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ISSN 1330-9862
(FTB-3387)

original scientific paper

Generation of New Genotypic and Phenotypic Features in Artificial and Natural Yeast Hybrids

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Received: March 29, 2013
Accepted: September 23, 2013

Summary

Evolution and genome stabilization have mostly been studied on the *Saccharomyces* hybrids isolated from natural and alcoholic fermentation environments. Genetic and phenotypic properties have usually been compared to the laboratory and reference strains, as the true ancestors of the natural hybrid yeasts are unknown. In this way the exact impact of different parental fractions on the genome organization or metabolic activity of the hybrid yeasts is difficult to resolve completely. In the present work the evolution of geno- and phenotypic properties is studied in the interspecies hybrids created by the cross-breeding of *S. cerevisiae* with *S. uvarum* or *S. kudriavzevii* auxotrophic mutants. We hypothesized that the extent of genomic alterations in *S. cerevisiae* × *S. uvarum* and *S. cerevisiae* × *S. kudriavzevii* should affect the physiology of their F1 offspring in different ways. Our results, obtained by amplified fragment length polymorphism (AFLP) genotyping and karyotyping analyses, showed that both subgenomes of the *S. cerevisiae* x *S. uvarum* and of *S. cerevisiae* × *S. kudriavzevii* hybrids experienced various modifications. However, the *S. cerevisiae* × *S. kudriavzevii* F1 hybrids underwent more severe genomic alterations than the *S. cerevisiae* × *S. uvarum* ones. Generation of the new genotypes also influenced the physiological performances of the hybrids and the occurrence of novel phenotypes. Significant differences in carbohydrate utilization and distinct growth dynamics at increasing concentrations of sodium chloride, urea and miconazole were observed within and between the *S. cerevisiae* × *S. uvarum* and *S. cerevisiae* × *S. kudriavzevii* hybrids. Parental strains also demonstrated different contributions to the final metabolic outcomes of the hybrid yeasts. A comparison of the genotypic properties of the artificial hybrids with several hybrid isolates from the wine-related environments and wastewater demonstrated a greater genetic variability of the *S. cerevisiae* × *S. kudriavzevii* hybrids. *Saccharomyces cerevisiae* × *S. uvarum* artificial and natural hybrids showed considerable differences in osmolyte tolerance and sensitivity to miconazole, whereas the *S. cerevisiae* × *S. kudriavzevii* hybrids exhibited differences also in maltotriose utilization. The results of this study suggest that chromosomal rearrangements and genomic reorganizations as post-hybridization processes may affect the phenotypic properties of the hybrid progeny substantially. Relative to their ancestors, the F1 segre-

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gants may generate different phenotypes, indicating novel routes of evolution in response to environmental growth conditions.

Key words: Saccharomyces cerevisiae, *S. uvarum*, *S. kudriavzevii*, yeast interspecies hybrids, AFLP, karyotyping

Introduction

Intensive development of molecular biology techniques and high-throughput sequencing technologies has improved the genetic characterization of yeasts immensely. The availability of different whole genome sequencing approaches has enabled us to study genome dynamics in a number of different yeast species and to understand the ways they evolve and their speciation. Whole genome comparisons have demonstrated that the acquisition of foreign genes is not a rare event among yeasts. Moreover, genome duplication, introgression and interspecies hybridization are considered to be some of the main molecular mechanisms of yeast genome evolution (*1,2*). Interspecies yeast hybrids have also become interesting in the study of cell adaptations to various environments in which the mode of genome stabilization and physiological cell responses are of particular interest. Hybridization and introgression processes are recognized within the asco- and basidiomycetous yeast genera (*Debaryomyces*, *Millerozyma*, *Zygosaccharomyces*, *Cryptococcus*), and the most comprehensive results come from the studies investigating hybridization among the species of the *Saccharomyces sensu stricto* complex (*3–10*).

Saccharomyces sensu stricto is a group of phylogenetically closely related species (*S. arboricolus*, *S. bayanus*, *S. cariocanus*, *S. cerevisiae*, *S. eubayanus*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus*, *S. pastorianus*, *S. uvarum*) that show weak prezygotic barriers and under specific environmental conditions exchange genetic material, generating double or even triple hybrids (*11–15*). Interspecies hybridization generates genomes of increased size, which evolve and stabilize over evolutionary time. These processes imply gross chromosomal rearrangements, translocations, inversions, and loss of DNA segments or entire chromosomes, resulting in aneuploid genomes (*4,8,16–19*). A high genetic diversity has been recognized among yeast hybrids isolated from fermentations, natural environments (vineyards, oak trees) or clinical materials, indicating that genome reorganization and selective pressure have shaped the genomes in different ways. The majority of the characterized yeasts are hybrids between *S. cerevisiae* and *S. bayanus*, *S. eubayanus*, *S. uvarum*, *S. kudriavzevii* or *S. paradoxus* (*5,15,20–25*). Among brewer's, wine and cider yeast isolates, triple hybrids have also been identified such as *S. cerevisiae* × *S. bayanus* × *S. kudriavzevii* and *S. cerevisiae* × *S. eubayanus* × *S. uvarum* (*5,26,27*). On the basis of the genome sequences, it has recently been recognized that even the type strain of *S. bayanus* var. *bayanus* CBS380 is also a hybrid, containing a complex genome composed of *S. uvarum* and *S. eubayanus* subgenomes, as well as some introgressed *S. cerevisiae* fragments (*27*). It can be assumed that many yeasts whose identification was based on rRNA coding sequences need to be reexamined by means of multigene sequences or even whole genome sequences. For a long time the taxonomy of yeasts as well as other microorganisms was based on the characterization of the conserved rRNA-encoding genes, which in the majority of cases was unable to disclose the hybrid nature of a number of established species. One of the early recognized hybrid yeasts was *S. pastorianus*, well known as lager brewer's yeast or *S. carlsbergensis*, which consists of *S. cerevisiae* and *S. bayanus*-like subgenomes (*28*). For a long time the non-*S. cerevisiae* genome portion was a subject of debate, since various genetic and molecular markers suggested that it was closely related but not identical to *S. bayanus* species. An ecological study and comparative genomic analysis has recently identified *S. eubayanus*, which shows 99.5 % sequence identity with the non-*S. cerevisiae* genome portion of *S. pastorianus* genome (*15*). The completely sequenced genome of the commercial brewer's yeast Weihenstephan 34/70 has shown that its genome contains three types of chromosomes, namely those which are characteristic for the parental species *S. cerevisiae* and *S. eubayanus*, and eight chimera chromosomes (*15,19*). These complex mosaic chromosomal structures have also been demonstrated by some *S. cerevisiae* × *S. kudriavzevii* hybrids isolated from beer, wine and vineyards (*8,9,18*). The whole genome sequence of the commercial strain VIN7 has shown that the strain is an allotriploid interspecies hybrid that contains a diploid *S. cerevisiae* and a haploid *S. kudriavzevii* genome (*29*). The rearrangement of parental chromosomes with several reciprocal translocations and chromosomal substitutions, and with minimal loss of the *S. kudriavzevii* genome, indicated that the genome of VIN7 is undergoing consolidation.

