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# Efficient use of DNA molecular markers to construct industrial yeast strains

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genetic mapping; DNA microarray; QTL; introgression; breeding; wine yeast.

## Abstract

*Saccharomyces cerevisiae* yeast strains exhibit a huge genotypic and phenotypic diversity. Breeding strategies taking advantage of these characteristics would contribute greatly to improving industrial yeasts. Here we mapped and introgressed chromosomal regions controlling industrial yeast properties, such as hydrogen sulphide production, phenolic off-flavor and a kinetic trait (lag phase duration). Two parent strains derived from industrial isolates used in winemaking and which exhibited significant quantitative differences in these traits were crossed and their progeny (50–170 clones) was analyzed for the segregation of these traits. Forty-eight segregants were genotyped at 2212 marker positions using DNA microarrays and one significant locus was mapped for each trait. To exploit these loci, an introgression approach was supervised by molecular markers monitoring using PCR/RFLP. Five successive backcrosses between an elite strain and appropriate segregants were sufficient to improve three trait values. Microarray-based genotyping confirmed that over 95% of the elite strain genome was recovered by this methodology. Moreover, karyotype patterns, mtDNA and tetrad analysis showed some genomic rearrangements during the introgression procedure.

## Introduction

The prime industrial microorganism *Saccharomyces cerevisiae* exhibits a huge genetic and genomic variability among individuals (Bidenne *et al.*, 1992; Winzeler *et al.*, 2003; Dunn *et al.*, 2005; Legras *et al.*, 2005), which generates metabolic diversity, resulting in the specialization of strains for specific industrial purposes, such as winemaking, brewing, distilling and baking (Oliver, 1991). Most industrial yeast traits are distributed in a complex, continuous way within a yeast population. This quantitative distribution results from the polygenic nature of those traits, and from the various allelic forms existing within a wide population. Generally unidentified, these loci are named quantitative trait loci (QTL) (Lynch & Walsh, 1998).

Historically, optimized yeast strains were first selected for their technological aptitudes by specific phenotypic screening of appropriate microorganism collections (Romano *et al.*, 1994; Murat *et al.*, 2001; Patel & Shibamoto, 2003). However, it is not possible to accumulate alleles determining optimal trait values with this methodology. Alternatively, suitable alleles were combined in a single strain by crossing strains with

complementary qualities (Clément & Loiez, 1982; Romano *et al.*, 1985; Prior *et al.*, 1999; Marullo *et al.*, 2006). Although efficient, these breeding programs suffered from the lack of knowledge of the loci/genes determining the desired trait value.

In agronomical science, quantitative genetics concepts and QTL-mapping approaches allowed the identification of numerous QTL in plants and animals (Grobet *et al.*, 1998; Frary *et al.*, 2000; Glazier *et al.*, 2002) despite the large genomes of agronomical species (50- to 2000-fold larger than yeast genome). This task was largely facilitated using molecular genetic markers to tag loci controlling economically relevant traits in plants and animals (Lander & Botstein, 1989). Recently, QTL-mapping strategies were applied to *Saccharomyces cerevisiae* using the powerful genomic tools of DNA-micro arrays (Winzeler *et al.*, 1998). Thanks to the compactness of the yeast genome and its sequence annotation, genes (Brem *et al.*, 2002; Steinmetz *et al.*, 2002; Sinha *et al.*, 2006), and even single nucleotide polymorphisms (SNP) (Deutschbauer & Davis, 2005; Marullo *et al.*, 2007) that control quantitative traits, have been identified. These advances are bridging the gap between genotypes and

phenotypes related to industrial yeast selection purposes. Once suitable alleles have been identified, they may be introduced into a strain of interest by DNA technologies to improve only the desired traits and conserving all their other qualities (Pellerin *et al.*, 2005). Nevertheless, in the food and beverage industry, genetically modified (GM) strains are poorly accepted by consumers (Pretorius & Bauer, 2002). An alternative approach, called introgression, consists of introducing by backcrossing suitable alleles in a recipient variety, called 'elite', which is generally one of the best ones on the market. This strategy generally assisted by molecular markers is routinely used in agronomy for plant and race improvement (Hospital & Charcosset, 1997).

In this work, we demonstrate that genetic mapping and marker-assisted introgression can also be applied to industrial yeast improvement. First, genetic mapping was used to identify genetic regions controlling three enological traits. Second, mapped loci were introduced by introgression in an 'elite' yeast strain by marker-assisted backcrosses.

## Materials and methods

### Description of yeast strains used

All strains used are listed in Table 1. To match winemaking practice, we used enological strains SB and GN. They were selected from among numerous strains for showing distinct, extreme phenotypes for the investigated traits. GN and SB were assumed to be totally homozygous, as they were obtained by tetrad micro-dissection of homothallic (*HO/*

*HO*) commercial strains. For QTL-mapping, the analyzed progeny population is derived from the BN hybrid. This strain was previously obtained by crossing a GN spore with *ho*-SB strain, a haploid SB derivative obtained by DNA engineering (Marullo *et al.*, 2006). One aim of this study was to propose an alternative for the use of GMO for yeast improvement. We therefore also constructed the F1-hybrid *HO*-BN by crossing SB and GN strains by spore-to-spore pairing using a Singer Manual micromanipulator (Singer Instruments, Roadwater, UK) (Naumov *et al.*, 1986). This non-GM hybrid was the starting genetic material for obtaining an optimized strain by marker assisted backcross. The backcrossed strains (BC1–BC6) were obtained by pairing SB spores with spores derived from progeny clones selected on the basis of their genotype (Table 1).

### Media and culture conditions

Yeasts were grown at 30 °C on complete YPD medium (1% yeast extract, 1% peptone, 2% dextrose) solidified with 2% agar, as necessary. Sporulation was induced on acetate medium (1% potassium acetate, 2% agar) after 3 days at 24 °C. Respiration-deficient strains (*petites*) were detected on YP-Glycerol medium (1% yeast extract, 1% peptone, 2% glycerol, 0.05% glucose).

