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Chromosome instability in industrial strains of *Saccharomyces cerevisiae* batch cultivated under laboratory conditions

B.T.L. Lucena[1,2], E.A. Silva-Filho[2], M.R.M. Coimbra[1], J.O.F. Morais[3], D.A. Simões[2,4] and M.A. Morais Jr.[1,2]

[1]Departamento de Genética, Universidade Federal de Pernambuco, Recife, PE, Brasil
[2]Setor de Biologia Molecular - LIKA, Universidade Federal de Pernambuco, Recife, PE, Brasil
[3]Departamento de Antibióticos, Universidade Federal de Pernambuco, Recife, PE, Brasil
[4]Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife, PE, Brasil
Corresponding author: M.A. Morais Jr.
E-mail: marcos.morais@pesquisador.cnpq.br

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ABSTRACT. Industrial ethanol fermentation is a complex microbiological process to which yeast cells must adapt for survival. One of the mechanisms for adaptation is thought to involve chromosome rearrangements. We found that changes in chromosome banding patterns measured by pulsed-field gel electrophoresis can also be produced in laboratory media under simulated industrial conditions. Based on analysis of their generational variation, we found that these

chromosome changes were specific to the genetic backgrounds of the initial strains. We conclude that chromosome rearrangements could be one of the factors involved in yeast cell adaptation to the industrial environment.

Key words: Chromosome instability, Ethanol fermentation, Karyotyping, Polymerase chain reaction-fingerprinting, Pulsed-field gel electrophoresis, Recombination rate

INTRODUCTION

In Brazil, the yeast *Saccharomyces cerevisiae* is used to produce ethanol from the fermentation of sugar cane juice and/or molasses throughout a five- to six-month harvesting period (da Silva-Filho et al., 2005a). Some industrial plants make repeated use of commercial yeast strains to provide starter cultures, whereas others use cells collected at the end of the previous fermentation cycle (da Silva-Filho et al., 2005b). In both cases, there is evidence that the initial population is replaced by strains that are introduced with the feeding substrate (da Silva-Filho et al., 2005a). However, whenever the industrial conditions are stable, cells from selected strains can be maintained at high levels within the yeast population (da Silva-Filho et al., 2005b). This stability is important for maintaining high industrial yields. Do these different genomic patterns correspond to new strains being added to the process or to chromosome rearrangements of the present cells? The answer to this question is important for the stability of the yeast population and for ethanol yield of the process. It has been postulated that chromosome rearrangements allow yeast strains to adapt to their new environment (Longo and Vezinhet, 1993; Dunham et al., 2002; Colson et al., 2004). For example, Mortimer et al. (1994) developed the "Genome Renewal" hypothesis to explain the rapid evolution of yeast strains during the production of wine. According to these authors, during the fermentation process multiple heterozygous diploid strains are replaced by diploid homozygous strains with higher fitness. Chromosome variations of this sort could also originate from gross chromosome rearrangements (GCR) between non-homologous parental chromosomes, i.e., involving rearrangements of huge chromosomal fragments by translocation, deletion or duplication (Fierro and Martin, 1999; Nadal et al., 1999).

Most of the GCR events are probably induced by duplicated elements, such as Ty transposons and minisatellites, spread all over the yeast genome (Umezu et al., 2002; Infante et al., 2003; Putnam et al., 2005). The different translocations produced along the cell generations could act synergistically to confer higher selective advantage (Colson et al., 2004). Compared to wine fermentation, which has been extensively studied, there are only a few scientific reports on chromosome rearrangements in yeast cells involved in ethanol-production fermentation (Schütz and Gafner, 1994). However, a high degree of chromosome polymorphism has been detected among yeast isolates collected from sugar cane fermentation tanks (Pataro et al., 2000; Guerra et al., 2001). These different chromosome patterns could be a result of GCR in the yeast genome induced by the environmental conditions.

Yeast chromosomes can be distinguished by pulsed-field gel electrophoresis (PFGE), which reveals the particular molecular karyotype of a given strain or species-specific chromosome patterns. It has long been used to identify wine-yeast strains (Longo and Vezinhet, 1993; Schuller et al., 2004; Antunovics et al., 2005) and cachaça-producing strains (Pataro et al., 2000; Guerra et al., 2001). We have found evidence that chromosome rearrangements can occur in industrial strains even under highly stable laboratory-cultivation conditions. These rearrangements were specific to the parental genetic background and were followed through successive cycles of the simulated industrial process.

