Abstract

The usefulness of four genetic fingerprinting methods (interdelta sequence typing, mitochondrial DNA restriction length polymorphism (mtDNA RFLP), chromosomal karyotyping and microsatellite analysis) was assessed in order to detect a commercial yeast strain (Zymaflore VL1, Lallemand). From 54 spontaneous fermentations performed with grapes collected in the Vinho Verde Wine Region in northwest Portugal, 101 isolates were recovered whose mtDNA RFLP were identical to strain Zymaflore VL1. Evaluation of polymorphisms was performed in comparison to 30 isolates of the corresponding original commercialized strain.

Microsatellite allelic polymorphisms were found in 12 natural isolates, two of them were characterized by complete loss of heterozygosity, whereas their chromosomal constitution showed a loss of structural heteromorphism. Major changes of chromosomal patterns were found among the natural isolates, apparent by the absence or changed position of bands in the presumable region of chromosomes VI and III. Interdelta amplification patterns depended on the primer pair used, and changes were apparent by additional bands. No correlations were found between interdelta sequence amplification patterns and chromosomal profiles or microsatellite typing patterns. The present data show that commercial yeast strains present a considerable genetic instability that can be assessed by distinct methods. Whether these changes are associated with the yeast's permanence in natural environments is subject of current investigations.

Genetic instability of a commercial Saccharomyces cerevisiae strain

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Introduction

Wild strains of Saccharomyces cerevisiae, *isolated from wine, cellars or vineyards are predominantly diploid, homothallic and mostly homozygous (65%), with low [1] to intermediate (30%) sporulation capacity [2] Aneuploid strains, with approximately diploid DNA contents, have been described [3] and meiosis seems not to be a common occurrence in their life cycle [1]. Such wine yeast strains present essentially an asexual life cycle and are characterized by high karyotype instability which is believed to be a potential source of genetic variability [4-7]. Haploid laboratory strains do not undergo by far such extensive changes [6]. Gross mitotic chromosomal rearrangements, such as large regions fusion between homologous and non-homologous chromosomes occur in wine yeast with frequencies around 10^{-3} [7].*

It was hypothesized that subtelomeric plasticity may allow rapid adaptive changes of the yeast strain to specific substrates [5]. The SSU1-R allele, generated by reciprocal translocation between chromosomes VIII and XVI, confers sulfite resistance to yeast cells and was described as first case of adaptive evolution, occurring probably as a consequence of the use for millennia of sulfite as a preservative in wine production [8,9]. Ty-promoted genome rearrangements are involved in karyotype alterations in natural and industrial strains, mediating reciprocal recombinations (chromosome I/III or III/VII) [5].

In view of the high genetic instability of S. cerevisiae *winery strains, the objective of the present work was to assess the usefulness of four genetic fingerprinting methods (interdelta sequence typing, mitochondrial DNA restriction fragment length polymorphism (mtDNA RFLP), chromosomal karyotyping and microsatellite analysis), to detect a commercial yeast strain (Zymaflore VL1, Lallemand) that derived either from isolates recovered from vineyards or the "original" commercialized strain and to evaluate whether the permanence of this strain in natural grapevine environments induced genetic changes.*

Materials and Methods

Fermentation and strain isolation

The natural isolates of the S. cerevisiae *strain Zymaflore VL1 were obtained from 54 grape samples collected at the harvest of the years 2001 – 2003 in different sampling sites close to 3 wineries located in the Vinho Verde Wine Region (northwest Portugal), that predominantly used this commercial yeast for the last five to ten years.*

About 0,5 l grape juice was obtained from 2 kg of aseptically smashed grapes and in most cases a spontaneous fermentations occurred (20ºC, 20 rpm). When must weight was reduced by 70 g/l, corresponding to the consumption of about 2/3 of the sugar content, diluted samples (10^{-4} and 10^{-5}) were spread on plates containing YPD medium. After incubation (2 days, 28ºC), 30 randomly chosen colonies were collected and used for molecular typing. Among the fermentative flora derived from 16 fermentations (8 in 2001, 2 in 2002, 6 in 2003), 101 isolates were obtained (N1 – N101). The original "mother strain" Zymaflore VL1, that is used for the production of the commercially available VL1 yeast was kindly provided from Lallemand and was used as reference. As above, 30 isolates (LM1 – LM30) were randomly chosen and used for molecular typing. All 131 isolates used throughout this work were kept in frozen stocks (glycerol, 30 %, v/v) at -80 ºC.

