



Glycolytic Functions Are Conserved in the Genome of the Wine Yeast *Hanseniaspora uvarum*, and Pyruvate Kinase Limits Its Capacity for Alcoholic Fermentation

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ABSTRACT *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*) is a predominant yeast on wine grapes and other fruits and has a strong influence on wine quality, even when *Saccharomyces cerevisiae* starter cultures are employed. In this work, we sequenced and annotated approximately 93% of the *H. uvarum* genome. Southern and synteny analyses were employed to construct a map of the seven chromosomes present in a type strain. Comparative determinations of specific enzyme activities within the fermentative pathway in *H. uvarum* and *S. cerevisiae* indicated that the reduced capacity of the former yeast for ethanol production is caused primarily by an ~10-fold-lower activity of the key glycolytic enzyme pyruvate kinase. The heterologous expression of the encoding gene, *H. uvarum* *PYK1* (*HuPYK1*), and two genes encoding the phosphofructokinase subunits, *HuPFK1* and *HuPFK2*, in the respective deletion mutants of *S. cerevisiae* confirmed their functional homology.

IMPORTANCE *Hanseniaspora uvarum* is a predominant yeast species on grapes and other fruits. It contributes significantly to the production of desired as well as unfavorable aroma compounds and thus determines the quality of the final product, especially wine. Despite this obvious importance, knowledge on its genetics is scarce. As a basis for targeted metabolic modifications, here we provide the results of a genomic sequencing approach, including the annotation of 3,010 protein-encoding genes, e.g., those encoding the entire sugar fermentation pathway, key components of stress response signaling pathways, and enzymes catalyzing the production of aroma compounds. Comparative analyses suggest that the low fermentative capacity of *H. uvarum* compared to that of *Saccharomyces cerevisiae* can be attributed to low pyruvate kinase activity. The data reported here are expected to aid in establishing *H. uvarum* as a non-*Saccharomyces* yeast in starter cultures for wine and cider fermentations.

KEYWORDS enology, genetic markers, chromosomes, ploidy, apiculatus yeast, glycolysis

Alcoholic fermentation in the wine industry is generally attributed to the activity of the wine, beer, and baker's yeast *Saccharomyces cerevisiae*, which, in large-scale fermentations, is routinely added as a starter culture with a cell density of approximately 10⁶ cells/ml (1). Even without the addition of such starter cultures, i.e., in spontaneous fermentations, *S. cerevisiae* usually dominates the fungal population after

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the first few days of vinification, explaining its importance for humankind over thousands of years (2, 3). Besides ethanol, *S. cerevisiae* produces several other compounds that determine the final wine quality, including glycerol, different esters, and fusel alcohols (4). Such compounds may be either desired or deleterious, frequently depending on their concentration. Acetate produced as a by-product of fermentation is generally regarded as unfavorable (5).

In contrast to its prevalence in the later stages of fermentation, *S. cerevisiae* displays a low abundance on the skin of grapes or in freshly prepared musts, with as few as one cell being found on 1,000 grapes (6). Instead, *Hanseniaspora uvarum* is the predominant yeast species and frequently constitutes more than 80% of the yeast population in must during early stages of fermentation (7, 8). *H. uvarum* is also still widely known for its imperfect form, *Kloeckera apiculata*, which led to the coining of the name “apiculate yeasts” due to its lemon-shaped cell morphology. It prevails within the first 48 h of fermentation, after which *S. cerevisiae* usually takes over and persists until the end of fermentation (9, 10). Initially, the decline of the *H. uvarum* population was attributed to its higher sensitivity to increasing ethanol concentrations during the course of fermentation. However, recent studies indicate that antimicrobial peptides secreted by *S. cerevisiae* are more important in reducing the population of non-*Saccharomyces* yeasts (11, 12). Nevertheless, *H. uvarum* forms a major part of the fungal microbiome in wine fermentations worldwide, including those in Austria (13), Brazil (14), China (15), France (16), Germany (17), Italy (18, 19), Portugal (20), Slovakia (21), and Spain (22). It should be noted that *H. uvarum* not only inhabits and ferments grapes but also resides on other fruits such as plums (23, 24) and apples, being a major factor in cider production (25, 26). This yeast is also found on more exotic substrates such as African coffee (27) and in chocolate production (28, 29). *H. uvarum* has further been suggested to be a useful agent in the biocontrol of molds such as *Botrytis cinerea*, one of the major plant pathogens (30). On the other hand, it is considered a spoilage yeast in orange juice, together with *S. cerevisiae* (31), and produces a killer toxin that is active against *S. cerevisiae* and *Candida albicans* (32, 33). These examples demonstrate the widespread nature, economical importance, and future potential of *H. uvarum* in food biotechnology.

Regarding vinification, different natural isolates of *H. uvarum* are known for their high capacities to form fruity esters but are also infamous for producing high levels of acetate and ethyl acetate (28, 34). Since during the first days of wine fermentation, *H. uvarum* can be present at cell densities similar to those of *S. cerevisiae*, even if the latter is added as a starter culture, its metabolism is expected to contribute significantly to the aroma profile and the final wine quality (5). In fact, after initial classifications as a spoilage yeast, different isolates have been tested for their performance in wine fermentations in mixed cultures with *S. cerevisiae*, with promising perspectives (35). In addition, a type strain of *H. uvarum* has been reported to be fructophilic, which could be of special importance regarding stuck fermentations attributed to an imbalance between glucose and fructose (19, 36).

Despite this obvious importance of *H. uvarum* in all kinds of fruit fermentations, data on its genetic makeup remained scarce. Thus, karyotyping approaches suggested the presence of 7 to 9 chromosomes, with high variability between different isolates (37, 38). As expected, *H. uvarum* also appears in deep-sequencing approaches addressing the microbiome in wine fermentations, since it dominates the yeast population and is readily identified by its ribosomal DNA (rDNA) sequences (39, 40). However, only a few genes have been isolated and sequenced, such as those encoding pyruvate decarboxylase or actin (40, 41). The mitochondrial genome of *H. uvarum* has an exceptional structure among fungi, as it is represented by a short, linear DNA molecule (42). Recently, two brief reports on whole-genome approaches have appeared for *H. uvarum* isolates, underlining the growing interest in this yeast, but with limited information regarding genome annotations (43) (GenBank accession number [JPP000000000](https://www.ncbi.nlm.nih.gov/nuccore/JPP000000000)).

Clearly, the determination of the physiological capacities of *H. uvarum*, with the future possibility of employing metabolic design to eliminate undesirable traits, would

profit from more detailed genetic studies. We therefore initiated a genome-sequencing project with a readily available *H. uvarum* type strain. By applying next-generation sequencing approaches, an estimated 93% of the total genome sequence was deciphered, with >80% being assigned to contigs of >50 kb. From the annotation of these data and additional manual sequencing of specific PCR products, we identified more than 4,000 predicted protein-encoding genes, 3,043 of which were assigned putative functions by comparison with characterized yeast genomes. This included the structure of the highly repetitive rDNA repeats. We applied this information to identify genes coding for hexose transporters, the entire glycolytic pathway, and the fermentative pyruvate decarboxylase and alcohol dehydrogenases. Specific enzyme activities relevant for alcoholic fermentation were determined by using crude extracts of *H. uvarum* and *S. cerevisiae*. The functionality of genes encoding key enzymes of this pathway was confirmed by complementation studies with the respective *S. cerevisiae* deletion mutants.

RESULTS AND DISCUSSION

Selection of nonflocculating *H. uvarum* cells from type strain DSM2768. Basic research on other yeast species, especially on the model yeasts *S. cerevisiae* and *Kluyveromyces lactis*, has profited from the use of small sets of laboratory strains after their original isolation from natural sources (44, 45). Therefore, we decided to start genetic work on *H. uvarum* using a readily available type strain (DSM2768; DMSZ [German Collection of Microorganisms and Cell Cultures], Braunschweig, Germany). As shown in Fig. 1A (left), cells of this strain tended to cluster in liquid cultures, which impedes the use of common microbiological and biochemical techniques such as the deduction of cell numbers from measurements of the optical density at 600 nm (OD_{600}). In order to avoid this problem, a selection regime that involved inoculation in liquid yeast extract-peptone-dextrose (YEPD) medium, shaking overnight at 30°C, sedimentation without agitation for 1 h at room temperature, and reinoculation from the upper medium phase was applied. This was repeated more than 60 times over a period of several weeks, resulting in a strain producing exclusively nonflocculating single budding cells in liquid cultures (Fig. 1A, right), which retained the original cell size and shape and was used for all further investigations. Cells measured approximately 2 by 4 μm and are thus considerably smaller than those of *S. cerevisiae*, which are in the range of 5 by 7 μm . It should be noted that the smaller size of *H. uvarum* cells is also reflected in the relationship of the optical density of the cultures to live-cell platings. Thus, we found that an OD_{600} of 1 equals approximately 10^8 CFU of *H. uvarum*, as opposed to 10^7 CFU for *S. cerevisiae*. This should be taken into account when using *H. uvarum* for inoculations in cocultures with *S. cerevisiae* for experimental wine fermentations.

Examination of genome composition. In order to assess the ploidy of the *H. uvarum* type strain employed here, we determined the DNA content per cell using Sybr green-stained G_0 cells and flow cytometry. According to a recent study, this method provides the best alternative for estimations of the ploidy of yeast cells (46). As controls, haploid and diploid strains of the milk yeast *Kluyveromyces lactis* were employed, which should have a haploid genome size of 10.7 Mbp, i.e., slightly larger than that of *H. uvarum*, which is approximately 9 Mbp. As evident from Fig. 1B, the DNA content per cell for the type strain of *H. uvarum* more closely resembled that of the diploid control cultures than that of the haploids. Moreover, we were unable to select colonies that were resistant to 5-fluorootic acid (5-FOA), i.e., a mutation in the *H. uvarum ura3* (*Huura3*) gene, after ethyl methanesulfonate (EMS) mutagenesis despite several attempts. Only after the prior induction of chromosome loss by nocodazole treatment, a method inspired by work on the diploid wine yeast *Zygosaccharomyces bailii* (47), were two *Huura3* mutants obtained in presumed aneuploid derivatives (Fig. 1B). Taken together, these findings suggested that the genome of the *H. uvarum* type strain employed here is probably diploid. This assumption is further supported by the data from genome sequencing, where variants in $50\% \pm 5\%$ of the reads were found in 10% of the annotated open reading frames (ORFs) of sequences with at least 20-fold read

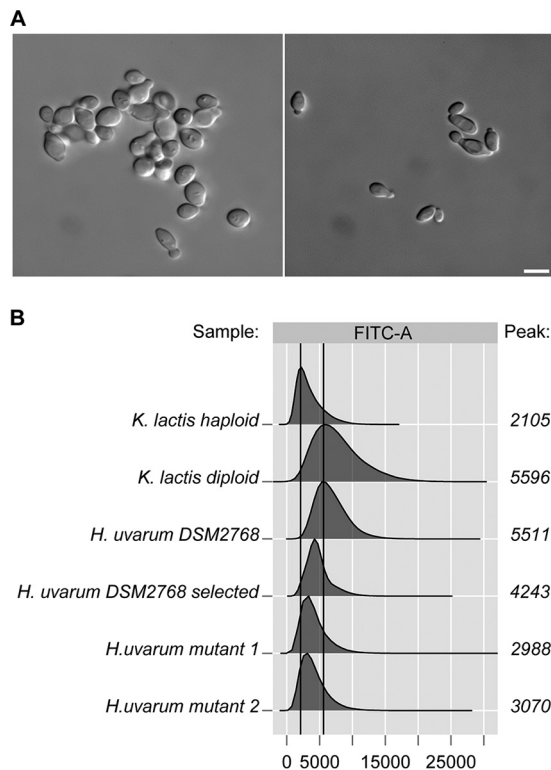


FIG 1 Countersélection of clustering in liquid cultures of *H. uvarum* type strain DSM2768. (A) Micrographs of cultures of the original *H. uvarum* type strain obtained from the stock culture (left) and after selection for nonclustering cells (right). Cells for image preparations were grown to logarithmic phase in YEPD medium. Bar, 2 μ m. Note that clustering of the original strain was also observed by FACS analysis. (B) Flow cytometric analyses of DNA contents of different yeast strains. FACS analysis was performed on the yeast indicated and as described in Materials and Methods. The x axis corresponds to the fluorescence intensity of the DNA dye, and the number of cells is depicted on the y axis. One million cells were measured for each experiment. The peak for each distribution was calculated and plotted next to the corresponding graph. *H. uvarum* mutants 1 and 2 refer to *Huura3* mutants selected on 5-FOA after prior treatment with nocodazole to induce aneuploidy. FITC, fluorescein isothiocyanate.

coverage. In addition, Fig. 2 shows that the mean of the fitted normal distribution of the percentage of all variant reads in open reading frames ($n = 1,717$) is very close to 50%. The fact that these mismatches are distributed over all large contigs assigned to the different chromosomes argues in favor of the yeast being entirely diploid rather than aneuploid for only some chromosomes.

