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original scientific paper

Sporulation in Diploid Transformants of *Saccharomyces cerevisiae* Obtained by Illegitimate Integration of Single-stranded DNA

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Summary

DNA sequence analysis and chromosomal polymorphism of natural and industrial yeast strains reveal important role of illegitimate recombination in the evolution of the yeast genome. The resulting genetic changes can be brought to the homozygous state by sporulation and mating between haploid progeny. In this work we compared sporulation of diploid transformants obtained by illegitimate integration of heterologous single-stranded (ss) DNA and transformants obtained with double-stranded (ds) plasmid that replicates autonomously in the yeast cell. The transformants that contained exogenous DNA integrated by illegitimate recombination had lower efficiency of sporulation, but the spore viability was decreased from 80% to 65% only among preselected fraction of transformants. Some of the surviving haploids contained transforming DNA integrated in their genome. Possible mechanisms that led to the decrease in sporulation efficiency and spore viability in transformants obtained by illegitimate integration of foreign DNA into the yeast genome are discussed.

Keywords: Saccharomyces cerevisiae, illegitimate recombination, sporulation

Introduction

Recent release of complete sequence of the yeast *Saccharomyces cerevisiae* genome offers indispensable source of information not only about genes and their functions but also about overall organization of the simple eukaryotic genome *(1)*. For example, it was shown that a typical yeast chromosome contains »waves« of cytosine/guanine-rich regions on both chromosome arms and it was suggested that this could be important for the stability and maintenance of chromosomes *(2)*. Another finding based on the analysis of the whole sequence was that almost one half of the yeast genome contains duplicated, but diverged regions. This was interpreted by ancient fusion of two diploids followed by genetic rearrangements that led to the loss of some parts of the genome and diversification of remaining duplications *(3)*. Illegitimate recombination, needed to produce such rearrangements, was well documented in higher eukaryotes but was also detected in *Sacch. cerevisiae (4–8)* and several genes involved in this process have been identified *(9–12)*. Due to the high gene density illegitimate events are expected to be very mutagenic in yeast, but they can also induce genetic changes that affect overall genome integrity *(8)*.

Different yeasts of the genus *Saccharomyces*, including the most important species *Sacch. cerevisiae*, are widely used in the production of alcoholic beverages, spirits and for dough leavening. The annual production of baking yeast is estimated to 2 million tons of compressed yeast *(13)*, corresponding to about 5×10^{22} individual cells. Such a massive cell propagation imposes considerable challenge to the genome integrity due to spontaneous mutations and genomic rearrangements. The dynamic aspect of genome structure was specifically addressed in several studies of natural wine-producing yeasts where genetic heterozygosity and chromosome polymorphism were analysed *(14–16)*. Mortimer *et al. (15)* found out that strains homozygous for the genetic markers analysed exhibited high spore viability, while

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significant decrease in viability could be related to the number of heterozygous loci detected. It was proposed that yeasts may enter meiosis even in the rich medium giving haploid progeny that will, for homothallic strains, eventually form stable homozygous diploids. This process, called genome renewal, was proposed to eliminate deleterious changes from a diploid yeast genome giving rise to homozygous clones with high growth rates *(15)*.

Yeast meiosis involves more than 150 genes that participate in different stages of this complex process leading to the formation of four haploid spores *(17)*. However, sporulation depends on both genetic and environmental factors and different natural and laboratory strains show different efficiency of sporulation only rarely approaching 100%. In the prophase stage of the first meiotic division homologous chromosomes have to pair and recombine in order to ensure proper segregation and formation of four viable meiotic products. This process is sensitive to the sequence divergence *(18)* and differences in the genome organization in the parental haploids. If identical sequences are present in non-allelic (ectopic) positions in two parental genomes, reciprocal recombination will produce chromosomal translocations that may be lethal in haploids *(19)*.

