth achieved for each wine yeast strain up to each time point. An average over all nitrogen sources is shown (*error bars* = SEM over all nitrogen sources). **b** Nitrogen sources were ranked based on the mean growth achieved of the four wine yeasts over all time points. Text color reflects the lab strain based classification into "good" (*green*), "intermediate" (*purple*), and "poor" (*red*) nitrogen sources (Godard et al. 2007). Allantoin and urea were not classified in this reference but are traditionally considered intermediate, non-preferred nitrogen sources. **c** Cell density change, from time $t = 0$ (inoculation) of each wine yeast strain in each nitrogen source by each time point. Color intensity depicts ~~growth~~degree of cell density change, *black* = no cell density change, *intense green* = high cell density change

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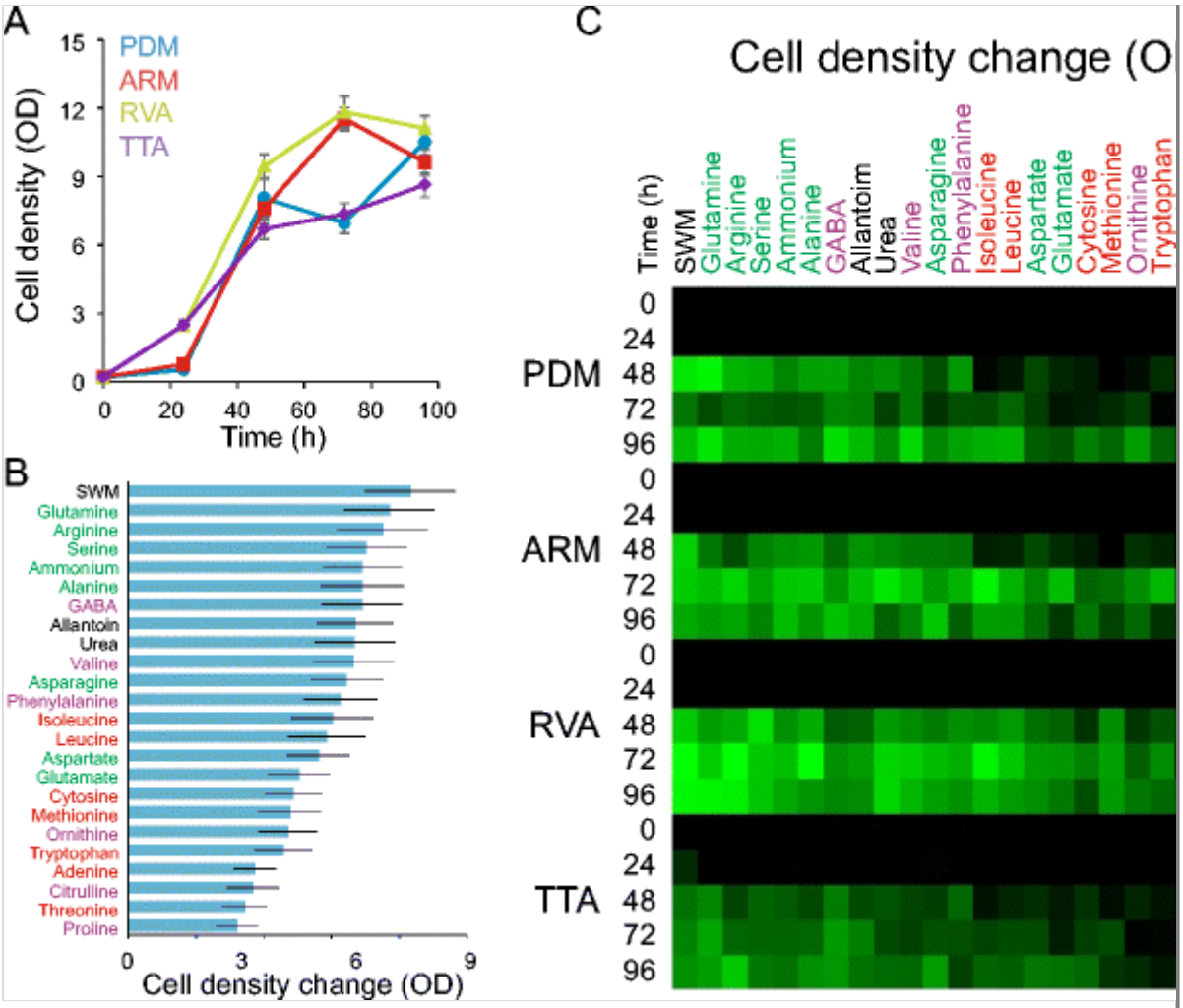