### Enological traits

Hydrogen sulfide production was estimated by the blackening of the yeast culture on BIGGY agar (Difco, Detroit,

**Table 1.** Yeast strains used

Strain	Descriptive	Relevant genotype*	Origin
X2180-1A	Laboratory strain	<i>ho, MATa</i>	YGSC Berkeley
GN	Parent strain	<i>HO/HO, YOL083-GN/YOL083-GN, pad1/pad1</i>	Marullo <i>et al.</i> (2006)
SB	Parent strain	<i>HO/HO, YOL083-SB/YOL083-SB, PAD1/PAD1</i>	Marullo <i>et al.</i> (2006)
<i>ho</i> SB	Parent strain for mapping	<i>Ho::KanMx4, YOL083-SB, PAD1</i>	Marullo <i>et al.</i> (2006)
BN	Hybrid <i>ho</i> SB × GN	<i>HO/ho::KanMx4, YOL083-SB/YOL083-GN, PAD1/pad1</i>	Marullo <i>et al.</i> (2006)
<i>HO</i> -BN	Hybrid SB × GN	<i>HO/HO, YOL083-SB/YOL083-GN, PAD1/pad1</i>	This study
<i>HO</i> -BN-2B	Progeny clone of <i>HO</i> -BN	<i>HO/HO, YOL083-GN/YOL083-GN, pad1/pad1</i>	This study
BC1	Hybrid <i>HO</i> -BN-2B × SB	<i>HO/HO, YOL083-SB/YOL083-GN, PAD1/pad1</i>	This study
BC1-1A	Progeny clone of BC1	<i>HO/HO, YOL083-GN/YOL083-GN, pad1/pad1</i>	This study
BC2	Hybrid BC1-1A × SB	<i>HO/HO, YOL083-SB/YOL083-GN, PAD1/pad1</i>	This study
BC2-2C	Progeny clone of BC2	<i>HO/HO, YOL083-GN/YOL083-GN, pad1/pad1</i>	This study
BC3	Hybrid BC2-2C × SB	<i>HO/HO, YOL083-SB/YOL083-GN, PAD1/pad1</i>	This study
BC3-5D	Progeny clone of BC3	<i>HO/HO, YOL083-GN/YOL083-GN, pad1/pad1</i>	This study
BC4	Hybrid BC3-5D × SB	<i>HO/HO, YOL083-SB/YOL083-GN, PAD1/pad1</i>	This study
BC4-2B	Progeny clone of BC4	<i>HO/HO, YOL083-GN/YOL083-GN, pad1/pad1</i>	This study
BC5	Hybrid BC4-2B × SB	<i>HO/HO, YOL083-SB/YOL083-GN, PAD1/pad1</i>	This study
BC5-1B	Progeny clone of BC5	<i>HO/HO, YOL083-GN/YOL083-GN, pad1/pad1</i>	This study
BC5-1C	Progeny clone of BC5	<i>HO/HO, YOL083-GN/YOL083-GN, PAD1/PAD1</i>	This study
BC6-hom	Hybrid BC5-1B × SB	<i>HO/HO, YOL083-GN/YOL083-GN, pad1/pad1</i>	This study
BC6-het	Hybrid BC5-1C × SB	<i>HO/HO, YOL083-SB/YOL083-GN, PAD1/pad1</i>	This study

Spore clones obtained by tetrad microdissection were noted as described by Wickner (1991).

\**pad1* refers to the *pad1-G213* allele described in text.

MI) after 3 days in culture (Mortimer *et al.*, 1994). Five colors were distinguished: 1, white; 2, light brown; 3, brown; 4, dark brown; 5, black. Duplicate determinations were carried out. The POF (phenolic off flavor) character was estimated by GC-MS, as previously described (Chatonnet *et al.*, 1993). Kinetic traits (lag phase duration and alcoholic fermentation time) were measured in batch fermentations carried out at 24 °C in 1.2 L locked – to maintain anaerobiosis – bioreactors, with permanent stirring. The fermentation medium used (KP-Medium) was detailed in a previous work (Marullo *et al.*, 2006) and mimicked a white grape juice. The amount of CO<sub>2</sub> released (g L<sup>-1</sup>) and the CO<sub>2</sub> production rate (g L<sup>-1</sup> h<sup>-1</sup>) were determined by automatic measurement of bioreactor weight loss at 20-min intervals (Marullo *et al.*, 2006). Lag phase (h) was the time elapsed between inoculation and the beginning of CO<sub>2</sub> release, reflecting the time required for a specific strain to adapt to the must. The alcoholic fermentation (AF) time (h) was the time during which CO<sub>2</sub> was released.

### Marker map construction

Genomic DNA was isolated, fragmented, labeled and hybridized to Affymetrix S98 microarrays, as previously described (Winzeler *et al.*, 1998). We applied this procedure to three independent ho-SB cultures, three independent GN cultures, and one culture of each of the 48 segregants to be genotyped. We then selected bi-allelic markers using the procedure described in Brem *et al.* (2002). Briefly the procedure consisted of three main statistical filters. First, 35 392 probe pairs exhibiting high hybridization differences between ho-SB and GN were selected using the Z and z statistics described in Brem *et al.* (2002). Second, 7167 probes pairs showing a 2:2 segregation in the segregating population were selected using a clustering algorithm described in Brem *et al.* (2002). Third, we removed markers with less than eight instances of the same genotype, highly unlikely by random segregation, and probe pairs for which genotype inference failed in over 15 hybridizations. Probe pairs positively selected in all the tests resulted in a set of 2212 bi-allelic markers spread throughout the genome.

### Search for relationships between markers and traits

Linkage between phenotype values and markers was analyzed as follows. At every marker position, segregants were separated into two groups, according to their genotype at the marker locus. The difference in phenotypic values between the two groups was then assessed by the Wilcoxon–Mann–Whitney (WMW) test. Genome-wide significance was addressed by permuting segregant phenotypes and rescanning the genome.

### Sequencing and PCR-RFLP fine mapping

Markers identified as QTLs were validated by sequencing a 500- to 800-bp fragment centered on map positions predicted by microarray. Sequence alignment of both strands was carried out by Millegen (Toulouse, France) using the following primers: *YOL083w* p118: 5'-CGAGTGTTCAGTTACAGGAGG-3' p119: 5'-TGCTTGATTCATCAGGGGAA-3', *ATG19* p120: 5'-GAAAAGTACGGACAATCAGGG-3' p121: 5'-TTTCATCAAGGGAGCGTTCT-3', *HAL9* p189: 5'-CAAATTGTATTTTAGGAACCG-3' p190: 5'-TGATGCC TTGAAACAGCTGTA, *IRA2* p155: 5'-CAAGAATTTCCG TATTTTG-3' p156: 5'-GGTGAAGTCAATGTAGTCGGT-3', *PAD1* p217: 5'-AAGATTGGTGATGGATAGAATTTCA-3' p218 5'-CAACTCTATTAGTAGTTGAGTAACGTA-3'. To readily follow SNPs found between parental strains, a restriction fragment length polymorphism (RFLP) analysis was carried out on PCR fragments, using the following restriction enzymes (New England Biolabs, Ipswich, MA). *YOL083w*: NlaIII cutting SB allele; *ATG19*: BpuAI cutting GN allele; *HAL9*: HinfI cutting GN and SB alleles once and twice, respectively; *IRA2*: ScaI cutting SB allele; *PAD1*: BglI cutting GN allele. Using these PCR/RFLP markers, detailed mapping was carried out on a large progeny population.

