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# The evolution and role of the periplasmic asparaginase Asp3 in yeast

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## ABSTRACT

The study of nitrogen assimilation in yeast is of interest from genetic, evolutionary, and biotechnological perspectives. Over the course of evolution, yeasts have developed sophisticated control mechanisms to regulate nitrogen metabolism, with domesticated lineages sometimes displaying particular specialisation. The focus of this study was on assimilation of asparagine, which is a significant nutritional source for some alcoholic fermentations. We were particularly interested in ASP3, which encodes a periplasmic asparaginase and that was proposed to have been acquired relatively recently in *S. cerevisiae* by horizontal gene transfer. We examined 1680 *S. cerevisiae* genome assemblies to evaluate the distribution and evolutionary trajectory of ASP3. Our findings suggest an alternative hypothesis that ASP3 is an ancient *Saccharomyces* gene that has generally been lost over the course of evolution but has been retained in certain fermentative environments. As asparagine is the major nitrogen source in apple juice, we explored whether the presence of ASP3 would confer a growth advantage. Interestingly, we found that although ASP3 enhances growth when asparagine is the sole nitrogen source, the same effect is not seen in apple juice. These data indicate that growth in pure culture may not reflect the original selective environment for ASP3<sup>+</sup> strains and highlight the role that complex regulation may play in optimising nitrogen assimilation in yeasts.

## Introduction

Although the evolution of yeast for alcoholic fermentation preceded human civilisation by millions of years, it is the trait that underpinned and drove the domestication of multiple species in the *Saccharomyces* genus (Dashko *et al.* 2014). Species of *Saccharomyces* were used in traditional food and beverage fermentations and were then domesticated for particular applications like beer, bread, wine, cider and more recently bioethanol fermentations (Verstrepen *et al.* 2001, Fleet 2008, Almeida *et al.* 2014, Gallone *et al.* 2016). There are a range of different types of genetic change that underpin the phenotypic changes and subsequent adaptation to human-related (anthropogenic) environments (Gallone *et al.* 2018, Giannakou *et al.* 2020). Within a genome, point mutations alter the structure/function of proteins and influence gene expression, while larger variations result in chromosomal rearrangements, segmental duplication, and variation of gene copy number. Introduction of new genes via horizontal gene transfer (HGT) is also an important means by which strains acquire new phenotypes; for example, the capacity to use particular nutrients (Hall *et al.* 2005, Novo *et al.* 2009, Galeote *et al.* 2010). Interspecific hybridization has also emerged as a critical means of adapting to anthropogenic environments, with multiple instances from the *Saccharomyces* genus (Alsammar and Delneri 2020) and from other budding yeasts (Solieri *et al.* 2021).

Nitrogen is a limiting factor in alcoholic beverage fermentation and there are several known examples where domesticated

*Saccharomyces* strains evolved more efficient nitrogen assimilation (Almeida *et al.* 2014, Marsit and Dequin 2015, Becerra-Rodríguez *et al.* 2020). Depending on the particular beverage, the ammonium salts, amino acids and small peptides that are assimilated by yeast are present in the fermentation substrates in different proportions. While free ammonium is a very good nitrogen source for yeast, it is generally only available in small amounts and most nitrogen is provided by free amino acids that are transported into the cell and then further catabolised. Amino acids that are more readily catabolised and sustain higher growth rates are typically used first by yeast and are considered preferred nitrogen sources (Boer *et al.* 2007, Ljungdahl and Daignan-Fornier 2012). *Saccharomyces cerevisiae* has sophisticated regulatory pathways to control and optimise acquisition of nitrogen, namely, TORC-regulated Nitrogen Catabolite Repression (NCR) (Magasanik and Kaiser 2002) and the SPS-sensor system (Ljungdahl 2009), which detect the nitrogen conditions in the medium and deploy signalling and transcriptional responses to induce or repress genes involved in the transport and metabolism of certain nitrogen compounds (Zhang *et al.* 2018).

In *S. cerevisiae*, asparagine, which is important for yeasts used for fermented food and beverages, is a preferred nitrogen source (Boer *et al.* 2007). Although, it is not a significant amino acid in grapes, it is found in other fruits and in beer wort. A study of the amino acid composition of fruit juices revealed that the quantity of asparagine in apple juice is 100 times higher than in grape juice (Dizy *et al.* 1992). Likewise, Ma *et al.*, (2018) analysed the amino acid

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content of 13 samples of apple juice from different cultivars, and reported that asparagine, aspartate and glutamine are the prominent amino acids in most of the apple juices, with asparagine being present at the highest concentration. Asparagine is a yeast nutrient in wort (Ferreira and Guido 2018), proved for the first time by Garza-Ulloa et al., (1986) who reported that yeast consumes asparagine during beer fermentation. The canonical pathway (Fig. 1) for asparagine assimilation starts with transport into the cell by one of the general amino acid transporters Agp1, Gnp1, Dip5 or Gap1 (Bianchi et al. 2019), all of which are transcriptionally regulated by NCR and the SPS sensor system (Ljungdahl and Daignan-Fornier 2012). Within the cytosol, a constitutively expressed asparaginase I (Asp1) hydrolyses the amide group of the side chain of asparagine generating one molecule each of aspartate and assimilable ammonia, which is the major nitrogen intermediate for amino acid biosynthesis (Dunlop et al. 1978, Sinclair et al. 1994). Some strains of *S. cerevisiae* have a second asparaginase encoded by ASP3. Asp3 is located in the periplasmic space and can degrade asparagine outside the cell (Jones and Mortimer 1973, Dunlop and Roon 1975, Dunlop et al. 1976). In this case, the resulting ammonium enters the cell via one of the dedicated Mep transporters and the aspartate is transported by the general transporters Gap1 and Dip5, which are under NCR and SPS control, or a dedicated transporter named Agp3. ASP3 is also controlled by NCR and its expression is upregulated in the absence of rich nitrogen sources to facilitate the utilization of extracellular asparagine (Oliveira et al. 2003, Scherens et al. 2006).

ASP1 is universally present in the *Saccharomycotina* (budding yeast) whereas ASP3 has only been reported in some *S. cerevisiae* strains and rarely elsewhere. The low prevalence and presence of a homologous gene in *Wickerhamomyces anomalus* led to the hypothesis that the presence of ASP3 in *S. cerevisiae* could be due to HGT from *W. anomalus* in biotechnological environments, where the yeasts co-occurred (League et al. 2012). In this regard, the fact that apple juice is rich in asparagine, and both *S. cerevisiae* and *W. anomalus* are frequently associated with spontaneous cider fermentations, points to an environment where such a transfer could take place. The rationale would be that the presence of Asp3 offered strains a growth advantage, whereby they would more efficiently assimilate asparagine during fermentation.