Different natural isolates of *H. uvarum* were previously investigated for their chromosomal constitution by pulse-field gel electrophoresis (PFGE) and yielded different numbers of chromosomes and genome sizes (37). In order to check the adapted type strain described above for these parameters, we used FIGE (field inversion gel electrophoresis) separations. As exemplified in Fig. 3A, these analyses revealed that the type strain used here most likely contains 7 chromosomes, with chromosomes VI and VII having similar sizes, so they could not be separated (the genome analysis data presented below strongly indicate that two different chromosomes form this signal). The electrophoretic mobility in FIGE analyses is inversely proportional to the chromosome size; i.e., the larger chromosomes migrated faster. Based on this, all chromosomes detected add up to a genome size of ~ 9 Mbp (Table 1). This correlates well with the 9.6-Mbp genome size calculated for another *H. uvarum* type strain (37). We further noticed a high degree of variation observed in karyotyping analyses of different natural isolates of *H. uvarum* with regard to the numbers of chromosomal bands and the total genome sizes, which we also found in our investigations of isolates from grapes from different locations in Germany and Spain (data not shown).

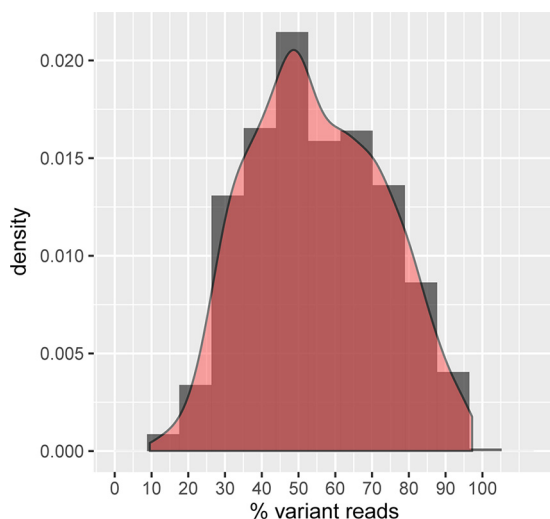


FIG 2 Reads in heterozygosity loci in the genome of the *H. uvarum* type strain. Shown is a histogram (dark gray) and a fitted normal distribution curve (red) of the percentage of reads displaying a variant in all loci showing heterozygosity ($n = 1,717$ loci). The peak at 50% variant reads suggests that the annotated genome is diploid.

Gels used for the karyotyping analysis described above were further employed for Southern blot analyses to assign the larger contigs obtained by genome sequencing as described below to specific chromosomal bands. Mixtures of three PCR-generated probes from each contig were used for this purpose (Fig. 3B). All Southern data obtained are presented in Fig. S1 in the supplemental material. These results combined with results of synteny analyses of the genome sequence (see below) using the Yeast Gene Order Browser (YGOB) server (<http://ygob.ucd.ie/>) (48) allowed us to create an approximate chromosome map of *H. uvarum* (Fig. 4) (for detailed descriptions of these analyses, see reference 49). An indication of the validity of these analyses was provided by the finding that outward primers designed for contigs 13 and 16 employed for PCR on genomic DNA of *H. uvarum* revealed that they were indeed located next to each other and separated by only a small gap of approximately 900 bp (Fig. S2).

Sequence and annotation of the *H. uvarum* genome. In order to facilitate future genetic manipulations and gain more insight into the phylogenetic position of *H. uvarum*, we decided to take advantage of the ease of modern sequencing techniques. Since longer reads significantly simplify the assembly of *de novo*-sequenced genomes, we utilized a combination of long PacBio RS reads with reads from a GS FLX run used for error correction, as described in Materials and Methods. As judged from the calculations of chromosome separations reported above, an estimated 93% of the total genome sequence could thus be obtained. In summary, 360 contigs with a total size of >9.7 Mb now represent the annotated genome.

Regarding its phylogenetic relationships, genome sequence and synteny analyses indicated that *H. uvarum* belongs to the group of yeasts not having undergone a whole-genome duplication (WGD) and is most closely related to *Kluyveromyces lactis*, more distantly related to *Ashbya gossypii* (*Eremothecium gossypii*), and clearly distinct from the *Saccharomyces sensu stricto* group of yeasts (Fig. 5). Interestingly, this positions *H. uvarum* quite close to the whole-genome duplication event in phylogenetic trees. It should be noted that our genome sequence available in GenBank (accession number [APLS01000000](https://www.ncbi.nlm.nih.gov/nuccore/APLS01000000)) was recently included in a highly detailed phylogenetic tree of yeast evolution, which supports these conclusions (50).

With respect to genome composition, relating the size of chromosome VII with the assigned contigs and the size of a single rDNA repeat determined by our separate analyses of cloned PCR products (see Fig. S3A in the supplemental material), we calculated a repeat number of approximately 40 rDNA units to be present in the *H.*

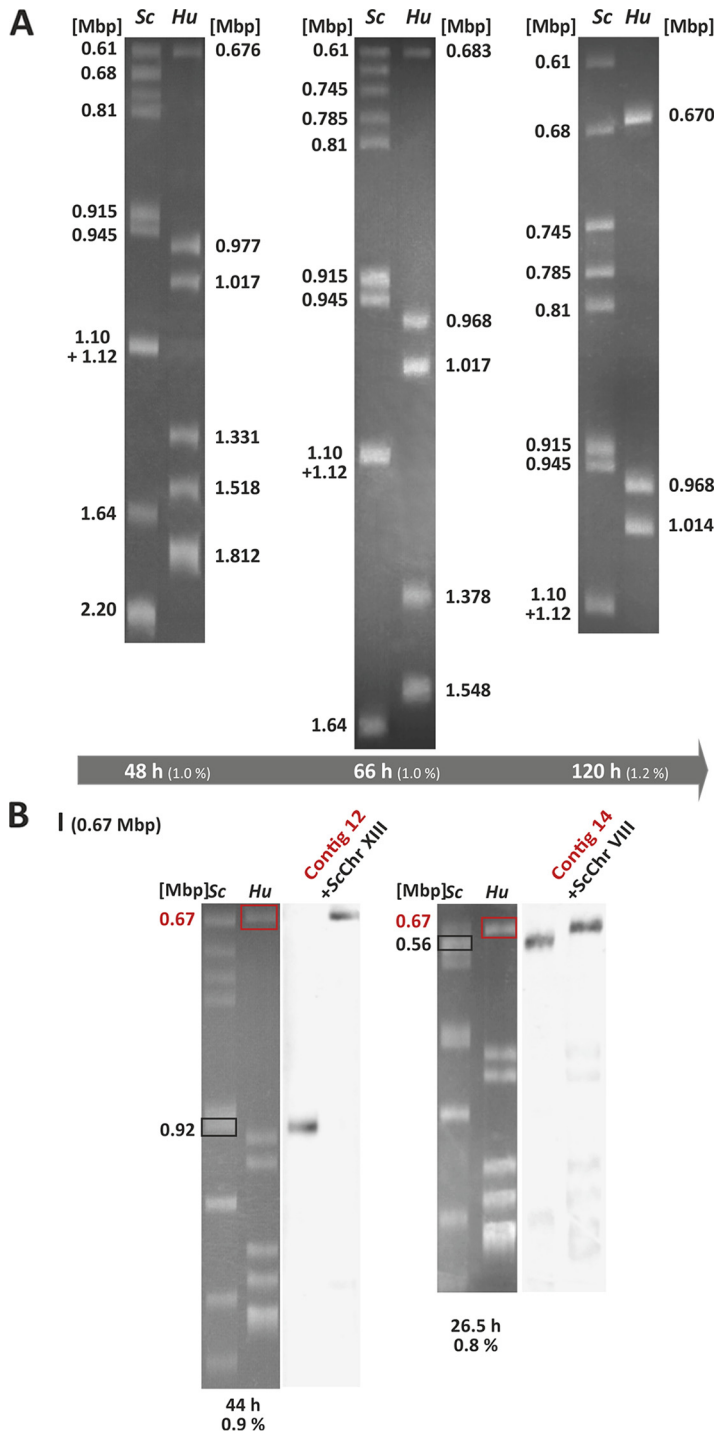


FIG 3 Karyotyping of *H. uvarum*. (A) PFGE analysis of the chromosomal constitution of the *H. uvarum* type strain. Chromosomal bands appearing after staining with ethidium bromide are shown, with three representative separation conditions (run times and agarose gel concentrations, as indicated at the bottom) being depicted. *Sc*, chromosomes from *S. cerevisiae* used as a size standard; *Hu*, chromosomes from *H. uvarum*. Numbers at the left and right indicate sizes in megabase pairs. (B) Example of Southern analysis for the assignment of contigs 12 and 14 to the smallest *H. uvarum* chromosome, chromosome I. DNA was stained with ethidium bromide (left) (white bands on a gray background). Gels were blotted and hybridized with a mixture of DIG-labeled probes with one probe for the indicated chromosomes of *S. cerevisiae* (right) (dark bands on a light background). Data from the entire set of experiments used to construct the genome map can be found in Fig. S1 in the supplemental material.

TABLE 1 Chromosomes and sizes deduced from FIGE gels and sequence analysis^b

Chromosome	Contig	Size (Mbp)	Coverage (%)	
I (0.67 Mbp)	12	0.227	66	
	14	0.215		
	Sum	0.44		
II (0.97 Mbp)	3	0.564	58	
	Sum	0.56		
III (1.02 Mbp)	2	0.572	97	
	4	0.412		
	Sum	0.98		
IV (1.36 Mbp)	7 ^a	0.345	81	
	8	0.323		
	10	0.263		
	18	0.162		
	Sum	1.09		
V (1.52 Mbp)	6	0.355	65	
	11	0.251		
	15	0.197		
	17	0.186		
	Sum	0.99		
VI and VII (1.77 Mbp)	VI	5	0.364	62
		9	0.309	
		13	0.226	
		16 ^a	0.193	
		Sum	1.09	
VII	VII	1	0.998	80
		rDNA	0.298	
		25	0.110	
		Sum	1.41	

^aContigs 7 and 16 give an additional signal on chromosome I.

^bPercent coverage gives the estimated percentage of the sequence obtained for each chromosome.

uvarum genome, which are located between contigs 1 and 25, presenting overlapping terminal sequences (Fig. 4). This is about 30% of the number of rDNA units reported for *S. cerevisiae* and may be attributed to the smaller size of *H. uvarum* cells (Fig. 1), which could explain the smaller number of ribosomes per cell.

Telomeres are also chromosomal structures that are difficult to assign by genome-sequencing approaches due to the large number of short nucleotide repeats. We found 28 small contigs with putative telomeric sequences. Their general composition is depicted in Fig. S3B. This number well exceeds the 14 telomeres expected from the presence of seven chromosomes and may be explained by the small sizes of the contigs on which these sequences are located, owing to their high degree of redundancy; i.e., several of these contigs may in fact constitute parts of the same chromosome ends.

As mentioned above, *H. uvarum* is the sexual form of anamorphic *K. apiculata* and should therefore undergo meiosis and produce spores (51). Despite several attempts with the *H. uvarum* type strain used here, we were unable to detect any hint of sporulation or sexual reproduction. In fact, genome analyses did not reveal the presence of a dimorphic mating type locus or of further silenced mating gene copies. Although the lack of these features could be assigned to the 7% of the genome not yet sequenced, alternative explanations are that sexual reproduction has been lost, at least in this type strain, or that the mating loci escaped detection by homology and synteny alignments due to high sequence divergence. In this context, it should be noted that a high degree of genetic variability was observed in numerous natural isolates of *H. uvarum* from wineries (52). Moreover, the loss of sexual reproduction may not be uncommon, as it is frequently found even in strains of *S. cerevisiae* used by fermentation industries (2).