Interspecies hybridization and introgression increase genetic variations in natural yeast populations and have a great impact on their metabolic diversity. It is well known that the hybrids containing *S. uvarum* or *S. kudriavzevii* subgenome can better adapt to low-temperature fermentations, whereas the *S. cerevisiae* parental part accomplishes sugar fermentation more efficiently (*30*). Different studies have also demonstrated the influence of environmental pressure on the acquisition of new advantageous attributes. Galeote *et al*. (*31*) have recently identified the *FSY1* gene in the EC1118 wine yeast that was acquired from another *Saccharomyces* species. The Fsy1p was characterized as a high-affinity fructose/H^+ symporter with kinetic properties similar to those of *S. pastorianus* Fsy1p. This protein should play an important role in alcoholic fermentations, especially at the end stages, as the *FSY1* gene is highly expressed at high ethanol and low glucose and fructose concentrations. Brewer's yeasts may show different abilities to ferment maltose and maltotriose, a property which is extremely important for beer production. These different attributes have recently been explained by copy number differences of the *MAL* genes and the presence of the *AGT1* permease gene in *S. pastorianus* strains that demonstrated an efficient utilization of maltotriose (*32*). Two commercial *S. cerevisiae* × *S.*

kudriavzevii hybrid strains showed an increased production of higher alcohols in comparison with a commercial *S. cerevisiae* strain, suggesting the importance of hybrid strains for the oenological properties of wines (*33*). On the other hand, *S. cerevisiae* × *S. kudriavzevii* hybrids isolated from Austrian vineyards produced wines with increased ester concentrations, and the fermentation of sugars was more efficient when compared to the parental strains (*21,34*). As a number of interspecies hybrids have been isolated in fermentation environments, it seems that these specific conditions (anaerobiosis, high alcohol and sugar concentration, high osmotic and hydrostatic pressure, and low temperature) induce their generation as a possible mode of adaptation (*35*). As the above-mentioned examples show, a number of yeast hybrids may show superior phenotypic properties in comparison with the parental strains, a fact that can be used in different biotechnological applications.

The impact of chromosomal rearrangements on the physiological properties of hybrids is difficult to monitor in natural yeast isolates, as their parents are unknown. We hypothesized that the extent of genomic changes after hybridization event depends on the hybridization partners, and that the hybrid offspring may develop quite different genetic and physiological properties compared to their ancestors. To evaluate the impact of the parental strains on the geno- and phenotype, we constructed a number of *S. cerevisiae* × *S. uvarum* and *S. cerevisiae* × *S. kudriavzevii* hybrids, and examined the changes to their genomes in the first filial generation as well as the contribution of these changes to physiological alterations. Our results demonstrated that the F1 offspring show different genotyping patterns as well as different chromosomal make-up in comparison with the hybrid and parental cells, suggesting that the genomes underwent distinct alterations after the hybridization event. The *S. cerevisiae* × *S. kudriavzevii* hybrid genome underwent more extensive modifications than the *S. cerevisiae* × *S. uvarum* genome, and these changes apparently influenced the phenotypic attributes of the first filial generation. Natural yeast hybrids demonstrated greater genotypic variability in comparison with artificial hybrid yeasts, and considerable physiological differences were also observed under some conditions.

Materials and Methods

Yeast strains

The *Saccharomyces* strains examined in this study are listed in Table 1. The strains *S. cerevisiae* HA2796, *S. uvarum* HA2786 and *S. kudriavzevii* HA2787 were used to generate auxotrophic mutants, hybridization experiments and hybrid isolation. The corresponding type strains were used to check their identity. The auxotrophic mutants were created using UV irradiation as described by Antunovics *et al*. (*16*), and those of *S. kudriavzevii* HA2787 were generated using α-aminoadipate as described by Arroyo-López *et al*. (*36*).

Generation of interspecies hybrids

The homothallic diploid strains (HA2786 and HA-2787) to be mated with the haploid *S. cerevisiae* HA2796 strain were grown on a sporulation medium (containing (in %): potassium acetate 1, yeast extract 0.1, glucose 0.05 and agar 2) at 26 °C for three days. A loop of the cell material was treated with 200 μg/mL of Zymoliase 20T (MP Biomedical, Aurora, OH, USA) at 37 °C for 15 to 30 min depending on the sensitivity of the ascus walls. To obtain the interspecies hybrids, the strains with different auxotrophies were mixed in liquid GYP medium (containing (in %): glucose 2, peptone 1, yeast extract 0.5) and incubated with shaking overnight. Samples of the hybridized strains were spread onto minimal medium (containing (in %): $(NH_4)_2SO_4$ 0.5, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.05, glucose 1, vitamin mix 1 and agar 2) and incubated at 25 °C for 3 to 5 days. The hybrid nature of the grown yeast cultures was verified by means of the

Table 1. Yeast strains used in the present study

Strain	Strain designation		Isolation source	Genotypic properties
	ACBR	Other		
S. cerevisiae	HA2764^T	CBS8803^T	S288C laboratory strain	
S. cerevisiae	HA2796	DEG 10-170	derivative of ATCC 204891	MATa, leu$^-$, heterothallic
S. uvarum	HA231^T	CBS395^T	juice of *Ribes nigrum*, The Netherlands	
S. uvarum	HA2786	DEG 10-522	wine must, Tokaj wine region	ura$^-$, homothallic
S. kudriavzevii	HA2261^T	CBS8840^T	decayed leaf, Japan	
S. kudriavzevii	HA2787	DEG 10-643	derivative of CBS8840^T	lys$^-$, homothallic
S. cerevisiae × *S. uvarum*	HA2797	DEG H10		HA2796 × HA2786
S. cerevisiae × *S. uvarum*	HA2560		wastewater, Mongolia	
S. cerevisiae × *S. kudriavzevii*	HA2828			HA2796 × HA2787
S. cerevisiae × *S. kudriavzevii*	HA1836		grape, Thermenregion, Austria	
S. cerevisiae × *S. kudriavzevii*	HA1842		grape, Thermenregion, Austria	.
S. cerevisiae × *S. kudriavzevii*	HA2654		Uvaferm CS2, Lallemand Inc.	

T=type strain; ACBR=Austrian Center of Biological Resources and Applied Mycology, Vienna, Austria; CBS=Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DEG=Department of Genetics and Applied Microbiology, University of Debrecen, Hungary

amplified fragment length polymorphism (AFLP) technique and pulsed-field gel electrophoresis.

Sporulation and ascus dissection of hybrid yeasts

In order to obtain F1 offspring tetrads, cultures of the hybrid strains were inoculated onto potassium acetate sporulation agar medium and incubated at 22 °C for 5 days. Ascospore formation was monitored in a drop of water on a microscope slide at 400× magnification. Strains unable to sporulate under these conditions were also tested at 12 °C on the same medium and on sodium acetate sporulation medium (containing (in %): sodium acetate 0.82, KCl 0.19, yeast extract 0.25, glucose 0.1, agar 2) at both temperatures. Strains unable to produce spores under any of these conditions were crossed with *MAT**a*** and *MATα* tester strains to test if they were heterothallic. Sporulated cultures were treated with Zymoliase 20T at 35 °C for 15 to 30 min and 20 μL of the culture were transferred onto a GYP agar plate. Tetrads were isolated and dissected by micromanipulator (Zeiss, Jena, Germany). The GYP plates were incubated at 25 °C for a week. Spore viability was calculated from ten tetrads and from each hybrid a fully viable tetrad was chosen for further geno- and phenotypic characterization.