We are interested in how yeasts adapt to domestic niches and decided to investigate the role and history of Asp3. The large number of genome sequences now available provide an opportunity to revisit the hypothesis of HGT. We were also curious to see whether Asp3 would be found in *Saccharomyces* species other than *S. cerevisiae*, especially in *Saccharomyces uvarum*, which is commonly used in cider fermentations. *S. uvarum* is a heterologous host that grows in environments where *S. cerevisiae* might have had a selective advantage in retaining the gene. In addition, we know that *S. uvarum* lacks ASP3 and it is more typically associated with apple juice fermentation. In this study, we first determined whether the HGT hypothesis is supported by more recent genomic data. The next question was whether the presence of ASP3 confers an advantage for yeast growth and nitrogen assimilation. And finally, we investigated whether fermentations of natural matrices (apple juice) support the idea that ASP3 confers a selective advantage. Our analysis led to the conclusion that ASP3 is most likely to be an ancestral gene that was not recently acquired but in fact has been lost in the majority of *S. cerevisiae* strains. We used heterologous expression of ASP3 in *S. uvarum* to establish that ASP3 does confer a growth advantage when asparagine was the sole nitrogen source, but unexpectedly this did not translate into better growth in apple juice where asparagine is the nitrogen source. The seem-

ingly contradictory findings that retention of ASP3 is enriched in strains used for beer or cider fermentations but the gene appears not confer a growth benefit in this environment, will be discussed.

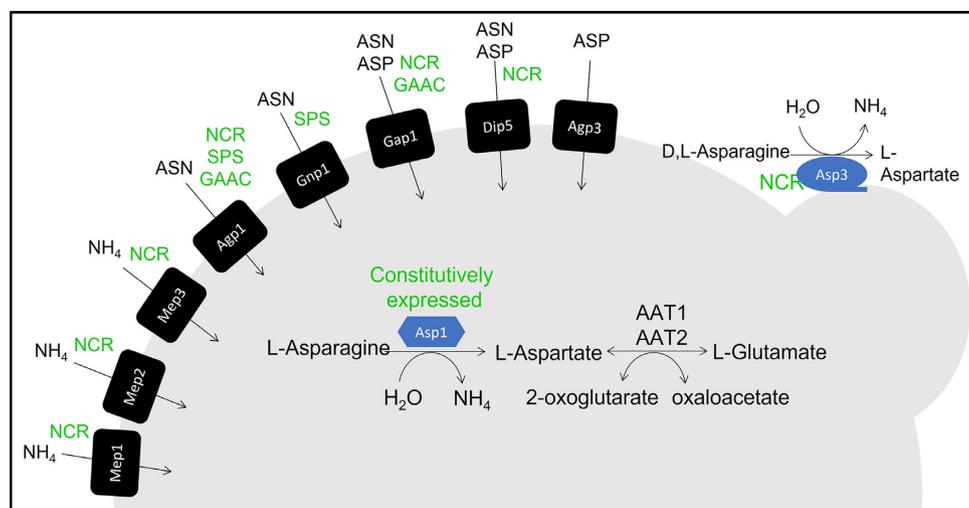
## Materials and methods

### Yeast strains and growth

The *Saccharomyces* strains isolated from different sources used for this study are listed in Table 1. Species identity of all strains was confirmed by sequencing the D1-D2 region of the 25S rRNA gene. (Kurtzman and Robnett 1997). The cryopreserved yeast strains were plated in Yeast Peptone Dextrose (YPD) agar and incubated during 48 h. For growth experiments, pre-cultures were grown in YPD broth at 28°C for 16 hours with shaking (180 rpm), then transferred to YNB media without ammonium and amino acids for 4 hours at 28°C to exhaust the yeast nitrogen reserves. The cells were washed with sterile 0.9% NaCl to remove all nitrogen residues and then resuspended in minimal synthetic medium (MM) (glucose 20 g/L, KH<sub>2</sub>PO<sub>4</sub> 3 g/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5 g/L, vitamin mix, and trace elements in concentrations adapted from Verduyn media) (Verduyn et al. 1992). The nitrogen source was provided as required for each experiment. This cell suspension was further used to inoculate fermenters or microtitre plates. For microtitre plate fermentations, precultures treated as described above were diluted in sterile water to A<sub>600</sub> 0.5 and then used to inoculate 200 µL fresh media at A<sub>600nm</sub> 0.05 (1:10 dilution). The 96 flat wells microplate was incubated at 20°C in the microplate reader CLARIOStar<sup>plus</sup> (BMG LABTECH, Germany). A<sub>600nm</sub> was measured for 60 cycles of 1 hour (24 flashes/cycle) with continual double orbital shaking (400 rpm) between measurements. This experiment was done with a minimum of three biological replicates of each strain in each media, using one blank per media. *Saccharomyces cerevisiae* CENPK113-7D was used as control in all the microtitre plates.

### Fermentation conditions

Batch fermentations were performed in 330 mL fermenters containing 250 mL of medium, equipped with airlocks to prevent the entry of air but allowing CO<sub>2</sub> release during fermentation. The fermenters were aerated for 20 minutes before inoculation to balance the dissolved oxygen content with the air. Yeasts were inoculated at 5 × 10<sup>5</sup> cells/mL (~0.04 A<sub>600</sub> for *S. uvarum* and ~0.05 A<sub>600</sub> for *S. cerevisiae*). The fermentations were conducted with continuous magnetic stirring (230 rpm) at 20°C. Fermentations were performed in biological triplicates of each strain. The fermentations were monitored by CO<sub>2</sub> release, weighting the fermenters to track record of weight loss. The CO<sub>2</sub> production rate (g/L/h) was calculated by polynomial smoothing of the last eight values of CO<sub>2</sub> production. The sampling points were determined depending on the methods to be performed afterwards. For frequent sampling, 1.5 mL of medium was taken to monitor the cell population with Coulter counter (BECKMAN®) and spectrophotometer (A<sub>600</sub>). The samples (6 mL) destined for HPLC and GCMS analysis were taken at the beginning of the exponential phase, at 40 g/L of CO<sub>2</sub> produced in the case of cider, and at the end of fermentation. Then, the samples were centrifuged to remove cells pellet and the supernatant was stored at -20°C for further HPLC and GCMS analysis. The cider fermentations were carried out on microfiltered apple juice A843 (Guillevic apple variety from Bretagne, France) provided by the Institut Francais des Productions Cidricoles (IFPC). The apple juice (pH 3.52) contained ~108 g/L of sugar at a ratio of glu-



**Figure 1.** Asparagine assimilation in *S. cerevisiae*. Asparagine (ASN) is imported into the cell through the transporters Agp1, Gnp1, Gap1 and Dip5; Aspartate (ASP) by Gap1, Dip5 and Agp3; and Ammonium (NH<sub>4</sub>) by the specific transporters Mep1, Mep2 and Mep3. Extracellular hydrolysis of asparagine is done by Asp3 localised in the cell wall, while Asp1 hydrolyses asparagine in the cytosol. Both enzymes catalyse the same reaction, producing aspartate and NH<sub>4</sub>. Intracellular aspartate is converted to glutamate and oxaloacetate, which are easily utilised by the cell through the central nitrogen metabolism. Nitrogen Catabolite Repression (NCR), the SPS-sensor system (SPS) and General Amino Acid Control (GAAC) are the regulation mechanisms of each protein shown in green while other genes are constitutively expressed.