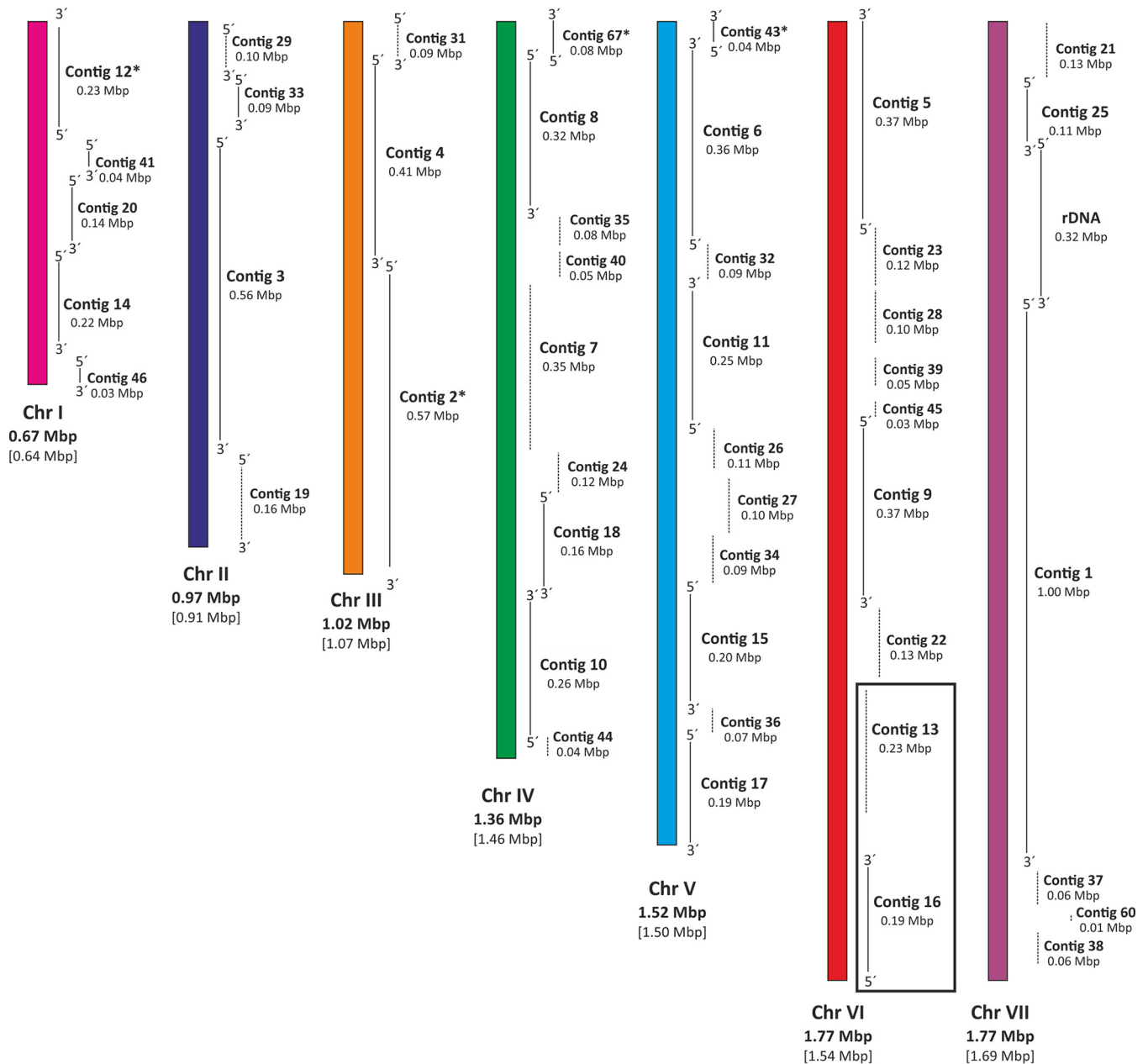


FIG 4 Draft of a genome map for *H. uvarum*. Sequence data, annotations, data from synteny analyses, and data from the Southern analyses described in the text and in Fig. S1 in the supplemental material were combined to assemble a predicted order of large contigs on the seven chromosomes. Contigs and contig sizes are indicated. Note that the gaps between contigs are most likely not drawn to scale, due to the lack of sequence information.

The annotation of 3,010 protein-encoding genes also indicated a number of physiological capacities with implications for wine making, which are compiled in Tables S1 to S3 in the supplemental material. Thus, homologs of all genes encoding enzymes necessary for glucose fermentation, including putative hexose transporters, were found. However, as expected from a nonduplicated genome, less redundancy was observed, with a maximum of 8 putative hexose transporter homologs compared to 20 in the *S. cerevisiae* genome (53) (Table S1). Likewise, only one homolog each of the two hexokinase and two enolase genes present in *S. cerevisiae* could be identified in the *H. uvarum* genome. Regarding possible effects on wine quality, we were able to identify 28 genes in *H. uvarum* with putative functions in the production of aroma compounds, which include esterases and glycosidases but also overlap the subset involved in

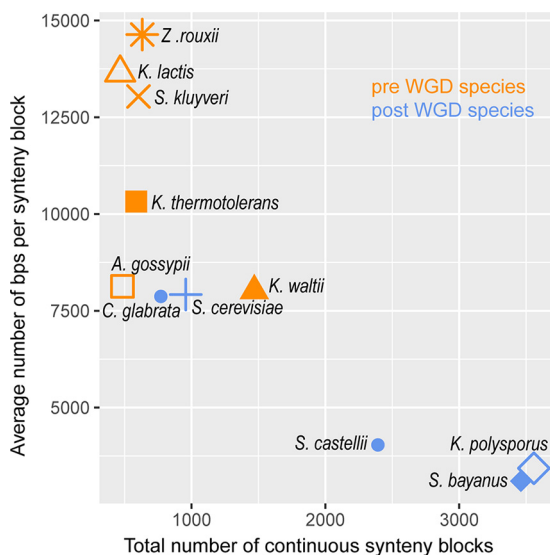


FIG 5 Synteny of the *H. uvarum* genome with the genomes of other yeast species. Relating the number of continuous synteny blocks to their average size, the annotated *H. uvarum* genome most closely resembles that of *Kluyveromyces lactis* and belongs to the group not having undergone a whole-genome duplication (pre- and post-WGD species). Genomes of the following species were obtained from YGOB (<http://ygob.ucd.ie/>) (48) (with YGOP file names and, if existent, alternative names of the species compared in parentheses): *Zygosaccharomyces rouxii* (Zrouxii_sequence.fsa), *Kluyveromyces lactis* (Klactis_sequence.fsa), *Saccharomyces kluyveri* (*Lachancea kluyveri*; Lkluyveri_sequence.fsa), *Kluyveromyces thermotolerans* (*Lachancea thermotolerans*; Lthermotolerans_sequence.fsa), *Ashbya gossypii* (*Eremothecium gossypii*; Egossypii_sequence.fsa), *Candida glabrata* (Cglabrata_sequence.fsa), *Saccharomyces cerevisiae* (Scerevisiae_sequence.fsa), *Kluyveromyces waltii* (*Lachancea waltii*; Lwaltii_sequence.fsa), *Saccharomyces castellii* (Ncastellii_sequence.fsa), *Kluyveromyces polysporus* (*Vanderwaltozyma polyspora*; Vpolyspora_sequence.fsa), and *Saccharomyces bayanus* (Suvarum_sequence.fsa).

central carbohydrate metabolism in the production of glycerol and aldehydes (Table S2). Interestingly, at least six putative genes for xylose-degrading activities were identified, which are represented by only one homolog in either *S. cerevisiae* or *Aspergillus nidulans*, supporting the notion that *H. uvarum* contributes significantly to the production of aroma compounds in the process of vinification. Finally, survival in vineyards and in wine fermentations requires the presence of various stress response pathways in yeast cells (54), which we also detected in *H. uvarum* (Table S3). These include homologs of most components of the yeast cell wall integrity (CWI) pathway, again with less redundancy of components of the downstream mitogen-activated protein kinase (MAPK) pathway (55). Interestingly, *H. uvarum* seems to have a single isoform of protein kinase C (Pkc), which, as in *S. cerevisiae*, displays a prototypic structure of Pkc isoforms found in higher eukaryotes, including the regulatory homology domains mediating interactions with a small GTPase located near the N-terminal end (56, 57). In addition, homologs encoding proteins of the high-osmolarity glycerol (HOG) pathway (58) were identified. Although some intermediary components have not been annotated, the homologs detected indicate that the pathway, like its counterpart in *S. cerevisiae*, is bifurcated in the upstream signaling components. We also found several genes with putative functions in the oxidative stress response (59) (Table S3). Although transcription factors such as Yap1 and Skn7 did not appear in this search, the presence of detoxifying enzymes such as Sod1 and Gsh1/Gsh2 indicates that *H. uvarum* encounters and is well equipped to cope with oxidative stress. Together, these findings suggest that *H. uvarum* disposes of the necessary mechanisms to respond properly to stresses encountered in the vinification process.

Comparative investigation of fermentative capacities of *H. uvarum* and *S. cerevisiae*. *H. uvarum* and other non-*Saccharomyces* yeasts that dominate the first stage of spontaneous wine fermentations have a limited capacity for alcohol production (60). We decided to address this question in more detail by determining the

TABLE 2 Comparative analysis of specific enzyme activities relevant for alcoholic fermentation in *H. uvarum* and *S. cerevisiae*^a

Enzyme	Abbreviation	Mean sp act (mU/mg protein) ± SD		Ratio of sp act of <i>S. cerevisiae</i> / <i>H. uvarum</i>
		<i>Hanseniaspora uvarum</i>	<i>Saccharomyces cerevisiae</i>	
Hexokinase	Hxk	525 ± 24	594 ± 38	1.1
Phosphoglucose isomerase	Pgi	1,192 ± 23	1,416 ± 118	1.2
Phosphofructokinase	PFK	361 ± 22	556 ± 84	1.5
Aldolase	Fba	420 ± 128	459 ± 87	1.1
Triosephosphate isomerase	Tpi	22,581 ± 2,847	26,804 ± 2,329	1.2
Triosephosphate dehydrogenase	Tdh	718 ± 305	937 ± 297	1.3
Phosphoglycerate kinase	Pgk	753 ± 157	1,033 ± 267	1.4
Phosphoglycerate mutase	Gpm	1,379 ± 243	1,987 ± 366	1.4
Enolase	Eno	1,251 ± 255	2,015 ± 326	1.6
Pyruvate kinase	Pyk	193 ± 61	3,263 ± 362	16.9
Pyruvate decarboxylase	Pdc	494 ± 169	707 ± 160	1.4
Alcohol dehydrogenase	Adh	1,727 ± 201	3,802 ± 699	2.2

^aSpecific enzyme activities are given in milliunits per milligram of protein at 30°C. The means of data from at least three biological and three technical replicates for each enzyme and yeast species were determined, and standard deviations were calculated. Cells were grown in rich medium with 1% glucose and 1% fructose (YEFD medium plus fructose) prior to the preparation of crude extracts. For a comparison of specific activities after growth with higher sugar concentrations and with ethanol as a carbon source, see Fig. S4 in supplemental material.

specific activities of all 12 enzymes involved in alcoholic fermentation in *H. uvarum* under different growth conditions and comparing them to those of a diploid laboratory strain of *S. cerevisiae* (HHD1). The latter is derived from the CEN.PK series (61), has a fermentation capacity and an aroma profile similar to those of commercial yeast strains, and is thus also suitable for spirit production (62).