AFLP fingerprinting

The genetic variability of the *Saccharomyces* hybrids was investigated by means of the amplified fragment length polymorphism (AFLP) technique using the AFLP™ Microbial Fingerprinting kit of Applied Biosystems (Foster City, CA, USA). The restriction/ligation steps, as well as the preselective and selective amplifications, were carried out as described by Lopandic *et al*. (*21*) with minor modifications. One primer pair, *EcoRI*-AC-FAM/*MseI*-C, was used for selective amplification. The fragments generated were analysed by electrophoresis on a 50-cm capillary column by an ABI 3130 genetic analyzer (Applied Biosystems). GeneMapper v. 4.0 software was used for extraction and comparison of the resulting electropherograms, and the Excel® macro program was used to convert the output into binary files (*37*). The tree topology was inferred using the simple matching genetic distance estimation and the UPGMA clustering method of the software TREECON v. 1.3b (*38*). Bootstrapping was performed with 1000 replicates.

Karyotype analysis by pulsed-field gel electrophoresis

Karyotype analysis was carried out according to Antunovics *et al*. (*16*). Separation of chromosomes was performed in a 1 % agarose gel (chromosomal grade, Bio-Rad, Hercules, CA, USA) by a counter-clamped homogenous electric field electrophoresis (CHEF-Mapper; Bio-Rad). The following running parameters were used: run time 24 h, voltage 6 V/cm, angle 120°, temperature 14 °C and pulse parameters 60 to 120 s. Gels were stained with ethidium bromide and washed in sterile water thereafter for 48 h before photographing with UV-transillumination.

Phenotypic assays

The ability of yeasts to grow on different carbohydrates and at altered concentrations of sodium chloride, urea and miconazole was tested with yeast nitrogen base medium (YNB; pH=5.6) containing 0.67 % yeast nitrogen base and 0.2 % amino acid mixture (ForMedium, Norfolk, UK). In order to test the yeast's ability to assimilate different carbohydrates, the YNB medium was supplemented with 2 % glucose, raffinose or maltotriose. Yeast growth at different amounts of sodium chloride (2.5, 5 and 10 %), urea (1, 2 and 5 %) and miconazole (0.01, 0.1 and 0.5 μg/mL) was carried out in YNB medium supplemented with 2 % glucose. Yeast cultures were pregrown on a starvation medium (containing (in %): glucose 0.1, peptone 1, yeast extract 0.5, agar 2) at 25 °C for 48 h and diluted with YNB medium to achieve an absorbance at 750 nm of 0.250. The media with the indicated test substances were inoculated with 1000 cells per mL and 200 μL of the obtained mixture were transferred into 96-well plates (Greiner Bio One GmbH, Frickenhausen, Germany) and incubated at 25 °C for several days. Yeast growth was measured by a microplate reader (Infinite® M1000, Tecan Group Ltd, Männedorf, Switzerland) at 750 nm using orbital shaking between measurements. All measurements were performed in quadruplicate and were reproducible. The mean values were calculated and the values at t_0 were subtracted from the values measured at different times to minimize the influence of different cell concentrations on the final results.

Results and Discussion

Genetic analyses of hybrid yeasts demonstrated that the alloploid genomes can undergo drastic changes during meiotic and mitotic divisions of the hybrid cells (*39,40*). Hybrid yeasts undergoing meiosis and producing viable offspring plausibly exhibit more genotypic and phenotypic changes from one generation to another than the clonally reproducing ones, although mitosis may also generate novel geno- and phenotypes over the course of many generations. Antunovics *et al*. (*16*) demonstrated by means of different genetic and molecular markers that hybrid genomes undergo a gradual reduction over several meiotic divisions. Genomic analyses of a number of natural hybrids also demonstrated the loss of distinct portions of the parent genomes (*8,9,17,18*). The genome stabilization processes affect the genome constitution, ploidy, fertility, spore viability and physiology (*39,40*). In the course of this study we constructed a number of *Saccharomyces* interspecies hybrids and selected one of each *S. cerevisiae* × *S. uvarum* and *S. cerevisiae* × *S. kudriavzevii* strain in order to monitor and correlate the changes of geno- and phenotypes in the F1 generation relative to their ancestors.

Generation and fertility control of interspecies hybrids and their offspring

Interspecies *S. cerevisiae* × *S. uvarum* and *S. cerevisiae* × *S. kudriavzevii* hybrids were created by crossing auxotrophic parental strains (a heterothallic *S. cerevisiae* and a homothallic *S. uvarum*/*S. kudriavzevii* strain) and select-

ed on the bases of their prototrophy. During this study numerous hybrid strains were generated and their ability to produce four-spore tetrads was simultaneously tested. Among the created hybrids, many were unable to sporulate and many produced nonviable spores or only partially viable tetrads. We selected the *S. cerevisiae* × *S. uvarum* HA2797 and *S. cerevisiae* × *S. kudriavzevii* HA-2828 strains due to their ability to produce fully viable tetrads. Both of these hybrids produced a similar number of asci after 5 days on potassium acetate medium: 38 % of HA2797 and 36 % of HA2828 cells created asci. However, while the spore viability of the former hybrid was very high at 98 %, only 53 % of the spores of the latter hybrid were viable. In both chosen tetrads, leu$^-$ auxotrophy of the *S. cerevisiae* parental strain segregated in a 2:2 manner (HA2797 F1b and F1c, HA2828 F1c and F1d were auxotrophic). As previously described, HA-2797 F1b and F1c spore clones were fertile, capable of generating viable offspring tetrads, while the other two F1 strains were non-sporulating (*41*). The *S. cerevisiae* × *S. kudriavzevii* HA2828 F1b, F1c and F1d spore clones were found to be non-sporulating under any tested conditions (they were also unable to mate with **a** or α mating tester strains), while F1a produced tetrads with fully viable F2 spores. Therefore, the segregation of fertility was 2:2 in the *S. cerevisiae* × *S. uvarum* and 1:3 in the *S. cerevisiae* × *S. kudriavzevii* hybrid. Highly viable F3 generations were also isolated from all viable F2 strains, showing that fertility and viability were stably inherited into the next generations in both hybrids. Sporulation and spore viability of natural hybrids were also tested. The *S. cerevisiae* × *S. uvarum* natural hybrid HA2560 showed no sporulation or mating with testers and the *S. cerevisiae* × *S. kudriavzevii* natural hybrid HA2654 produced only deformed asci with non-separable and nonviable spores. The other *S. cerevisiae* × *S. kudriavzevii* natural hybrids (HA1836 and HA1842) were able to sporulate, but the spores were not viable.