### Pulsed field gel electrophoresis (PFGE)

Chromosomal DNA was prepared from overnight yeast cultures in agarose plugs, as previously described (Bellis *et al.*, 1987). Chromosomes were separated on 1% multi-purpose agarose gel (Roche, Mannheim, Germany) using a CHEF DRII apparatus (Biorad, Richmond, CA). Electrophoresis was carried out at 200 V and 14 °C for 16.5 h, with a switching time of 60 s, then for 10 h with a switching time of 90 s. Chromosomal DNA of X2180-1A was used as *S. cerevisiae* standard karyotype.

### mtDNA extraction

mtDNA was purified as previously described (Defontaine *et al.*, 1991). Polymorphisms between strains were visualized by EcoRV (New England Biolabs, Ipswich, MA) restriction digest.

### Statistical analysis

Biometric data set and trait differences between strains were statistically analyzed using statistic tests currently used in biology (Berry & Lindgren, 1995). The significance level of WMW, and Pearson, tests were assayed by a randomization test of 1000 permutations computed by R software ([www.r-project.org](http://www.r-project.org)).

## Results

### Study design

This work was divided into two parts. First, we carried out a QTL-mapping by establishing a link between phenotypic values and genetic markers within a large lineage. The phenotype data set used was derived from a previous investigation (Marullo *et al.*, 2006). The genetic marker set was obtained by a comparative genome hybridization approach described below. Second, we used the mapped loci to improve a reference strain using introgression assisted by molecular markers.

### Enological traits

In a previous study, a vast progeny derived from the BN hybrid was obtained and assayed for several winemaking traits (Marullo *et al.*, 2006). These progeny clones and relative phenotypic data were used to investigate the genetic bases of three enological traits i.e. hydrogen sulfide production, phenolic off-flavor production and lag phase duration. Table 2 summarizes parent and hybrid values of each trait, together with their value ranges and distribution shapes observed within BN progeny. When assaying hydrogen sulfide production by plating strains on BIGGY agar, the higher the H<sub>2</sub>S production, the blacker the colonies (range: 1–5). Values up to level 2 (GN strain) are acceptable for winemaking, while strains over level 4 (SB strain) are unsuitable for winemaking (Romano *et al.*, 1985; Jiranek *et al.*, 1995; Marullo *et al.*, 2004). Despite the small number of phenotypic classes, trait segregation showed a continuous distribution for 170 progenies ( $n=170$ ), consistent with polygenetic control. The lag phase duration (h) was estimated by measuring the time between medium inoculation and the initial moment of CO<sub>2</sub> release. Parent strains showed a significant difference in lag phase, i.e. 20 and 11 h for SB and GN, respectively. This measurement was carried out on 51 segregants ( $n=51$ ), and the values obtained ranged from 9 to 38 h, with a highly right-skewed distribution. The L-shaped distribution observed is only apparent and likely due to the fact that the first class (9–14 h) is too large to reveal the left tail of the distribution. A tetrad

analysis on BN progeny suggested that one main locus controlled this trait (Marullo *et al.*, 2006). From an enological point of view, a short lag phase is necessary to ensure colonization of nonsterile grape juice by the industrial strain. Finally, a POF assay revealed that SB was a POF producer (POF+), while GN was not (POF–). POF+ strains produce vinyl-4-phenol and vinyl-4-guaiacol that are unpleasant in white wine (Chatonnet *et al.*, 1993). This trait was previously described as monogenic and controlled by the *PADI* gene (Goodey & Tubb, 1982). This was consistent with the monogenic, Mendelian (1:1) segregation observed in BN progeny.

### Genetic markers map

The marker map used for QTL-mapping was constructed by comparative genome hybridization. Briefly, the genomic DNA of parent strains (ho-SB and GN) and 48 BN progenies was hybridized on high-density oligonucleotide microarrays for genotyping (Winzeler *et al.*, 1998). First, oligonucleotides showing differential signals between GN and ho-SB were primarily selected by hybridizing genomic DNA from both strains on microarrays in triplicate. Second, the entire hybridization data set of the parents and 48 segregants was used to generate a marker map, by applying the algorithms described in 'Materials and methods'. A dense coverage of the genome was obtained, with 2212 reliable markers.

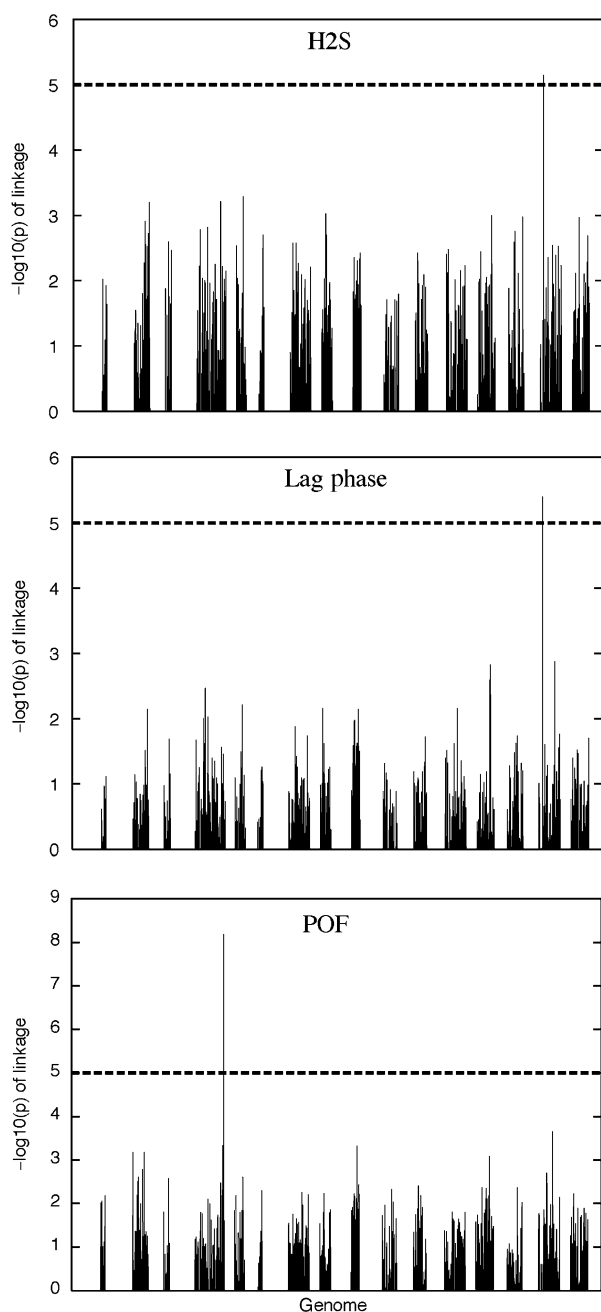
### Association test between traits values and markers