As summarized in Table 2, most of the specific enzyme activities measured in extracts from cells grown in the presence of 1% glucose plus 1% fructose are in the same range for *H. uvarum* and *S. cerevisiae*; i.e., differences are <2-fold. This indicates a general conservation and similar capacities of the glycolytic pathways and the subsequent reactions leading to ethanol production in the two species. However, pyruvate kinase shows a striking difference, with *H. uvarum* having a >15-fold-lower specific activity than that of *S. cerevisiae*. This is especially interesting since pyruvate kinase catalyzes the second irreversible step specific for glycolysis after phosphofructokinase (PFK), thus being an ideal target to control metabolic flux. In fact, pyruvate kinase has been suggested to be a major determinant at the branching point of respiratory and fermentative sugar degradation (63). Of note, fructose-1,6-bisphosphate, the product of the PFK reaction, is a potent allosteric activator of pyruvate kinase in *S. cerevisiae*, thus connecting the two controlling steps of sugar degradation (64). We conclude that the reduced activity of pyruvate kinase in *H. uvarum* may be a major factor explaining the lower fermentative capacity of this yeast than of *S. cerevisiae* and also its classification as a Crabtree-negative yeast (65). It should be noted that specific pyruvate kinase activities are increased in both *S. cerevisiae* (approximately 2-fold) and *H. uvarum* (approximately 10-fold) when cells are grown in the presence of 20% as opposed to 2% sugar, i.e., under conditions similar to those of must fermentations (Fig. S4). Nevertheless, activities are still 3-fold lower in *H. uvarum* than in *S. cerevisiae* and are expected to decrease during the course of wine fermentations, as sugars are degraded. Although the specific activities of several other enzymes tested after growth with high sugar concentrations were also higher, the increases were similar for *H. uvarum* and *S. cerevisiae*, not affecting the ratios of <2-fold differences between the two species. The same is true for the generally lower specific activities measured after growth on 2% ethanol as a nonfermentable carbon source (Fig. S4).

From data obtained for a different *H. uvarum* isolate grown in chemostat cultures at different dilution rates, it was previously concluded that glucose degradation is limited by a step “before pyruvate formation,” which is in good agreement with our findings (66). Those authors determined the specific activities of a number of fermentative enzymes and those of pyruvate dehydrogenase and acetyl coenzyme A (acetyl-CoA) synthase, concluding that the low activity of the latter is a major cause of acetate

production. It should be noted that the specific activities of the fermentative enzymes reported here differ considerably from those determined in the work cited above, which may be attributable to different assay conditions. Besides the use of batch cultures here, fructose-2,6-bisphosphate and fructose-1,6-bisphosphate, potent allosteric activators of phosphofructokinase and pyruvate kinase, respectively, for example, were apparently not added to mixtures in the enzymatic assays reported previously (66). Nevertheless, the aim of the present study was to compare specific enzyme activities of the *H. uvarum* type strain and *S. cerevisiae*. Since the same assay mixtures were used to assess activities in crude extracts from both species, it allowed us to assess the relative contribution of each step to alcoholic fermentation.

Functional analyses of genes encoding phosphofructokinase and pyruvate kinase. The data described above suggested crucial differences in the activity of pyruvate kinase in *H. uvarum* compared to that in *S. cerevisiae*. These differences may be attributed to either different regulatory circuits governing *PYK1* gene expression in the two yeast species or inherent properties of the enzyme itself. In order to distinguish between these possibilities, we decided to express the *HuPYK1* gene in a *pyk1* deletion mutant of *S. cerevisiae*. Indeed, *HuPYK1* introduced on a *CEN/ARS* vector rescued the glucose-negative growth phenotype of the deletion strain VWH3B (*pyk1::HIS3*), indicating functional complementation. This was confirmed by enzyme assays of cultures grown in rich medium with 2% glucose, yielding specific pyruvate kinase activity of 465 ± 100 mU/mg protein, as determined by using two independent transformants carrying plasmid pJJH2144 and three technical replicates. This value is higher than the specific activity determined for the wild-type *H. uvarum* strain described above under similar growth conditions (193 ± 39 mU/mg protein) (Table 2) but only approximately 15% of that observed for transformants with a plasmid carrying the *S. cerevisiae* *PYK1* (*ScPYK1*) gene ($3,179 \pm 270$ mU/mg protein) or diploid wild-type strain HHD1 ($3,087 \pm 163$ mU/mg protein). However, placement of the *HuPYK1* open reading frame under the control of the *ScPYK1* promoter yielded $1,982 \pm 82$ mU/mg protein when it was introduced into VWH3B (*pyk1::HIS3*), demonstrating that the low specific activity is probably due to a low level of gene expression rather than being determined by the structure of the heterologously produced enzyme.

Finally, the key glycolytic enzyme PFK of *S. cerevisiae* is a hetero-octamer, composed of four α - and four β -subunits, which are encoded by the genes *PFK1* and *PFK2*, respectively (67). While mutations in either of these two genes do not prohibit growth on glucose, *pfk1 pfk2* double mutants are glucose negative (68, 69). This has been attributed to the conservation of all catalytic and allosteric domains in each of the encoded subunits, retaining the activity of homooligomeric forms *in vivo* but not *in vitro* (70). For *H. uvarum*, we also identified two homologous genes in the annotated sequence and designated them *HuPFK1* and *HuPFK2* based on sequence alignments and synteny analyses. These genes encode proteins with deduced molecular masses of 103,428 Da and 85,351 Da, respectively. It should be noted that the annotated genome sequence of *HuPFK1* is lacking an adenine nucleotide at position +442 relative to the correct ATG translation start codon, as confirmed by cloning and Sanger sequencing (GGAAGCCACCAAAAAAGAAAAAATTGCTG). Thus, the underlined sequence in fact contains five rather than four nucleotides, which leads to a considerable extension of the 5' end of the predicted *HuPFK1* open reading frame. For use in further studies, we therefore again obtained the entire coding sequences by Sanger sequencing of cloned PCR products and submitted the sequences of the genes under the accession numbers given in Materials and Methods.

When individually introduced on *CEN/ARS* vectors into an *S. cerevisiae* *pfk1 pfk2* double-deletion mutant (HD114-8D [*pfk1::HIS3 pfk2::HIS3*]), either gene from *H. uvarum* complemented the glucose-negative phenotype, indicating that the subunits are functionally conserved and that they also exert catalysis *in vivo*. However, as for *S. cerevisiae* single-deletion mutants, PFK activity was below detectable levels in enzyme assays of crude extracts from strains carrying either of the *HuPFK* genes, even when introduced on multicopy vectors (Table 3). However, the introduction of both heterologous genes

TABLE 3 Specific activities of phosphofructokinase^a

Strain (relevant genotype)	Plasmid	PFK gene(s) introduced	Mean sp act of PFK (mU/mg protein) ± SD
<i>H. uvarum</i> (HuPFK1 HuPFK2)	None	None	413 ± 25
HHD1 (PFK1 PFK2)	None	None	326 ± 33
HOD114-8D (<i>pfk1::HIS3 pfk2::HIS3</i>)	None	None	<1
	YCp111u	ScPFK1	<1
	pJH2203	ScPFK2	<1
	pJH2200	ScPFK1 ScPFK2	384 ± 27
	pJH2185	HuPFK1	<1
	pJH2208	HuPFK1	<1
	pJH1868	HuPFK2	<1
	pJH2209	HuPFK2	<1
	pJH1856new	HuPFK1 HuPFK2	63 ± 6
	pJH2211	HuPFK1 HuPFK2	2,999 ± 232

^aSpecific PFK activities were determined from at least three biological replicates and three technical replicates, with the exception of pJH1856new, for which only two biological replicates were employed. Cells were grown in rich medium with 2% glucose.

together on a *CEN/ARS* vector restored *in vitro* PFK activity in the double-deletion mutant, which amounted to approximately one-third of the specific activity determined for either the *H. uvarum* or *S. cerevisiae* wild-type strain. As expected, overproduction by the expression of the two genes from a multicopy vector yielded much higher activities. This indicates that PFK of *H. uvarum* is also an oligomeric enzyme and may form a hetero-octamer, like its counterparts in *S. cerevisiae* and *K. lactis* (71). The overall domain structure of the subunits, which retain both the catalytic and allosteric substrate binding sites, is consistent with the observation that sufficient glucose can be metabolized *in vivo* by either HuPfk1 or HuPfk2. However, as in *S. cerevisiae*, heterooligomerization seems to stabilize the enzyme structure, so *in vitro* activity can be observed only if both subunits are produced in one cell (70). The lower specific activity measured in the transformants with the two *HuPFK* genes carried on a *CEN/ARS* vector than in the wild-type strains of both species could be due to a lower expression level caused by the native *HuPFK* gene promoters in *S. cerevisiae*, analogous to the data presented above for *HuPYK1*. It should also be noted that heterooligomeric complexes between subunits of the two species either do not form or are not stable, since transformants of single *Scpfk1* or *Scpfk2* deletion mutants with the respective *HuPFK* genes did not restore measurable enzyme activity in crude extracts (data not shown).

The enzymatic determinations and data from sequence analyses were further substantiated by performing Western blot analyses using antiserum raised against ScPFK, which also detects the subunits of *K. lactis* PFK (72). As shown in Fig. 6, the *HuPFK* subunits migrate according to their deduced molecular weights, with the β -subunit encoded by *HuPFK2* migrating much faster than its counterpart from *S. cerevisiae*. While all PFK subunits from different yeasts and fungi analyzed so far carry an N-terminal elongation of approximately 200 amino acids compared to their bacterial or mammalian homologs, this extension is missing in the β -subunit of *H. uvarum*. Determinations of PFK activities after controlled proteolysis, which cleaved off the N-terminal parts of the *S. cerevisiae* enzyme, demonstrated that these extensions are actually not required for catalysis (73). It is thus rather surprising that sequences encoding the N termini of PFK subunits should have been eliminated only during the evolution of the *HuPFK2* gene and should not have occurred in other yeasts. With the growing number of reported genome sequences of other non-*Saccharomyces* wine-related yeasts, it will be interesting to see if this is indeed a single "evolutionary accident" confined to *H. uvarum*. If so, this would indicate a selective advantage of larger subunits whose biological significance remains to be elucidated.

Conclusions. Here we present a comprehensive analysis of the genome of an *H. uvarum* type strain, including extensive annotation. This and other non-*Saccharomyces*

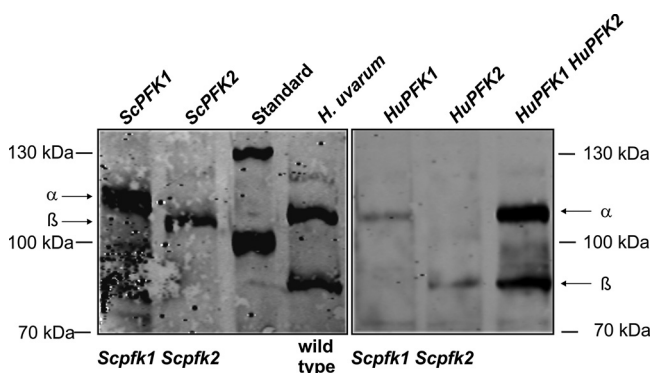


FIG 6 Western blot analysis of yeast phosphofructokinase. Total protein was prepared from strain HD114-8D (*Scpfk1 Scpfk2*) (see Materials and Methods for the complete genotype) carrying plasmids with the *PFK* genes indicated above the blots or from the adapted wild-type *H. uvarum* strain. Samples were separated by SDS-PAGE, blotted, and subjected to immunological detection with ScPFK antiserum as described in Materials and Methods. α and β refer to the PFK subunits encoded by *PFK1* and *PFK2* for *S. cerevisiae* (left) and *H. uvarum* (right). Plasmids employed were YCp111u (*ScPFK1*), pJH2203 (*ScPFK2*), pJH2208 (*HuPFK1*), pJH2209 (*HuPFK2*), and pJH2211 (*HuPFK1 HuPFK2*). Note that for reasons of detectability, ~ 10 -fold-larger amounts of protein were loaded for the samples with *H. uvarum* PFKs than for those containing the *S. cerevisiae* subunits, with the exception of the *HuPFK1 HuPFK2* overproducer, for which only double the amount of total protein was loaded. *HuPFK* subunits produced from *CEN/ARS* vectors in the *S. cerevisiae* *pfk1 pfk2* double-deletion strain, although barely detectable, confirmed the subunit sizes depicted here (data not shown).

Yeasts are of growing interest for the production of fermented beverages, especially wine, due to their important contribution of desired aroma compounds (5). The genome sequence presented here provides the basis for future manipulations of the underlying pathways, e.g., for increasing the production of desired and reducing that of undesired metabolites. It also allows the assessment of physiological capacities. Thus, our studies indicate that in *H. uvarum*, pyruvate kinase activity could be a limiting step in alcoholic fermentation. This also has implications with regard to climate change and the corresponding increase in grape sugar content. Thus, cofermentations with non-*Saccharomyces* yeasts have been suggested as a measure to control increasing ethanol concentrations in wine (74). Although our functional studies of key glycolytic enzymes demonstrate the usefulness of the annotated genome sequence, they can be considered just a proof of principle. Further functional studies on enzymes involved in the production of aroma compounds, such as esterases or glycosylases, will follow. Moreover, this genome sequence provides the basis to study wine-related phenotypes, such as the capacity to flocculate and the signaling pathways involved in coping with environmental stress conditions during vinification (54). All these studies would greatly profit from the development of a system for the targeted genetic manipulation of *H. uvarum*, which is the most urgent subject to be addressed in future research on this yeast.