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Wine yeast nitrogen uptake much precedes growth

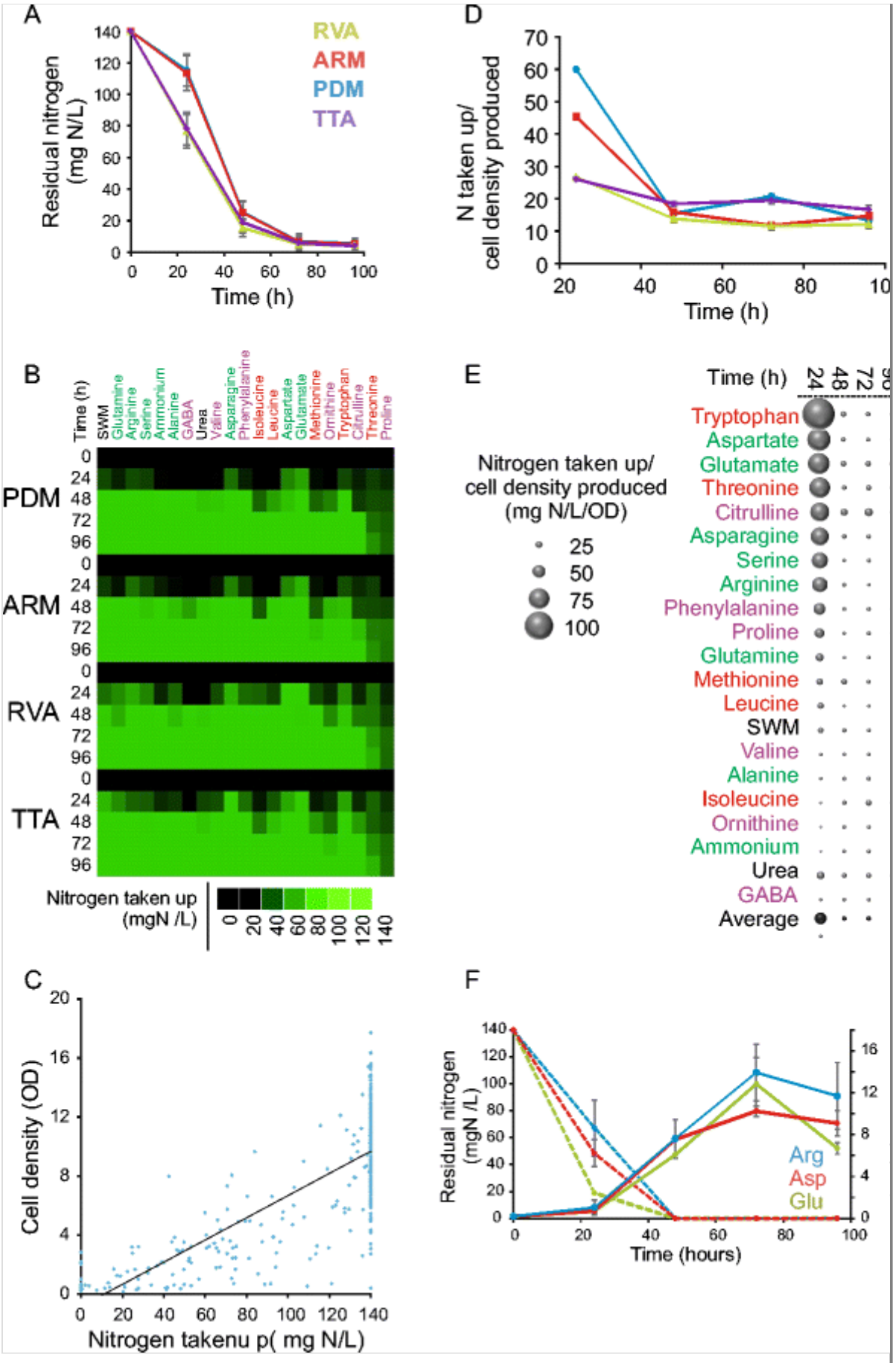
To investigate to what extent variations in the growth supported by different nitrogen sources depended on variations in nitrogen source assimilation, we followed nitrogen source uptake from the medium over time, in each strain-amino acid combination. In general, strains RVA and TTA took up nitrogen faster than ARM and PDM (Fig. 2a, Student's *t* test, $p < 0.0025$ at 24 h), but the dynamics of nitrogen uptake were affected by strain-by-nitrogen source interactions (Fig. 2b). Overall, nitrogen assimilation and the capacity of a nitrogen source to support growth was clearly correlated (Fig. 2c; Spearman rank, $r^2 = 0.56$), but variation around the linear trend-line was substantial. This variation was accompanied by a temporal mismatch, as nitrogen uptake tended to precede growth. This tendency became abundantly clear when considering the nitrogen taken up per cell density unit