The 2212 microarray-derived markers obtained were further used to find loci statistically linked to trait values. At every marker position, a statistical linkage was tested by splitting the segregants according to their genotype (ho-SB or GN) and by applying the WMW test for phenotypic difference between the two groups. In accordance with previous recommendations (Kruglyak & Lander, 1995), we preferred the WMW test to the classical Student's test because the lag phase trait presented an asymmetric distribution with a non-normal distribution of residues. For H<sub>2</sub>S production, we found a peak linkage (nominal  $P$ -value =  $7.12 \times 10^{-6}$ ) at a marker located on chromosome XV, position 166 601 bp

**Table 2.** Enological traits distribution

	SB	GN	BN	Trait distribution among BN progeny		
				$n$	Range	Distribution shape
H <sub>2</sub> S production (BIGGY)	4	2	3	170	1–5	Gaussian-shape
Lag phase duration (h)	20	11	12	51	9–38	Asymmetric distribution (L-shaped-like)
POF production	+	–	+	51	+ / –	1 : 1 segregation

Phenotypic values of parent (SB, GN) and F1-hybrid (BN) are shown for each enological trait. Values were means of three determinations and showed in all cases an CV lower than 5%. Trait distribution shapes and progeny range values were obtained through the analysis of  $n$  progenies.



**Fig. 1.** Genetic mapping of loci involved in H<sub>2</sub>S production, lag phase duration, and POF character. The physical genome is represented on the x-axis with gaps between consecutive chromosomes. Each peak corresponds to the nominal *P*-value of linkage between a marker located at position *x* and the tested parameter. The significance level mentioned in the text is shown as a dashed line.

on the reference S288c genome (Fig. 1). Surprisingly, we found a linkage peak for lag phase duration at the same marker position (nominal *P*-value =  $4 \times 10^{-6}$ ) (Fig. 1). This locus will be referred to as *LP<sub>H2S</sub>*. For the POF character, a single marker (*YDR534w*) located on chromosome IV,

**Table 3.** Fine mapping of *LP<sub>H2S</sub>* locus with lag phase and H<sub>2</sub>S production

	<i>Locus name</i> (position on XV in bp)			
	<i>HAL9</i> (155754)	<i>YOL083w</i> (165695)	<i>ATG19</i> (169707)	<i>IRA2</i> (173548)
<b>H<sub>2</sub>S production</b>				
Analyzed progenies	172	113	107	174
SB inherited progeny	76	55	53	91
GN inherited progeny	96	58	54	83
WMW score ( <i>n</i> = 106)	$3.9 \times 10^{-9}$	$3.2 \times 10^{-10}$	$4.6 \times 10^{-9}$	$4.2 \times 10^{-8}$
<b>Lag phase</b>				
Analyzed progenies	47	49	48	50
SB inherited progeny	26	24	22	25
GN inherited progeny	21	25	26	25
WMW score ( <i>n</i> = 46)	$3.4 \times 10^{-4}$	$3.5 \times 10^{-5}$	$3.3 \times 10^{-5}$	0.4

The statistic linkage between phenotype values and PCR/RFLP markers neighboring *LP<sub>H2S</sub>* locus was tested using the nonparametric Wilcoxon–Mann–Whitney (WMW) statistics. For both traits (H<sub>2</sub>S production and lag phase duration) progeny clones were divided into two groups according to their SB or GN inheritance. The WMW scores (*P*-value) shown for each marker were calculated using the same number of progenies (*n*).

position 1503534 bp was identified (nominal *P*-value =  $6.4 \times 10^{-9}$ ) (Fig. 1). For all traits, the genome-wide statistical significance was empirically assessed by permuting segregant indexes and rescanning for QTLs. Among 1000 permutation tests, only 79 genome scans successfully mapped one of the three phenotypes at  $P < 10^{-5}$ , indicating that the detected loci were significant.

### Fine mapping of *LP<sub>H2S</sub>* locus assisted by PCR/RFLP

Correlation between H<sub>2</sub>S production and lag phase values was tested by comparing their Pearson correlation coefficient to the coefficients obtained after data set permutations. We found a significant but weak correlation i.e.  $r = 0.34$ , *P*-value = 0.017. Notably, some progeny clones presenting a short lag phase ( $\leq 14$  h) and a high H<sub>2</sub>S production ( $\geq 4$ ) were found. The poor correlation is likely due to the presence of other QTLs specifically controlling H<sub>2</sub>S production or lag phase duration. In order to refine the genetic mapping for lag phase and H<sub>2</sub>S production, we looked for additional markers in the region by systematic sequencing. We found four markers (located in *HAL9*, *YOL083w*, *ATG19* and *IRA2* genes) harboring RFLP that discriminated between GN and SB. A larger progeny sample (*n* = 170) was genotyped on the basis of these PCR/RFLP markers. H<sub>2</sub>S production in these progenies (*n* = 106) was measured using the BIGGY agar test, and linkage increased to *P*-value =  $4 \times 10^{-10}$ , thus narrowing the candidate region to 17 kb (Table 3), with *YOL083w* as the best marker. For lag phase duration, linkage analysis was carried out with only 51

segregants (Table 3), narrowing the locus to 14 kb and excluding a linkage with the *IRA2* marker. Finally, we looked for clones showing a crossing over between *HAL9* and *IRA2* among 170 genotyped progenies. Eight recombinants were found and both traits were measured. As in the whole genome analysis, the highest linkage was at the *YOL083w* marker locus suggesting that these traits are in part determined by very close (or identical) genetic regions. In the mapped region, eight genes/ORF (*HAL9*, *MPD2*, *YOL087c*, *ADH1*, *PHM7*, *YOL083w*, *ATG19* and *IRA2*) were found (www.yeastgenome.org/). None of these had any obvious relation with H<sub>2</sub>S production or lag phase duration, preventing us from proposing a plausible candidate gene. To definitively identify the gene(s) involved in the investigated traits, the deletion effect of each gene of *LP\_H<sub>2</sub>S* locus needs to be assayed in both parent backgrounds.