MATERIALS AND METHODS

Strains, plasmids, media, and culture conditions. In this study, type strain DSM2768 of *H. uvarum* was obtained from the German Collection of Microorganisms. It is equivalent to ATCC 9774 (American Type Culture Collection). Presumably aneuploid derivatives with mutations in the *HuURA3* gene were obtained by treatment with nocodazole prior to selection on medium containing 5-FOA. Haploid and diploid reference strains of *Kluyveromyces lactis* employed in fluorescence-activated cell sorter (FACS) analyses were type strains CBS2359 and KHO70, respectively (44). For the preparation of *S. cerevisiae* chromosomes as a size standard, strain BY4743 (Euroscarf, Frankfurt, Germany) was used. For comparisons of fermentative enzyme activities, we used diploid *S. cerevisiae* strain HHD1 (*MAT α /MAT α ura3-52/URA3 LEU2/leu2-3,112*) as a reference, which is a derivative of the CEN.PK series applied previously in fermentations for spirit production (62). HD114-8D (*MAT α ura3-52 leu2-3,112 his3-11,15 pfk1::HIS3 pfk2::HIS3*) (75) served as a recipient strain for *PFK* gene expression, and VWH3B (*MAT α ura3-52 leu2-3,112 his3 Δ 1 trp1-289 pyk1::HIS3*), also a derivative of the CEN.PK series, was used for the expression of genes encoding pyruvate kinase.

Standard procedures for the handling of yeast, *Escherichia coli* strain DH5 α , and DNA were followed, as described previously (76). Genes from *H. uvarum* were amplified by PCR using the High Fidelity *Taq*

TABLE 4 Oligonucleotides used to generate contig-specific probes used in Southern analyses^a

Contig	Forward primer	Reverse primer
1	TTCAATTCGCAAGTGATGG CCATACATTTGCCAAGGTTT CGCAAGCCAATGTACCAAAG	TATAGAGGCAGGTAACGTGAG CTTACCACCAAAGATCATT CACCGTTACACAACCTACC
2	AACGCTAACAAATTCTACAAG ATATTGAAGTACCGCAATG TCACCAACCACATCCAGTAG	TTAGGCCCTATAGTCAAATC TCTTGGTAACCATGACTAAC AAGCATCTCTCAACTGTAG
3	ATTCTCCACTTAGGACTTAC GCATCAACTCGTCAAGATAG GTTCTGTTCTGGTGAATC	ATTCATCACCACCAGTATTG GTAGACAGTGAGGTTGAAAG CTAGGCTCAGTCTCTACTTC
4	TGTTCTCTGTTGGAGGATTC CAACAACCAACTGGTTTCTC TGCCGTTGATAGATTCTG	GAGTTGCAAGATGCTGTTTC AGCAGCTATGACTGCTAGAG ATGTAGATGCAGAATCTACC
5	TTTCTACTTCGCTTAATTGC TGAACATGACCAGGAATTAG AATTCGCTGTGGCAAATTGG	TCGGTACTTCAAGAAATGG TCCAGTATGAGGACATTTC GTCAATCACCTTTGTTATCG
6	TTGCATTAACAGCGCAAAG TAAGACAGAGTCGTCTAGAG TGGGTGCAAGAAATAGATGG	ACCAAATCCAACGACCAATG AATTGCGGCTGATGAGTAAG AACGTCTCCACTGCTCTATC
7	AATCTGCTTCAGGCTGATAC TCTTATCAGCCACATGTCAC CTTGCCCTGGGTTGTTGATAG	ATACCACTTCTTCTGGAATG ATGGACTATTAGGCGATAAC TCCTCCCTTAGAGAAGTTG
8	TCTACCATAGCCAATCAAAG GCTTACAGCACCTAAAGATG TTGACATGTTGAGGTTGTTT	CCAGACTTAGAGCTGATGAC GGCTCAAATTTAGCACCATC GATCCCTGCTTACTTGAC
9	AACTAAGGCAGTATCCAAAG TGCCTACCTTAAATTCTCAC TGAATAACGGAGTGAATAC	GGTTTAGCAGAATCTTTAGC GCAGCTGATCTATCACTTTG GTCATGTTCTTACCATACC
10	AAATGGCCGAAACCATTGGG ATCATCGCCGTTATTAACAC CCTGCATTGTGAATTTCTTG	CTTGTGTTGTTGCTGAATAC GGAAATTAATCCCTGACTTG GCTATATCGCTTGAAGAAGG
11	CGGCAATTTGCGAGTATTTT CATTGCTGCTGGTACTTATG TTAGAGAACCGCTGTTATC	TTGGTCCACAGACCAAAG GCAATTCAGGTGCTCTATAC TACCGTTGTTGTAACAAAG
12	GTCTGAAATATGTGCACTTG CAGAACTGATGCCTTTAATG AGAATCTGCTTCTGCTTCTG	CATGAACCAACTTGAATAG GGTATTTAGGCCACAGTATC CTTGGCCATAAGTAGATTG
13	AATCTGTTGCCTTGTGTTTG CAGCAACTTGCTCGTTATAC AGGTTGACCTTGAGTGAATG	GTATACCTGTGGAGATATGG ACTGATCCGTACATCGAAAG ATCTGCTTTAGCCAACAAGG
14	TAGAGTTACCACTGCTGTTT GCCAACGTAGCACTTTCTTC TCAGATGGACCATTCTTAC	CTGGTATTGCCCCAGTAAC AAAGACCGTTTAAATCAGATG ACTCATCATGACCATCTTC
15	CTCTTTCACAAGCTTCTAAC AAAGTTTGGGATGCTTATG CTACCCAGAAGAATTCAAAG	TGACTTGTTGGCTTCATTTC AACTTCATCAACAGGCTTAC CCAAAGAAGTTGTGGTAGTC
16	GTTAGAGACGCTTTAAACAG CTGACTTCCAGAAAGTATTG ACCCATGCTGTATCCCATTC	AAACGGTTCTAACACCCTTG TCTGCCTTTATATCCATTGC GTACAGTTGTACGAGCATTTC

(Continued on next page)

TABLE 4 (Continued)

Contig	Forward primer	Reverse primer
17	TTTGAATGGACCCATATTG TTGTTTTGAAGACACAAATG TCTATTGGTGGAGCTGAATC	ATTGCTGAGTACCAATTGAC GTCCAAACAAGGCTCATAAC CTTGATAGCCTTGTGAATAG
18	GTATTGACCAGGAACAATAC ATATCAAGCCGTTAGTAAGC AAGGTTACTGGAGCTTTGTG	ATGAGCATCAAATCCTCATC AAAGTACCAGTACTCTATC TGTAGATGATGCTGGTATTG
25	ATATACCTGTCCATCCAAAG TGCAGCAGATAACTCAGAAC CCAATGTAAGCACTGTATTC	AAGATCCTGGCGAATATGTC AACATTGCTTACAGTGTAG TGCTTCTAGCCGCTTCTG

^aMixtures of three probes for each contig generated by the oligonucleotide pairs indicated were used for Southern analyses on blots from FIGE-separated genomic DNA to assign contigs to chromosomes. Sequences are all given in the 5'-to-3' direction.

polymerase kit from Roche (Mannheim, Germany) and cloned by restriction/ligation into yeast-*E. coli* shuttle vectors by either using natural restriction sites or adding the recognition motifs to the 5' ends of the primer sequences, if required. Plasmids employed were based on the *CEN/ARS* vector YCplac33 (77) and the 2 μ m vector YEp352 (78). Genes with their flanking regions were amplified from genomic DNA preparations by PCR with appropriate primer pairs and cloned by conventional restriction/ligation. Sequences of oligonucleotides and the resulting plasmids are available upon request. Specifically, the *PYK1* gene from *S. cerevisiae* was cloned as a BamHI/HindIII fragment into YCplac33 to yield pJH2137. The gene from *H. uvarum* (*HuPYK1*) was also cloned as a BamHI/HindIII fragment into the same vector, yielding pJH2144. To place the open reading frame of *HuPYK1* between the promoter and terminator regions of *ScPYK1*, pJH2145 was obtained by *in vivo* recombination using pJH2137 as a recipient plasmid and a PCR fragment with *HuPYK1* and the appropriate flanking regions for recombination. For phosphofructokinase clones, *ScPFK1* was cloned as an SphI fragment into YCplac33 to yield YCp111u, and *ScPFK2* was cloned as a SacI/SalI fragment to yield pJH2203. The latter fragment was also inserted into YCp111u to give pJH2200, which carries both *ScPFK* genes on the *CEN/ARS* vector. *PFK* genes from *H. uvarum* were obtained as follows. *HuPFK1* was obtained as a SacI/HindIII fragment and cloned into YCplac33 (pJH2185) and YEp352 (pJH2208). *HuPFK2* was obtained as a SacI/PstI fragment and cloned into YCplac33 (pJH1868) and YEp352 (pJH2209). Plasmids containing both *HuPFK* genes were obtained by cloning *HuPFK2* as a Sall/SphI fragment into pJH2208 to yield the multicopy plasmid pJH2211 and by simultaneously cloning *HuPFK1* as a BamHI/SacI fragment and *HuPFK2* as a SacI/PstI fragment into YCplac33 linearized with BamHI/PstI to yield the *CEN/ARS* plasmid pJH1856new.

Rich media were based on 1% yeast extract and 2% Bacto peptone (Difco) with 2% glucose (wt/vol) as a carbon source (YEPD), if not stated otherwise. Synthetic media were based on yeast nitrogen base with ammonium sulfate, supplemented with 2% glucose and a mixture of amino acids and bases as described previously (79), omitting compounds for plasmid selection as required. Mixtures of 10% (wt/vol) glucose plus 10% (wt/vol) fructose were used to mimic the initial stages of grape must fermentation, and 1% (wt/vol) each sugar was used for later stages.

Preparation of genomic DNA and sequencing. The preparation of larger amounts of genomic DNA for sequencing of the *H. uvarum* genome was carried out with the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The resulting DNA was eluted in sterile, distilled water and showed an absorbance ratio at 260/280 nm of ≥ 1.8 and an absorbance ratio at 260/230 nm of ≥ 1.9 .

LS454 sequencing using GS FLX Titanium technology (Roche, Basel, Switzerland) was done by Microsynth AG (Balgach, Switzerland), additional PacBio RS sequencing was performed by GATC Biotech (Constance, Germany), and data were combined to generate the final assembly. The assembly was performed by GATC Biotech using a hybrid approach for error correction of PacBio reads (80), with final assembly being performed by using Celera Assembler (May 2013 version).

Custom Sanger sequencing was performed by GATC Biotech (Cologne, Germany) with oligonucleotide primers obtained from Metabion (Munich, Germany).