**Table 1.** Yeast strains used in growth studies. Additional strains are listed in supplementary material.

Yeast	Strain	Geographical origin	Environment	ASP3 status
<i>S. cerevisiae</i>	S288c	-	Laboratory	4 copies
<i>S. cerevisiae</i>	CEN.PK113-7D	-	Laboratory	1 copy
<i>S. cerevisiae</i>	EC1118	Champagne, France	Wine	Absent
<i>S. uvarum</i>	BMV58/MTF3098	Valencia, Spain	Wine	Absent
<i>S. uvarum</i>	CBS7001/OS24	Avila, Spain	Insect Mesophylax adopersus	Absent
<i>S. uvarum</i>	OS472	Marlborough, New Zealand	End of Sauvignon Blanc Wine fermentation	Absent
<i>S. uvarum</i>	CBS395	The Netherlands	Juice of Ribes nigrum	Absent
<i>S. uvarum</i>	CLIB1050	France	Cider must	Absent
<i>S. uvarum</i>	CLIB393	Japan	Cider brewery	Absent
<i>S. uvarum</i>	CLIB501	Normandie, France	Industrial sweet cider	Absent
<i>S. uvarum</i>	ΔHO::ASP3	-	-	1 copy

cose to fructose of 3:7, and ~127 mg/L of nitrogen, made up of asparagine (95 mgN/L), aspartate (10 mgN/L), traces of other amino acids, and NH<sub>4</sub> (0.6 mgN/L). Conditions and measurements were as described for batch fermentations except that the experiments were performed at 16°C.

### Construction of overexpression strains

To express ASP3 in *S. uvarum*, the gene was amplified by PCR from *S. cerevisiae* CENPK113-7D using Q5 polymerase (New England Biolabs (NEB) Inc., MA, USA) and cloned into the pGREG-505-TEF1 plasmid (Varela et al. 2017) via Gibson Assembly (New England Biolabs (NEB) Inc., MA, USA). All primers and plasmids are listed in Tables. The assembly reaction was transformed into *E. coli* and then plated on LB medium supplemented with ampicillin 100 μg/mL. The resulting plasmid pGREG-505-TEF1-ASP3 was recovered and verified with HindIII enzymatic digestion (NEB) and sequencing (Eurofins Genomics, Germany). pGREG-505-TEF1-ASP3 was transformed into *S. uvarum* MTF3098 using the LiAC/SS carrier DNA/PEG procedure (Gietz and Schiestl 2007) with selection on YPD agar supplemented with G418 200 μg/mL. ASP3 was also integrated into the HO locus of *S. uvarum*

MTF3098. The integration fragment, called GREG-ASP3 (1843 bp), consisting of the TEF1 promoter, ASP3 coding sequence and CYC1 terminator, was PCR amplified from pGREG-505-TEF1-ASP3 with primers containing homology regions to the HO gene. A plasmid was constructed to introduce a CRISPR Cas9-mediated double stranded break at the HO locus. For this, oligonucleotides containing the target HO sequence were cloned into pUDP002-HH using Golden Gate assembly as previously described (Rajkumar et al. 2019). Finally, the CRISPR plasmid targeting HO and the GREG-ASP3 repair fragment were co-transformed into *S. uvarum* MTF3098. The cells were plated on YPD supplemented with hygromycin 200 ng/μL and the correct integration of ASP3 at the HO locus was confirmed via PCR with diagnostic primers.

### Analytical methods

Free amino acid content in the media was measured by cation exchange chromatography (Biochrom 30, Biochrom, Cambridge, UK) as previously described (Crépin et al. 2012). The measurement of glucose, fructose, ethanol and other central carbon metabolites was done with High-Performance Liquid Chromatography (HPLC) on Phenomenex Rezex ROA column (HPLC HP1100 Infinity, Ag-

ilent Technologies) as described by Rollero et al. 2015. Volatile compounds were measured by gas chromatography/mass spectrometry (GC-MS) as described in detail by Rollero et al. 2015. In brief, a liquid extraction of supernatants to recover higher alcohols, fatty acids, acetate esters and ethyl esters was performed with dichloromethane  $\text{CH}_2\text{Cl}_2$ , the organic phase was recovered, dried, and concentrated with nitrogen gas before injection onto a Hewlett Packard 6890 gas chromatograph (Agilent Technologies, Santa Clara, California, USA) equipped with a CTC Combi PAL Autosampler AOC-5000 (Shimadzu, Columbia, USA), coupled to a HP 5973 mass spectrometry detector (HP, now Agilent Technologies, Santa Clara, California, USA).

### Bioinformatic and molecular analysis of ASP3

ASP3 sequences were identified *in silico* with BLASTP and TBLASTN using the *S. cerevisiae* S288c ASP3 sequence (Agarwala et al. 2016), the BLAST was done in all Ascomycota and the species that carry the gene were graphed in Fig. 2B. Strains without annotations were annotated using Augustus (Stanke and Morgenstern 2005). The Peter et al., (2018) tree and other phylogenetic trees were displayed using Iroki (<https://www.iroki.net/>) with custom tables to highlight taxa (Moore et al. 2020). Phylogenetic trees for Asp3 protein sequences were generated with RAxML-NG (Kozlov et al. 2019) using the model parameters LG + G4. Synteny analysis at species level was performed using the MCSCAN pipeline as part of JCVI utility libraries (Tang et al. 2008). Synteny within *S. cerevisiae* was performed with a series of python scripts incorporating BLAST, annotations and contigs/scaffold information. Additionally, PCR was used to detect the presence of ASP3 in *Saccharomyces* species. PCR amplification was done using the OneTaq master mix (New England Biolabs (NEB) Inc., MA, USA) with conserved interspecies primers designed to amplify ASP3 in different yeast species, including *S. cerevisiae*, *Z. parvabailii* and *W. anomalus* (Table S1). The thermocycling conditions were as follows: an initial enzyme activation of 1 minute at 95°C, followed by 30 cycles of denaturation for 15 seconds at 95°C, annealing at 52°C for 15 seconds and extension for 90 seconds at 65°C, with a final elongation step of 5 minutes at 68°C. The evaluated strains included 10 *S. cerevisiae* strains, 5 *S. uvarum* strains and other *Saccharomyces* species such as *S. kudriavzevii*, *S. arboricola*, *S. eubayanus*, *S. jurei*, *S. paradoxus* and *S. mikatae* (Table S2). The expected ASP3 fragments 642 bp long were detected via 1% gel electrophoresis.

### Statistical analysis

The statistical analysis of the growth kinetics data was performed using R Studio software, version 1.3.1093 (RStudio Team. 2020) with GrowthCurver package (Sprouffske and Wagner 2016). The reported growth kinetics are expressed in the metric area under the curve (AUC) that integrates information from the logistic parameters: lag phase length, growth rate, final time and maximum biomass produced (Fig. S6). The AUC metric is a measure of growth that has been used in previous nitrogen preference studies (Su et al. 2020). For the fermentation data, the graphs and statistical tests were performed using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California, USA).

