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GENETIC RECOMBINATION AFTER PROTOPLAST FUSION IN *Candida* sp.

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ABSTRACT

The objective of this work was to describe the parasexual cycle of *Candida* sp, which is generally considered to exist in the asexual state. The conditions for protoplast isolation and regeneration were established for the wild-type strain of *Candida* sp. Protoplasts prepared from the auxotrophic strains (pab bio and ura arg) were fused with polyethylene glycol. Prototrophic derivatives formed by this fusion protocol were selected by complementation on minimal medium. From a selected nuclear fusion product, several monoauxotrophic recombinants were obtained, whose nuclear diameter remained similar to the parental ones. This fact suggested that the auxotrophic recombinants arose from chromosome loss.

INTRODUCTION

The absence of a sexual cycle in yeasts of the genus *Candida* has long been a limiting factor in genetic studies. After the discovery of the fusogenic property of polyethylene glycol (PEG) by Kao and Michayluk (1974), PEG started to be widely used to induce protoplast fusion in yeasts. This technique has permitted genetic recombination in yeasts of the genus *Candida*, which cannot be obtained in experiments conducted on intact cells (Gaillardin and Heslot, 1971). Intraspecific fusions have been reported thus far for *C. albicans* (Sarachek *et al.*, 1981; Poulter *et al.*, 1981; Kakar and Magee, 1982; Pesti and Ferenczy, 1982; Sarachek and Weber, 1984; Gil *et al.*, 1988) and *C. tropicalis* (Fournier *et al.*, 1977; Vallin and Ferenczy, 1978). Interspecific and intergeneric fusions

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have also been reported for *C. tropicalis* x *C. albicans* (Kucsera and Ferenczy, 1979); *C. lipolytica* x *C. utilis* (Horitsu *et al.*, 1989), *C. tropicalis* x *Pichia guilliermondii* (Vallin *et al.*, 1979) and *C. tropicalis* x *Saccharomycopsis fibuligera* (Provost *et al.*, 1978).

Candida sp is a yeast with biotechnological potential because it produces an enzyme complex called Renital which is of interest for milk coagulation in the manufacturing of cheese (Menezes and Nagato, 1974). The objective of the present study was to elaborate methods for the derivation, regeneration and fusion of *Candida* sp protoplasts and to determine the possibility of obtaining recombinant genotypes after protoplast fusion. This recombination process may be useful in future programs of genetic breeding of *Candida* sp.

MATERIAL AND METHODS

Strain

A wild *Candida* sp strain supplied by the Industrial Fermentation Section of the "Instituto de Tecnologia de Alimentos" (Food Technology Institute - ITAL), Campinas, SP, was used to determine the methods for obtaining protoplast regeneration. According to Menezes and Menezes (1974), this yeast does not correspond to any of the species described by Lodder (1971) or Davenport (1974). The species most closely resembling the yeast studied in the present investigation are *C. muscorum* and *C. scottii*. The auxotrophic mutants *pab bio* and *ura arg*, obtained by UV light irradiation of the parental strain at a dosage permitting 5% survival, were used in the protoplast fusion experiments (Fungaro, 1990).

Media and solutions

The complete medium (CM) used was YEPD described by Mortimer and Hawthorne (1969) and the minimal medium (MM) was that described by Reaume and Tatum (1949), with some modifications. When necessary, MM was supplemented with vitamins (1-5 µg/ml), amino acids (50-100 µg/ml) and nucleic acids (50 µg/ml). For protoplast regeneration experiments, CM was prepared replacing water with 0.2 M sodium phosphate buffer, pH 5.8, and adding 0.8, 1.0 or 1.2 M sorbitol. For regeneration of fusion products, MM was prepared replacing water with 0.2 M sodium phosphate buffer, pH 5.8, and adding 1.2 M sorbitol.

The medium used to induce haploidization had the following composition; 30 ml potato broth; 4 g glucose, 170 ml 0.2 M sodium phosphate buffer, pH 5.8, 10 µg *p*-aminobenzoic acid, 5 µg biotin, 1 mg uracil, 2 mg arginine and 10 mg pFA.