In our previous work we demonstrated that illegitimate integration of heterologous single-stranded (ss) DNA into the yeast genome may induce different genetic changes in mitotic cells *(8)*. Using pulsed-field gel electrophoresis we detected chromosomal rearrangements and transformation-induced chromosome instability among some transformants as well as the simultaneous presence of transforming DNA on several chromosomes. Here we investigated if such rearrangements could also affect yeast meiosis, so we analysed sporulation efficiency and spore viability in diploid transformants obtained by illegitimate integration of foreign DNA. Several transformants exhibiting low efficiency of sporulation and decreased spore viability were further characterised by Southern analysis of intact chromosomes separated by pulsed-field gel electrophoresis.

Materials and Methods

Yeast strain

In this work we used the diploid yeast strain DAU4 (*MATa/MATα, arg4::URA3/arg4::URA3, ura3–52/ura3–52, trp1/+, leu2–3,119/+, his7/+, cyh^r/cyh^r*) transformed with the integrative plasmid pCW12 in the single-stranded form. The plasmid pCW12 contains the *ARG4* gene which was deleted from the host strain, so transformants could be generated only by illegitimate recombination with the yeast genome. As a control, we used the same yeast strain transformed with the replicative double-stranded plasmid pATUD (*ARG4, URA3, TRP1*). The strain, plasmid construction and transformation procedure were described previously *(8, 20)*.

Determination of sporulation efficiency and spore viability

Individual transformants were transferred to the agar plates with minimal supplemented medium and replica-plated onto the sporulation medium after incubation for three days at 30 °C. Four days later, the cultures were examined under the light microscope and vegetative cells and asci were scored. For each culture at least hundred cells/asci were counted. Sporulated cultures were further treated with β-glucuronidase (Glusulase, Endo Laboratories, New York) and individual spores were separated on the YPDA plates. Colony-forming spores were detected after four days of incubation at 30 °C. We used standard growth conditions, media and yeast manipulation procedures *(20)*.

Pulsed-field gel electrophoresis and Southern blotting

The detailed protocols for the preparation of genomic DNA, electrophoresis and Southern blotting have been described previously *(8)*. Electrophoresis was performed using Pulsaphor system (Pharmacia) at an applied voltage of 160 V. Pulse durations of 16 h, 5 h and 6 h with pulse switch times of 800, 100 and 120 s, respectively, were used. Labeling of the probe (pCW12) with digoxigenine-11-dUTP, hybridization and detection were performed as suggested by the manufacturer (Boehringer, Mannheim).

Results and Discussion

In order to investigate whether genome rearrangements induced by illegitimate integration of ss DNA may affect yeast meiosis we first analysed sporulation efficiency of transformants obtained with heterologous ss integrative plasmid. The results presented in Fig. 1

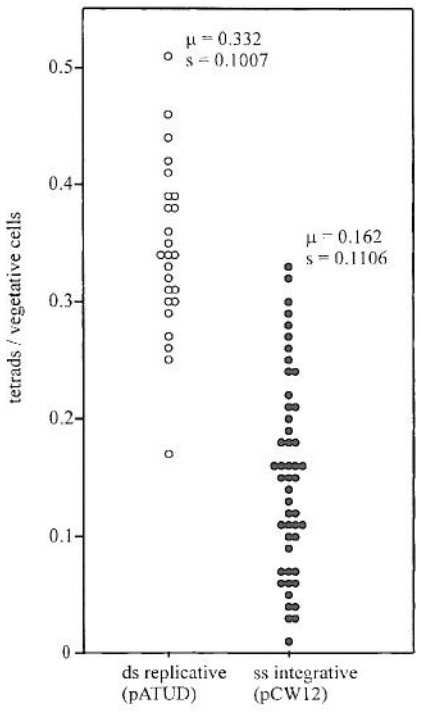

Fig. 1. Sporulation efficiency of transformants. Each point represents the proportion of asci in a single sporulating culture; (○) transformants obtained with 5 ng of ds replicative plasmid pATUD, (●) transformants obtained with 200 μg of ss heterologous integrative plasmid pCW12. The mean (μ) and the standard deviation (s) are indicated.