## Results

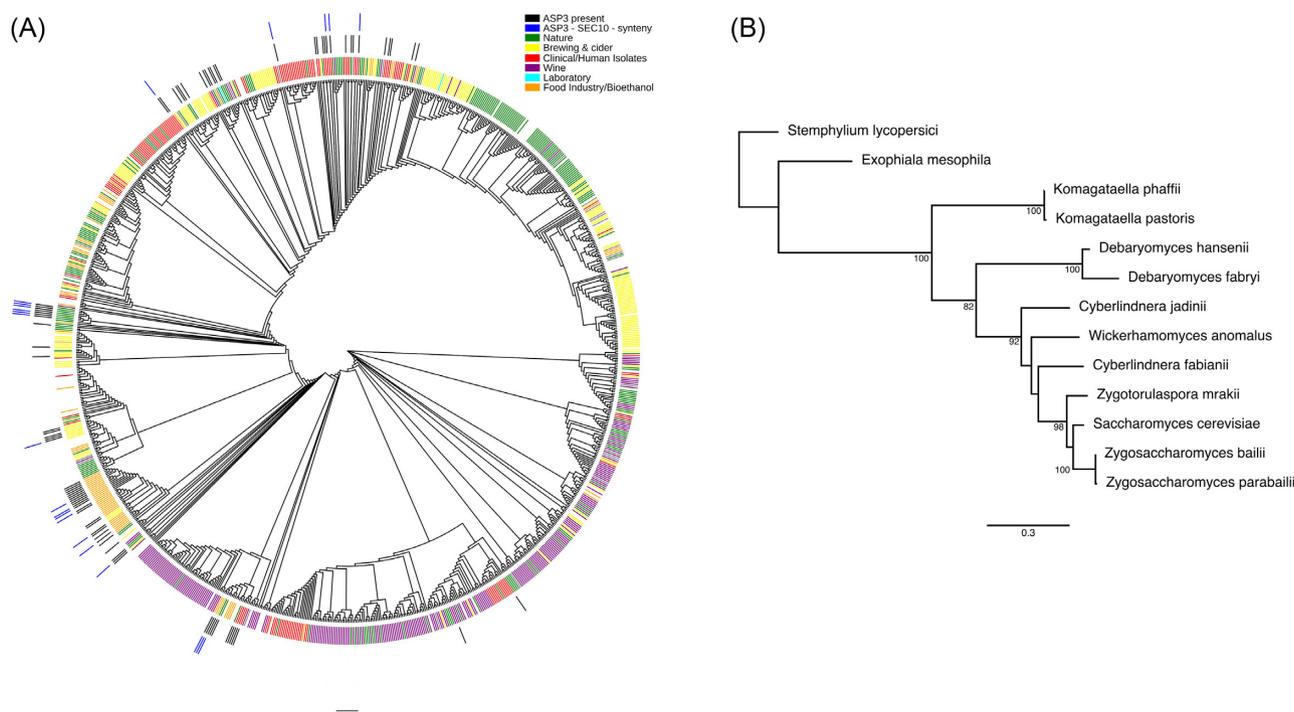
### ASP3, an ancestral gene of *S. cerevisiae* lost throughout evolution

To study the distribution of ASP3 within *S. cerevisiae* strains, a database with 1680 strains was constructed using publicly avail-

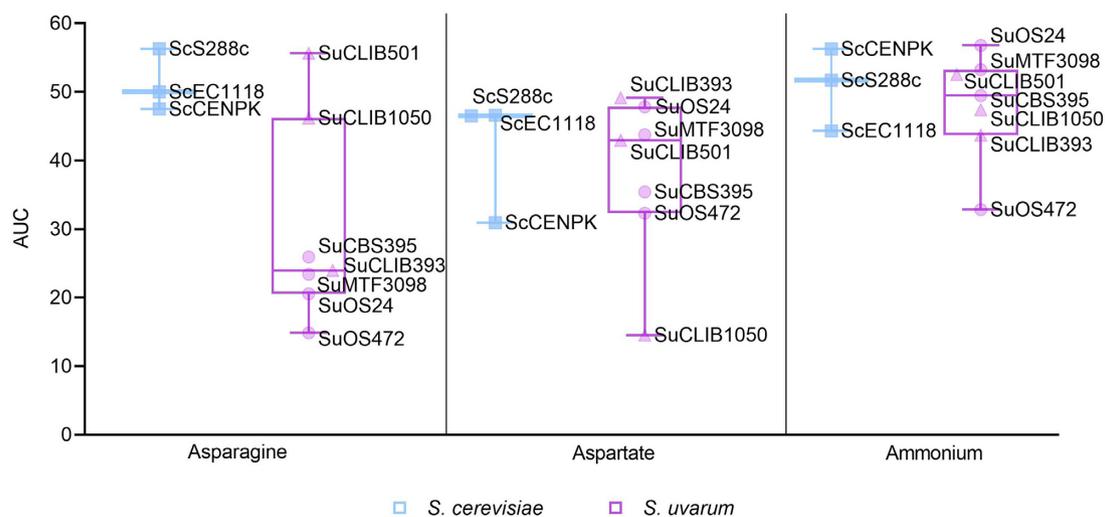
able genome assemblies. Using BLASTP, we detected the presence of at least one copy of ASP3 in the genomes of 117 strains (~7%). These data are mapped onto the previously published *S. cerevisiae* phylogenetic tree (Peter et al. 2018), which allowed us to investigate the distribution of ASP3-containing strains (Fig. 2A; for expanded tree including all *S. cerevisiae* strains see Fig. S1). For many of these strains, the isolation source is known allowing us to ask whether the distribution of ASP3 showed any patterns (Table S3). Although there is not an absolute correlation between the presence of ASP3 and the environmental or geographical origin of the strains, ASP3 is predominantly found in strains associated with the bioethanol & food industry, brewing & cider making, and research laboratories. It is interesting to see that ASP3 is largely absent in strains isolated from wine environments.

To examine the relationship of the *S. cerevisiae* ASP3 genes to each other and to ASP3 in other ascomycetes, we first interrogated databases to identify homologous genes using a combination of BLASTP and TBLASTN to ensure that both annotated and unannotated genes were found. In this way, we identified ASP3 homologues in *Stemphylium lycopersici*, *Exophiala mesophila*, *Komagataella phaffii*, *Komagataella pastoris*, *Debaryomyces hansenii*, *Debaryomyces fabryi*, *Cyberlindera jadinii*, *Cyberlindera fabianii*, *Zygorulasporea mrakii*, *Zygosaccharomyces parvabailii*, and *Wickerhamomyces anomalus* (where it was already known). We aligned the protein sequences and drew phylogenetic trees to see the relationships. This revealed that all the *S. cerevisiae* Asp3 sequences are more similar to each other than any other Asp3 and thus derive from a common ancestor (Fig. 2B) (see Fig. S9 that includes 117 *S. cerevisiae* strains and ASP3 identity analysis). It is also seen that the distribution of Asp3 amongst yeast species largely follows the pattern that would be expected in a yeast species phylogenetic tree and *S. cerevisiae* Asp3 is most related to the Asp3 found in *Zygosaccharomyces/Zygorulasporea* species. Next, we examined the contig/scaffold containing ASP3 of each *S. cerevisiae* strain to determine whether ASP3 is present in a conserved locus. It was only possible to perform this synteny analysis in forty strains because in most of the genome assemblies, ASP3 is the sole protein-coding gene on that contig/scaffold. In all forty cases, however, ASP3 is adjacent to MAS1, PUS5 and SEC10 (ASP3-MAS1-PUS5-SEC10) showing that ASP3 is located within a conserved location that we refer to as the ASP3-SEC10 locus (Supplementary Fig. 2). We added these to the phylogenetic tree (Fig. 2A, outer ring) and, as with ASP3 itself, this syntenic locus is distributed across the tree. We also analysed the ASP3 synteny in other yeast species but failed to find any relationship between ASP3 location in any of these species (Fig. S2). Considering the conserved synteny and the sequence similarity of the protein sequences, it is concluded that ASP3 is ancestral in *S. cerevisiae* and, while its prevalence seems enriched in strains from certain human-associated environments, the gene was not recently acquired by horizontal gene transfer. We do not report the actual frequencies of ASP3 positive strains from different environments as there is some uncertainty as to whether there are duplicate strains in the collection. The low number of available sequences and lack of synteny preclude any strong conclusion for other yeast species, but the phylogenetic pattern is most consistent with ancestral carriage of ASP3 in the *Saccharomycotina* and subsequent widespread loss.