Estimation of genomic alterations in the hybrids and their meiotic segregants

Using anonymous AFLP markers and karyotyping analysis, we estimated variations of both, parental genomes in the hybrids and their F1 segregants. The genome alterations were qualitatively very simply observable by AFLP markers, the amplification of which is directly affected by point mutations, deletions, insertions and duplications. Fig. 1 shows the cluster analysis of the hybrid and parental yeast strains based on the AFLP genotyping analysis. Two main clusters were recognized that correlate with two studied hybrid types, namely *S. cerevisiae* × *S. kudriavzevii* and *S. cerevisiae* x *S. uvarum*. The resulting tetrads of both hybrid types segregated in a 2:2 manner for the parental AFLP markers. Natural yeast hybrids were closely related to the corresponding experimental hybrids, but at the same time demonstrated significant genetic variations. The AFLP analysis of the *S. cerevisiae* × *S. uvarum* HA2797 and *S. cerevisiae* × *S. kudriavzevii* HA2828 hybrid strains generated 80 and 77 fragments respectively, between 60 and 450 bp. A pairwise comparison of the banding patterns revealed that the hybrid yeasts contained almost all AFLP markers that are diagnostic for their parental strains. As Tables 2

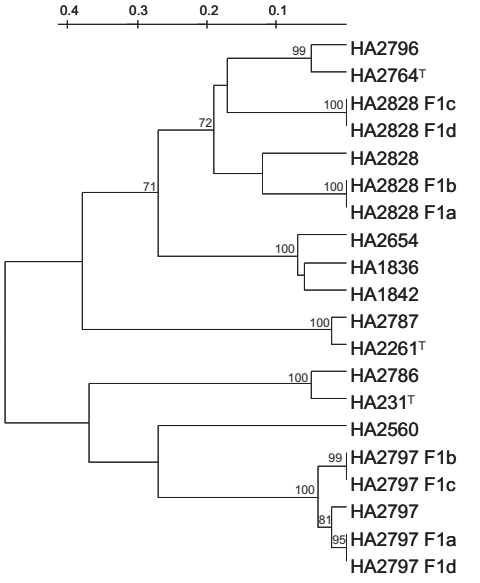

Fig. 1. Cluster analysis based on the AFLP molecular markers depicting genomic similarity between artificial and natural hybrid yeasts and parental strains. The scale bar indicates relative genetic distances between the investigated strains. Bootstrap analysis was carried out with 1000 replicates, and values above 70 % are given at nodes

and 3 show, the interspecies hybridization event was followed by a loss of several markers of *S. cerevisiae* and non-*S. cerevisiae* origins in both hybrids. Additional fragments, designated as gained because they were not detected in the electropherograms of any of the parental strains, were also observed. The process of the genome changes was observed in all the spore clones and was characterized by losses and gains of some additional AFLP markers (Tables 2 and 3). We believe that the changes of the AFLP profiles are correlated mainly with the alterations in the DNA sequences that can be influenced by chromosomal rearrangements, translocations, inversions and gene mutations. The spore clones lost many more AFLP markers than the corresponding hybrid strains, suggesting that the additional more serious genomic reorganizations followed meiosis. Interestingly, the meiotic segregants of *S. cerevisiae* × *S. kudriavzevii* HA2828 hybrid underwent more extensive alterations than those of *S. cerevisiae* × *S. uvarum* HA2797 hybrid (Tables 2 and 3). Particularly *S. kudriavzevii* subgenome underwent major changes, as could be judged from the greater number of lost AFLP markers. Similarly, the chromosomal changes in the *S. cerevisiae* × *S. uvarum* hybrid F1 segregants were minor, limited to the loss of the chromosome 2, while the *S. cerevisiae* × *S. kudriavzevii* hybrids exhibited major changes in the karyotypes of the F1 generation (Figs. 2a and b). The observed loss of the *S. uvarum* chromosome 2 in the HA2797 hybrid strain has recently been demonstrated by additional genetic and molecular markers too (*41*). The HA2828 hybrid itself seemed to lack chromosome XV of the *S. kudriavzevii* subgenome, or at least distinct rearrangements induced the loss of large DNA segments, changing the size and mobility of the chromosomes in the agarose gel. Besides the loss or alteration of the *S. kudriavzevii* parental chromosomes XV, the genome stabilization of the *S. cere-*

Table 2. Changes of the AFLP patterns of the *S. cerevisiae* × *S. uvarum* artificial hybrid strain HA2797 and its offspring. As a comparison, one natural hybrid isolate (HA2560) was used. All numerals indicate the sizes (bp) of the amplified fragments

	S. cerevisiae × *S. uvarum* HA2797 hybrid												
S. cerevisiae alleles lost						121	139						
S. uvarum alleles lost						148	259						
AFLP alleles gained						77	81						
Tetrad F1	F1a=F1d								F1b=F1c				
S. cerevisiae alleles lost		81	121	139				81	121	139			
S. uvarum alleles lost			148	259				148	219	259			
AFLP alleles gained			77	81			77	298	323	349			
	S. cerevisiae × *S. uvarum* HA2560 native hybrid												
S. cerevisiae alleles lost				81	108	121	215	310	311				
S. uvarum alleles lost	63	66	90	120	137	152	161	192	217	233	234	248	
	252	259	320	322	330	359	375	385	386	387	440		
AFLP alleles gained	77	81	91	107	163	176	201	299	233	323	332	349	

Table 3. Changes of the AFLP patterns of the *S. cerevisiae* × *S. kudriavzevii* artificial hybrid strain HA2828 and its offspring. As a comparison, two natural hybrid isolates (HA1836, HA1842) and one commercial strain (HA2654) were used. All numerals indicate the sizes (bp) of the amplified fragments

	S. cerevisiae × *S. kudriavzevii* HA2828 hybrid																								
S. cerevisiae alleles lost										423	446														
S. kudriavzevii alleles lost									102	183	204	272													
AFLP alleles gained									68	323	349														
Tetrad F1	F1a=F1b																F1c=F1d								
S. cerevisiae alleles lost				139	423	446											139	423	446						
S. kudriavzevii alleles lost	69	87	88	89	91	97	102	111	179	183		63	65	69	77	102	110	119	134	160	172	183			
	202	204	210	270	272	297	370	376	399			199	204	222	269	272	290	304	340	337	365	367			
AFLP alleles gained					323	349									68	172	323	349							
	S. cerevisiae × *S. kudriavzevii* native hybrids																								
S. cerevisiae alleles lost																									
1836									83	187	310	311													
1842									83	187	310	311	446												
2654										187	310	311													
S. kudriavzevii alleles lost																									
1836								69	102	183	204	270	304		376										
1842								69	102	183	204	270	304		376										
2654								69	102	183	204	270	304	367	376										
AFLP alleles gained																									
1836		68	82		95		146	172	186	201	262	314		336		359	374	402	441	442					
1842		68	82	84	95	129	146	172	186	201	262	314	323	336	349	359	374								
2654		68			95		146	172	186	201		314	323		349	359	374		441	442					