DNA isolation

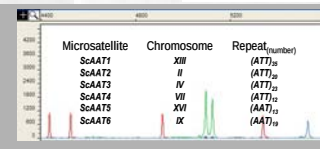
Each isolate was cultivated in 5 ml YPD medium (36 h, 28ºC, 200 rpm) and DNA isolation was performed using a previously described method [10]. The progress of cell lysis was dependent on the strain and could last between 1 to 3 hours. DNA was quantified and used for interdelta sequence typing, mitochondrial RFLP and microsatellite analysis.

Interdelta sequence typing

Amplification reactions were performed on a BioRad iCycler thermal cycler, using the primers δ1 (5'-CAAAATTCACCTATATCT-3') and δ2 (5'-GTGGA TTTTTATTCCAAC-3') (primer pair A) [11] or δ12 (5'-TCAACAATGGAATCC CAAC-3') and δ2 (primer pair B) [12] as described [13].

Chromosomal polymorphisms

Yeast chromosomal DNA was prepared in plugs as previously described [4]. Pulse gield gel electrophoresis was performed using the TAFE (transverse alternating field electrophoresis) system (Geneline, Beckman). The gels were run for 26 h: 6 h at 250 V with 35 s pulse time followed by 20 h at 275 V with 55 s pulsed time, at a constant temperature (14ºC).



Microsatellite amplification

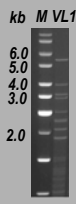
The six trinucleotide microsatellite loci described as ScAAT1, ScAAT2, ScAAT3, ScAAT4, ScAAT5 and ScAAT6 were amplified [14]. Samples were separated in the ABI Prism 310 DNA sequencer (Applied Biosystems) and analyzed with the corresponding GENESCAN software. The equivalence of this typing method to previously described ones has been shown for the case of commercial S. cerevisiae strains [13].

Mitochondrial DNA restriction profiles (mtDNA RFLP)

From the total genomic DNA, isolated as described, 17 µl were digested with 0.5 µl of the restriction endonucleases HinfI (10 U/µl, MBI Fermentas), 2 µl of the appropriate 10x buffer and 0.5 µl of RNAse (MBI Fermentas) overnight at 37ºC. Restriction fragments were separated on a 1.5 % agarose gel containing ethidium bromide, visualized and photographed.

RESULTS

Mitochondrial DNA restriction fragment length polymrphism (mtDNA RFLP)



- *From 16 spontaneous fermentations, performed with grapes collected in vineyards of the Vinho Verde Region, 101 isolates derived from natural environments (N1-N101) were obtained showing the mtDNA RFLP of the starter strain Zymaflore VL1 (Lallemand).*
- *These isolates revealed a unique and stable banding pattern (range of 1.8 to 5.5 kb). They were selected for further analysis by the other typing methods.*
- *All 30 isolates obtained from the original commercial (LM) strain showed the same pattern.*

Microsatellite analysis of S. cerevisiae Zymaflore VL1 isolates recovered from natural environments (N) in comparison to the original commercial strain.