### Identification of a relevant single nucleotide polymorphism on *PAD1*

The monogenic POF – character is already known to result, in some strains, from a lack of phenylacrylic acid decarboxylase activity encoded by the *PAD1* gene (*YDR538w*) (Clausen et al., 1994). This is consistent with our linkage results (*YDR534c* marker), within 7 kb of *PAD1*. Comparison of the *PAD1* sequence from both parent strains with the S288c reference strain revealed several sequence polymorphisms (Table 4). In seven positions, both wine yeast strains shared a common nucleotide sequence different from S288c. Two other SNPs (positions 550 and 638) differentiated GN and SB. For both SNPs, the POF+ strains (SB and S288c) shared the same sequence, while the POF – strain (GN) was different. The first SNP (position 550) was synonymous and the second (position 638) generated an aspartate to glycine conversion in GN (D213G). Protein alignment with other bacterial or yeast phenylacrylic acid decarboxylases (GI17932869, GI56553716, GI75235662, GI68475725, GI42520414) showed that the D213 aspartate residue was

strongly conserved. This substitution was the only significant polymorphism on the *PAD1* gene between SB and GN strains and was therefore assumed to be responsible for the POF – character. A PCR/RFLP test was set up to easily follow the GN *pad1-G213* allele without any physiological measurement (see ‘Materials and methods’).

### Introgression of two loci in the elite recipient strain SB

The GN allelic forms of mapped QTLs were introduced in the SB strain by marker-assisted backcrosses. This approach is more and more used in plant and animal breeding when loci need to be specifically introduced to improve a recipient variety. During the backcross procedure, the use of molecular markers allows eliminating phenotypic tests necessary to select appropriate progeny clones on the basis of their GN genotype. This was monitored by PCR/RFLP at *PAD1* and *YOL083w* loci. Progeny clones from HO-BN were isolated by tetrad microdissection and genotyped. One clone (HO-BN-2B) with both desired alleles was selected. Since all strains used were derived from homothallic spore clones (*HO/HO*), they produced fully homozygous diploids by self-crossing. After meiosis, HO-BN-2B spores were isolated and paired with SB spores. After zygote formation, the resulting strain, BC1 was isolated and spread on to ACK medium to induce sporulation. Tetrads were micro-dissected and the resulting progeny clones (BC1-x) were genotyped. Clone BC1-1A was selected as it harbored both desired GN alleles. Strains BC1, BC2, BC3, BC4 and BC5 were obtained in turn by the same procedure (Table 1). Statistically, these successive backcrosses should carry from 75% to 98.4% of the SB genome and be heterozygous for introgressed loci. The genome of a BC5-segregant (BC5-4C) was analyzed by DNA-microarray hybridization (Fig. 2). SB-inherited markers were strongly overrepresented (> 95%), confirming the nearly SB-isogenic nature of BC5. As verified by PCR/RFLP analysis, *PAD1* and *YOL083w* alleles were inherited from GN, thus confirming the efficiency of introgression.

**Table 4.** Sequence polymorphisms of *PAD1*

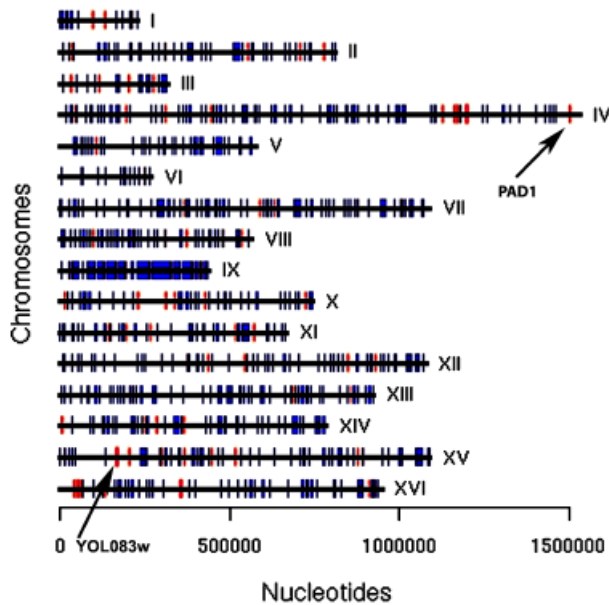
Strains	Nucleotide position								
	112	140	177	252	354	447	550	609	638
S288C	C	C	C	C	T	C	C	G	A
SB	T	T	T	A	C	T	C	T	A
GN	T	T	T	A	C	T	G	T	G

Strains	Corresponding amino acid residues								
	38	47	59	84	118	149	183	203	213
S288C	H	A	V	T	R	I	H	A	D
SB	Y	V	V	T	R	I	H	A	D
GN	Y	V	V	T	R	I	H	A	G

### Validation of introgressed loci effects

The recipient strain SB showed optimal fermentative aptitudes, but presented a long lag phase and some off-flavor productions (H<sub>2</sub>S and POF) (Table 2), which are not suitable for winemaking. The donor strain GN is an aromatically neutral strain (POF –, Low H<sub>2</sub>S production) with weak fermentation capacities. Due to the nearly SB-isogenic nature of BC5, BC5 progeny clones with homozygous *pad1-1* and *H<sub>2</sub>S\_LP* allele of GN were expected to harbor the technological aptitudes of SB with the following improvements: first a shorter lag phase coupled with lower



**Fig. 2.** Physical map of a BC5 segregant. The map was obtained by hybridization of genomic DNA isolated from a BC5 segregant (BC5-4C) on an oligonucleotide microarray. The genomic positions of the 2212 markers are shown by blue and red vertical ticks, reflecting SB and GN inheritance, respectively. This strain showed > 94% SB alleles as expected. The relative position of *PAD1* and *YOL083w* markers are indicated by arrows.

$H_2S$  production (contributed by the GN allele of *LP\_H2S* locus); second, a POF – character (contributed by the *pad1* allele of GN).