MATERIAL AND METHODS

Yeast strains and media

Yeast strains were isolated from the fermentation must of a fuel-ethanol distillery at Japungu Agroindustrial (Santa Rita, PB, Brazil) throughout the harvesting period, as described by da Silva-Filho et al. (2005b). The IA1238 strain was used as starter strain and the JP1 strain was the dominant strain at the end of the fermentation process (da Silva-Filho et al., 2005b). The MF1(1) strain was isolated at the beginning of the fermentation period. The three strains were used as platforms for industrial yeast chromosome instability, two with a suspected genetic relationship and one of different clonal origin. Yeast cells were stored in slants containing WLN medium immersed in sterile mineral oil at room temperature. Other industrial strains were isolated by plating diluted-must samples onto WLN medium containing ampicillin and nalidixic acid at 50 µL/mL each. Yeast colonies were picked and the cells were submitted to both molecular karyotyping and DNA fingerprinting, as described below.

The following media were used for the fermentation assays: YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose), Sabouraud (10 g/L peptone, 20 g/L glucose), WLN (4 g/L yeast extract, 5 g/L casein, 50 g/L glucose, 10 mL 100X salt solution, 10 mL 100X trace-element solution) and fermentation medium (120 g/L sucrose, 5 g/L yeast extract, 2 g/L casein, 2 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 1 mL 100X trace-element solution). For solid media, agar was added at 20 g/L. The media were sterilized by autoclaving at 121°C for 20 min. Sterile 100X trace-element solution (13 g/L $CaCl_2 \cdot 7H_2O$, 13 g/L $MgSO_4 \cdot 7H_2O$, 0.25 g/L $FeCl_3 \cdot 6H_2O$, 0.25 g/L $MnSO_4 \cdot 4H_2O$) was added after medium autoclaving.

Fermentation assays

Pre-cultures of IA1238, MF1(1) and JP1 strains were used to inoculate assay tubes (17 mm x 200 mm) containing 20-mL fermentation medium to 10^5 cells/mL for non-aerated cultivations. A sample was immediately taken, diluted and plated onto solid Sabouraud medium for zero generations. The tubes were incubated at 35°C without agitation. Two aliquots of 4 mL were withdrawn at each 24-h cultivation for inoculation of new tubes containing fresh 16-mL fermentation medium and for plating onto Sabouraud medium (20 CFU per plate), respectively. This procedure was consecutively repeated for 150 days.

Culture cell density was spectrophotometrically measured at 660 nm from samples taken at the beginning and the end of batch cultivations to calculate the mean specific growth rate and generation number. Cells from yeast colonies at these generations were used for polymerase chain reaction (PCR)-fingerprinting and PFGE karyotyping.

Molecular methods

For PCR-fingerprinting analysis, DNA from 20 yeast colonies isolated at different culture generations was extracted and used as a template for PCR with $(GTG)_5$ primer (da Silva-Filho et al., 2005a). For yeast karyotyping, cells from the same yeast colonies were suspended into 60-µL SEM buffer (1 M sorbitol, 50 mM EDTA, 28 mM β-mercaptoethanol) containing 2.5 mg/mL lyticase and 60 µL melted agarose (1.2% low-melting-point agarose in SEM buffer) was added afterwards. The mixtures were immediately transferred to the solidification blocks. The agarose blocks were immersed in 300-µL SEM buffer containing 2.5 mg/mL lyticase and incubated for 24 h at 37°C. After incubation, the agarose blocks were washed in 500-µL EST buffer (100 mM EDTA, pH 8.0, 1% N-laurylsarcosyl, 10 mM Tris, pH 8.0). Deproteinization was done in 500-µL EST buffer containing 1 mg/mL proteinase K for 48 h at 50°C. After washing in 500 mM EDTA, pH 8.0, the blocks were stored in this solution at 4°C until use.

Yeast chromosomes were separated on 1.3% agarose gel by PFGE in the Gene Navigator system (Amersham-Pharmacia Biotech). Electrophoretic runs were performed in 1X TBE at 12°C, using the following migration conditions: interpolated pulses from 140 to 20 s for 35 h and 75 to 15 s for 7 h. Gels were stained in ethidium bromide and recorded on Polaroid film. Yeast YNN295 strain PFGE marker (Amersham-Pharmacia Biotech) was used to calculate the apparent molecular weight of the chromosome bands of the strains and their variations. The number of chromosome variants was scored at every sample and used to calculate the rate of rearrangements according to the formula: $R = 1 - P_{(n)}^{1/n}$, where $P_{(n)}$ represents the number of parental karyotypes at "n" generations (Carro and Piña, 2001).