visiae × *S. kudriavzevii* HA2828 hybrid strain resulted in the loss of the chromosomes V and VIII in F1a and F1b, chromosome X in F1b, F1c and F1d, as well as chromosome XII in F1a, F1b and F1d spore clones (Fig. 2b). As already mentioned, the *S. cerevisiae* × *S. uvarum* hybrid HA2797 displayed 98 % spore viability, and the *S. cerevisiae* × *S. kudriavzevii* HA2828 only 53 %. This difference may be related to the fact that the former hybrid produced F1 strains with much more consistent AFLP patterns and karyotypes than the latter. These results also indicate that meiosis operated with a more precise outcome in the *S. cerevisiae* × *S. uvarum* HA2797 than in the *S. cerevisiae* × *S. kudriavzevii* HA2828 hybrid. This is also supported by the observations of the other examined tetrads of HA2797 (*41*), as well as by the karyotype analyses of additional HA2828 F1 segregants (not shown). The lower spore viability of HA2828 may in fact be the result of the inability of the chromosomes to pair, preventing normal meiotic division and generating incorrect meiotic products. It has recently been shown that the interspecies barrier of F1 sterility in the *S. cerevisiae* × *S. uvarum* artificial hybrids can be broken down by the loss

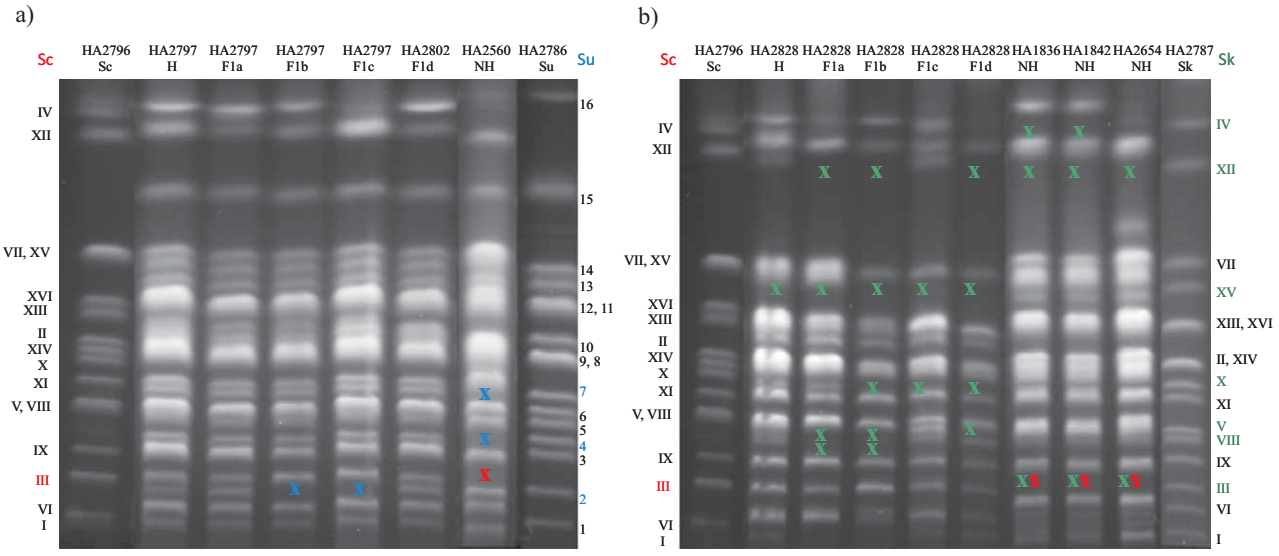

Fig. 2. Chromosomal patterns of: a) *S. cerevisiae* × *S. uvarum*, and b) *S. cerevisiae* × *S. kudriavzevii* artificial (H) and natural hybrids (NH). Chromosome bands of S. *cerevisiae* and *S. kudriavzevii* are indicated in Roman numerals, and those of *S. uvarum* in Arabic numerals. The missing chromosomes or those with supposed altered sizes are indicated with red (*S. cerevisiae*), blue (*S. uvarum*) and green (*S. kudriavzevii*) crosses

of the chromosome of the *S. uvarum* parental species that bears the mating type *MAT*-allele. The resulting alloaneuploid strains, which only possess one *MAT*-allele from the *S. cerevisiae* parent, are capable of mating-type switch and zygote formation, enabling correct meiosis and the generation of F2 hybrid offspring, resulting in heritable fertility (*41*).

In order to assess the extent of genomic variations in natural hybrids in comparison with the artificial ones, we assumed that their parental strains are different but closely related to *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii*, which were used for constructing the artificial hybrids (Fig. 1). The genome of *S. cerevisiae* × *S. uvarum* HA2560, isolated from wastewater, was characterized by a greater number of lost and gained AFLP markers in comparison with the artificial hybrid HA2797 and its progeny (Table 2). An increase in the number of lost *S. uvarum*-like AFLP alleles was particularly observed in the HA2560 strain. The *S. cerevisiae* × *S. uvarum* HA2560 isolate also showed a different chromosomal pattern when compared to those of the artificial hybrids (Fig. 2a). More chromosomes with altered size (*S. cerevisiae* chromosome III, and *S. uvarum* chromosomes 4 and 7) were identified in comparison with those of the artificial hybrids. The genomes of three wine-associated *S. cerevisiae* × *S. kudriavzevii* hybrid strains lost more AFLP markers from both parental genomes in comparison with the artificial hybrid HA2828, but the number of lost *S. kudriavzevii*-like AFLP markers was lower when compared to those in the HA2828 F1 strains (Table 3). The most significant differences between the artificial and natural *S. cerevisiae* × *S. kudriavzevii* hybrids were observed in the form of a greater number of new AFLP markers acquired by the natural strains. Natural hybrids also demonstrated a significant chromosomal polymorphism in comparison with the artificial ones (Fig. 2b). *Saccharomyces kudriavzevii*-like chromosomes III, IV and XII appear to be absent, or their size was changed by the genome modifications. An additional large chromosome between the chromosomes VII and XII was also observed in the commercially available *S. cerevisiae* × *S. kudriavzevii* HA-2654 strain, suggestive of chromosomal translocations or brakes. All these results indicated that the genomes of the natural hybrid isolates underwent more severe reorganizations under environmental pressures. However, as in the case of the *S. cerevisiae* × *S. uvarum* HA2560 strain, it should be borne in mind that the true parental strains of the *S. cerevisiae* × *S. kudriavzevii* natural hybrids are unknown. Natural *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* strains may show rather heterogenous karyotypes (*42*,*43*), so that their direct comparison with the strains used in the present study should be interpreted carefully. Most likely the fertility is not a key factor for the survival of hybrids in natural environments, or wine and brewing industries, since many hybrids from these habitats are sterile, as the four natural hybrids analyzed in this study also are. These clonal (sterile) hybrids may loose some chromosomes of their alloploid chromosome sets in the course of their mitotical generations, but the rare fertile hybrids are probably much more prone to large-scale genomic changes that could be mediated by the recombination mechanism during meiosis (*18*).