produced (Fig. 2d). For all strains, but particularly so for ARM and PDM, substantially more nitrogen was taken up per cell density unit produced early in growth. The trend was evident for most, but not for all, nitrogen sources (Fig. 2e), with the quality of the nitrogen source being of little relevance. The good nitrogen sources aspartate, asparagine, and glutamate all saw massive early assimilation of nitrogen that failed to manifest in growth. However, so did the poor nitrogen sources tryptophan, threonine, and citrulline. Correspondingly, the good nitrogen sources ammonium and alanine were taken up early on and immediately channeled into new cells, with no apparent time delay. ~~However~~ , but so were the poor nitrogen sources ornithine and isoleucine. In nitrogen complete synthetic wine must, where nitrogen is available in many forms, nitrogen was both rapidly taken up and rapidly transformed into growth with little delay (Fig. 2e). To better illustrate the temporal mismatch between nitrogen assimilation and growth, we detailed aspartate, glutamate, and arginine assimilation and growth in strain ARM. At 48 h, virtually all of the glutamate, aspartate, and arginine had been taken up but only marginal population growth had occurred (Fig. 2f). All nitrogen sources ultimately did support strong growth. However, the bulk of this growth was delayed until after complete assimilation of external nitrogen.

Fig. 2

Replenishment and mobilization of intracellular nitrogen pools decouples nitrogen uptake from wine yeast growth. Wine yeast uptake of 20 nitrogen sources was followed by measuring residual amino groups in the medium ($n = 3$). Synthetic wine must was included as reference. **a** Mean residual nitrogen remaining at each time point for each wine yeast strain. The mean was calculated over all nitrogen sources. *Error bars* = SEM over all nitrogen sources. **b** Residual nitrogen for each wine yeast strain in each nitrogen source at each individual time point. Color intensity depicts residual nitrogen, *black* = initial nitrogen concentration (140 mg N/L), *intense green* = no residual nitrogen. **c** Cell density achieved as a function of nitrogen taken up from the medium. All amino acids, time points (except $t = 0$) and strains were considered. Linear regression is displayed. **d** Mean nitrogen taken up per unit of cell density produced for each strain. The mean was calculated over all amino acids. **e** Mean nitrogen taken up per cell density produced for each amino acid.

The mean was calculated over all strains. **f** Time-resolved growth (*right axis*, *full lines*) and nitrogen assimilation (*left axis*, *broken lines*) profiles for ARM growing in glutamate, aspartate, and arginine. *Error bars* = SEM


A
Residual nitrogen (mg N/L)
Time (h)
RVA
ARM
PDM
TTA
D
N taken up/ cell density produced
Time (h)
B
Time (h)
SWM
Glutamine
Arginine
Serine
Ammonium
Alanine
GABA
Urea
Valine
Asparagine
Phenylalanine
Isoleucine
Leucine
Aspartate
Glutamate
Methionine
Ornithine
Tryptophan
Citrulline
Threonine
Proline
PDM
ARM
RVA
TTA
Nitrogen taken up (mgN /L)
E
Nitrogen taken up/ cell density produced (mg N/L/OD)
25
50
75
100
Time (h)
Tryptophan
Aspartate
Glutamate
Threonine
Citrulline
Asparagine
Serine
Arginine
Phenylalanine
Proline
Glutamine
Methionine
Leucine
SWM
Valine
Alanine
Isoleucine
Ornithine
Ammonium
Urea
GABA
Average
C
Cell density (OD)
Nitrogen takenu p(mg N/L)
F
Residual nitrogen (mgN /L)
Arg
Asp
Glu
Time (hours)