The apparent enrichment of ASP3 in fermentation-associated though not wine isolates of *S. cerevisiae* led us to assess whether the gene might be present, but unidentified, in other species of *Saccharomyces*. Using primers that would amplify ASP3 in different yeast species (Table S1), we performed PCR on genomic DNA



**Figure 2.** ASP3 phylogeny in budding yeasts. **(A)** The distribution of ASP3 in *S. cerevisiae* is shown as black bars in the second ring that is superimposed in a multigenic phylogenetic tree of 963 *S. cerevisiae* strains (from Peter et al. 2018). The isolation source of the strains is highlighted in the first ring using the colours: green (nature), yellow (brewing & cider), red (clinical/human isolates), purple (wine), light blue (laboratory) and orange (bioethanol & food industry). The outer blue bars mark strains with conserved ASP3 synteny based on identification of a conserved ASP3-MAS1-PUS5-SEC10 locus. **(B)** The evolutionary relationship of ASP3 in budding yeast was determined by aligning protein sequences and drawing a phylogenetic tree. Only bootstrap values above 70% are displayed on the phylogenetic tree.



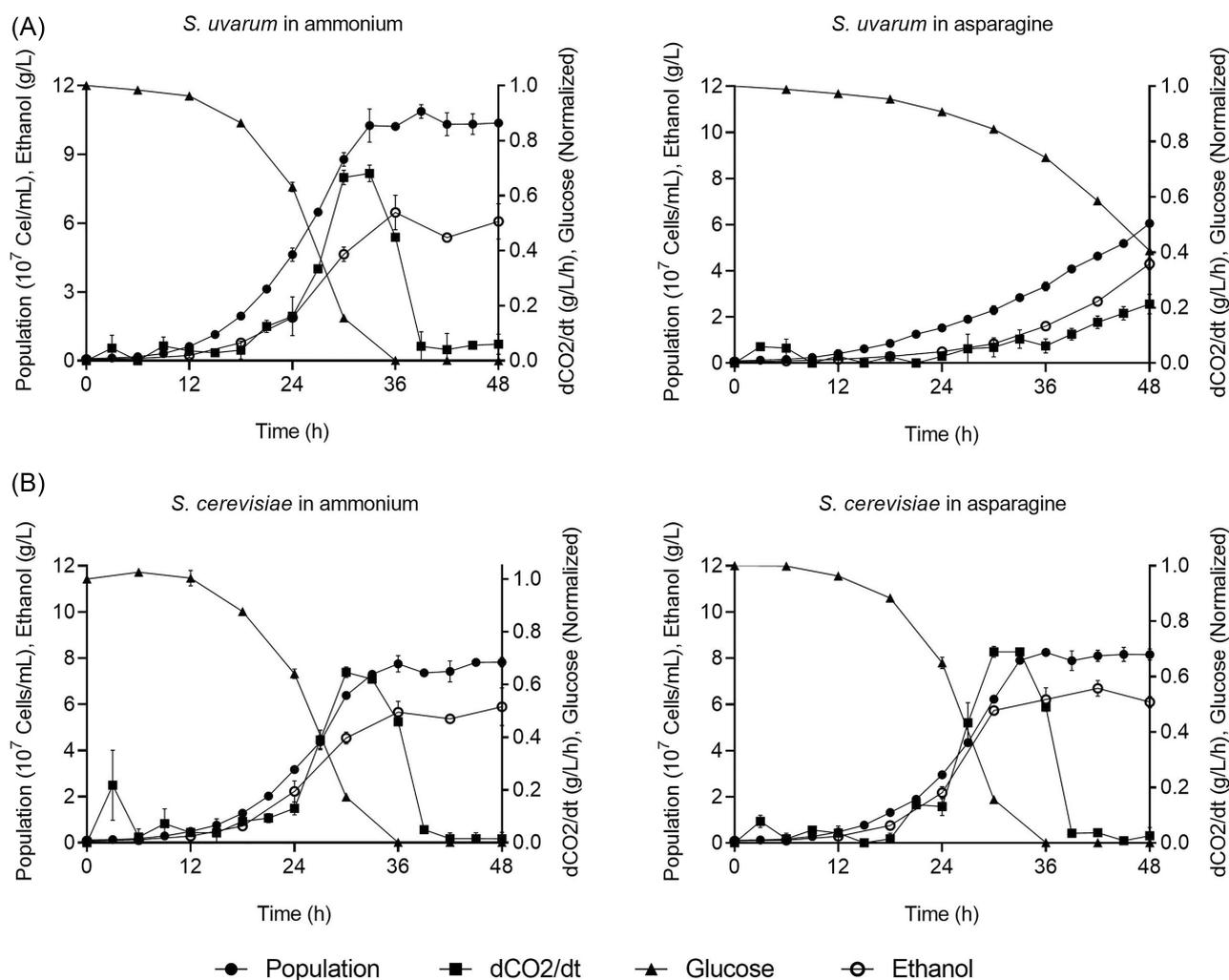
**Figure 3.** Preference of the nitrogen source by *S. cerevisiae* and *S. uvarum* strains. Growth of three *S. cerevisiae* and seven *S. uvarum* strains on minimal media with asparagine, aspartate, or ammonium as sole nitrogen source (1059 mgN/L) was measured. Growth is represented by the parameter 'area under the curve (AUC)'. Data are presented as boxplots and each point represent the mean of three biological replicates. The origin of the *S. uvarum* strains are represented with circles (wine) or triangles (cider). In *S. cerevisiae* strains the origin is not discriminated. One-way Anova was performed for each nitrogen source, revealing significant difference between *S. cerevisiae* and *S. uvarum* species only on asparagine with Adj P-value of 0.02.

from eight different *Saccharomyces* species (21 strains) and found that ASP3 was only present in the control *S. cerevisiae*. Specifically for *S. uvarum*, as well as the five strains examined by PCR, we performed *in silico* analysis of a further 24 strains, and again failed to detect ASP3 (Supplementary method). Although, it must be acknowledged that a much larger set of strains for each species would need to be examined to rule out carriage at a low fre-

quency, the data indicate that *S. uvarum* appears not to encode ASP3.