Protoplast derivation and regeneration

Cells were grown in YEPD medium up to the log phase by incubating at 30°C with shaking (150 rpm) for 18 hours. Cells were washed twice in 0.2 M phosphate buffer solution, pH 5.8, and resuspended in phosphate-sorbitol buffer (PSB). Novozym enzyme was added to the suspension (10%, w/v) and the mixture was incubated at 37°C for one hour with gentle shaking. The protoplasts obtained were collected by centrifugation and washed three times in PSB. The percentage of cells converted to protoplasts was estimated by the ratio of the number of cells to the number of protoplasts present in three fields of the Neubauer chamber. For regeneration, protoplasts were plated onto the following culture media by the pour-plate technique: YEPD, YEPD-0.8 M PSB, YEPD-1.0 M PSB, and YEPD-1.2 M PSB. After 5 days of incubation, the colonies were counted and the percentage of regeneration in the media of different osmolarity was estimated on the basis of the following ratio: (number of colonies obtained from protoplasts/number of protoplasts plated) x 100.

Protoplast fusion

Protoplast fusion was induced with PEG (MW, 6000). One ml of 1.2 M $CaCl_2$ solution was added to 4 ml of PSB solution containing the parental protoplasts. These were mixed 1:1 and centrifuged at 1000 rpm for 10 minutes. The pellet was resuspended in 1 ml PEG solution (40%, w/v), the mixture was incubated at 30°C for 30 minutes and three successive washings in PSB were performed. The protoplasts were plated onto MM-1.2 M PSB and YEPD-1.2 M PSB by the pour-plate technique. After 8 days of incubation, the colonies present in the two media were counted and the frequency of cytoplasmic fusion was estimated using the following equation: number of colonies developed in MM-1.2 M PSB/number of colonies developed in YEPD-1.2 M PSB.

Selection of the nuclear fusion product

After 8 days of growth in selective regeneration medium (MM-1.2 M PSB), the fusion products were transferred to plates containing MM. After 4 days of incubation, the colonies capable of growing in this culture medium were counted. Cells from one of the colonies growing in MM (FP1) were diluted in saline (0.85% NaCl, w/v) and plated onto CM and MM. After 3 days of incubation, the colonies present in the two types of medium were counted. A similar number of colonies present in MM and CM was one of the criteria used to select the nuclear fusion product. An additional criterion used for nuclear fusion product selection was the comparison of the cell length and nuclear volume of the fusion products and parental strains.

Nuclear staining

Cells were grown in liquid MM up to the stationary phase. They were then washed and small aliquots dribbled on to slides which were dried at room temperature for 15 minutes and fixed in absolute methanol for 15 minutes. The slides were washed with distilled water, soaked in 1 N HCl for 6 minutes and then in 1 N HCl at 60°C for 5 minutes, and washed again with distilled water. The material was stained by placing a film of 0.02 M sodium phosphate buffer, pH 6.9, on the slides and dribbling with a 1% Giemsa solution. After 4 minutes, the slides were washed with running water and mounted with coverslips for light microscopy. Nuclear diameters were measured with an ocular micrometer.

Segregant isolation

Two procedures were used for the isolation of auxotrophic segregants: 1) the cells of the nuclear fusion product were cultivated three times consecutively in pFA-free haploidization medium for 48 hours at 150 rpm. The cells thus obtained were plated onto CM and incubated for 48 hours. The colonies were transferred to 26-point plates containing MM and those unable to grow in MM were characterized in terms of auxotrophy. 2) An identical procedure was used, except that the cells of the nuclear fusion product were cultivated only once in haploidization medium containing pFA for 48 hours at 150 rpm.

RESULTS AND DISCUSSION

Protoplast derivation, regeneration and fusion

The methodology used for protoplast formation premitted 99% conversion of cells to protoplasts. Percent protoplast regeneration was higher with increased buffer molarity (Table I). However, even with 1.2 M PSB, the percentage of *Candida* sp protoplast regeneration was relatively low when compared with that obtained for *C. albicans*, which ranged from 20.5 to 58.1% (Pesti and Ferenczy, 1982).