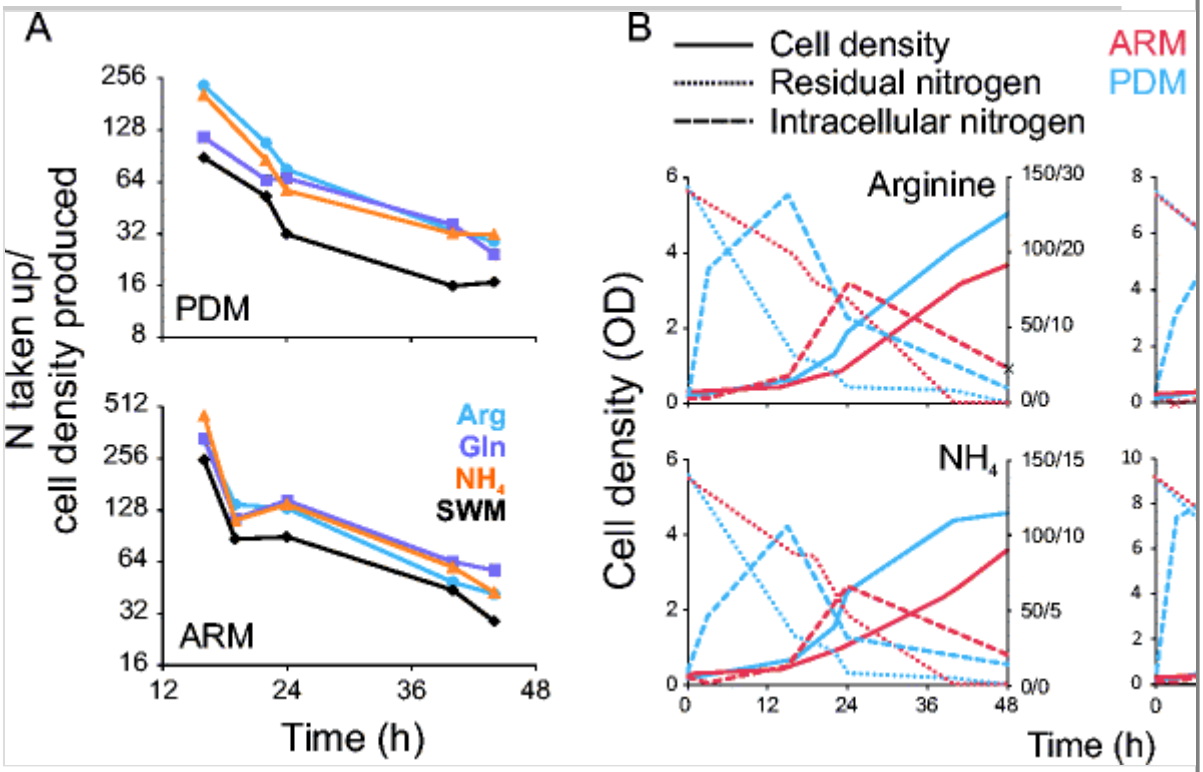
Replenishment and later mobilization of intracellular nitrogen pools decouples nitrogen uptake from population growth

To provide an independent verification of that nitrogen assimilation often precedes growth and ensures that it holds true also for more wine production-like conditions, we repeated a subset of experiments using rehydrated dry yeast inoculated directly into the experimental synthetic wine must. Single good nitrogen sources, arginine (Arg), glutamine (Gln), and ammonium (NH_4) were used, with arginine representing one of the most common nitrogen sources in grape must. Surveying PDM and ARM nitrogen uptake and growth at higher time resolution, we confirmed that nitrogen assimilation and growth indeed were mismatched such that assimilation preceded growth (Fig. 3a). Early assimilation of nitrogen without comparable growth can be explained in two ways: either by loss of the nitrogen back into the medium in a form that evades detection by the assay (that only detects free amino acids and ammonia) or by accumulation inside cells. Accumulation could either occur in dedicated storages, such as in the vacuole, or as metabolic pools in catabolism or anabolism. To distinguish between loss back to the medium and intracellular accumulation, we tracked the total free intracellular nitrogen accumulated during arginine, glutamine, ammonium, and synthetic wine must growth in PDM and ARM. Confirming the accumulation model, free intracellular nitrogen concentrations increased rapidly from a severely nitrogen-depleted initial state once the external nitrogen was assimilated. Intracellular free nitrogen concentrations reached a maximum at around 12 h (PDM) or 24 h (ARM) (Fig. 3b), well before the bulk of the growth occurred. This provides direct evidence that early assimilated nitrogen is accumulated intracellularly. Moreover, as the external nitrogen approached depletion, the intracellularly accumulated nitrogen was mobilized to support substantial growth in the absence of external nitrogen. Growth on the intracellular nitrogen rapidly depleted the intracellular pools, leaving them essentially empty at 48 h.