Chromosome banding patterns were analyzed by NetWork 4.1.0.8 program (www.fluxus-engineering.com) treated as binary data for matrix calculation using Median Joining. This program was originally developed for geographical distribution of haplotypes and was used by us to predict the evolutionary flow of chromosome rearrangements. The nodes represent the variant forms and their size represents the frequency of each variant form in the population. The distance between two nodes is proportional to the number of steps (chromosome rearrangements) that separates them.

RESULTS

Chromosome pattern of industrial strains

More than one hundred colonies were collected from the sugar cane-fermenting distillery of Japungu Agroindustrial and checked for their identity by PCR-fingerprinting. By using the molecular data base described by da Silva-Filho et al. (2005a), it was possible

to identify them as *S. cerevisiae* and to classify them by the fingerprint patterns (data not shown). Some representatives of the industrial isolates were submitted to molecular karyotyping, which revealed a huge chromosome polymorphism especially observed in the mid- and small-sized chromosomes, ranging from 300 to 800 kbp (Figure 1). The distillery usually uses the strain IA1238 as starter. The strain designated JP1 was previously isolated as dominant strain of that process, and has been used since then (da Silva-Filho et al., 2005b). Therefore, we chose both strains, and one industrial isolate, for testing the predicted chromosome instability.

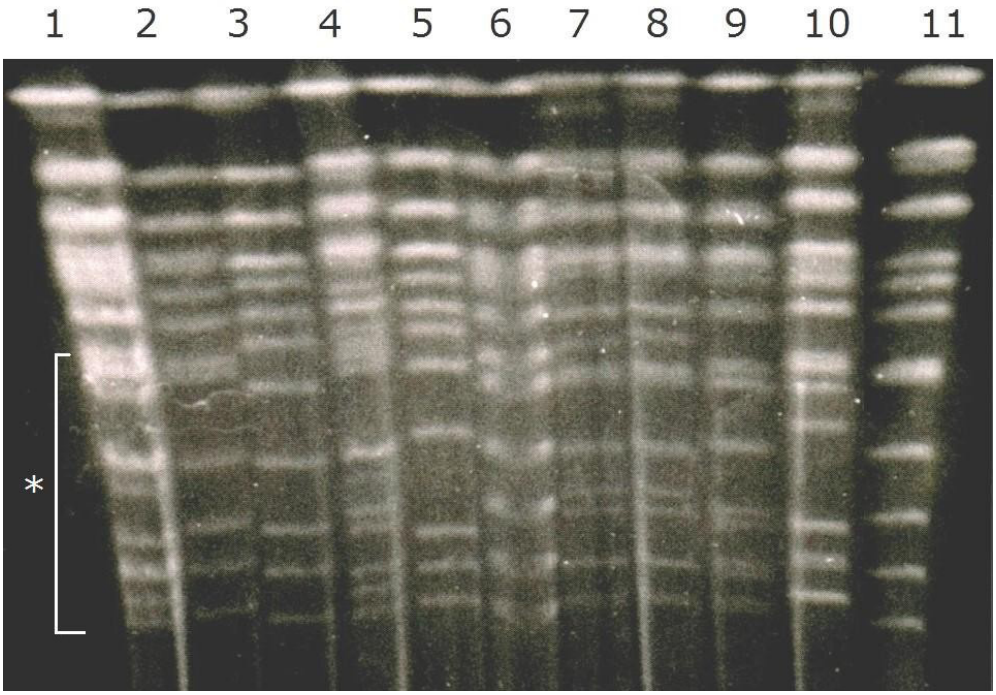

Figure 1. Molecular karyotype representative of industrial isolates of *Saccharomyces cerevisiae* collected directly from fuel-ethanol fermentation must (*lanes 1-5* and *7-11*). Cells were spread onto WLN medium and the yeast colonies were treated as in Material and Methods. The molecular marker YNN295 strain is shown (*lane 6*) and yeast chromosomes ranging from 350 to 800 kbp are marked with asterisk.

All three industrial yeast strains had their own distinct chromosome patterns (Figure 2A). Strain IA1238 presented 17 chromosome bands, one more than the standard yeast pattern for laboratory strain YNN295 (Figure 2A). The MF1(1) strain presented almost the same chromosome pattern as strain IA1238, except for the lack of the 430-kbp chromosome band present in strain IA1238 (Figure 2A). On the other hand, JP1 strain had 15 chromosome bands, with many variations compared to strain IA1238 (Figure 2A). The chromosome bands at 1400, 940 and 820 kbp were not present in JP1, while additional bands at 850, 760 and 540 kbp were visualized in this strain. In addition, the three chromosome bands ranging from 400 and 500 kbp were slightly smaller in JP1 than in strain IA1238.