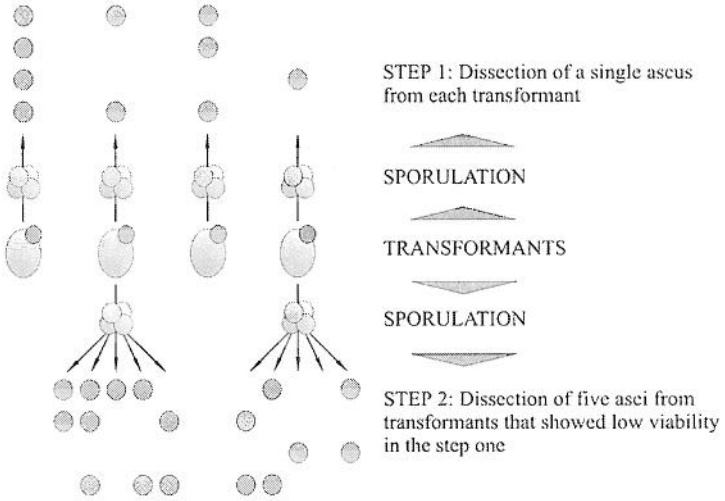

Fig. 2A. Spore viability of yeast transformants obtained by illegitimate integration of ss plasmid pCW12. The selection procedure.

show that formation of asci is greatly reduced in transformants obtained after incubation of protoplasts with high concentration of heterologous ss DNA. Since we used as a control transformants obtained with replicative ds plasmid, this could not be attributed to the transformation procedure itself, but rather to the different mechanisms of transformation giving rise to the Arg^+ colonies.

We also wondered whether the viability of meiotic progeny will be affected among some transformants obtained with ss DNA. In order to detect such transformants we developed a two-step dissection procedure (Fig. 2A). In the first step, a single ascus was dissected from a number of individual transformants obtained with 5 μg of ss pCW12, 200 μg of ss pCW12 and 5 ng of pATUD (which was used as a control). Altogether 192 asci were dissected and we found no significant difference in the overall spore viability among these three classes of transformants (not shown). In the second step we dissected only those transformants from the first dissection that gave 0, 1 or 2 viable meiotic products: 16 transformants obtained with 5 μg of ss pCW12, 34 obtained with 200 μg of ss pCW12 and 22 obtained with 5 ng of pATUD. After sporulation, five tetrads from each transformant were dissected and the results of this analysis are presented in Fig. 2B. The overall spore viability was as follows: 65.3% for transformants obtained with 5 μg of ss pCW12, 64.3% for transformants obtained with 200 μg of ss pCW12 and 80.2% for transformants obtained with the control plasmid pATUD. This difference is statistically significant ($P<0.01$) indicating that the spore survival was indeed affected among transformants selected by the two-step dissection procedure. This was due to a decrease in the number of asci with four viable spores and an increase in the number of asci containing only one or two viable spores (Fig. 2B).

Using this two-step dissection protocol we were able to select several transformants that produced only 20–50% of viable spores and exhibited low sporulation efficiency. Eight such transformants, obtained with 200 μg of ss pCW12, were further characterised by pulsed-field

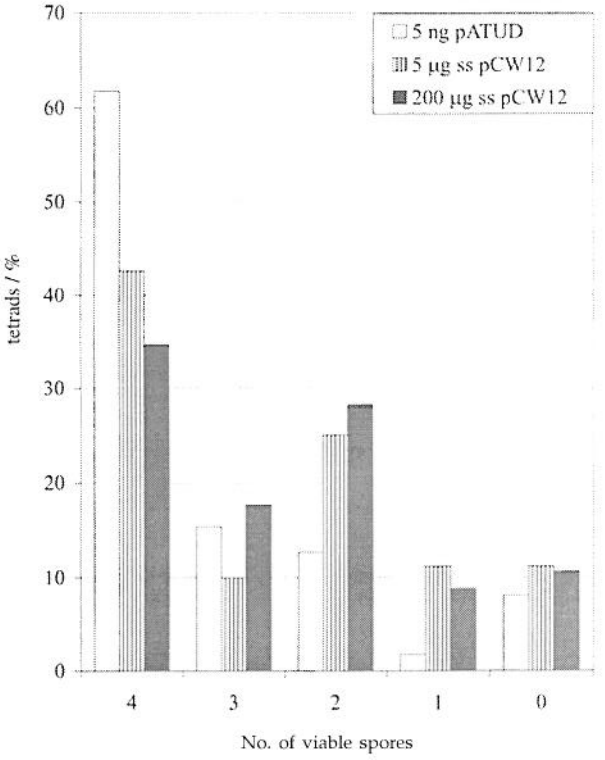

Fig. 2B. Spore viability of yeast transformants obtained by illegitimate integration of ss plasmid pCW12.
The results of tetrad analysis after second dissection. The percentage of tetrads giving 4, 3, 2, 1 or 0 viable spores is presented.