We used microarray-based comparative genomic hybridization (CGH) to evaluate global changes in genomic DNA of the natural hybrid yeasts *S. cerevisiae* × *S. uvarum* HA2560 and *S. cerevisiae* × *S. kudriavzevii* HA-1836 (not shown). Our preliminary results of CGH analysis, where the genome of the natural hybrid yeast HA-2560 was compared with the laboratory haploid *S. cerevisiae* strain S288C, identified a number of locations on chromosomes (III, V, VI, VII, VIII and XVI) where changes in the ratio of hybridisation (ROH) were observed. Such changes in ROH are indicative of recombination sites between parental chromosomes or locations where gene deletion or amplification have occurred. Similar results were obtained by Bond *et al.* (*4*), who used CGH analysis

to elucidate DNA copy number changes in two bottom-fermenting lager strains (*S. pastorianus*). The specific points with observed changes in ROH most likely represent intra- and/or interchromosomal recombinations between *S. cerevisiae* and *S. uvarum* homologous chromosomes. Our results also revealed chromosomes (III, VI, VIII, X, XI, XII and XV) with depleted DNA segments at subtelomeric regions. The low sequence homology with the control strain S288C indicated that the identified regions differ notably or are absent from natural hybrid yeasts. On the basis of these results we concluded that various chromosomal rearrangements may influence the karyotype of the HA2560 strain. It is posible that the *S. cerevisiae* chromosomes with large depleted DNA segments changed their size and hence their position in agarose gel (Fig. 2a). The CGH analysis of the *S. cerevisiae* × *S. kudriavzevii* HA1836 strain against the *S. cerevisiae* S288C microarray did not reveal any large depleted DNA regions or changes in ROH compared to the *S. cerevisiae* × *S. uvarum* HA2560 strain (not shown). The investigated *S. cerevisiae* × *S. kudriavzevii* HA1836 natural strain contained a complete set of *S. cerevisiae* chromosomes, which appear most probably in two copies. Peris *et al.* (*8,9*) have recently analysed five additional hybrid yeasts isolated from Austrian vineyards and came to similar conclusions. The CGH analysis showed that one strain used in the present study (HA1842) contained all the *S. cerevisiae* and *S. kudriavzevii* chromosomes. This corroborated our assumption that the parental chromosomes of the natural hybrids may show a considerable polymorphism, and they should not align perfectly with the reference strains (Figs. 2a and b).

Phenotypic properties of the hybrid yeasts

After remarkable genomic alterations were observed, we wondered how these genomic changes would affect the physiological properties of the hybrid yeasts and their offspring relative to parental strains. We monitored the fitness of the hybrid yeasts under the conditions of different carbohydrate supplementation and with increasing concentrations of osmolytic and antifungal compounds. The first observation was that *S. cerevisiae* species determined the growth rates of all investigated hybrids under almost all tested conditions. The inhibited growth was detected only on raffinose, where *S. cerevisiae* achieved around 67 % of the growth rate of *S. uvarum* after 96 h (Fig. 3). The *S. cerevisiae* × *S. uvarum* HA2797 and *S. cerevisiae* × *S. kudriavzevii* HA2828 hybrids exhibited similar fitness under the majority of investigated conditions. The two hybrid strains showed noteworthy differences only in the assimilation of raffinose and their sensitivity against miconazole. The HA2828 utilized raffinose less efficiently than the HA2797 strain (Fig. 3) and showed an increased resistance against miconazole (Fig. 4). The inhibited growth of the *S. cerevisiae* × *S. kudriavzevii* HA2828 hybrid may be explained by a decreased ability of the parental strains to assimilate raffinose. On the other hand, a somewhat greater growth rate in comparison with their ancestors was shown by the *S. cerevisiae* × *S. uvarum* HA2797 hybrid and its offspring, which could be explained by a synergistic activity of both parental strains. The meiotic segregants of the two hybrid types displayed quite different behaviour. While the *S. cerevisiae* × *S. uvarum* HA2797 spore clones as well as a natural isolate HA2560 strain exhibited the same growth rate as the hybrid HA2797 with all the tested sugars, the *S. cerevisiae* × *S. kudriavzevii* HA2828 F1 segregants showed significant variability in their carbohydrate consumption (Fig. 3). Sugar utilization was strain dependent, with the HA2828 strain always showing the highest growth rate, *i.e.* that of *S. cerevisiae*. A remarkable increase in the growth of HA2828 hybrid strain was observed on glucose at the beginning of incubation (t_{24}). A shorter lag and accelerated growth phases were also observed at 0.5 % glucose (not shown), indicating that better growth was not a random but intrinsic attribute of the *S. cerevisiae* × *S. kudriavzevii* HA2828 strain. The spore clones demonstrated different growth abilities; for instance, HA2828 F1a grew slowly and could not reach the growth level on glucose and raffinose of its ancestors after 96 and 144 h, respectively. This and the HA2828 F1d strain also showed delayed growth on maltotriose. As *S. kudriavzevii* showed no growth on maltotriose, the delayed growth of the HA2828 F1 strains may indicate that some changes in the *S. cerevisiae* genome influenced the expression of the genes involved in the maltotriose metabolism.

Fig. 4 shows the growth patterns of the interspecies hybrid yeasts at different amounts of sodium chloride, urea and miconazole after 96 h of incubation. In general, the growth of all investigated strains declined upon increasing the concentration of the osmolitic or antimicrobial substances in the medium, and the most significant differences among the strains were demonstrated at the highest concentrations. The artificial HA2797 and HA-2828 hybrids demonstrated the ability to grow at 10 % sodium chloride and 5 % urea. The growth of HA2797 and HA2828 strains at an increased concentration of urea is also remarkable, because it was not inherited from any of the parental species, but was characterized as a novel phenotype. The *S. cerevisiae* × *S. uvarum* HA2797 F1 segregants exhibited positive growth at 10 % sodium chloride, but the *S. cerevisiae* × *S. kudriavzevii* HA2828 F1 segregants showed an incomplete inheritance of the growth ability (only HA2828 F1d showed a positive growth). Similarly, the growth of all the *S. cerevisiae* × *S. kudriavzevii* HA2828 segregants was inhibited by 5 % urea. The *S. cerevisiae* x *S. uvarum* HA2797 F1 segregants demonstrated 2:2 segregation of urea phenotype. This phenotype correlated with the presence of the *S. uvarum* chromosome 2; the other two strains lacking this chromosome did not exhibit this phenotype (Fig. 2a). *Saccharomyces cerevisiae* has frequently been used as a model organism for studying cellular and molecular mechanisms of osmolyte tolerance. Yeast cells accumulate glycerol to counteract high ion concentrations, and use proton gradients to control ion fluxes under stress (*44–46*). Less is known about the accumulation of glycerol and the maintenance of ion homeostasis in hybrid yeasts. As hybrid yeasts are exposed to similar stress in distinct natural environments, or food and fermentation industries, it would be of interest to investigate how the non-*S. cerevisiae* parental fraction influences their adaptation to osmotic and ionic stress. Under the stress conditions caused by the high concentration of the antimicotic compound