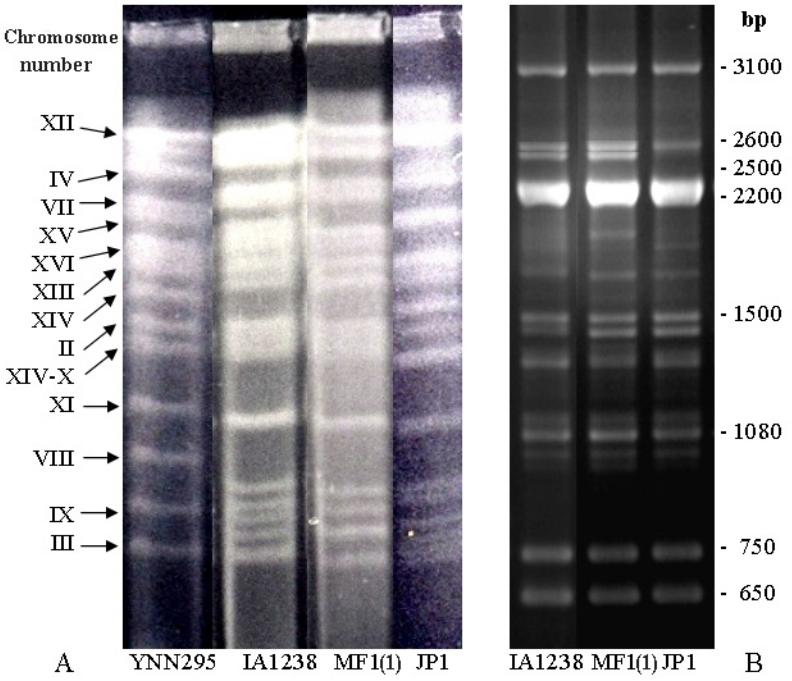

Figure 2. Molecular karyotype and PCR-fingerprinting of *Saccharomyces cerevisiae* industrial strains. **A.** PFGE of *S. cerevisiae* industrial strains IA1238 and JP1 and the industrial isolate, MF1(1). Chromosome indication for the marker strain YNN295 is shown on the left. **B.** Amplification of DNA from IA1238 and JP1 strain and the industrial isolate, MF1(1) with $(GTG)_5$ primer. The length of the amplified bands is shown on the right.

Strains IA1238 and MF1(1) presented the same PCR-fingerprint pattern characterized by the presence of both 2500- and 2600-bp bands. On the other hand, the 2500-bp band was absent in strain JP1 (Figure 2B). Therefore, it was assumed that IA1238 and MF1(1) strains correspond to the same clonal lineage. Thus, it was assumed that MF1(1) is a possible GCR-derivative of IA1238 generated in the industrial process. On the other hand, JP1 represents a different clonal lineage, with a distinct genetic background present in the industrial process.

Chromosome instability under laboratory cultivation

Cells of the three industrial strains were long-term cultivated under non-aerated conditions and collected at different generations for molecular analysis by both PCR-fingerprinting and PFGE. Three chromosome pattern variations were observed for the IA1238 strain, henceforth designated IAR1, IAR2 and IAR3 (Figure 3A). These variant forms were observed only after 435 generations, with IAR1 (5% of the isolates) losing the 670-kbp parental band, IAR2 (35% of the isolates) losing both 670- and 480-kbp bands, and IAR3 (60% of the isolates) losing the chromosome bands of 670, 480 and 430 kbp. This could represent the evolutionary flow in this population (IAR1 > IAR2 > IAR3), to produce the most adapted genome array that allowed its cells, in this case IAR3, to dominate the yeast population at the end of the cultivation. We did not detect any yeast colony presenting the parental karyotype pattern at the end of the cultivations, which could be either below 5% (our limit of detection) or absent in the population.