After protoplast fusion of the auxotrophic *pab bio* and *ura arg* strains it was possible to obtain prototrophic colonies in regeneration MM. Considering that the frequency of reversion of the pab, bio, ura and arg markers is low (less than 1 in 10^{-6}, Fungaro, 1990), we may assume that the colonies developing in regeneration MM are products of cytoplasmic fusion. Thus, the frequency of fusion between regenerated cells (number of colonies in MM/number of colonies in CM) was approximately 1.55×10^{-3}. This frequency was much higher than that detected by Fournier *et al.* (1977) between

Candida tropicalis mutants (3.0 x 10^{-5}) but similar to that obtained for *C. albicans* by Gil *et al.* (1988), which ranged from 4 x 10^{-4} to 8 x 10^{-3}.

Table I - Percent conversion of cells to protoplasts and percent regeneration obtained at three different molar concentrations of PSB (mean of three replications).

PSB molarity	% Conversion of cells to protoplasts	% Regeneration
0.8	99.95	4.92
1.0	99.91	8.26
1.2	99.97	14.69

Only 13% of the cytoplasmic fusion products obtained in regeneration MM maintained the ability to grow when transferred to MM containing no stabilizer. Those products unable to continue to grow in MM are probably unstable heterokaryons which dissociate the auxotrophic parental nuclei. An identical situation was encountered by Fournier *et al.* (1977) when working with *C. tropicalis* protoplast fusion. According to these authors, approximately 30% of the colonies obtained in regeneration MM immediately segregated the parental markers and, therefore, were unstable heterokaryons.

When cells of one of the fusion products (FP1) which had maintained the ability to grow in MM were plated onto CM and MM, the mean number of colonies obtained in the two media was similar (75.66 in CM and 71.33 in MM). This indicates the stability of the fusion product obtained and suggests the occurrence of nuclear fusion.

The fusion product (FP1) and parental cells were 100% uninucleate. As is also the case for *C. albicans* (Sarachek *et al.*, 1981), the cells of the nuclear fusion product of *Candida* sp were almost twice as large as the parental cells. Mean nuclear diameter of FP1 cells was 1.26 times that of the parental cells (Table II). These results agree with those obtained by Suzuki *et al.* (1986), who reported that the nuclear size of the *C. albicans* x *C. guilliermondii* hybrid was larger than that of either parent.

Segregant isolation

As also reported by Fournier *et al.* (1977) in a study of segregation of *C. tropicalis* fusion products, spontaneous (i.e., unaided by a haplodizing agent) production of segregant colonies was obtained. The segregant genotypes obtained from FP1 were arg (0.234%) and bio (0.117%).

Table II - Means and standard deviations for cell length and nuclear diameter of the nuclear fusion product and of the parental strains (mean of 15 replications).

Strain	Cell length (μm)	Nuclear diameter (μm)
ura arg	7.06 ± 1.19	1.93 ± 0.20
pab bio	6.61 ± 0.64	1.85 ± 0.12
PF1	11.14 ± 1.87	2.39 ± 0.11

The frequency of production of auxotrophic *C. tropicalis* segregants was considerably lower than the frequency obtained in the present study, which ranged from 0.02 to 0.07%. This discrepancy was possibly due to the different methodologies used. In the study by Fournier *et al.* (1977), the fusion product was cultivated only once in CM before auxotrophic analysis was undertaken. In contrast, in the present study, as already mentioned in the Methods section, the fusion product was cultivated three consecutive times in CM, possibly leading to an increased probability of the formation of auxotrophic segregants.

The induction of haploidization with *p*-fluor-phenylalanine has been reported for several organisms. As demonstrated both genetically and cytologically by Lhoas (1961, 1968), this compound may act by inducing successive chromosome losses during the cell division process. Sarachek *et al.* (1981) and Fournier *et al.* (1977) reported the use of a pFA concentration of 5 mg/ml in attempts to obtain haploid products of *C. albicans* and *C. tropicalis*, respectively.