Loci	Alleles (bp) of distinct microsatellite patterns							
	M1	M2	M3	M4	M5	M6	M7	M8
ScAAT1	209/219	219	209	209	209/219	209/219	209/219	209/219
ScAAT2	367/381	373	381	381	381	373	367/381	367/381
ScAAT3	265	265	265	265	265	265	265	265
ScAAT4	320	320	320	320	320	320	320	320
ScAAT5	219/222	222	219	219	219/222	219/222	222	219/222
ScAAT6	256/259	259	256	256	256/259	256/259	256/259	259
N° of natural isolates (N)	89	1	1	1	1	1	2	5
N° of original VL1 isolates (LM)	30	0	0	0	0	0	0	0

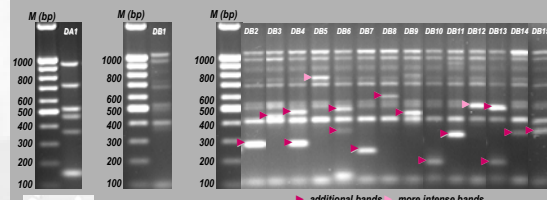
- *All isolates derived from the Lallemand reference strain VL1 show a characteristic allelic distribution (pattern M1). This pattern was also found in the majority of the isolates recovered from nature (89 of 101 isolates).*
- *Patterns M2, M3 and M4 from three natural isolates were characterized by complete loss of heterozygosity (LOH), but maintained their diploid chromosomal constitution (data obtained by flow cytometry, not shown).*
- *Pattern M4 is characterized by a trinucleotide increment from 381 bp to 384 bp in locus ScAAT2, while patterns M7 and M8 are characterized by the absence of alleles 219 and 256 (ScAAT5 and ScAAT6). These changes could be result of microsatellite expansion due to the hypothesized "replication-slippage" model [15], giving raise to alleles 222 and 259 respectively. The disappearance of alleles 372 and 381 (ScAAT2) in patterns M5 and M6 may be associated with other mechanisms.*
- *Pattern M8 corresponds to the absence of allele 256 (ScAAT6) and was the most frequent variation, but the isolates could be clonal since four of them derived from the same fermentation.*

Patterns for interdelta sequence amplification using primer pair A or B among isolates derived from natural environments (N) and from the "original" VL1 strain (LM).

Primer pair	Pattern	Additional bands (bp)	Number of isolates from population: Natural isolates (N)	Original VL1 isolates (LM)
A	DA1	-	101	30
	DB1	-	76	29
	DB2	290	1	
	DB3	450	1	
	DB4	280, 480	1	
	DB5	760	1	
	DB6	360, 500	1	
	DB7	240	1	
B	DB8	610	1	
	DB9	480	1	
	DB10	190	1	
	DB11	330	1	
	DB12	530	1	
	DB13	190, 320	1	
	DB14	340	1	
	DB15	320		1

- *Patterns DA1 and DB1 are characteristic for strain Zymaflore VL1.*
- *All isolates showed the characteristic VL1-pattern when primer pair A was used, independent of their origin.*
- *Amplification with primer pair B generated a more polymorphic banding pattern when compared to primer pair A.*
- *Variant amplification patterns DB2-DB14, characterized by additional bands (see image below) were apparent in 14% of the isolates recovered from winery environments, whereas only one (3%) of the original VL1 isolates showed the variant pattern DB15.*

Patterns obtained by interdelta sequence analysis with primer pairs A and B



Microsatellite pattern	Numbers of isolates with patterns DB1 – DB15														
	DB 1	DB 2	DB 3	DB 4	DB 5	DB 6	DB 7	DB 8	DB 9	DB 10	DB 11	DB 12	DB 13	DB 14	DB 15
MS 1	76	1	1	1	0	1	1	1	1	1	1	0	1	1	1
MS 2	1														
MS 3	0											1			
MS 4	1														
MS 5	1														
MS 6	1														
MS 7	1				1										
MS 8	5														
N° of natural isolates (N)	86	1	1	1	1	1	1	1	1	1	1	1	1	1	0
N° of original VL1 isolates (LM)	29	0	0	0	0	0	0	0	0	0	0	0	0	0	1