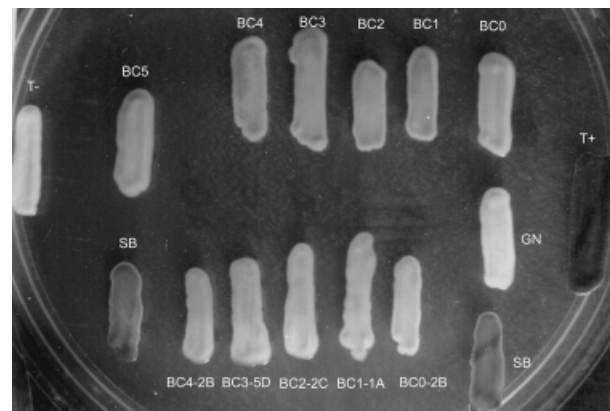
$H_2S$  production and lag phase duration were measured for 12 BC5 progenies. As shown in Table 5, phenotypes and genotypes cosegregated perfectly for  $H_2S$  production. SB, GN, HO-BN and the backcrosses (BC) had blackening levels of 4, 2 and 3, respectively, indicating additivity for this locus (Fig. 3). Although all BC-segregants carrying the GN allele presented a low blackening level, they were slightly darker (noted 2+) than the donor GN parent. These findings confirmed that, although *LP\_H2S* was the main QTL explaining this phenotype, other minor loci on the SB genome also enhanced  $H_2S$  production, explaining the slight phenotypic difference between GN and improved BC5 progenies. The lag phase duration of BC5 (13.6 h) showed that the *LP\_H2S*-GN allele was dominant in an SB background (Table 5). Moreover, segregation analysis of 12 BC5 progenies showed a significant relationship between GN/SB inheritance and short/long lag phases (Table 5), confirming the *LP\_H2S*-GN effect on the SB-like background. However, the L-shaped distribution of this trait and the comparable values of progenies with different haplotypes (BC5-5C vs. BC5-8B) suggested that other minor loci were also involved in phenotype variance. Thanks to introgression, we analyzed the genetic effects of *LP-H2S* locus in an SB-like background,

**Table 5.** Phenotypic analysis of BC5-segregants for *YOL083w* and *PAD1* loci

Strain	YOL083w		Lag phase		PAD1	
	Genotype	H <sub>2</sub> S			Genotype	POF
SB	SB/SB	4	20.6	SB/SB	+	
GN	GN/GN	2	12.7	GN/GN	–	
BC5	GN/SB	3	13.6	GN/SB	+	
BC5-1B	SB/SB	4	41.3	SB/SB	+	
BC5-1C	GN/GN	2+	14.7	GN/GN	–	
BC5-2A	SB/SB	4	66.7	GN/GN	–	
BC5-2C	GN/GN	2+	12.7	SB/SB	+	
BC5-3C	GN/GN	2+	13.7	SB/SB	+	
BC5-4C	GN/GN	2+	14.3	GN/GN	–	
BC5-5B	SB/SB	4	30.7	SB/SB	+	
BC5-5C	SB/SB	4	18.6	SB/SB	+	
BC5-7B	GN/GN	2+	14.3	SB/SB	+	
BC5-8B	GN/GN	2+	17.7	GN/GN	–	
BC5-8C	SB/SB	4+	24.7	GN/GN	–	
BC5-8D	GN/GN	2	13	SB/SB	+	
Mean for SB allele		4	34.5		100% (+)	
Mean for GN allele		2+	14.4		100% (–)	
Significance		***	**		***	

\*\* $P < 0.01$  (Student's *t*-test).

\*\*\* $P < 0.001$  ( $\chi^2$  test).



**Fig. 3.** Hydrogen sulfide production of parent strains, BC hybrids, and related progeny clones.  $H_2S$  production was measured by the blackening of colonies on BIGGY medium. T-, GN, HO-BN, SB and T+ strains had levels of 1, 2, 3, 4 and 5, respectively. Hybrids BC 1–5 had the same  $H_2S$  production level as HO-BN (level 3), while their descendants that inherited *YOL083*-GN had a level of 2+, slightly darker than GN (lower row).

excluding most of the effects of GN alleles (> 95%). This convincingly proved that this locus was linked to the investigated traits, at least in SB background.

Finally, the segregation of the *PAD1* alleles was verified. As expected, in 12 BC5 progenies, the *pad1-G213* allele correlated perfectly with the POF – trait (Table 5). Due to its monogenic nature, POF – character may be introgressed by



**Table 6.** Tetrad analysis and mtDNA RFLP pattern of BC backcrosses

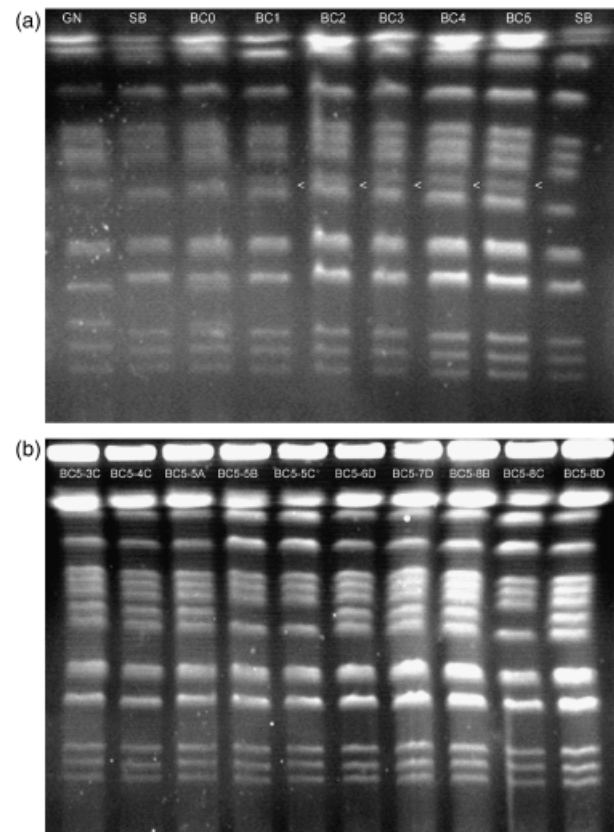
Strain	Dissected tetrads	Germination ratio (%)	Tetrad analysis					'Petites' (%)	mtDNA
			T	t	d	m	0		
HO-BN	12	58	4	0	6	0	2	0	H
BC1	10	49	0	2	4	2	2	0	H
BC2	8	50	3	0	3	0	2	0	H
BC3	9	53	2	1	2	4	0	10	H
BC4	10	49	0	0	9	1	0	25	H
BC5	12	39	2	0	6	0	4	30	H
BC5-1C	10	100	10	0	0	0	0	40	H
BC5-1B	10	100	10	0	0	0	0	40	H
BC6-het	10	52	2	0	3	5	0	42	H
BC6-hom	8	95	7	1	0	0	0	50	H
GN	15	100	15	0	0	0	0	0	GN
SB	19	97	17	2	0	0	0	3	SB

The germination ratios of HO-BN hybrid and BC backcrosses were calculated as the number of viable spores divided by the total number of dissected spores. For each strain the number of dissected tetrads is given. T, t, d, m and 0 indicate that 4, 3, 2, 1 and 0 viable spores per tetrad were obtained, respectively. The ratio of dissected spores identified as petites was calculated as the number of spore clones unable to grow on YP-Gly medium divided by the total number of viable spore clones obtained. The mtDNA RFLP pattern shown in Fig. 5 is indicated in the last column. H, GN and SB indicate mtDNA patterns similar to HO-BN, GN and SB, respectively.

tracking *pad1* alleles without phenotypic measurement. Introgression and breeding programs on other genetic backgrounds gave similar results using the *pad1-G213* locus (data not shown).