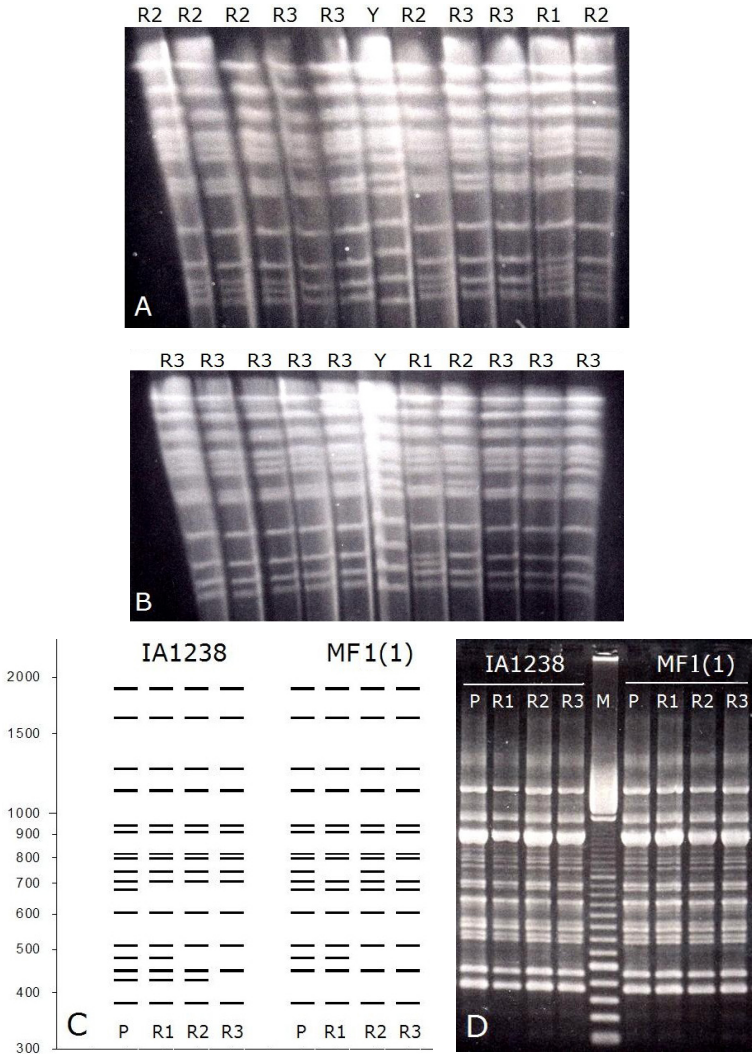

Figure 3. Molecular karyotype of yeast colonies from IA1238 (panel A) and MF1(1) (panel B) strains cultivated under non-aerated conditions. Isolates representative of the rearranged patterns IAR1 or MFR1 (*lane R1*), IAR2 or MFR2 (*lane R2*) and IAR3 or MFR3 (*lane R3*) are shown. A yeast chromosome marker YNN295 was used (*lane Y*). The different chromosome variant patterns produced by both strains were graphically represented, with the molecular weight of yeast chromosomes calculated from the yeast marker shown in the left (panel C). PCR-fingerprinting analysis was performed for all chromosome variant forms and compared to their parentals (panel D). P = parental strain; M = 100-bp molecular marker (Amersham-Pharmacia).

Strain MF1(1) produced three variations appearing late in the cultivation, henceforth designated MFR1, MFR2 and MFR3 (Figure 3B). The variant MFR1 (5% of the isolates) did not have the 745-kbp parental band, while variant MFR2 (5% of the isolates) lacked the 480-kbp parental band. Meanwhile, variant MFR3 (90% of the isolates) lost both chromosome bands (Figure 3B). This latter variant form could have originated as the second modification of either MFR1 or MFR2, which dominated the yeast population at the end of the cultivation. As shown for IA1238, the parental array was not observed in the population at the end of the cultivation. Variant forms of both parental strains had polymorphism in mid- and small-sized chromosomes (Figure 3C), as predicted by our hypothesis of environmentally induced genome arrangement.

Despite the chromosome polymorphism, all rearranged isolates produced PCR-fingerprint patterns similar to their parentals (Figure 3D). First, it is an indicative that the observed chromosome variations were originated from the parental IA1238 and MF1(1) strains and did not include the karyotypes of contaminant cells. Second, it showed that the amplification pattern generated by $(GTG)_5$ primer, which includes the amplification of small extensions in the yeast genome, was not disturbed by larger structural alterations imposed by genome rearrangements.

Cells of strain JP1 displayed 12 chromosome rearrangements observed over 519 cell generations, henceforth called JPR1 to JPR12 (Figure 4A). After 107 cell generations, the parental pattern decreased to 30% of the population, in which nine of the 12 variations (JPR1 to JPR9) were already observed. At 519 generations, three new arrays JPR10, JPR11 and JPR12 were observed, while arrays JPR6, JPR7 and JPR9 disappeared from the culture, or decreased their cell number to below the limit of detection. Chromosome patterns generated by the JP1 derivatives revealed two variable banding regions, a highly variable region ranging from 350 to 610 kbp and a partially variable region ranging from 700 to 800 kbp (Figure 4B). Similar to two other industrial strains, DNA-fingerprint patterns of variant isolates were similar to the parental JP1 strain (Figure 5), confirming their clonal origin.