gel electrophoresis of their chromosomal DNA, followed by Southern analysis. The results of this analysis, including the blots of several viable haploids, are presented in Fig. 3. Plasmid DNA was always detected associated with yeast chromosomes and no extrachromosomal elements were observed, while we previously found that 30% of haploid transformants contained the *ARG4* gene present on autonomously replicating elements (8). For

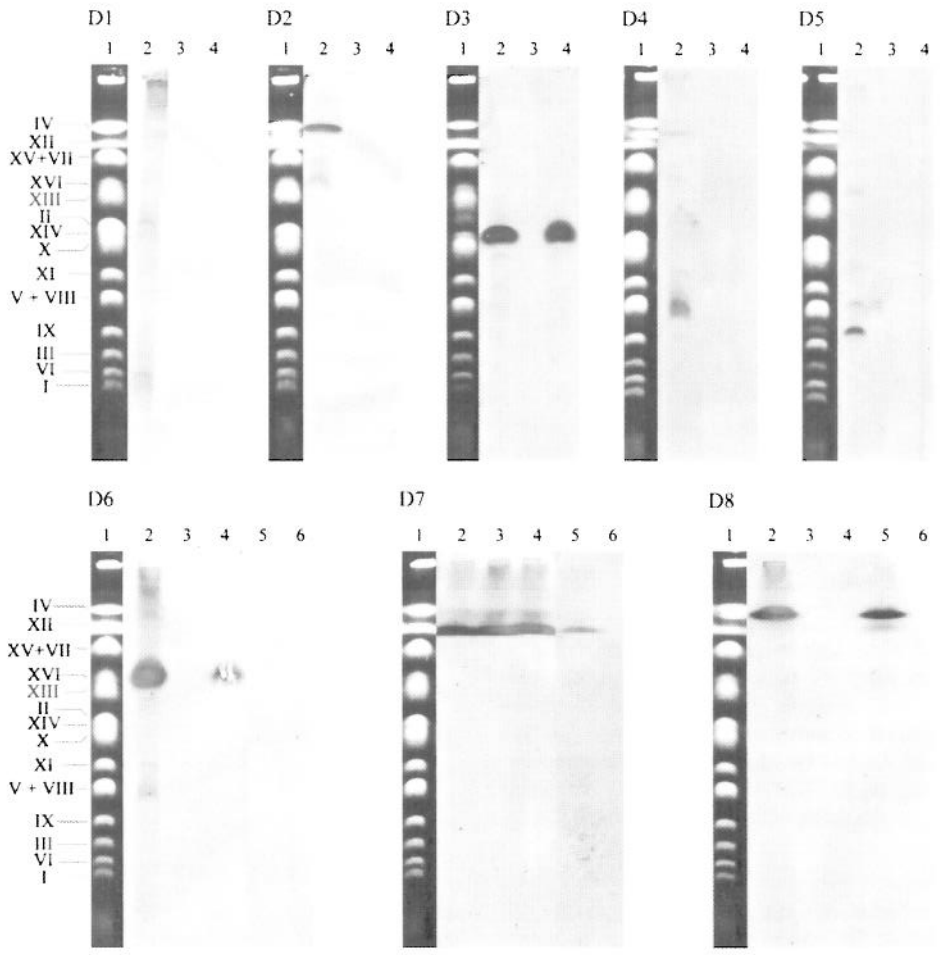

Fig. 3. Molecular analysis of selected transformants and their meiotic progeny. Genomic DNA of eight diploid transformants (D1 through D8) and their monosporic clones were resolved by pulsed-field gel electrophoresis and hybridised to the labelled plasmid pCW12. Ethidium bromide stained chromosomes of diploids are presented in lane 1 and corresponding Southern blots are shown in lane 2. Lanes 3 trough 6 show Southern blots of meiotic progeny.