In the present study, the attempt to obtain haploid segregants with pFA was made using treatment of FP1 with pFA at the concentration of 50 μg/ml, since the rate of growth inhibition obtained with this concentration was already quite high, i.e., approximately 93.5%. At a concentration of 5 mg/ml, pAF inhibited the cell growth of *Candida* sp in 100%, in contrast to the results obtained for *C. albicans* by Sarachek *et al.* (1981) who did not observe any growth-inhibiting effect. The following segregants were obtained: *ura* (0.09%), *pab* (0.045%) and *arg* (0.045%). It is interesting to note that the frequency of spontaneously obtained auxotrophic segregants (0.352%) was higher than that obtained with pFA (0.18%). Considering that, in addition to the presence or absence of pFA, the number of cultures in CM differed in the two experiments, it is difficult to compare the frequencies obtained in the two cases. Thus, doubts persist as to whether the production of segregants that occurred when pFA was used is associated or not with the action of the drug.

Comparison of mean nuclear diameters by the Scheffé test (Table III) showed that the R1, R2, R3, R4, R5 and R6 recombinants differ from PF1 but do not differ statistically from the parental strains. As was concluded for *Aspergillus nidulans* (Pontecorvo and Roper, 1952) the results of the present work indicate that these segregants arose by haploidization during vegetative growth. The difference in nuclear diameter between segregant R7 and the fusion product were statistically nonsignificant. However, the nuclear diameter of this segregant differed from only one of the parental strains (pab bio). Thus, we may assume that the R7 segregant is an aneuploid that did not yet complete its haploidization or that became stable in this condition, since high tolerance to aneuploidy occurs in other yeasts, such as *Saccharomyces cerevisiae* (Parry and Cox, 1970).

Table III - Nuclear diameter and respective standard deviations of the segregant and parental strains and of the nuclear fusion product.

Strains	Nuclear diameter (µm)
Parental	
ura arg	1.933 ± 0.207
pab bio	1.858 ± 0.128
Fusion product	
PF1	2.393 ± 0.113
Spontaneously obtained recombinants	
R1: ura^+ arg pab^+ bio^+	1.955 ± 0.097
R2: ura^+ arg pab^+ bio^+	1.980 ± 0.183
R3: ura^+ arg^+ pab^+ bio	2.023 ± 0.181
Recombinants obtained with pFA	
R4: ura arg^+ pab^+ bio^+	2.032 ± 0.093
R5: ura arg^+ pab^+ bio^+	1.997 ± 0.185
R6: ura^+ arg^+ pab bio^+	2.028 ± 0.131
R7: ura^+ arg pab^+ bio^+	2.127 ± 0.225

The description of the parasexual cycle is the first step to establish linkage groups in species without sexual recombination. Although a series of additional studies, such as induction of mitotic crossing-over, is still needed for full elucidation of the parasexual cycle in this yeast, the present results clearly indicate the possibility of inducing genetic recombination in *Candida* sp. However, in a novel species, based on seven recombinants only, the conclusions about whether or not the genes concerned are on different linkage groups, would be merely tentative. Efforts are being made to obtain more recombinants and in this way determine the genetic map of *Candida* sp.

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RESUMO

O objetivo deste trabalho foi descrever o ciclo parassexual em *Candida* sp. uma levedura que não apresenta ciclo sexual. As condições para isolamento e regeneração de protoplastos foram estabelecidas para a linhagem selvagem de *Candida* sp. O polietileno glicol foi utilizado para induzir a fusão dos protoplastos isolados a partir das linhagens auxotróficas (pab bio e ura arg). As colônias prototróficas originadas após fusão foram selecionadas por complementação em meio mínimo. A partir de um produto de fusão nuclear selecionado, foram obtidos vários recombinantes monoauxotróficos, os quais tiveram diâmetro nuclear similar às linhagens parentais. Este fato sugere que os recombinantes auxotróficos se originaram de perdas cromossômicas do produto de fusão nuclear.

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