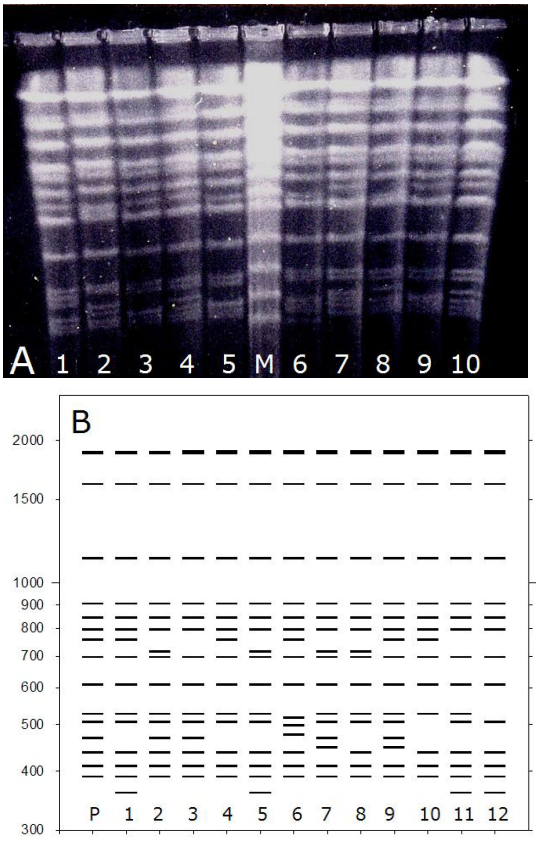

Figure 4. Molecular karyotyping of cells from the JP1 strain cultivated under non-aerated conditions. **A.** Yeast isolates representative of the JP1 yeast karyotype variations JPR6 (*lane 1*), JPR7 (*lane 2*), JPR4 (*lanes 3* and *9*), JP1 (*lanes 4* and *10*), JPR8 (*lane 5*), JPR5 (*lane 6*), JPR9 (*lane 7*), and JPR2 (*lane 8*). The yeast marker YNN295 strain was used (*lane M*). **B.** Graphic representation of all chromosome variation patterns JPR1 to JPR12 (*lanes 1* to *12*) observed along the cultivation of JP1 cells (*lane P*) in non-aerated tubes.

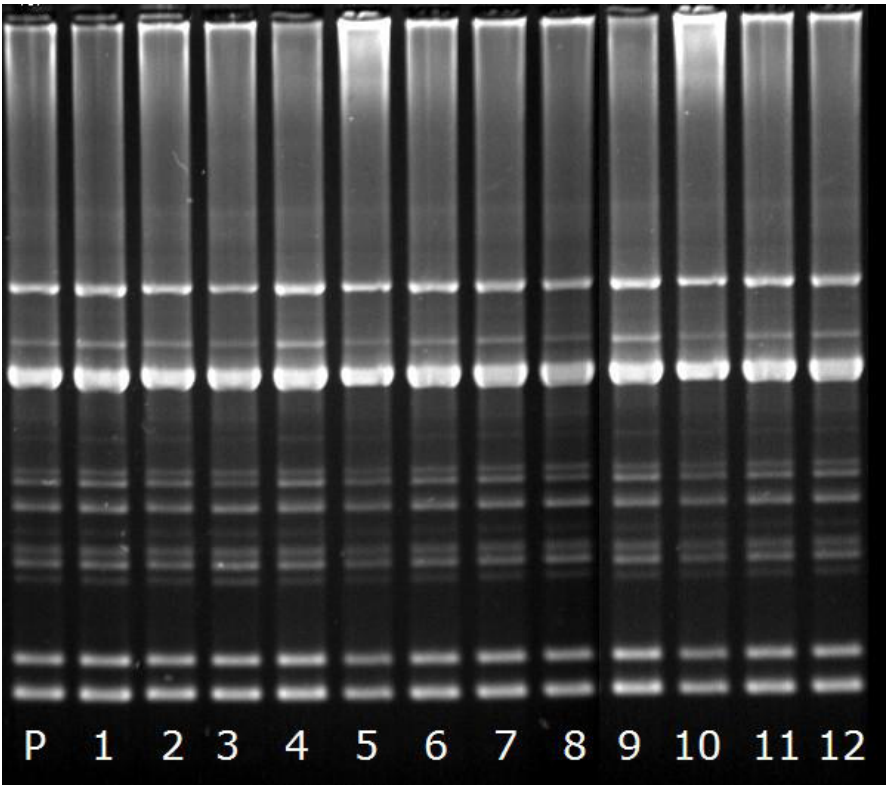

Figure 5. PCR-fingerprinting of the chromosome variation patterns JPR1 to JPR12 (*lanes 1* to *12*) observed along the cultivation of JP1 cells (*lane P*) in non-aerated tubes.