Fig. 3

Replenishment and mobilization of intracellular nitrogen pools decouples nitrogen uptake from growth also in rehydrated wine yeasts. Nitrogen assimilation, growth, and free intracellular nitrogen were followed in

parallel in rehydrated dry yeast (PDM and ARM) inoculated directly into synthetic wine must, with different nitrogen sources ($n = 3$). **a** Nitrogen taken up per cell density unit produced in PDM and ARM as a function of time. **b** Cell density (*left y-axis*, *full line*), residual nitrogen in the medium (*right y-axis*, *numbers before slash*, *dotted line*) and intracellular nitrogen per biomass unit (*right y-axis*, *numbers after slash*, *dashed line*) in PDM (*blue*) and ARM (*red*) as a function of time



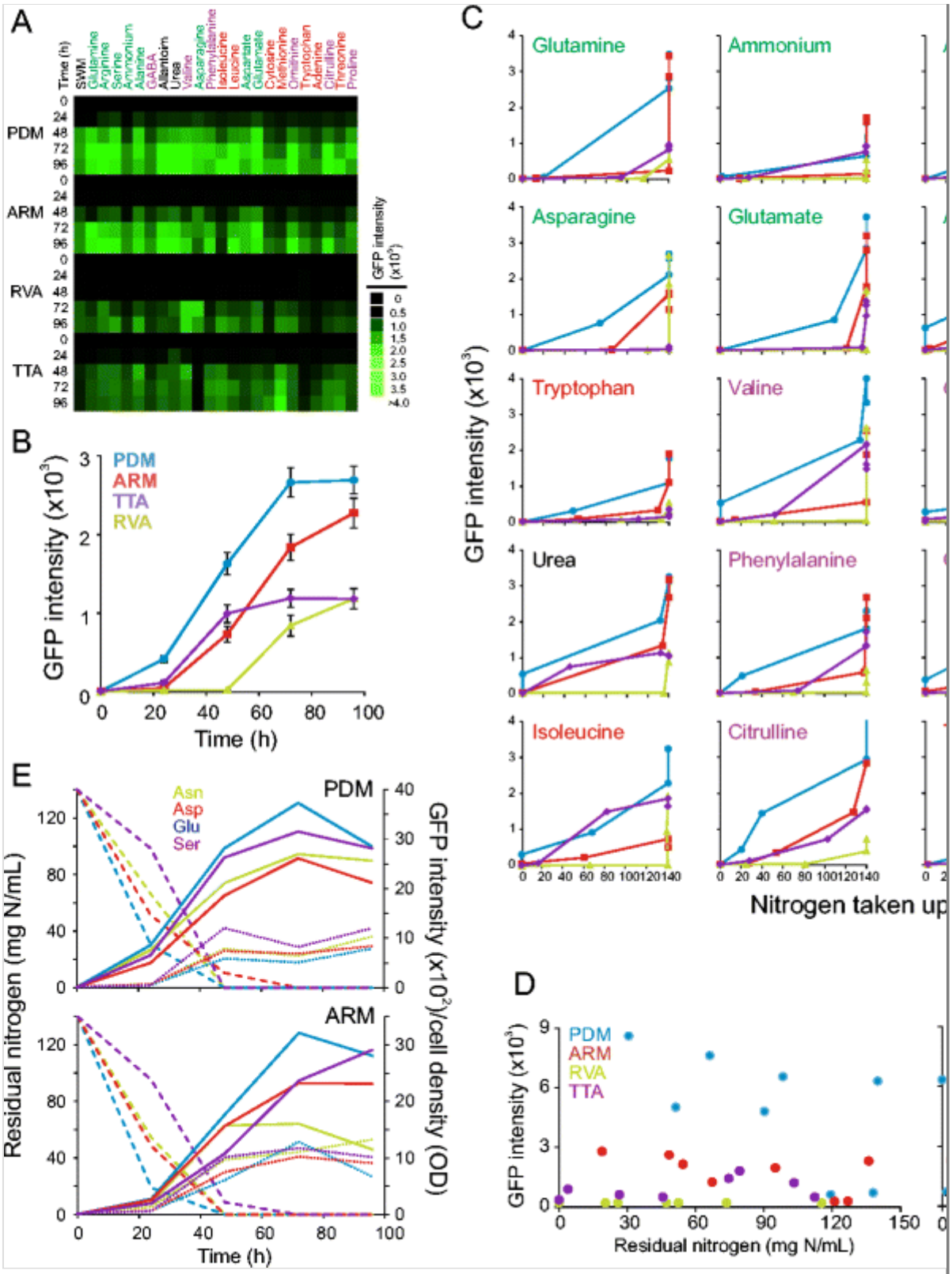
NCR repression of *GAP1* transcription is partially decoupled from nitrogen uptake and population growth in wine strains