### Asparagine consumption profile is different in *S. cerevisiae* and *S. uvarum* species

*S. uvarum* grows in niches where the yeast species that carry Asp3 might have had a selective advantage to retain the gene. To in-

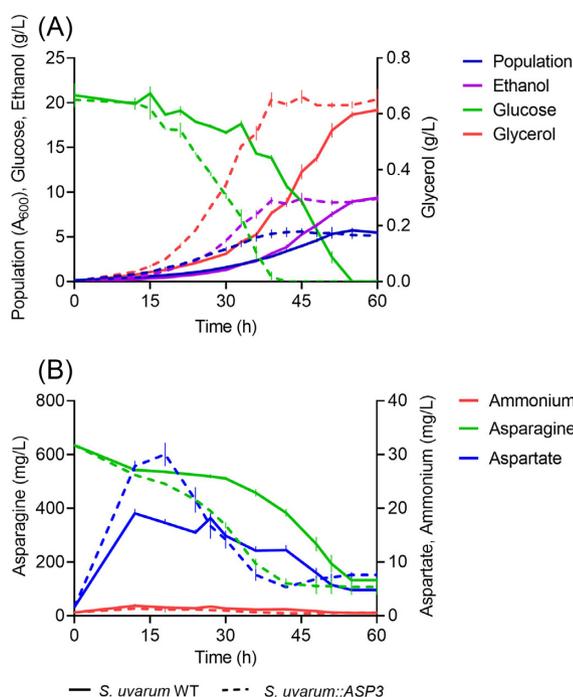


**Figure 4.** Fermentation profile of *S. uvarum* and *S. cerevisiae* strains on ammonium and asparagine. Fermentations of *S. uvarum* MTF3098 (panel A) and *S. cerevisiae* CENPK113-7D (panel B) were carried out at 30°C, on minimal medium with excess of asparagine (right) and ammonium (left) as sole nitrogen sources (1059 mgN/L) and glucose (20 g/L) for 48 h. Each graph shows the progress of glucose consumption normalized to 1 (initial), ethanol production expressed in g/L, the CO<sub>2</sub> production rate expressed in g/L/h and the cell population size expressed in cells/mL. The error bars were calculated from biological duplicates.

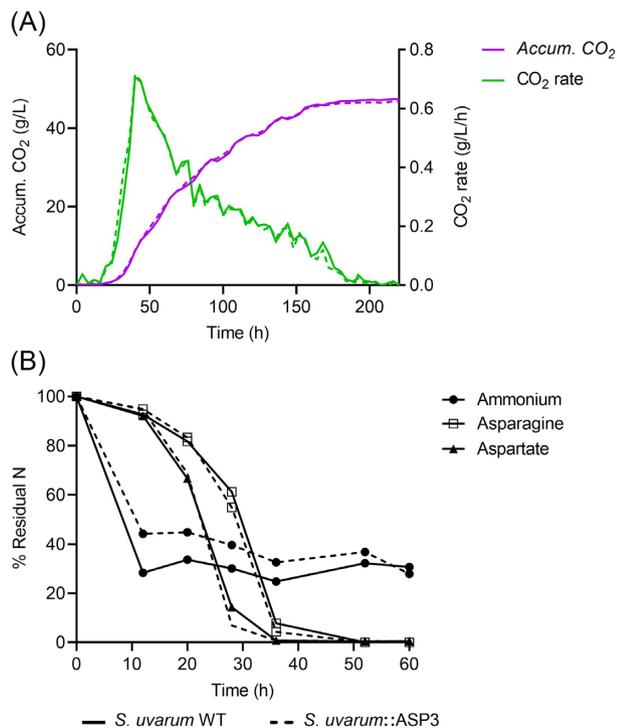
investigate the asparagine assimilation of *S. cerevisiae* and *S. uvarum* species, we performed growth tests on a set of 10 *Saccharomyces* strains using asparagine, aspartate or ammonium as the sole nitrogen source (Fig. 3). The strains came from wine or cider sources and included three isolates of *S. cerevisiae* (carrying 1, 4 or no copies of ASP3) and seven *S. uvarum* isolates (all ASP3 negative). All strains encode Asp1 and so can use asparagine as a sole nitrogen source, but the questions were whether the presence of Asp3 would confer an advantage, whether there was a difference between the species, and whether the origin of the strain had an effect. For *S. cerevisiae*, it is seen that asparagine and ammonium are preferred to aspartate, although the statistical test did not show significance, but the strain lacking Asp3 (EC1118) did not stand out as growing less than the other strains on asparagine, thus there is no evident advantage to ASP3 strains. In the case of *S. uvarum*, a uniform pattern was not seen regarding preferences for nitrogen sources. For example, the strain CLIB1050 grew poorly on aspartate but well on asparagine and ammonium, whereas the strain OS24 grew poorly on asparagine but well on the other two nitrogen sources. Overall, with the exception of the slow growing CLIB1050 (on aspartate), the growth of both species was similar

on aspartate and ammonium (there is not a significant difference between the species with Adj P-value 0.96 in aspartate and 0.97 in ammonium), but a difference was seen on asparagine (Adj P-value 0.02). Here, the *S. uvarum* strains bifurcated with two strains growing similarly to *S. cerevisiae* and five strains growing much less well. The striking observation was that the two better growing *S. uvarum* strains were isolated from cider fermentations and four of the strains that grew poorly specifically on asparagine, came from the wine environment. Although numbers of strains included in this analysis are low, it could indicate that cider strains of *S. uvarum* have adapted to use asparagine more efficiently despite the absence of ASP3.

To further investigate the differences in the capacity of *S. cerevisiae* and *S. uvarum* to use asparagine as sole nitrogen source, two strains were selected for deeper analysis. *S. cerevisiae* CEN.PK113-7D (ASP1, ASP3) and *S. uvarum* MTF3098 (ASP1) were used for the batch fermentations in MM containing either asparagine or ammonium as the sole nitrogen source (Fig. 4). Fermentations were monitored for 48 hours measuring the population size, CO<sub>2</sub> production rate, glucose consumption and ethanol production. No substantial differences were seen in the fermentation profile of *S.*



**Figure 5.** Evaluation of fermentation performance of *S. uvarum::ASP3*. *S. uvarum* wild type (*S. uvarum* WT) and the strain carrying ASP3 (*S. uvarum::ASP3*) were grown in minimal medium containing glucose (20 g/L) and asparagine (100 mgN/L) at 30°C. **(A)** Fermentation parameters include population size expressed in  $A_{600}$ , glucose, ethanol and glycerol levels expressed in g/L. **(B)** Nitrogen consumption of asparagine, aspartate and ammonium are shown in mg/L. The continuous line represents *S. uvarum* WT and the dash line represents *S. uvarum::ASP3*. The error bars were calculated from biological triplicates. **(C)** Table of rates of population, consumption and production of compounds. Statistical analysis done with Multiple t-tests using the Holm-Sidak method.



**Figure 6.** Fermentation profile in apple juice. Apple juice fermentations with *S. uvarum* wild type (WT) and *S. uvarum* carrying ASP3 in the genome (*S. uvarum::ASP3*). **(A)** The progress of the fermentation is presented by showing the rate of  $CO_2$  production ( $CO_2$  rate) and the total accumulated  $CO_2$  (Accum.  $CO_2$ ). The curves represent the mean of three replicates and were smoothed with 4 neighbouring points to average. **(B)** Nitrogen consumption during 60 hours of fermentation, shows the residual nitrogen through time expressed in percentage. The error bars were calculated from triplicates.