By ordering the chromosome rearrangements by the time course of fermentation, and assuming the parsimony method, the clonal origin of JP1 variation forms can be suggested (Figure 6). In this picture, three rearrangements independently arose from the parental pattern: JPR3 and JPR4 with a single modification and JPR9 with two modifications. The network calculation assumed that JPR4 and JPR10 were ancestors of JPR6. Since JPR10 was observed later in the culture than JPR6, it indicates that this variant might be present early in the culture but remained at a low cell count number for a long time. The JPR6 variant form, which presented four rearrangements from the JPR10 pattern, disappeared from the population, or decreased in cell number, along the batch cultivation. This happened to JPR7 and JPR9 variants, which presented two rearrangements compared to their ancestors. The JPR11 variant should have two possible origins, similar to that suggested above for the MFR3 variation form. The JPR4 variant lost the parental 470-kbp band (Figure 4B) and seemed to be the basis of a long stretch of genetic modifications (Figure 6). The absence of this chromosome band was also detected for variants of IA1238 and MF1(1) strains, indicating its high instability in industrial strains. However, other chromosome rearrangements were specific for a given parental background.

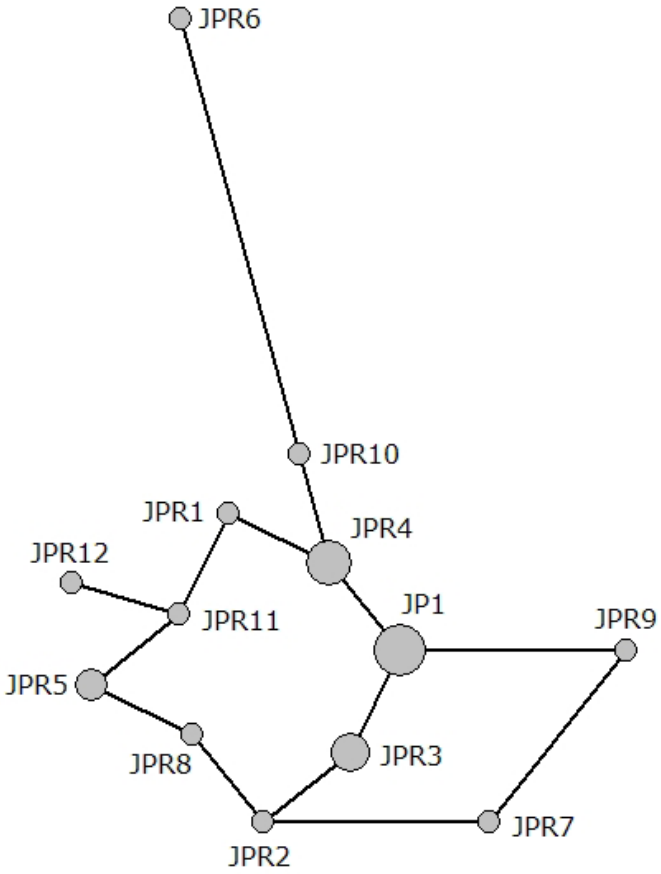

Figure 6. Diagram of network analysis presenting the flow of chromosome rearrangements detected upon cultivation of the parental strain JP1 under non-aerated conditions. The nodes represent the variant forms and their size represents the frequency of each variant form in the population. The distance between two nodes is proportional to the number of steps (chromosome rearrangements) that separates them.