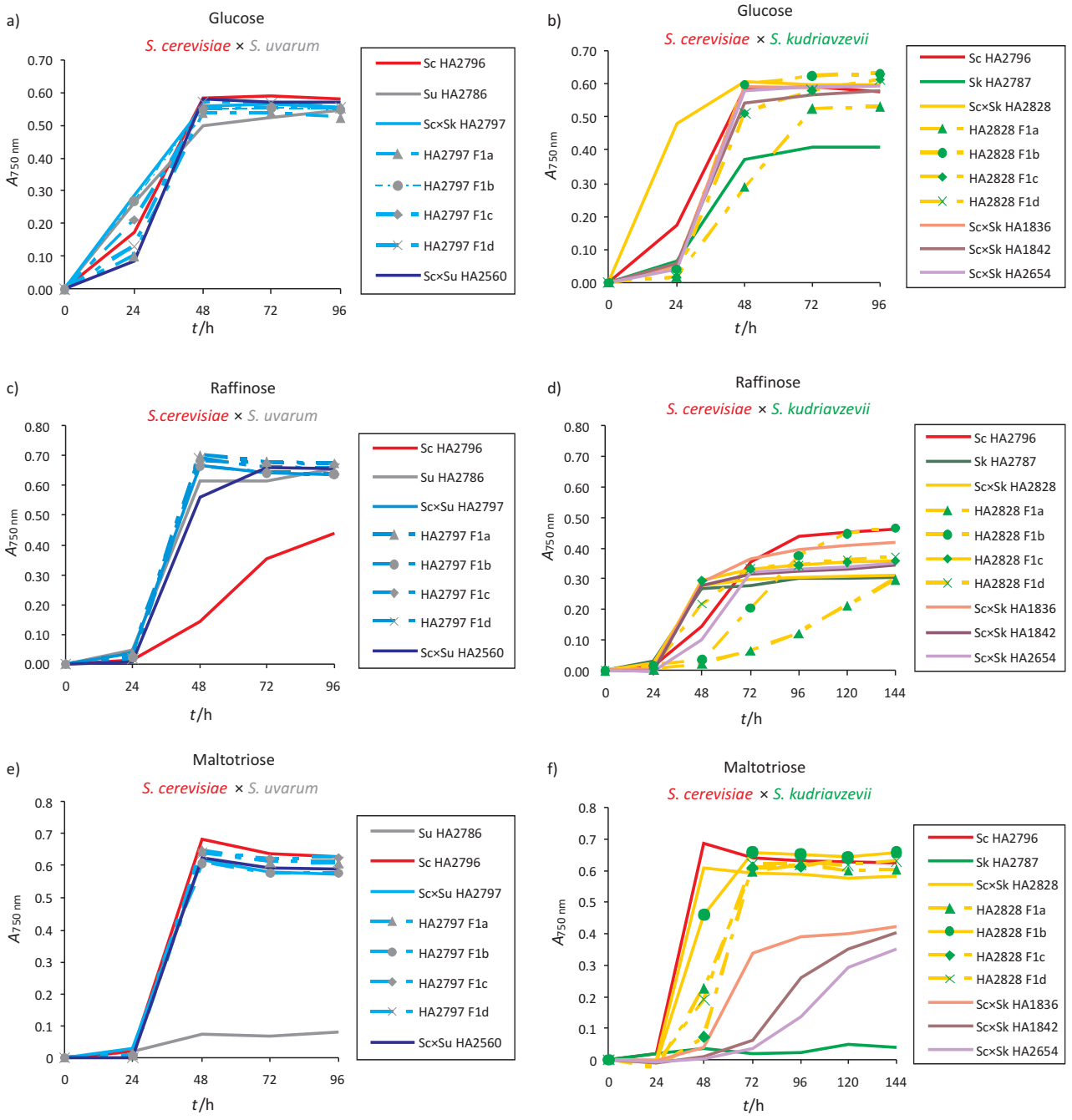

Fig. 3. Utilization of glucose (a and b), raffinose (c and d) and maltotriose (e and f) by *S. cerevisiae* × *S. uvarum* and *S. cerevisiae* × *S. kudriavzevii* strains

miconazole, the *S. cerevisiae* × *S. kudriavzevii* HA2828 hybrid demonstrated a considerable resistance comparable to its ancestors (Fig. 4). In the HA2828 F1 generation, growth capability varied greatly, from no growth to full tolerance, and an even somewhat better resistance was observed in the HA2828 F1a and F1d than in the parental strains. A comparison of these results with those generated by the *S. cerevisiae* × *S. uvarum* hybrids indicated that in addition to *S. cerevisiae*, *S. kudriavzevii* HA2787 played a significant role in the ability of the *S. cerevisiae* × *S. kudriavzevii* hybrids to develop resistance against miconazole (Fig. 4). By contrast, the *S. cerevisiae* × *S. uvarum* HA2797 and its offspring demonstrated a considerable sensitivity against miconazole, suggesting that one of the mechanisms regulating resistance against the antimicotic drug in this hybrid was affected by hybridization (*47–49*).

Three wine-associated strains: HA1836, HA1842 and HA2654 displayed a very similar ability to consume glucose and raffinose like the artificial hybrid HA2828, but quite different profiles of maltotriose utilization (Fig. 3). These strains exhibited different growth rates compared to each other, and utilized maltotriose less efficiently than the artificial hybrids. As we already supposed above, the most reasonable explanation would be that the natural yeast isolates contain *S. cerevisiae* subgenomes of differ-