Genome annotation and synteny analysis. The main objective of the genome annotation was to accomplish high data reliability. As a first step, we therefore used the Yeast Genome Annotation Pipeline (YGAP) (81) (<http://wolfe.ucd.ie/annotation/>). This pipeline employs preexisting annotations of related species and also includes synteny information. YGAP resulted in a compilation of putative ORFs and a list of tRNA genes. The YGAP list contained 4,300 entries. We decided to further scrutinize this list since it also contained incomplete ORFs (e.g., those with missing start or stop codons). In addition, we found that automated annotation of intron-containing ORFs was not reliable, and we thus excluded all entries of ORFs that were continuous between a start codon and a stop codon. To this end, a list was generated from the assembly by using GetORF (part of the EMBOSS package) (82), and only entries present in the YGAP and GetORF lists were incorporated in further annotations. This resulted in the annotation of 3,010 protein-encoding sequences. Deduced tRNA genes were then added to the resulting list. Annotation of ribosomal DNA was performed with a BLAST search (83) using a sequence of *H. uvarum* rDNA as a query, which was obtained by the cloning of PCR-amplified DNA fragments and conventional Sanger sequenc-

TABLE 5 Oligonucleotides used to generate specific probes for *S. cerevisiae* chromosomes in Southern analyses^a

Gene	Forward or reverse primer	<i>S. cerevisiae</i> chromosome (size [Mb])
<i>BUD14</i>	gcgtcctgcagCTATTAAGAGCTGATGGAATCATCTTTCGAA CTGGATAGCGCCGATAAGG	I (0.2)
<i>SLA1</i>	GAACCAGTTCACCTGGTGGAG CTTAGGGTCGACTCCACCATTTC	II (0.81)
<i>TRP1</i>	GCGGCTTGACAGCACAGAGG GTCTCCACACCTCCGC	IV (1.61)
<i>GEA2</i>	gcgtaccgggAAACCACAGCCACATTAAC GGTGCCGTTGGAATCACTG	V (0.61)
<i>PFK1</i>	ggatctcgagTCAATCTCAAGATTCATGCTACGG gagcgtcgagTATTCAGTACCTGGAACG	VII (1.12)
<i>GEA1</i>	gcgtcgcctgcGGTATAAATGCCACCGTCCG CAGCCAAAGACCGCCAATTTGC	X (0.745)
<i>HXT1</i>	GGTACCATTGTTTTCCAGGCTGTCCG GCCGGTGAAGGTCAAGAAGTAG	VIII (0.56)
<i>GPM1</i>	GAAGCCGCTAGAGCCGGTG GGCAACAGCAGCGGCACCAGC	XI (0.68)
<i>MID2</i>	GTGGAACGTAAAGCACTCG CCTCAAGTGCTGACTCATCTTCCC	XII (1.9)
rDNA	gcgcgaattcGCTAGTACCGATTGAATGGCTTAG gcgcgatccGATGCGGAGAACCAAGAGATCCG	XII (1.9)
<i>PFK2</i>	ggatctcgagTACTGTTACTACTCCTTTTGTG cagcgtcgagTATTCAGTACCTGGAAC	XIII (0.915)
<i>WSC2</i>	GTGGAATATGCACCTAGATCTC CCACAACCACACTACTAC	XIV (0.785)
Noncoding sequence	GCGTTTATTGTATCCCTTGAC GGTAGATAGCTTGAGGCAC	XV (1.09)
<i>GAL4</i>	GGTCTCCGCTGACTAGGGCAC CCCCCTTATACACCAGGCTCC	XVI (0.945)

^aFor *S. cerevisiae* chromosomes, probes corresponding to the genes indicated were obtained by PCR with the oligonucleotide pairs given. Lowercase type indicates sequences added to create restriction sites for cloning, which do not hybridize to the target DNA. Sequences are all given in the 5'-to-3' direction.

ing. The sequence assembly and annotation were formatted according to GenBank guidelines. Further analyses of the data were performed by using software tools from the EMBOSS package and R statistical language v 3.3.2 (84). Synteny analysis was performed by using MUMmer version 3 (85). The SAMtools package (86) was used for variant analysis.

FIGE and Southern analysis. Chromosome sizes in FIGE gels were determined by using LabImage 1D (Kapelan Bio-Imaging Solutions, Leipzig, Germany). A total of 25 gels obtained under various electrophoresis conditions were used to determine mean chromosome sizes. For Southern analyses, three different target sequences were chosen from each contig for PCR amplification with the oligonucleotides listed in Table 4. Probes for the detection of a number of *S. cerevisiae* chromosomes were also prepared by PCR with the oligonucleotides listed in Table 5. Strain BY4743, a diploid derivative of the S288C strain employed for the genome-sequencing project, was used as a source for the *S. cerevisiae* size standard (Euroscarf Collection, Frankfurt, Germany). Probes were prepared with the hexanucleotide digoxigenin (DIG) DNA labeling kit from Roche (Mannheim, Germany).

Flow cytometry measurements of DNA content. The fixation and staining of cells were performed according to methods described previously (46). Samples were measured on a BD FACSAria II flow cytometer (Becton Dickinson, Heidelberg, Germany). R statistical language v 3.3.2 (84) and flow cytometry packages from Bioconductor (87) were used for data analysis and the generation of graphics.

Determination of specific enzyme activities. To obtain crude extracts for the determination of specific enzyme activities, yeast cells were washed and broken with glass beads, and the supernatant was

TABLE 6 Compositions of mixtures for determination of enzyme activities^a

Enzyme	Composition of assay mixture	Substrate (concn [mM])
Hexokinase	IB, 0.4 mM NADP, 1 mM ATP, 10 mM MgCl ₂ , 0.2 mM EDTA, 0.3 U/ml glucose-6-phosphate dehydrogenase	Fructose (5)
Phosphoglucose isomerase	IB, 0.4 mM NADP, 1 mM ATP, 10 mM MgCl ₂ , 100 mM KCl, 0.1 mM EDTA, 0.3 U/ml glucose-6-phosphate dehydrogenase	Fructose-6-phosphate (5)
Phosphofruktokinase	PB, 0.2 mM NADH, 1 mM ATP, 1 mM AMP, 10 mM MgCl ₂ , 5 μM fructose-2,6-bisphosphate, 1 U/ml fructose-1,6-bisphosphate aldolase, 0.5 U/ml each of glycerol-3-phosphate dehydrogenase and triosephosphate isomerase	Fructose-6-phosphate (2.5)
Aldolase	PB, 0.2 mM NADH, 5 mM MgCl ₂ , 30 mM KCl, 10 mM NH ₄ Cl, 0.3 mM MnCl ₂ , 0.2 mM Zn-acetate, 0.5 U/ml each of glycerol-3-phosphate dehydrogenase and triosephosphate isomerase	Fructose-1,6-bisphosphate (2)
Triosephosphate isomerase	IB, 0.2 mM NADH, 10 mM MgCl ₂ , 0.1 mM EDTA, 0.5 U/ml glycerol-3-phosphate dehydrogenase	Glyceraldehyde-3-phosphate (5)
Triosephosphate dehydrogenase	PB, 0.2 mM NADH, 1 mM ATP, 10 mM MgCl ₂ , 5 mM cysteine, 1 U/ml 3-phosphoglycerate kinase	3-Phosphoglycerate (4)
Phosphoglycerate kinase	PB, 0.2 mM NADH, 10 mM ATP, 10 mM MgCl ₂ , 100 mM KCl, 5 mM cysteine, 1 U/ml glyceraldehyde-3-phosphate dehydrogenase	3-Phosphoglycerate (10)
Phosphoglycerate mutase	IB, 0.2 mM NADH, 1 mM ADP, 10 mM MgCl ₂ , 0.5 U/ml enolase, 1 U/ml pyruvate kinase, 1 U/ml lactate dehydrogenase	3-Phosphoglycerate (10)
Enolase	PB, 0.2 mM NADH, 1 mM ADP, 10 mM MgCl ₂ , 100 mM KCl, 1 U/ml pyruvate kinase, 1 U/ml lactate dehydrogenase	2-Phosphoglycerate (10)
Pyruvate kinase	PB, 0.2 mM NADH, 0.6 mM ADP, 10 mM MgCl ₂ , 100 mM KCl, 1 mM fructose-1,6-bisphosphate, 1 U/ml lactate dehydrogenase	Phosphoenolpyruvate (10)
Pyruvate dehydrogenase	CB, 0.2 mM NADH, 10 mM MgCl ₂ , 100 mM KCl, 1.7 mM cysteine, 1.6 mM thiamine pyrophosphate, 1 U/ml alcohol dehydrogenase	Pyruvate (5)
Alcohol dehydrogenase	PPB, 2 mM NAD, 1 mM glutathione	Ethanol (0.6)

^aThe following assay conditions were modified from those described previously: hexokinase, glucose isomerase, triosephosphate isomerase, phosphoglycerate kinase, and enolase (92); phosphofruktokinase (93); aldolase (94); triosephosphate dehydrogenase; (95); phosphoglycerate mutase (96); pyruvate kinase (63); pyruvate dehydrogenase (97), and alcohol dehydrogenase (98). Ancillary enzymes and reagents were obtained from Roche (Mannheim, Germany), as far being as available in ammonium sulfate suspensions and used far beyond the indicated expiration date, partially due to the lack of current commercial resources. Alternatively, enzymes and substrates were obtained from Sigma-Aldrich. IB, 50 mM imidazole buffer, pH 7.0; PB, 50 mM sodium phosphate buffer, pH 7.0; CB, 50 mM sodium citrate buffer, pH 7.0; PPB, 85.5 mM sodium pyrophosphate buffer, pH 7.0.

obtained after 10 min of centrifugation in a microcentrifuge at 13,000 × *g* at 4°C as described previously (88). Specific activities were obtained by coupling the enzyme reactions in question to either NAD(P)H oxidation or reduction with ancillary enzymes, as described previously (89). Tests were performed with a Beckmann DU800 photometer, recording kinetics at 30°C and determining the slope after constant reaction rates were reached. As controls, kinetics were recorded without the presence of substrates prior to the start of the reactions. Buffers, concentrations of reagents, and ancillary enzymes employed are listed in Table 6. Protein concentrations in crude extracts were determined by the Micro-Biuret method, using bovine serum albumin as a standard (90).

Immunological detection of phosphofruktokinase subunits. Crude extracts prepared with glass beads as described above were obtained from cells grown in YEPD medium, boiled with loading buffer, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) on gels with 7.5% acrylamide. A polyclonal antiserum raised against PFK from *S. cerevisiae* was employed for the immunological detection of *H. uvarum* PFK subunits, applied at a dilution of 1:10,000 (68). Since the reactivity against the heterologous enzyme is much weaker than that against *S. cerevisiae* PFK, approximately 250 μg of protein from crude extracts prepared from *H. uvarum* cells or *S. cerevisiae* *pfk1 pfk2* double-deletion mutants transformed with either *HuPFK1* or *HuPFK2* carried on multicopy plasmids was loaded into each lane of the SDS-PAGE gel. For transformants carrying a multicopy plasmid with both heterologous *PFK* genes, only approximately 50 μg of protein was loaded. This concentration was also used for strains carrying endogenous *ScPFK* genes. Secondary anti-rabbit antibodies coupled to an infrared dye with fluorescence at 700 nm were then employed for the detection of PFK signals using the Odyssey imaging device as described previously (91). Contrast, brightness, and frames of the images were adjusted by using Corel PhotoPaint, which was applied only to the entire images shown in the figures and not to single bands.

Accession number(s). The annotated genome sequence was submitted to GenBank (<https://www.ncbi.nlm.nih.gov/GenBank/>) under accession number APLS01000000. The two *HuPFK* sequences were obtained again independently and submitted under GenBank accession numbers MF509744 (*HuPFK1*) and MF509745 (*HuPFK2*).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01580-17>.

SUPPLEMENTAL FILE 1, PDF file, 2.0 MB.