DISCUSSION

The bioethanol fermentation process can be considered a very stressing industrial environment, in which yeast cells are constantly submitted to oscillations in sucrose and ethanol concentrations, as well as temperature fluctuations and variations of the pH in the medium. In such an environment, cells are continuously recycled in the course of the harvesting period, which can last over six months (da Silva-Filho et al., 2005b). This imposes a continuous periodic selection that favors the establishment of dominant strains that are more adapted than their counterparts (da Silva-Filho et al., 2005a). Industrial yeast strains have a complex genome constitution with variable chromosome number and a ploidy state that may contribute to that adaptation. While laboratory strains of *S. cerevisiae* tend to present 16 chromosomes, this number may vary in industrial strains (Schütz and Gafner, 1994; Nadal et al., 1999; Pataro et al., 2000; Guerra et al., 2001; Schuller et al., 2004; Antunovics et al., 2005). Whether

chromosome polymorphism is a cause or a consequence of such an adaptation is still a matter of divergence among scientific reports. We identified chromosome rearrangements in distilling yeast cultivated under mildly stressing conditions in successive laboratory batches. We observed a huge variation in chromosome patterns in the yeast population in terms of number and length of the chromosomes along the cell generations. The polymorphism affected mainly the small- and mid-size chromosomes. This contrasts with the results of Carro and Piña (2001) that showed that the large chromosome XII was hypervariable among the wine yeast isolates. We found three main features of fuel-ethanol fermenting strains: 1) no instability for the largest chromosomes XII, IV, VII, and XV; 2) high instability of small-size chromosomes, and 3) presence of an extra set of chromosomes compared to the standard laboratory strain. These additional chromosomes result from unequal events of recombination, such as non-reciprocal translocations. It has been stated that the extra set of chromosomes might give rise to more vigorous strains, reflecting optimal fitness in an industrial environment (Adams et al., 1992; Dunham et al., 2002; Pérez-Ortín et al., 2002; Colson et al., 2004) and the maintenance of chromosome polymorphism is an important mechanism for yeast adaptation (Puig et al., 2000). However, the results of Codon et al. (1998) on DNA content analysis of several industrial strains did not support this hypothesis. Therefore, even with accumulating evidence on the molecular mechanisms of such diversity, the significance of chromosome polymorphism in industrial strains remains unclear (Lockhart et al., 2002). There is convincing evidence that karyotype instability is a genetically defined characteristic that segregates among the descendents as a dominant phenotype, and that some strains are more prone to chromosome rearrangements than others (Schütz and Gafner, 1994). This would explain the difference in the type and frequency of chromosome rearrangements among the three distilled alcohol strains that we analyzed.

Despite the different chromosome patterns derived from the three strains, some similarities were observed, such as the instability of the 480-kbp chromosome band. This recurrent genomic rearrangement should have an adaptive significance (Dunham et al., 2002). Our hypothesis is that once a new chromosome rearrangement is acquired the yeast cells acquire a new genetic background that drives further different rearrangement patterns. However, the results for JP1 strain also suggest that convergent chromosome rearrangements can appear from different genome backgrounds, as shown for the variant forms JPR7 and JPR11. Another common aspect is that all variant forms decreased their chromosome number, similar to previous reports showing that mitotic chromosome rearrangements lead to karyotype simplification (Bidenne et al., 1992; Fierro and Martin, 1999).

Meiotic recombination, in accordance with the "Genome Renewal" hypothesis, may be the source of an adaptive mechanism that generates chromosome arrays with higher fitness in the industrial environment. The inconvenience of this hypothesis is its need for cell sporulation, which is difficult to achieve at such high-sugar and low-nitrogen environments of beverage and fuel-ethanol fermentations. Therefore, mitotic recombination might be the primary source of chromosome rearrangements in such an environment. The fact is that industrial yeast cells cultivated in successive batches under different conditions produced a heterogeneous pattern of chromosome variation that could arise depending on the strain's genetic background and culture conditions. This argues in favor of adaptive evolution of the yeast population (Puig et al., 2000). Experiments of microbial population evolution have been carried out in chemostats and in successive batches, in which cells are cultivated under limiting conditions of one

nutrient, allowing the selection of specific phenotypes over generations. New phenotypes should replace the old ones in a process called periodic selection (Finkel and Kolter, 1999), similar to that observed here for industrial yeast strains collected from an industrial plant that are cultivated in YPD medium. Colson et al. (2004) showed lower fitness for translocations in mineral medium, both in batch and chemostat cultivations, than for YPD medium. Moreover, it has been postulated that accumulation of adaptive mutations with epistatic interactions can cause a decrease in the fulfillment of the whole population (Adams et al., 1992). However, the molecular nature of such an adaptation is still a matter of controversy. In any case, the higher chromosome variation observed among yeast isolates collected directly from the industrial plants (Figure 1) compared to isolates collected from laboratory cultivations strengthen the periodic selection hypothesis in the sense that higher stress conditions should impose a selective pressure that induces gross chromosome rearrangements to occur in order to produce genome arrays with higher fitness to that environmental condition.

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