To uncover to what extent nitrogen uptake and growth were associated to NCR activity, we followed transcriptional activation of the *GAP1* and *DAL4* promoters. In lab strains, these are key targets of NCR (Jauniaux and Grenson 1990; Stanbrough and Magasanik 1996). In diploid versions of our four wine strains, one of the *GAP1* or *DAL4* loci was altered such that the native ORFs were replaced by the ORF of green fluorescent protein (GFP), creating heterozygotic *GAP1*/*pGAP1-GFP* and *DAL4*/*pDAL4-GFP* strains. Change in GFP expression over time was then followed in each strain-nitrogen source combination. The long time spent in stationary phase in the pre-culture ensured that the

expression from the *GAP1* and *DAL4* promoters, which are strongly but transiently activated in early stationary phase, were off at experiment start and that GFP proteins had been degraded. Thus, an increase in GFP signal over time reflects an absence of activation of the NCR activity that represses transcription at the *GAP1* and *DAL4* promoters. Transcription from *DAL4* varied enormously between wine strains, being almost undetectable in three lineages but rapidly induced to high levels in TTA (Fig. S1 in the Supplementary Material). This made it unsuitable as NCR reporter. The *GAP1* promoter, driving expression of the broad-specificity general amino acid permease, was activated in all strains and nitrogen environments, albeit with large variations in timing and degree of activation between strains and nitrogen sources (Fig. 4a). Generalizing overall nitrogen sources, PDM relaxed NCR repression of *GAP1* transcription faster than other strains, whereas RVA maintained complete *GAP1* repression until the 48-h mark (Fig. 4b). In all environments except for proline and threonine, *GAP1* repression in RVA lasted until all external nitrogen was depleted (Fig. 4c). Given a strict interpretation of NCR, we expected high-quality nitrogen sources, but not low-quality nitrogen sources, to repress *GAP1* activation until complete depletion of external nitrogen. Within the analytical restrictions imposed by sampling density, this was true for glutamine and ammonium. In RVA and TTA, *GAP1* expression was typically also suppressed until near complete depletion of external nitrogen on other good nitrogen sources. However, in PDM, the *GAP1* repression was relaxed well before nitrogen depletion (Fig. 4c). Variations between strains in relaxation of *GAP1* repression on different good nitrogen sources (*GAP1* expression at 24 h), were uncorrelated to variations in how much nitrogen remained in the medium (Fig. 4d; Pearson, $r = 0.12$, $p = 0.50$). In contrast, they were well correlated to the nitrogen taken up per cell produced (Fig. 4d; Pearson, $r = 0.64$, $p < 0.0001$). Thus, the relaxation of *GAP1* repression on good nitrogen sources is associated to, and potentially triggered by, the build-up of intracellular nitrogen that follows from the temporal mismatch between nitrogen uptake and growth. As growth continued beyond external nitrogen depletion supported by intracellular nitrogen, *GAP1* expression was further reinforced (Fig. 4e) and remained high for many days after depletion of external and internal nitrogen (data not shown).

Fig. 4

NCR repression of *GAP1* transcription is partially decoupled from nitrogen uptake and growth in wine yeast. NCR repression of transcription from the *GAP1* promoter was followed separately in four wine yeast strains and 23 nitrogen sources and synthetic wine must using a GFP reporter coupled to the *GAP1* promoter and a time series of FACS measurements of GFP signal intensity. Each measure corresponds to the median GFP intensity of 10,000 cells (FACS events). **a** GFP signal intensity (*GAP1* expression) for each wine yeast strain in each nitrogen source at each time point. Color intensity depicts GFP signal intensity, *black* = initial GFP intensity (intensity = 0, in all environments), *intense green* = high GFP signal intensity. **b** Mean GFP signal intensity (*GAP1* expression) over all nitrogen sources for each strain and time point. *Error bars* = SEM. **c** GFP signal intensity (*GAP1* expression) as a function of nitrogen remaining in the medium. Connecting lines capture the order of measurements over time. For visualization purposes, measurements above 4000 in GFP in signal intensity have been set to 4000. **d** GFP signal intensity (*GAP1* expression) on good nitrogen sources at 24 h, as a function of nitrogen remaining in the medium (*left panel*) and as a function of nitrogen assimilated/cell density gained (*right panel*). *Black line* shows linear regression. **e** Time-resolved view of growth (*dotted line*, *right y-axis*), nitrogen uptake (*dashed lines*, *left y-axis*), and GFP signal intensity (*GAP1* expression, *full line*, *right y-axis*) during ARM and PDM growth on aspartate, asparagine, glutamate, and serine.