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REFERENCES

- Boulton RB, Singleton VI, Bisson LF, Kunkel RE. 1999. Principles and practices of winemaking. Springer, New York, NY.
- Chambers PJ, Pretorius IS. 2010. Fermenting knowledge: the history of winemaking, science and yeast research. *EMBO Rep* 11:914–920. <https://doi.org/10.1038/embor.2010.179>.
- Heard GM, Fleet GH. 1985. Growth of natural yeast flora during the fermentation of inoculated wines. *Appl Environ Microbiol* 50:727–728.
- Bisson LF, Karpel JE. 2010. Genetics of yeast impacting wine quality. *Annu Rev Food Sci Technol* 1:139–162. <https://doi.org/10.1146/annurev.food.080708.100734>.
- Jolly NP, Varela C, Pretorius IS. 2014. Not your ordinary yeast: non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res* 14:215–237. <https://doi.org/10.1111/1567-1364.12111>.
- Mortimer R, Polsinelli M. 1999. On the origins of wine yeast. *Res Microbiol* 150:199–204. [https://doi.org/10.1016/S0923-2508\(99\)80036-9](https://doi.org/10.1016/S0923-2508(99)80036-9).
- Wang C, Garcia-Fernandez D, Mas A, Esteve-Zarzoso B. 2015. Fungal diversity in grape must and wine fermentation assessed by massive sequencing, quantitative PCR and DGGE. *Front Microbiol* 6:1156. <https://doi.org/10.3389/fmicb.2015.01156>.
- Zott K, Miot-Sertier C, Claisse O, Lonvaud-Funel A, Masneuf-Pomaredé I. 2008. Dynamics and diversity of non-*Saccharomyces* yeasts during the early stages in winemaking. *Int J Food Microbiol* 125:197–203. <https://doi.org/10.1016/j.jfoodmicro.2008.04.001>.
- Capece A, Fiore C, Maraz A, Romano P. 2005. Molecular and technological approaches to evaluate strain biodiversity in *Hanseniaspora uvarum* of wine origin. *J Appl Microbiol* 98:136–144. <https://doi.org/10.1111/j.1365-2672.2004.02434.x>.
- Fleet GH. 2003. Yeast interactions and wine flavour. *Int J Food Microbiol* 86:11–22. [https://doi.org/10.1016/S0168-1605\(03\)00245-9](https://doi.org/10.1016/S0168-1605(03)00245-9).
- Albergaria H, Francisco D, Gori K, Arneborg N, Girio F. 2010. *Saccharomyces cerevisiae* CCM1 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains. *Appl Microbiol Biotechnol* 86:965–972. <https://doi.org/10.1007/s00253-009-2409-6>.
- Wang C, Mas A, Esteve-Zarzoso B. 2015. Interaction between *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* during alcoholic fermentation. *Int J Food Microbiol* 206:67–74. <https://doi.org/10.1016/j.jfoodmicro.2015.04.022>.
- Lopandic K, Tiefenbrunner W, Gangl H, Mandl K, Berger S, Leitner G, Abd-Ellah GA, Querol A, Gardner RC, Sterflinger K, Prillinger H. 2008. Molecular profiling of yeasts isolated during spontaneous fermentations of Austrian wines. *FEMS Yeast Res* 8:1063–1075. <https://doi.org/10.1111/j.1567-1364.2008.00385.x>.
- Bezerra-Bussoli C, Baffi MA, Gomes E, Da-Silva R. 2013. Yeast diversity isolated from grape musts during spontaneous fermentation from a Brazilian winery. *Curr Microbiol* 67:356–361. <https://doi.org/10.1007/s00284-013-0375-9>.
- Li SS, Cheng C, Li Z, Chen JY, Yan B, Han BZ, Reeves M. 2010. Yeast species associated with wine grapes in China. *Int J Food Microbiol* 138:85–90. <https://doi.org/10.1016/j.jfoodmicro.2010.01.009>.
- Beltran G, Torija MJ, Novo M, Ferrer N, Poblet M, Guillamon JM, Rozes N, Mas A. 2002. Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. *Syst Appl Microbiol* 25:287–293. <https://doi.org/10.1078/0723-2020-00097>.
- Brysch-Herzberg M, Seidel M. 2015. Yeast diversity on grapes in two German wine growing regions. *Int J Food Microbiol* 214:137–144. <https://doi.org/10.1016/j.jfoodmicro.2015.07.034>.
- Caruso M, Capece A, Salzano G, Romano P. 2002. Typing of *Saccharomyces cerevisiae* and *Kloeckera apiculata* strains from Aglianico wine. *Lett Appl Microbiol* 34:323–328. <https://doi.org/10.1046/j.1472-765X.2002.01090.x>.
- Ciani M, Faticenti F. 1999. Selective sugar consumption by apiculate yeasts. *Lett Appl Microbiol* 28:203–206. <https://doi.org/10.1046/j.1365-2672.1999.00505.x>.
- Teixeira A, Caldeira I, Duarte FL. 2015. Molecular and oenological characterization of Touriga Nacional non-*Saccharomyces* yeasts. *J Appl Microbiol* 118:658–671. <https://doi.org/10.1111/jam.12727>.
- Brezna B, Zenisova K, Chovanova K, Chebenova V, Krakova L, Kuchta T, Pangallo D. 2010. Evaluation of fungal and yeast diversity in Slovakian wine-related microbial communities. *Antonie Van Leeuwenhoek* 98:519–529. <https://doi.org/10.1007/s10482-010-9469-6>.
- Clavijo A, Calderon IL, Paneque P. 2010. Diversity of *Saccharomyces* and non-*Saccharomyces* yeasts in three red grape varieties cultured in the Serrania de Ronda (Spain) vine-growing region. *Int J Food Microbiol* 143:241–245. <https://doi.org/10.1016/j.jfoodmicro.2010.08.010>.
- Janisiewicz WJ, Jurick WM, II, Peter KA, Kurtzman CP, Buyer JS. 2014. Yeasts associated with plums and their potential for controlling brown rot after harvest. *Yeast* 31:207–218. <https://doi.org/10.1002/yea.3009>.
- Vadkertiova R, Molnarova J, Vranova D, Slavikova E. 2012. Yeasts and yeast-like organisms associated with fruits and blossoms of different fruit trees. *Can J Microbiol* 58:1344–1352. <https://doi.org/10.1139/cjm-2012-0468>.
- Morrissey WF, Davenport B, Querol A, Dobson AD. 2004. The role of indigenous yeasts in traditional Irish cider fermentations. *J Appl Microbiol* 97:647–655. <https://doi.org/10.1111/j.1365-2672.2004.02354.x>.
- Pando Bedrinana R, Querol Simon A, Suarez Valles B. 2010. Genetic and phenotypic diversity of autochthonous cider yeasts in a cellar from Asturias. *Food Microbiol* 27:503–508. <https://doi.org/10.1016/j.fm.2009.11.018>.
- Masoud W, Cesar LB, Jespersen L, Jakobsen M. 2004. Yeast involved in fermentation of *Coffea arabica* in East Africa determined by genotyping and by direct denaturing gradient gel electrophoresis. *Yeast* 21:549–556. <https://doi.org/10.1002/yea.1124>.
- Batista NN, Ramos CL, Dias DR, Pinheiro AC, Schwan RF. 2016. The impact of yeast starter cultures on the microbial communities and volatile compounds in cocoa fermentation and the resulting sensory attributes of chocolate. *J Food Sci Technol* 53:1101–1110. <https://doi.org/10.1007/s13197-015-2132-5>.
- Illegheems K, De Vuyst L, Papalexandratou Z, Weckx S. 2012. Phylogenetic analysis of a spontaneous cocoa bean fermentation metagenome reveals new insights into its bacterial and fungal community diversity. *PLoS One* 7:e38040. <https://doi.org/10.1371/journal.pone.0038040>.
- Liu HM, Guo JH, Cheng YJ, Liu P, Long CA, Deng BX. 2010. Inhibitory activity of tea polyphenol and *Hanseniaspora uvarum* against *Botrytis cinerea* infections. *Lett Appl Microbiol* 51:258–263. <https://doi.org/10.1111/j.1472-765X.2010.02888.x>.
- Renard A, Gomez di Marco P, Egea-Cortines M, Weiss J. 2008. Application of whole genome amplification and quantitative PCR for detection and quantification of spoilage yeasts in orange juice. *Int J Food Microbiol* 126:195–201. <https://doi.org/10.1016/j.jfoodmicro.2008.05.021>.
- Radler F, Schmitt MJ, Meyer B. 1990. Killer toxin of *Hanseniaspora uvarum*. *Arch Microbiol* 154:175–178. <https://doi.org/10.1007/BF00423329>.
- Schmitt MJ, Poravou O, Trenz K, Rehfeldt K. 1997. Unique double-stranded RNAs responsible for the anti-*Candida* activity of the yeast *Hanseniaspora uvarum*. *J Virol* 71:8852–8855.
- Ciani M, Beco L, Comitini F. 2006. Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *Int J Food Microbiol* 108:239–245. <https://doi.org/10.1016/j.jfoodmicro.2005.11.012>.
- Tristezza M, Tufariello M, Capozzi V, Spano G, Mita G, Grieco F. 2016. The oenological potential of *Hanseniaspora uvarum* in simultaneous and sequential co-fermentation with *Saccharomyces cerevisiae* for industrial wine production. *Front Microbiol* 7:670. <https://doi.org/10.3389/fmicb.2016.00670>.
- Schütz M, Gafner J. 1993. Sluggish alcoholic fermentation in relation to