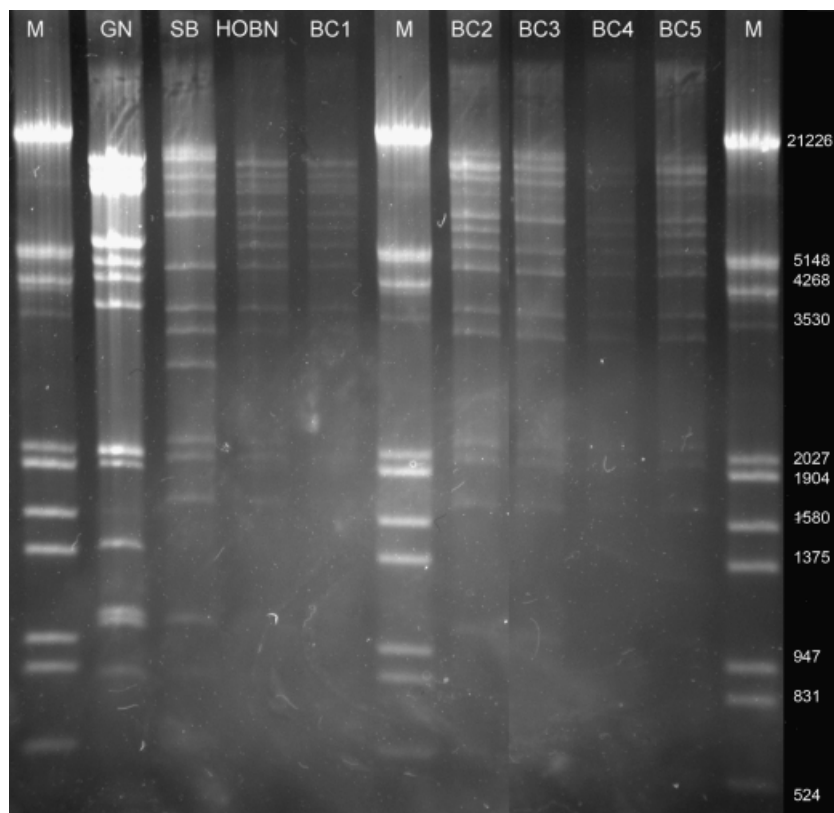
#### Tetrad analysis, chromosomal and mitochondrial patterns of BC backcrosses

We observed a low germination rate in BC5 spores (39%), despite its nearly-isogenic SB background (Table 6). Poor germination (50%) had already been observed in HO-BN (Table 6) and BN spores (Marullo *et al.*, 2006). Generally, with other unrelated genetic backgrounds, the germination ratio increased up to nearly 100% after three backcrosses. In contrast, the germination ratio leveled off at 50% in all BC hybrids throughout introgression. We controlled chromosomal patterns of parent strains (SB, GN) hybrid HO-BN and backcrosses BC1–BC5 by PFGE (Fig. 4a). As previously shown (Marullo *et al.*, 2006), parent strains SB and GN showed a few dimorphic chromosome bands that were combined in HO-BN. Following several supervised backcrosses, the chromosomal patterns of BC hybrids became identical to those of the recipient SB strain. However, a supernumerary band clearly appeared in BC1 and persisted through BC5 at the site of chromosome XI. This band was absent in both parent strain and HO-BN hybrid, but had been previously observed in some BN progeny clones (Marullo *et al.*, 2006). We speculated that this band resulted



**Fig. 4.** Chromosomal pattern of introgressed BC-hybrids (a) and BC5 progeny clones (b). The analysis of parent and BC hybrids (a) showed the persistence of a chromosomal band at the level of chromosome XI (marked by <). This supernumerary band segregated with the *YOL083W* marker (indicated in parentheses) in spores derived from BC5 (b).

from a reciprocal translocation that occurred during HO-BN meiosis, linked to a molecular marker used during the introgression procedure. To test this hypothesis, we analyzed the chromosomal patterns of BC5-progeny clones (Fig. 4b). The presence of this band perfectly correlated with the GN inheritance at *YOL083w* locus in 17 BC5-segregants. Consistent with these findings, the germination ratio of BC hybrid spores was affected according to the presence/absence of this band: strain BC6-hom resulting from BC5-1B × SB presented a germination ratio close to 100% (Table 6) and showed no supernumerary band in its karyotype (data not shown). Furthermore, strain BC6-het resulting from BC5-1C × SB exhibited both a 50% germination rate (Table 6) and the supernumerary band (data not shown). We conclude that a reciprocal translocation occurred near *YOL083w* in the first meiosis step of introgression and was transmitted with *YOL083w* in BC (genetic hitch-hiking effect) (Smith & Haigh, 1974). These conclusions were supported by the tetrad germination patterns observed in HO-BN and BC1–BC5 backcrosses.



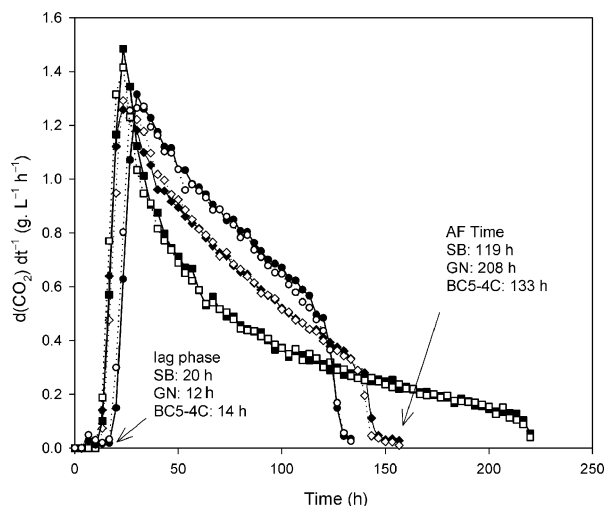
**Fig. 5.** Agarose electrophoresis of EcoRV digested mtDNA of GN, SB, HO-BN and BC(1–5) backcrosses. Lane M, HindIII/EcoRI restriction fragments of  $\lambda$  DNA.