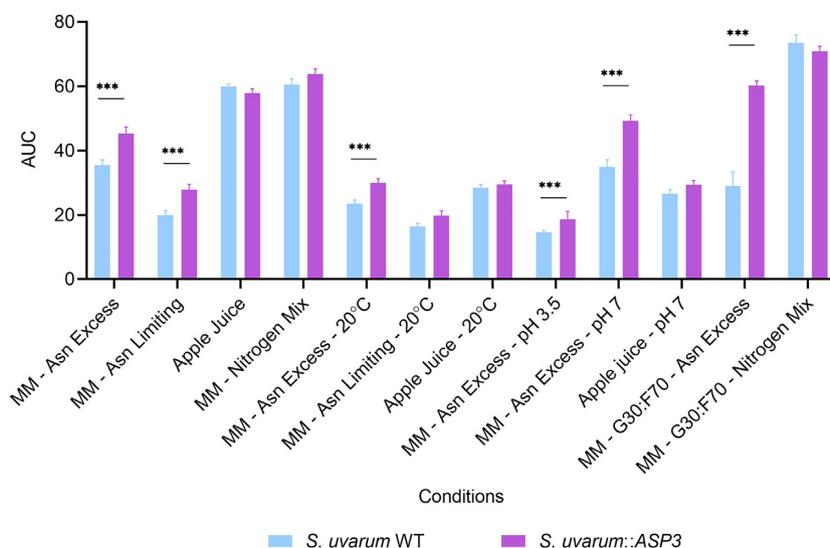
**(C)**

	Compound	Mean $\mu_{max}$ ASP3	Mean $\mu_{max}$ WT	Adj p-value	Significant
Consumption	Ammonium	0.02	0.03	0.16	No
	Asparagine	11.38	9.93	0.16	No
	Aspartate	0.55	0.34	0.06	No
	Glucose	0.55	0.39	0.00	Yes
Production	Population	0.15	0.09	0.00	Yes
	Ethanol	0.19	0.10	0.00	Yes
	Glycerol	0.20	0.10	0.00	Yes
	Aspartate	1.80	1.66	0.69	No

*cerevisiae* in asparagine and ammonium (Fig. 4B) and *S. uvarum* in ammonium (Fig. 4A). In those cases, the cells reached stationary phase and the maximum rate of  $CO_2$  production after approximately 32 hours of fermentation, glucose was depleted after 36 hours, and the ethanol production was approximately 6 g/L. It is noted that *S. uvarum* does not achieve a larger population size than *S. cerevisiae* on ammonium. In contrast, for the fermentations with *S. uvarum* in asparagine, after 48 hours the cells were still in an active growth phase and 40% of glucose remained in the media. The maximum specific growth rate ( $\mu_{max}$ ) for *S. uvarum* in asparagine was  $0.10\ h^{-1}$ , whereas it was  $0.27\ h^{-1}$  for *S. cerevisiae*. In *S. cerevisiae* a similar population size was reached on both nitrogen sources but for *S. uvarum*, after 48 hours, the population size on asparagine was substantially lower than that on ammonium. These data confirm that asparagine is not a preferred nitrogen source for the reference strain *S. uvarum* MTF3098.

### The role of ASP3 in growth and the production of secondary metabolites in *S. uvarum*

We next wanted to investigate whether ASP3 could confer an advantage for yeast growth with asparagine as a nitrogen source as that could provide an explanation for selective retention in some lineages. As it was apparent from the data in Fig. 3 that strain background would be a confounding aspect, we decided to work in a congeneric background and heterologously expressed ASP3 in *S. uvarum* MTF3098, which lacks an endogenous copy. We evaluated the growth of three *S. uvarum* strains: the wild type (WT), a strain with ASP3 on a plasmid and a strain carrying ASP3 in the genome (constitutive promoter in both cases). Fermentations were conducted in MM with asparagine or ammonium provided in excess. Initial experiments found that growth in ammonium



**Figure 7.** Evaluation of the growth of *S. uvarum* strains under different conditions. *S. uvarum* wild type (*S. uvarum* WT) and the *S. uvarum* strain carrying ASP3 (*S. uvarum::ASP3*) were grown in different fermentation conditions for 60 hours and growth is presented using the 'area under the curve (AUC)' parameter. The standard conditions were: Minimal medium (MM) containing 20 g/L glucose, excess concentration of nitrogen source (1059 mgN/L), pH of 4.6, at 30°C. When mentioned, just some factors varied from the standard conditions. Mix of nitrogen compounds (Nitrogen Mix) mimics the nitrogen composition of apple juice (127 mgN/L from which 95 mgN/L is asparagine). Limiting concentrations of asparagine represent 100 mgN/L (Asn Limiting). To variate the C:N ratio, 30 g/L of glucose and 70 g/L of fructose (G30:F70) were used mimicking the sugar composition of apple juice. The pH was tested at 3.5 and 7. The error bars were calculated from five biological replicates. The \*\*\* show significant strain effect where P-value was < 0.001.

was similar for the three strains but, in asparagine, the strains expressing ASP3 grew more efficiently (Supplementary Fig. 3). This led us to perform a more comprehensive in-depth comparison of the fermentation profiles in MM containing 100 mgN/L asparagine of the wild-type strain with *S. uvarum::ASP3*, which carries a single constitutively expressed copy of ASP3 in its genome (Fig. 5). Comparison of four key growth parameters showed that *S. uvarum::ASP3* (dashed line) consumed glucose faster, accumulated ethanol and glycerol more rapidly, and achieved maximum population size earlier than the wild-type (WT) *S. uvarum* (Fig. 5A). The faster fermentation of *S. uvarum::ASP3* was also evident in the production of other central carbon metabolites and volatile compounds, where concentrations of propanol, 2-methylpropanol and 2,3-methylbutanol were higher in *S. uvarum::ASP3* after 30 hours but both strains showed similar profile of metabolites produced at the end of fermentation (Supplementary Fig. 4). We also followed how the concentrations of asparagine, aspartate and ammonium varied over the fermentation (Fig. 5B). Both strains consumed the same amount of asparagine within 60 hours, but they displayed very different pattern of consumption. *S. uvarum* WT showed low consumption of asparagine during the first 30 hours of fermentation, with low accumulation of aspartate in the medium (below 20 mg/L). In contrast, during the first 18 hours of the *S. uvarum::ASP3* fermentations there was rapid consumption of asparagine and a higher accumulation of aspartate (30 mg/L). Ammonium concentration was constant during the 60 hours for both strains, so the cells consumed it immediately as it was not accumulated in the media.

### Role of Asp3 in fermentation of apple juice

The growth advantage of *S. uvarum::ASP3* with asparagine as a nitrogen source led us to investigate how this strain would perform in apple juice, where it is known that asparagine is present at higher concentrations than other amino acids. In the natural apple juice we used, the concentrations of asparagine, aspartate and  $\text{NH}_4$  were 95 mgN/L, 10 mgN/L, and 0.6 mgN/L,

respectively. Despite the presence of asparagine as the main nitrogen source, however, the fermentation profiles of WT and *S. uvarum::ASP3* were indistinguishable (Fig. 6A). Furthermore, the consumption patterns for the different nitrogen sources present were largely identical, with ammonium depleted first, then aspartate and finally asparagine (Fig. 6B). There were also no detectable differences in the production of central carbon metabolites and the only variation at all was a transient reduction in  $\alpha$ -ketoglutarate production in *S. uvarum::ASP3* (Fig. 5S).