In contrast to the rather consistent behavior of the *GAP1* promoter on good nitrogen sources, no clear patterns were found on poor and intermediate nitrogen sources. In particular, an early relaxation of NCR repression of *GAP1* expression was not uniformly observed. During growth on proline and threonine, NCR was relaxed before the bulk of

the nitrogen had been consumed in all strains. For other poor and intermediate nitrogen sources, *GAP1* was repressed in one or more strains until most of the nitrogen had been assimilated. In tryptophan and GABA, *GAP1* repression was strong and consistent until complete depletion of nitrogen. In contrast to the situation during growth on good nitrogen sources, no correlation between relaxation of *GAP1* expression and either external nitrogen or the decoupling between nitrogen uptake and growth was found during growth on poor or intermediate nitrogen sources ($r < 0.1$, data not shown).

Discussion

We showed that nitrogen uptake and growth were surprisingly decoupled in wine yeasts. In particular, at early time points, nitrogen assimilation did not translate into a comparable population expansion. Thus, nitrogen must either have been stored internally in non-dividing cells or lost back to the environment. When supplied in toxic concentrations, nitrogen may be taken up and then secreted back to the environment, in free amino acid form (Hess et al. 2006). Metal stressed yeasts are also known to secrete short peptides, such as glutathione, to bind and detoxify the stress agent outside the cell (Thorsen et al. 2012). However, we ~~could not~~ detected no amino groups remaining in the medium. Secretion of free amino acids or short peptides therefore cannot explain the discrepancy between early nitrogen uptake and reproduction. Measurements of intracellular nitrogen also confirmed that early assimilated nitrogen was channeled into internal nitrogen pools, not lost back to the environment. This is largely in line with a recent report (Crepin et al. 2014) showing that early accumulated nitrogen can be stored as arginine in the vacuole and later mobilized to support growth after depletion of external nitrogen. This prioritization of replenishment of internal nitrogen pools over reproduction ~~is~~may best be seen in the light of the depletion of such pools during the preceding stationary phase. Protein synthesis continues in the stationary phase, albeit at a much reduced rate, even though no nitrogen is available externally (Fuge et al. 1994). These nitrogen demands are satisfied by increasing mobilization of internal storages, including by autophagic degradation of organelles (Gray et al. 2004). Rebuilding degraded organelles is likely to be a prerequisite for entering the mitotic

cell cycle and may account both for the long lag phase and a need to channel early assimilated nitrogen away from reproduction. It has also been argued that fast uptake of nitrogen compounds circumvents negative consequences for nitrogen uptake of rising ethanol concentrations (Boulton et al. 1996). For example, uptake of amino acids via the Gap1 low affinity permease is almost completely inhibited by high ethanol concentrations (Ferreras et al. 1989). Fast nitrogen uptake could have evolved at least partially to avoid ethanol short-circuiting reproduction.

Fast nitrogen assimilation may also provide *S. cerevisiae* with a competitive edge relative to other *Saccharomyces* and non-*Saccharomyces* yeasts present in fermenting musts (Crepin et al. 2012; Garcia-Rios et al. 2014). Such a strategy has two potential benefits. First, it deprives competitors of the crucial nutrient they need to increase in population size. Secondly, once nitrogen storages have been refilled, the strategy allows *S. cerevisiae* to switch from a fast to an efficient, but slower, mode of population expansion without having to worry about faster growing competitors depriving them of the limited nitrogen. We found the early channeling of nitrogen into intracellular pools to often be accompanied by strong population expansion after exhaustion of external nitrogen. In fact, after peaking in late lag-/early growth phase, intracellular nitrogen decreased rapidly back to initial levels. Mobilization of intracellularly stored nitrogen rather than direct uptake from the environment, therefore, necessarily supported much of the later population expansion. This is a non-trivial observation as it directly opens up for selection on efficient, rather than fast, growth strategies. In absence of privatization of nitrogen by intracellular accumulation, nitrogen saved for later by enhanced nitrogen efficiency is equally accessible to all members of the population (MacLean 2008). Increasing the efficiency and leaving nitrogen for later therefore confers no selective advantage to a genotype and it cannot increase in frequency, except by chance. Reproduction based on internally stored nitrogen circumvents this dilemma. Nutrients that have been rapidly accumulated can be catabolized and anabolized efficiently as the stored resources are private to the efficient genotype and cannot be accessed by others.