transformants D3, D6, D7 and D8 it was shown that the heterologous transforming DNA could be transmitted to the haploid progeny. In other words, increased spore lethality was not related to the insertional inactivation of an essential gene, but rather to some other transformation-induced change in the yeast genome. The absence of any signal in the haploid progeny of other transformants might be due to insertional mutagenesis of essential genes, but this interpretation could only be supported by the analysis of large samples of monosporic clones. However, it should be noted that transformants D1, D4 and D5 showed smearing of the signal on the blot, which had been previously attributed to the transformation-induced chromosomal instability giving rise to genetically heterogenous colonies *(8)*. In transformant D5, the strongest signal corresponded to the position of a 'new' chromosome on the gel, migrationg between chromosomes *IX* and *V*. Haploid spores resulting from reductional division of such unbalanced genomes may be expected to lack some function(s) affecting their viability. Transformant D1 also showed poor growth and very low efficiency of sporulation (<1%).

Our results show that the genetic changes induced in diploids by illegitimate integration of foreign DNA may lead to a decreased sporulation efficiency and spore viability. Transformation with heterologous ss DNA may introduce different genetic alterations in the host genome, including multiple tandem integrations, simultaneous integrations in different loci, chromosomal rearrangements and formation of mitotically unstable chromosomes *(8)*. The results presented here suggest that such transformation-induced changes could block normal progression through meiotic division leading to the decreased formation of asci. In mitotic cells, the presence of a single double-strand break in DNA is known to induce cell cycle arrest *(22)*. In cells committed to meiosis, some other signal(s) could induce return to the mitotic growth. For example, strains homozygous for the *rad50S* mutation are deficient in processing of meiotic double-strand breaks and this leads to a 100-fold decrease in asci formation *(23)*. In our case, such signal could be related to difficulties in homologous pairing and formation of synapses due to the genomic changes induced by illegitimate integration of transforming DNA. For exam-

ple, illegitimate events could interrupt high-ordered chromosome structures and the presence of ectopic sequences could lead to ambiguous chromosome pairing. Some transformants selected for low spore viability also exhibited very low efficiency of sporulation, suggesting that these two phenomena could be related. The main source of spore lethality was not insertional inactivation of essential genes, consistent with the finding that only a small fraction of yeast genes is essential for growth *(24)*.

Industrial yeast strains show very reduced sporulation efficiency and low spore viability *(25)*. Based on our results obtained with artificially-induced illegitimate rearrangements, we suggest that spontaneous illegitimate events can contribute to the suppression of sexual reproduction after prolonged vegetative growth typical for yeast-based technologies. However, in natural yeasts, such rearrangements could become stabilised in homozygous diploids through the sporulation-based process of genome renewal *(15)*.

Acknowledgments

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Sporulacija u diploidnih transformanata kvasca *Saccharomyces cerevisiae* dobivenih ilegitimnom integracijom jednolančane DNA

Sažetak

Analiza sekvencije DNA kao i polimorfizam kromosoma prirodnih i industrijskih sojeva kvasca upućuju na mogućnost ilegitimne rekombinacije u evoluciji kvaščeva genoma. Nastale genetičke promjene mogu postati homozigotne nakon sporulacije i križanja između haploidnog potomstva. U ovom je radu uspoređivana sporulacija diploidnih transformanata dobivenih ilegitimnom integracijom heterologne jednolančane DNA i transformanata dobivenih s dvolančanim plazmidom koji se samostalno replicira u stanici kvasca. Transformanti koji su sadržavali stranu DNA integriranu ilegitimnom rekombinacijom pokazali su smanjenu sposobnost sporulacije, dok je preživljavanje spora bilo smanjeno s 80% na 65% samo u prethodno selekcioniranoj skupini transformanata. Neki preživjeli haploidi u genomu sadržavali su integriranu transformirajuću DNA. Razmotreni su mogući mehanizmi koji dovode do smanjene sposobnosti sporulacije i preživljavanja spora u transformanata dobivenih ilegitimnom integracijom strane DNA u genom kvasca.