To understand why heterologous expression of ASP3 conferred a growth advantage in MM but not in apple juice where asparagine is the main source of nitrogen, we performed a series of experiments varying different parameters, namely temperature, pH, nitrogen concentration and sugar content (Fig. 7). These experiments compared wild-type and *S. uvarum::ASP3* using growth as the sole measurement of strain performance. The previous observation that *S. uvarum::ASP3* had a growth advantage in MM with asparagine as a sole nitrogen source was evident regardless of when asparagine was limiting (100 mgN/L) or in excess (1059 mgN/L), though the slower growth at 20°C partially obscured the effect at that temperature. When a mixture of nitrogen compounds that mimic the nitrogen content of apple juice was used, the results were the same as with the apple juice. An effect of pH was excluded since raising pH of apple juice from pH 3.5 to pH 7 or reducing the pH of the MM to pH 3.5 had no impact. The final variable assessed was sugar concentration, so MM was prepared with sugar at 100 g/L as opposed to 20 g/L. The concentration of 100 g/L and the glucose:fructose ratio of 30:70 was designed to mimic that of apple juice. As before, in the nitrogen mix, both strains grew equally well and an advantage was seen for *S. uvarum::ASP3* when asparagine was limiting. It was interesting to note that of all the conditions tested, the advantage conferred by ASP3 was greatest in this final condition, an indication perhaps that either the sugar concentration or the ratio of carbon to nitrogen might have some effect. The overall conclusion remains, however, that a benefit of

carrying ASP3 was only seen in circumstances where asparagine is the sole nitrogen source.

## Discussion

There are many potential nitrogen sources available to fermenting yeast and cells have tight control mechanisms to enable efficient use of available resources. When provided with a mixture of inorganic and organic nitrogen, as is typical in a natural environment, these mechanisms allow cells sequentially use nitrogen sources in order of preference (Beltran et al. 2004, Crépin et al. 2012). In such beverage fermentations, nitrogen is generally limiting and the capacity to assimilate available nitrogen determines the rate of fermentation and the profile of AA-derived volatiles that are synthesised. The study of these processes is therefore of interest from genetic, evolutionary, and biotechnological perspectives. Our focus was on the assimilation of asparagine and the evolution and role of the periplasmic asparaginase encoded by ASP3. Previous studies reported that this gene was only rarely found in *S. cerevisiae* and an attractive hypothesis was presented whereby *S. cerevisiae* acquired ASP3 by HGT from *W. anomalus*, another yeast found in biotechnological environments (League et al. 2012). We tested this hypothesis bioinformatically and, in fact, the evidence was not supportive. Whilst we confirmed the low prevalence (~7%) of ASP3 in *S. cerevisiae* by examining 1680 genome sequences, the distribution of ASP3 in multiple clades of the *S. cerevisiae* phylogenetic tree and the syntenic location in all genomes where this could be determined, point to an ancestral origin with multiple losses being more likely than recent HGT in human associated fermentations. ASP3 was not found in > 20 genomes of the related species *S. uvarum* but the experience with *S. cerevisiae* indicates that many more genomes would be required to exclude the possibility that a percentage of strains encode Asp3. The detection of ASP3 in the genomes of other budding yeasts could be taken as an indication that this gene is ancestral in this sub-phylum, but again more genome sequences are required before forming definitive conclusions.

Although we did not find evidence of HGT, with the exception of wine, there was enrichment in isolates from fermentative environments. This suggests that there was selection for ASP3 positive strains, or for retention of ASP3, in strains used for brewing beer or making cider. The distinction between different beverage fermentations can be explained since asparagine is a prominent free amino acid in beer wort and apple juice, but not in grape must. Despite this, there is not any evidence that ASP3 confers an advantage to *S. cerevisiae* since we found that strains with or without ASP3 grew equally well when asparagine was the sole nitrogen source (Fig. 3). It is possible, however, that the different genetic backgrounds of these strains occlude effects of Asp3. To more specifically address the hypothesis that Asp3 would confer a growth advantage in apple juice, we expressed ASP3 in a strain of *S. uvarum* that lacked an endogenous copy. Indeed, we found that growth was enhanced when asparagine was the sole nitrogen source but were surprised that this effect was not also observed in apple juice when asparagine is by far the most abundant source of nitrogen. Detailed analysis of the fermentation kinetics of the strain expressing ASP3 on synthetic medium confirmed that Asp3 cleaved extracellular asparagine to ammonium and aspartate. As a preferred nitrogen source, the NH<sub>4</sub> was immediately assimilated via a Mep transporter and aspartate accumulated outside the cell to be assimilated later in the fermentation by either a specific or a general amino acid permease. It is not certain why the same benefit is not seen when a mixture of nitrogen sources is provided

but we postulate that it relates to regulation via the NCR and SPS systems. Asparagine is not a preferred nitrogen source for *S. uvarum*, and we propose that when an alternative nitrogen source is present at low concentration, it is used first, allowing growth but also triggers the release from NCR as its availability drops. This increases expression of the general AA permeases GAP1 and DIP5, and the asparagine-preferring permeases ACP1 and GNP1, which allows the rapid uptake of asparagine and catabolism via Asp1 and thus no growth impairment is seen.

Other factors that should be considered when interpreting the data are species differences, genetic background of the strains, and additional selective pressures that have shaped strain domestication and evolution. The explanation for why differences are seen when asparagine is the sole nitrogen source or is simply the dominant nitrogen source, highlights the importance that regulation can play. Genetic regulatory circuits are complex systems and already there are multiple examples of rewiring over the course of yeast evolution (Solieri et al. 2021). Thus, while components are conserved, it cannot be assumed that nitrogen regulatory systems function identically in *S. cerevisiae* and *S. uvarum*. Within *S. uvarum*, strain-specific variation is already evident as it was seen that some strains are more effective assimilators of asparagine than others (Fig. 3). Although the total numbers of strains tested was low (7), it was notable that two strains isolated from cider fermentations exhibited far better growth on asparagine than four strains from wine fermentations. This could indicate that, even without ASP3, cider strains have evolved to more efficiently use asparagine, the main source of nitrogen in apple juice, possibly by reprogramming regulatory circuits.

While our study provided some insights on Asp3 and its possible role in alcoholic fermentations, important questions remain. More genome sequences are needed to resolve whether ASP3 truly is ancestral in budding yeasts, and, if so, why has it apparently been lost from so many species? This suggests that its original purpose is generally no longer relevant. Yet, even within *S. cerevisiae*, a minority of strains have retained the gene. The enrichment of ASP3 positive strains in beer and cider fermentations would generally be taken to indicate an advantage for these strains but we did not see this. It should also be recognised, however, that historical selection of strains did not take place with pure cultures and interactions with other microbes could have been important. For example, in a competitive environment, ASP3 strains may outcompete others for asparagine assimilation. Our data showing transient extracellular accumulation of aspartate was also interesting and it could be envisioned that another microbe would use this amino acid and provide a nutrient in return. Although we do not have evidence for this, similar symbiotic interactions are well-documented in fermented beverages (Stadie et al. 2013, Tran et al. 2020, Comitini et al. 2021). Indeed, it was shown that extracellular amino acids can play an important role in *Saccharomyces* community dynamics (Mülleder et al. 2016, Campbell et al. 2018). The possibility that Asp3 has alternative functions also cannot be excluded. Glutaminase activity for instance, has been reported to be a minor activity of asparaginases of yeast, and especially in bacteria and plants (Imada et al. 1973, Borek and Jaskólski 2001, Michalska and Jaskólski 2006, Sanches et al. 2012), as well as the ability to transport dipeptides (Homann et al. 2005). It is possible that some Asp3 variants improved one of these complementary activities in strains that retained the gene. It is clear that further investigations are required but, in overall terms, our study demonstrates how the integration of bioinformatic and experimental approaches can test evolutionary hypothesis and yield new insights of relevance for biotechnology.

## Supplementary data

Supplementary data are available at [FEMSYR](https://femsyr.onlinelibrary.wiley.com/doi/10.1111/1567-1364.12161) online.

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