- alterations of the glucose-fructose ratio. *Chem Mikrobiol Technol Leb-ensm* 15:73–78.
37. Cadez N, Raspor P, de Cock AW, Boekhout T, Smith MT. 2002. Molecular identification and genetic diversity within species of the genera *Hanseniaspora* and *Kloeckera*. *FEMS Yeast Res* 1:279–289. <https://doi.org/10.1111/j.1567-1364.2002.tb00046.x>.
 38. Esteve-Zarzoso B, Peris-Toran MJ, Ramon D, Quero A. 2001. Molecular characterisation of *Hanseniaspora* species. *Antonie Van Leeuwenhoek* 80:85–92. <https://doi.org/10.1023/A:1012268931569>.
 39. Belda I, Ruiz J, Alastruey-Izquierdo A, Navascues E, Marquina D, Santos A. 2016. Unraveling the enzymatic basis of wine “flavorome”: a phylo-functional study of wine related yeast species. *Front Microbiol* 7:12. <https://doi.org/10.3389/fmicb.2016.00012>.
 40. Kurtzman CP, Robnett CJ. 2003. Phylogenetic relationships among yeasts of the ‘*Saccharomyces* complex’ determined from multigene sequence analyses. *FEMS Yeast Res* 3:417–432. [https://doi.org/10.1016/S1567-1356\(03\)00012-6](https://doi.org/10.1016/S1567-1356(03)00012-6).
 41. Holloway P, Subden RE. 1994. The nucleotide sequence and initial characterization of pyruvate decarboxylase from the yeast *Hanseniaspora uvarum*. *Yeast* 10:1581–1589. <https://doi.org/10.1002/yea.320101207>.
 42. Pramateftaki PV, Kouvelis VN, Lanaridis P, Typas MA. 2006. The mitochondrial genome of the wine yeast *Hanseniaspora uvarum*: a unique genome organization among yeast/fungal counterparts. *FEMS Yeast Res* 6:77–90. <https://doi.org/10.1111/j.1567-1364.2005.00018.x>.
 43. Sternes PR, Lee D, Kutyna DR, Borneman AR. 2016. Genome sequences of three species of *Hanseniaspora* isolated from spontaneous wine fermentations. *Genome Announc* 4:e01287–16. <https://doi.org/10.1128/genomeA.01287-16>.
 44. Heinisch JJ, Buchwald U, Gottschlich A, Heppeler N, Rodicio R. 2010. A tool kit for molecular genetics of *Kluyveromyces lactis* comprising a congeneric strain series and a set of versatile vectors. *FEMS Yeast Res* 10:333–342. <https://doi.org/10.1111/j.1567-1364.2009.00604.x>.
 45. Schacherer J, Ruderfer DM, Gresham D, Dolinski K, Botstein D, Kruglyak L. 2007. Genome-wide analysis of nucleotide-level variation in commonly used *Saccharomyces cerevisiae* strains. *PLoS One* 2:e322. <https://doi.org/10.1371/journal.pone.0000322>.
 46. Delobel P, Tesniere C. 2014. A simple FCM method to avoid misinterpretation in *Saccharomyces cerevisiae* cell cycle assessment between G₀ and sub-G₁. *PLoS One* 9:e84645. <https://doi.org/10.1371/journal.pone.0084645>.
 47. Rodrigues F, Ludovico P, Sousa MJ, Steensma HY, Corte-Real M, Leao C. 2003. The spoilage yeast *Zygosaccharomyces bailii* forms mitotic spores: a screening method for haploidization. *Appl Environ Microbiol* 69: 649–653. <https://doi.org/10.1128/AEM.69.1.649-653.2003>.
 48. Byrne KP, Wolfe KH. 2005. The Yeast Gene Order Browser: combining curated homology and syntenic context reveals gene fate in polyploid species. *Genome Res* 15:1456–1461. <https://doi.org/10.1101/gr.3672305>.
 49. Langenberg AK. 2016. Genetische und physiologische Charakterisierung von *Hanseniaspora uvarum*. PhD thesis University of Osnabrück, Osnabrück, Germany.
 50. Shen XX, Zhou X, Kominek J, Kurtzman CP, Hittinger CT, Rokas A. 2016. Reconstructing the backbone of the Saccharomycotina yeast phylogeny using genome-scale data. *G3 (Bethesda)* 6:3927–3939. <https://doi.org/10.1534/g3.116.034744>.
 51. Barnett JA, Payne RW, Yarrow D. 2000. *Yeasts: characteristics and identification*. Cambridge University Press, Cambridge, England.
 52. Albertin W, Setati ME, Miot-Sertier C, Mostert TT, Colonna-Ceccaldi B, Coulon J, Girard P, Moine V, Pillet M, Salin F, Bely M, Divol B, Masneuf-Pomarede I. 2015. *Hanseniaspora uvarum* from winemaking environments show spatial and temporal genetic clustering. *Front Microbiol* 6:1569. <https://doi.org/10.3389/fmicb.2015.01569>.
 53. Boles E, Hollenberg CP. 1997. The molecular genetics of hexose transport in yeasts. *FEMS Microbiol Rev* 21:85–111. <https://doi.org/10.1111/j.1574-6976.1997.tb00346.x>.
 54. Heinisch JJ, Rodicio R. 2017. Stress response in wine yeast. In König H, Uden G, Fröhlich J (ed), *Biology of microorganisms on grapes, in must and wine*, 2nd ed, in press. Springer-Verlag, Berlin, Germany.
 55. Jendretzki A, Wittland J, Wilk S, Straede A, Heinisch JJ. 2011. How do I begin? Sensing extracellular stress to maintain yeast cell wall integrity. *Eur J Cell Biol* 90:740–744. <https://doi.org/10.1016/j.ejcb.2011.04.006>.
 56. Schmitz HP, Heinisch JJ. 2003. Evolution, biochemistry and genetics of protein kinase C in fungi. *Curr Genet* 43:245–254. <https://doi.org/10.1007/s00294-003-0403-6>.
 57. Schmitz HP, Lorberg A, Heinisch JJ. 2002. Regulation of yeast protein kinase C activity by interaction with the small GTPase Rho1p through its amino-terminal HR1 domain. *Mol Microbiol* 44:829–840. <https://doi.org/10.1046/j.1365-2958.2002.02925.x>.
 58. Hohmann S. 2002. Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev* 66:300–372. <https://doi.org/10.1128/MMBR.66.2.300-372.2002>.
 59. Matallana E, Aranda A. 2017. Biotechnological impact of stress response on wine yeast. *Lett Appl Microbiol* 64:103–110. <https://doi.org/10.1111/lam.12677>.
 60. De Benedictis M, Blevé G, Grieco F, Tristezza M, Tufariello M. 2011. An optimized procedure for the ecological selection of non-*Saccharomyces* starter cultures. *Antonie Van Leeuwenhoek* 99:189–200. <https://doi.org/10.1007/s10482-010-9475-8>.
 61. Nijkamp JF, van den Broek M, Datema E, de Kok S, Bosman L, Luttk MA, Daran-Lapujade P, Vongsangnak W, Nielsen J, Heijne WH, Klaassen P, Paddon CJ, Platt D, Kotter P, van Ham RC, Reinders MJ, Pronk JT, de Ridder D, Daran JM. 2012. De novo sequencing, assembly and analysis of the genome of the laboratory strain *Saccharomyces cerevisiae* CEN.PK113-7D, a model for modern industrial biotechnology. *Microb Cell Fact* 11:36. <https://doi.org/10.1186/1475-2859-11-36>.
 62. Schehl B, Müller C, Senn T, Heinisch JJ. 2004. A laboratory yeast strain suitable for spirit production. *Yeast* 21:1375–1389. <https://doi.org/10.1002/yea.1189>.
 63. Boles E, Schulte F, Miosga T, Freidel K, Schluter E, Zimmermann FK, Hollenberg CP, Heinisch JJ. 1997. Characterization of a glucose-repressed pyruvate kinase (Pyk2p) in *Saccharomyces cerevisiae* that is catalytically insensitive to fructose-1,6-bisphosphate. *J Bacteriol* 179: 2987–2993. <https://doi.org/10.1128/jb.179.9.2987-2993.1997>.
 64. Jurica MS, Mesecar A, Heath PJ, Shi W, Nowak T, Stoddard BL. 1998. The allosteric regulation of pyruvate kinase by fructose-1,6-bisphosphate. *Structure* 6:195–210. [https://doi.org/10.1016/S0969-2126\(98\)00021-5](https://doi.org/10.1016/S0969-2126(98)00021-5).
 65. Rodicio R, Heinisch JJ. 2017. Carbohydrate metabolism in wine yeast. In König H, Uden G, Fröhlich J (ed), *Biology of microorganisms on grapes, in must and wine*, 2nd ed, in press. Springer-Verlag, Berlin, Germany.
 66. Venturin C, Boze H, Moulin G, Galzy P. 1995. Glucose metabolism, enzymic analysis and product formation in chemostat culture of *Hanseniaspora uvarum*. *Yeast* 11:327–336. <https://doi.org/10.1002/yea.320110405>.
 67. Heinisch J, Ritzel RG, von Borstel RC, Aguilera A, Rodicio R, Zimmermann FK. 1989. The phosphofructokinase genes of yeast evolved from two duplication events. *Gene* 78:309–321. [https://doi.org/10.1016/0378-1119\(89\)90233-3](https://doi.org/10.1016/0378-1119(89)90233-3).
 68. Heinisch J. 1986. Construction and physiological characterization of mutants disrupted in the phosphofructokinase genes of *Saccharomyces cerevisiae*. *Curr Genet* 11:227–234. <https://doi.org/10.1007/BF00420611>.
 69. Breitenbach-Schmitt I, Schmitt HD, Heinisch J, Zimmermann FK. 1984. Genetic and physiological evidence for the existence of a second glycolytic pathway in yeast parallel to the phosphofructokinase-aldolase reaction sequence. *Mol Gen Genet* 195:536–540. <https://doi.org/10.1007/BF00341459>.
 70. Arvanitidis A, Heinisch JJ. 1994. Studies on the function of yeast phosphofructokinase subunits by *in vitro* mutagenesis. *J Biol Chem* 269: 8911–8918.
 71. Bär J, Schellenberger W, Kopperschläger G. 1997. Purification and characterization of phosphofructokinase from the yeast *Kluyveromyces lactis*. *Yeast* 13:1309–1317.
 72. Heinisch J, Kirchrath L, Liesen T, Vogelsang K, Hollenberg CP. 1993. Molecular genetics of phosphofructokinase in the yeast *Kluyveromyces lactis*. *Mol Microbiol* 8:559–570. <https://doi.org/10.1111/j.1365-2958.1993.tb01600.x>.
 73. Kopperschläger G, Bar J, Stellwagen E. 1993. Limited proteolysis of yeast phosphofructokinase. Sequence locations of cleavage sites created by the actions of different proteinases. *Eur J Biochem* 217:527–533. <https://doi.org/10.1111/j.1432-1033.1993.tb18273.x>.
 74. Ciani M, Morales P, Comitini F, Tronchoni J, Canonico L, Curiel JA, Oro L, Rodrigues AJ, Gonzalez R. 2016. Non-conventional yeast species for lowering ethanol content of wines. *Front Microbiol* 7:642. <https://doi.org/10.3389/fmicb.2016.00642>.
 75. Raben N, Exelbert R, Spiegel R, Sherman JB, Nakajima H, Plotz P, Heinisch J. 1995. Functional expression of human mutant phosphofructokinase in yeast: genetic defects in French Canadian and Swiss patients with phosphofructokinase deficiency. *Am J Hum Genet* 56:131–141.
 76. Rodicio R, Koch S, Schmitz HP, Heinisch JJ. 2006. KIRHO1 and KIPKC1 are essential for cell integrity signalling in *Kluyveromyces lactis*. *Microbiology* 152:2635–2649. <https://doi.org/10.1099/mic.0.29105-0>.

77. Gietz RD, Sugino A. 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* 74:527–534. [https://doi.org/10.1016/0378-1119\(88\)90185-0](https://doi.org/10.1016/0378-1119(88)90185-0).
78. Hill JE, Myers AM, Koerner TJ, Tzagoloff A. 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast* 2:163–167. <https://doi.org/10.1002/yea.320020304>.
79. Zimmermann FK. 1975. Procedures used in the induction of mitotic recombination and mutation in the yeast *Saccharomyces cerevisiae*. *Mutat Res* 31:71–86. [https://doi.org/10.1016/0165-1161\(75\)90069-2](https://doi.org/10.1016/0165-1161(75)90069-2).
80. Bashir A, Klammer AA, Robins WP, Chin CS, Webster D, Paxinos E, Hsu D, Ashby M, Wang S, Peluso P, Sebra R, Sorenson J, Bullard J, Yen J, Valdovino M, Mollova E, Luong K, Lin S, LaMay B, Joshi A, Rowe L, Frace M, Tarr CL, Turnsek M, Davis BM, Kasarskis A, Mekalanos JJ, Waldor MK, Schadt EE. 2012. A hybrid approach for the automated finishing of bacterial genomes. *Nat Biotechnol* 30:701–707. <https://doi.org/10.1038/nbt.2288>.
81. Proux-Wera E, Armisen D, Byrne KP, Wolfe KH. 2012. A pipeline for automated annotation of yeast genome sequences by a conserved-synteny approach. *BMC Bioinformatics* 13:237. <https://doi.org/10.1186/1471-2105-13-237>.
82. Rice P, Longden I, Bleasby A. 2000. EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet* 16:276–277. [https://doi.org/10.1016/S0168-9525\(00\)02024-2](https://doi.org/10.1016/S0168-9525(00)02024-2).
83. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol* 215:403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2).
84. R Core Team. 2016. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
85. Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, Antonescu C, Salzberg SL. 2004. Versatile and open software for comparing large genomes. *Genome Biol* 5:R12. <https://doi.org/10.1186/gb-2004-5-2-r12>.
86. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25:2078–2079. <https://doi.org/10.1093/bioinformatics/btp352>.
87. Huber W, Carey VJ, Gentleman R, Anders S, Carlson M, Carvalho BS, Bravo HC, Davis S, Gatto L, Girke T, Gottardo R, Hahne F, Hansen KD, Irizarry RA, Lawrence M, Love MI, MacDonald J, Obenchain V, Oles AK, Pages H, Reyes A, Shannon P, Smyth GK, Tenenbaum D, Waldron L, Morgan M. 2015. Orchestrating high-throughput genomic analysis with Bioconductor. *Nat Methods* 12:115–121. <https://doi.org/10.1038/nmeth.3252>.
88. Breitenbach I, Schmitt HD, Heinisch J, Zimmermann FK. 1984. Yeast mutants without phosphofructokinase activity can still perform glycolysis and alcoholic fermentation. *Mol Gen Genet* 195:530–535. <https://doi.org/10.1007/BF00341458>.
89. Ciriacy M, Breitenbach I. 1979. Physiological effects of seven different blocks in glycolysis in *Saccharomyces cerevisiae*. *J Bacteriol* 139:152–160.
90. Zamenhoff S. 1957. Preparation and assay of deoxyribonucleic acids from animal tissue. *Methods Enzymol* 3:702–704.
91. Kock C, Artl H, Ungerermann C, Heinisch JJ. 2016. Yeast cell wall integrity sensors form specific plasma membrane microdomains important for signalling. *Cell Microbiol* 18:1251–1267. <https://doi.org/10.1111/cmi.12635>.
92. Maitra PK, Lobo Z. 1971. A kinetic study of glycolytic enzyme synthesis in yeast. *J Biol Chem* 246:475–488.
93. Rodicio R, Strauss A, Heinisch JJ. 2000. Single point mutations in either gene encoding the subunits of the heterooctameric yeast phosphofructokinase abolish allosteric inhibition by ATP. *J Biol Chem* 275:40952–40960. <https://doi.org/10.1074/jbc.M007131200>.
94. Boles E, Zimmermann FK. 1993. *Saccharomyces cerevisiae* phosphoglucose isomerase and fructose bisphosphate aldolase can be replaced functionally by the corresponding enzymes of *Escherichia coli* and *Drosophila melanogaster*. *Curr Genet* 23:187–191. <https://doi.org/10.1007/BF00351494>.
95. McAlister L, Holland MJ. 1985. Differential expression of the three yeast glyceraldehyde-3-phosphate dehydrogenase genes. *J Biol Chem* 260:15019–15027.
96. Heinisch JJ, Muller S, Schluter E, Jacoby J, Rodicio R. 1998. Investigation of two yeast genes encoding putative isoenzymes of phosphoglycerate mutase. *Yeast* 14:203–213.
97. Schmitt HD, Zimmermann FK. 1982. Genetic analysis of the pyruvate decarboxylase reaction in yeast glycolysis. *J Bacteriol* 151:1146–1152.
98. Ciriacy M. 1975. Genetics of alcohol dehydrogenase in *Saccharomyces cerevisiae*. II. Two loci controlling synthesis of the glucose-repressible ADH II. *Mol Gen Genet* 138:157–164.