We also observed differences in colony morphology between SB and BC5 dissected spores. An increasing number (10–50 %) of spores grew as *petites* from BC3 to BC6 hybrid progenies (Table 6). In this case, no correlation was observed with any introgressed allele. The respiration deficiency of these *petite* progeny clones was confirmed on YP-glycerol respiratory medium. The high frequency of *petite* spores contrasted with the absence of *petites* among GN and HO-BN spores and the small number (3%) of *petites* in SB progenies (Table 6). The HO-BN hybrid and all the BC (1–6) strains showed a very similar mtDNA restriction map which was different from the SB and GN ones (Fig. 5). Surprisingly, this new, recombined mtDNA appeared in the F1 HO-BN hybrid and persisted during the backcross procedure despite the input of new SB mitochondria. Consequently, the BC5 and SB strains shared nearly the same genomic DNA, but harbored different mtDNA genomes. This genetic difference may be responsible for the higher frequency of *petite* spores observed in BC5 (50% vs. 3%).

#### Loss of a genetic determinant for AF time during introgression

One of the main advantages of introgression is to add new, interesting alleles to the elite strain, without losing its

existing qualities, e.g. here the good fermentation behavior of SB strain. As expected, the final strain BC5-4C exhibited the improved traits of interest: H<sub>2</sub>S production, lag phase and POF character (Table 5), as well as a fermentation profile similar to that of SB elite strain (Fig. 6). Although this optimization was a success in industrial terms, the AF time was not completely preserved after five backcrosses. In fact, BC5-4C reached AF in a time 11% longer than SB i.e. 133 vs. 119 h respectively, and showed a lower fermentation rate at mid-fermentation. Surprisingly, this slight reduction in kinetics did not segregate in the BC5 hybrid, as, statistically, all 12 BC5 progenies exhibited the same phenotype (AF time = 127–134 h). The high level of homozygosity of BC5 and the absence of segregation suggested that one or more genetic determinants responsible for the efficient fermentation rate in the SB strain had been lost during the backcross procedure. One possible explanation was the loss of mitochondrial sequences present in the SB genome but absent in the BC5 hybrid and its related progeny. In fact, the distinct restriction profiles of mtDNA and the high frequency of *petite* colonies in BC5 segregants suggested that mtDNA defects were a plausible hypothesis. These changes in mtDNA genome may have indirect consequences on the kinetic performances of BC5 strain during AF.



**Fig. 6.** Comparative kinetic profiles of SB, GN, and BC5-4C strains. The  $\text{CO}_2$  production rate,  $d\text{CO}_2 dt^{-1}$ , was plotted against the time for strains SB (circles), GN (squares), and BC5-4C (diamond) (open and dark symbols correspond to two independent repetitions). Trait values for lag phase and AF time are indicated on the graph.

## Discussion

For three decades, industrial yeast strains have continuously been genetically improved using breeding methods. However, both the quantitative genetic bases of industrial traits and the homothallic nature of the strains drastically reduced the efficiency of this strategy. Breeding programs needed to be rationalized to take advantage of the genetic potential of the *Saccharomyces cerevisiae* species (i.e. genetic diversity and short sexual cycle). In a previous study, we showed how the selection of appropriate progenies was effective for fixing numerous properties in a single individual (Marullo *et al.*, 2006). However, the hybrids generated may exhibit unexpected defects that were not investigated during the selection program. Introgression is effective for conserving all the qualities of the elite strain while improving specific traits without using GM. The main concept is not to construct *de novo* a new strain, but to improve an already good strain called 'elite'. Although quite popular in plant breeding (Tanksley & Nelson, 1996; Hospital & Charcosset, 1997), this methodology had rarely been used for yeast selection purposes. Pioneering research showed the efficient conversion of wine yeast to heterothallism by crossing a laboratory strain with homothallic industrial ones (Bakalinsky & Snow, 1990). Another program improved a quantitative trait (glycerol production) via two recurrent backcrosses between industrial strains and relevant natural isolates (Prior *et al.*, 1999). However, in these studies, the introgression procedure was strongly delayed and impaired by the need to measure trait values, to select appropriate progenies at each step. To facilitate simultaneous introgression of many traits,

genetic molecular markers were used here instead of trait measurements. We used PCR/RFLP as a rapid, cheap, robust method to follow the different alleles. In this study, we demonstrated that QTL mapping was an effective approach for identifying the main loci controlling trait values. We optimized three desirable traits in an elite strain using molecular-marker-assisted introgression. Global mapping detected only one major QTL for the two quantitative traits analyzed ( $\text{H}_2\text{S}$  production and lag phase duration). However, many genes determining the same character may be found in the same mapped locus (Steinmetz *et al.*, 2002) and QTL often present a cluster organization (Chen & Tanksley 2004). Moreover, other loci may remain undetected, due to their minor contribution to phenotype variation, as suggested as an example by slight blackening of BC5 progenies compared to GN. A more extensive genotyping effort is required to increase QTL-detection power and thus enhance the impact of this methodology. Finally, despite the SB-isogenic nature of BC5, some of the 'elite' strain fermentation aptitudes were lost during backcrosses. We hypothesized that those mtDNA alterations may affect the kinetic performances of BC5 strain during AF. Preliminary experiments showed that *petites* fermented slowly than their congeneric  $\text{rho}^+$  strains (data not shown). This may be due to the reduced of biomass production in *petite* mutants even observed in fermentable carbon source. Moreover, *petite* mutations have been reported to affects the mitochondrial volume and morphology (Stevens, 1981). As an indirect consequence, some essential metabolic reactions occurring in mitochondria (e.g. amino acid and or sterol synthesis) may be reduced affecting the fermentation performances of the strains. This hypothesis will be tested using  $\text{rho}^\circ$  derivatives. If confirmed, new derivatives would correct the problem by restoring the genuine SB mtDNA genome. In addition to mtDNA rearrangements, our results showed that successive backcrosses may also generate chromosomal translocation that should be monitored closely by pulsed field gel electrophoresis. These rearrangements frequently occur in industrial and wild yeast strains (Bidenne *et al.*, 1992; Miklos *et al.*, 1997; Puig *et al.*, 2000) and may have considerable impact on complex phenotypes (Dunham *et al.*, 2002; Perez-Ortin *et al.*, 2002). Taken together, our results illustrate the power, as well as the difficulty, of using molecular markers coupled with introgression to optimize industrial strains.

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