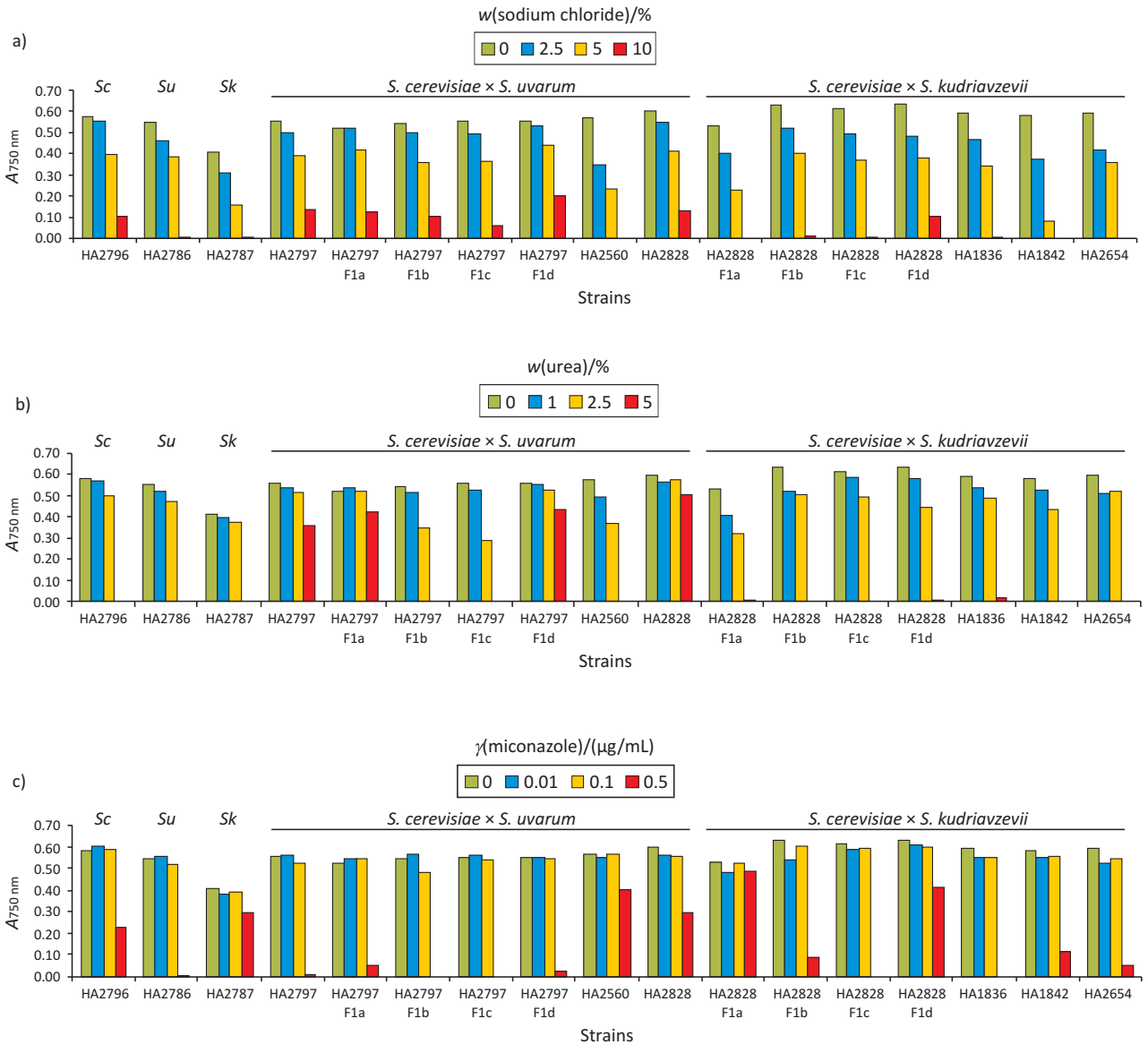

Fig. 4. Growth of *S. cerevisiae* (*Sc*) × *S. uvarum* (*Su*) and *S. cerevisiae* × *S. kudriavzevii* (*Sk*) strains in the presence of increasing amounts of: a) sodium chloride, b) urea and c) miconazole

ent origins which utilize maltotriose with different efficiency. Duval *et al*. (*32*) have recently stressed the importance of the *AGT1* gene for efficient maltotriose utilization in *Saccharomyces* strains. Our preliminary microarray-based comparative genomic hybridization analysis indicated that the *S. cerevisiae* × *S. kudriavzevii* HA1836 hybrids had an increased copy number of *MAL31* gene on chromosome II, encoding for maltose permease, and a depleted copy number of the *AGT1* gene on chromosome VII in comparison with the control S288C *S. cerevisiae* strain, indicating that the *AGT1* gene may be absent. This may explain a slow and delayed growth of the natural *S. cerevisiae* × *S. kudriavzevii* strains observed in the medium with maltotriose (Fig. 3). The same analysis showed that in addition to an increased copy number of *MAL31* gene, *S. cerevisiae* × *S. uvarum* HA2560 strain had the permease-encoding *AGT1* gene too. Although these results need additional experimental verifications, they are in full agreement with the findings of Duval *et al*. (*32*) and may likely explain differences in maltotriose utilization between the natural and artificial *S. cerevisiae* × *S. kudriavzevii* strains.

The increased fractions of sodium chloride and urea inhibited the growth of the natural *S. cerevisiae* × *S. uvarum* and *S. cerevisiae* × *S. kudriavzevii* hybrid yeasts (Fig. 3). Differently from the artificial hybrids, the natural *S. cerevisiae* × *S. kudriavzevii* strains showed no or weak resistance to 0.5 μg/mL of miconazole. On the other hand, the *S. cerevisiae* × *S. uvarum* HA2560 isolate from wastewater exhibited considerable growth in comparison with the corresponding artificial hybrids. As this strain was isolated from a specific ecological niche, which most likely also contains the azole antifungals among other antimicrobial substances, it could be assumed that this strain has developed resistance against miconazole over time.

Conclusions

In the present study we used two *Saccharomyces* interspecies hybrids to correlate the evolution of their geno- and phenotypes. The results demonstrate that the hybridization of different yeast genomes generates segregants with different viability, genomic and physiological properties. Relative to their ancestors, the hybrids stabilize their genomes and acquire similar or novel phenotypic attributes. The genomes of two species may recombine in such a way that the metabolic activity of the hybrid cells is influenced by one parental part, or both species may act synergistically, or even novel phenotypes may appear. Results of the present study suggest that the artificial *S. cerevisiae* × *S. kudriavzevii* HA2828 hybrid yeast underwent more extensive genome changes after meiotic segregation than the *S. cerevisiae* × *S. uvarum* HA2797, and the generation of the new genotypes obviously had a remarkable influence on phenotypic diversity. We believe that the observed phenotypic diversity may be attributed to genomic modifications of the former hybrids. Assuming that the hybrid strains examined here reach the environment, selective pressure will further modify their genomes. Distinct genotyping profiles and karyotypes in artificial and natural hybrid isolates, as well as an increase in miconazole resistance observed in the *S. cerevisiae* × *S. uvarum* HA2560 and decreased maltotriose utilization ability identified in the *S. cerevisiae* × *S. kudriavzevii* natural hybrids, support the view that environmental growth conditions greatly determine genome constitution and gene expression. The importance and application of the studied yeasts in alcohol fermentations should be tested by additional experiments under circumstances characteristic for these processes. It can be assumed that the genomic alterations of the artificial hybrids will be more drastic in harsh fermentation than under laboratory conditions. This environment may increase the loss of genes and alter metabolic pathways which are important for fermenting yeasts. We suppose that the wine-associated hybrids used in this study lost important genes involved in maltotriose metabolism during their genome evolution. It was shown that the *S. cerevisiae* × *S. kudriavzevii* natural isolates from a vineyard (HA1836 and HA1842) and a commercial wine strain (HA2654) are less suitable for brewing, as deduced from their reduced ability to utilize maltotriose. Different behaviour of the F1 segregants of the two hybrid types at increased amounts of sodium chloride, urea and miconazole suggests that their tolerance mechanisms to osmolyte and antimicotic compounds should be investigated more comprehensively using the genome-wide screening approaches. In order to understand evolution of the phenotypic traits and genetic background of the differences which arise due to a hybridization event in more detail, it would be of interest to explore the whole genome sequences in the hybrids and their progeny. Comparative analysis of the complete sequences can help link genetic changes to phenotypic modifications and better understand the role of specific genes in various metabolic and regulatory pathways.

Acknowledgements

This work was partially supported by the grant 83ÖU12 from the Stiftung Aktion Österreich-Ungarn. We wish to thank E. Barrio, A. Querol and U. Rentsendorj for providing strains.

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