- *One hundred and five (76 + 29) isolates showing the VL1- characteristic microsatellite pattern MS1 showed the VL1-characteristic interdelta sequence pattern DB1.*
- *No correlations were apparent between microsatellite typing patterns and interdelta sequence amplification patterns.*

Karyotype analysis (PFGE)

- *Chromosomal polymorphisms were analyzed in 45 of the 101 isolates derived from natural environments. The most abundant pattern K 1 was considered to be characteristic of strain Zymaflore VL1, since it was identical with the commercialized strain VL1 (not shown).*
- *Two isolates showed a chromosomal constitution similar to the expected pattern for a haploid derivative (KD), characterized by loss of structural heteromorphism for example for chromosomes III and VI.*
- *Major changes of chromosomal patterns were evident by the absence of one band in the presumable region of chromosomes VI (K 2) and III (K 4).*
- *Minor chromosomal changes, in the same chromosomal regions, were assigned to patterns K 3 (Chr. VI), K 5 (Chr. III) and K 6 (both Chr. III and VI) are characterized by double bands closer or more distant than in pattern K1.*
- *One strain (pattern K 7) is characterized by changes in chromosomal regions III and V-VIII.*

Microsatellite pattern	Numbers of isolates with patterns K 1 – K 7						
	KD	K 1	K 2	K 3	K 4	K 5	K 6	K 7
MS 1		10		8	2	12	2	1
MS 2	1							
MS 3								
MS 4	1							
MS 5								
MS 6		1						
MS 7		1	1	1				
MS 8						1		
N° of natural isolates (N)	2	15	1	9	2	13	2	1
N° of original VL1 isolates (LM)	0	0	0	0	23	0	2	0

- *For natural isolates, recovered from sites close to the wineries where yeast strain Zymaflore VL1 has been used, most of the isolates showed the karyotype pattern K1, that matched the corresponding microsatellite patterns MS 1 (with 2 exceptions, MS 6 and MS 7).*
- *Surprisingly, 28 of 30 isolates that derived from Lallemands VL1 "mother strain" corresponded to chromosomal pattern K 4, and pattern K 1 was not represented in this population.*
- *Isolates with loss of structural heteromorphism (KD) corresponded to microsatellite patterns M2 and M4, that are characterized by complete loss of heterozygosity.*
- *No further correspondence were apparent for karyotype patterns K 2 – K 7 and microsatellite patterns.*

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Conclusions

Microsatellite allelic polymorphisms were found among natural isolates of strain VL1. They are mainly characterized by loss of heterozygosity (LOH) and trinucleotide expansions which are referred as microevolutionary changes. LOH can be explained by the previously described "genome renewal" [2] i.e. the occurrence of sporulation and subsequent "self-diploidization".

The PCR-based interdelta amplification patterns also showed a high level of polymorphisms among natural isolates, characterized mainly by the appearance of additional bands. Contrarily, isolates derived from the "original" VL1 strain did not show by far such a high genetic variability.

Considerable chromosomal DNA polymorphisms were observed, most evident for the smaller chromosomes III and VI, rearrangements, that are considered to be involved in adaptive evolution. Populations recovered from natural environments and from the original reference strain showed different predominating karyotypes (K1 / K 5 and K 4, respectively). Whether the observed genetic changes represent microevolutionary changes with relevance for the yeast's survival in nature needs further investigation.

No correlations regarding variant patterns were found between different combinations of typing methods. Although gross chromosomal rearrangements may be mediated by delta sequences flanking Ty elements, variations in delta sequence chromosomal positions apparent by additional bands was not correlated with karyotype variability.

Our data show that isolates of commercial strains recovered from environments surrounding the wineries where they were used, present a considerable genetic instability that can be assessed by interdelta sequence typing, microsatellite analysis and electrophoretic karyotyping. Mitochondrial DNA RFLP revealed to be a very stable marker, whereas interdelta sequence analysis depends on the primer pair used. Electrophoretic chromosome karyotyping is still the method of choice for evaluation of chromosomal rearrangements.

This poster is available at

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