Using rehydrated dry yeast inoculated directly into the synthetic wine must, we confirmed that the early replenishment and late mobilization of nitrogen storages holds true also for more winery-like situations. Nevertheless, extrapolation of results to wineries should be done with some caution. Oxygen is near absent in most wine-fermenting vessels but was here present in small amounts. Oxygen limitation in wineries also requires the addition of essential aneurobiosis factors whose intracellular synthesis requires oxygen. Finally, although the capacity of nitrogen sources to support growth was comparable to that reported using 1 % of the cultivation volume (Gutierrez et al. 2013a), results may not necessarily be similar when scaling many orders of magnitude upwards to wine production scale.

The majority of measured nitrogen traits, including the decoupling of nitrogen uptake and growth, were subject to strong strain-nitrogen source interactions. This should be seen in the light of *S. cerevisiae* nitrogen metabolic genes being known to be genetically polymorphic (Bergström et al. 2014; Liti et al. 2009). Several of these sequence variants have now also been linked to the capacity of wine or natural strains to take up, or grow on, different nitrogen sources (Gutierrez et al. 2013a; Jara et al. 2014). For instance, variations in the glutamate synthase *GLT1* alter aspartic and glutamic acid uptake, while variations in the amino acid permeases *AGP1* and *ASI1* alter uptake of aspartic acid, phenylalanine and serine, threonine, and glutamine. There have been indications of nitrogen use polymorphisms also in lab strains. Ammonia is preferred over proline by Σ1278b, but not by S288c (Rytka 1975). This difference is explained by the proline catabolism regulating Mpr1/2 N-terminal acetyltransferases (Takagi et al. 2000), which are absent in S288c but present in Σ1278b. The polymorphic nature of nitrogen associated traits is in line with generally high levels of phenotypic polymorphism in *S. cerevisiae*, which exceeds that of other yeasts (Brown et al. 2011; Warringer et al. 2011). Considered in this light, it is remarkable that the general order in which nitrogen sources are assimilated from a complex nitrogen environment appears to be reasonably well conserved (Crepin et al. 2012).

Following transfer to an environment rich in a preferred nitrogen source, cells must first sense this abundance of preferred nitrogen,

activate NCR, and thereby repress target promoters of genes encoding proteins that allow use of less preferred nitrogen source. There is no clear consensus on what the NCR trigger is, but increase in intracellular glutamine and ammonium levels have been suggested as primary candidates (Magasanik and Kaiser 2002; ter Schure et al. 2000). Once the preferred nitrogen source is taken up and approaches depletion, NCR must be deactivated in order to allow uptake of, and growth on, less preferred nitrogen. Again, there is no consensus around what the deactivation signal is. The basic empirical requirement for this signal ~~must be~~is that the signal intensity ~~of the NCR relaxing signal should~~ co-var~~y~~ies with degree of expression of NCR targets~~,~~ in the time window when the NCR deactivation occurs. Here, 24 h was the time point at which variation in *GAP1* repression between strains and between good nitrogen sources began to manifest. At this time point, the degree of *GAP1* repression correlated well with the degree of decoupling between nitrogen taken up and growth achieved, i.e., with the nitrogen build-up inside cells. One interpretation of this correlation is that intracellular nitrogen build-up triggers *GAP1* de-repression. From this perspective, the filling up of intracellular nitrogen pools signals that there is no longer any fitness benefit of taking-up (privatizing), and using, good over poor nitrogen, and NCR is relaxed. Nevertheless, correlation is not causation. It cannot be excluded that both *GAP1* de-repression and intracellular nitrogen build-up depend on a third variable. Data strongly argues against that external concentrations of good nitrogen sources constitute such a third variable. However, intracellular levels of one or more internal nitrogen species, such as ammonium or glutamine, may possible control both the build-up of intracellular nitrogen overall and the relaxation of NCR.

In summary, we here reported the strain and nitrogen source-dependent build-up of intracellular nitrogen in wine yeast due to a decoupling between nitrogen uptake and growth and the potential influence of this build-up on NCR activity. These findings are relevant for both wine production and for our general understanding of nitrogen use.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Electronic supplementary material

ESM 1

(PDF 204 kb)

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