Genetic System of *Schwanniomyces alluvis* Determined by Diad Analysis of Fusion Products

ALLEN P. JAMES* AND DIANA M. ZAHAB

Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada

Received 21 May 1984/Accepted 10 September 1984

The genetics of *Schwanniomyces alluvis*, a yeast that secretes α-amylase, were investigated. No mating types have been detected in this haploid organism. Hybrids were produced by protoplast fusion, and these were subjected to diad analysis by using two-spored ascii. Results showed that the diploidy introduced by cell fusion persists through successive spore generations. It was concluded that in this organism, sporulation is preceded by the fusion of mitotic products, regardless of the ploidy of the latter. Routine procedures for constructing novel strains would, no doubt, be hampered by this failure of the sporulation process to restore haploidy. Nevertheless, chromosomal instability of hybrids, as indicated both by heterogeneity of fusion products and by a high frequency of morbidity among their segregants, may permit the use of classical genetic techniques for strain construction.

Many microorganisms that have not been subjected to very intense investigations in the past are nevertheless known to possess attributes of potential industrial or medical value. Some of these organisms are now being scrutinized with particular care either in the hope that they may provide a source of genetic material through the utilization of recombinant DNA techniques or, alternatively, in the hope that they may prove to be useful in their own right. Among these are species of *Schwanniomyces*, one of the few yeasts known to produce α-amylase (1). This yeast is now being studied in several laboratories (2, 3, 8, 10). If its usefulness is verified, as now appears to be likely, then some knowledge of the more efficient methods of manipulating it genetically would no doubt be advantageous.

*Schwanniomyces alluvis*, the species under investigation here, is a budding yeast. Typically it sporulates to produce single-spored ascii. Early studies were more taxonomic than genetic in their thrust, although one investigation (6) has dealt with unsuccessful attempts to induce hybridization by methods of prototroph selection. More recently, an important first step toward clarification of the genetics of this yeast was taken by Wilson et al. (10), who used protoplast fusion of auxotrophic mutants to create prototrophic fusion products. Strong evidence that these products were true hybrids was provided by analyses of induced and spontaneous mitotic segregants; these were found to include both parental and recombinant phenotypes.

In the investigation described here, diad analysis was utilized to demonstrate that the diploidy achieved by cell fusion is not reduced to haploidy by meiosis. Instead, a degree of diploidy is maintained through successive spore generations.

**MATERIALS AND METHODS**

**Yeast strains.** The yeast used in this study was *S. alluvis* (ATCC 26074). Single and double auxotrophs were obtained by mutagenesis with *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine (7). Care was taken that mutant strains selected for fusion experiments had low spontaneous reversion rates and did not suffer from the reduced ability to sporulate.

**Media.** Standard YEPD medium was used for general purposes. The medium for sporulation (YEP) contained 1.3% (wt/vol) yeast extract, 0.5% peptone, 1.5% dextrose, and 2% agar. The pH was adjusted to 4.5. Plates of sporulating cells were incubated at 19°C for 20 days. For genetic characterization, omission media were prepared from a basal medium of 0.67% yeast nitrogen base without amino acids.

**Protoplast fusion.** Cells (80 ml) were harvested in mid-log phase (3 × 10⁷ cells per ml) by centrifugation at 874 × g with an International HN-S centrifuge and washed once with 10 ml of 1.2 M sorbitol. The cells were then suspended in 10 ml of protoplasting buffer (1.2 M sorbitol plus 0.1 M phosphate) to which 4 mg of zymolyase 60000 was added. After incubation at 30°C for 20 min, protoplasts were washed three times at 492 × g in 1.2 M sorbitol. The pellet was suspended in 0.2 ml of a 1.2 M sorbitol–10 mM CaCl₂ solution. The viability of protoplasts at this step averaged 10%. A 0.1-ml fraction of each of two strains was mixed and incubated for 15 min at 23°C. Two milliliters of fusion buffer (20% [wt/vol] polyethylene glycol plus 10 mM Tris-hydrochloride [pH 7.5] with 10 mM CaCl₂ added just before use) was gently overlaid on the mixture and incubated an additional 20 min at 23°C. The cell mixture was subsequently centrifuged at 874 × g, suspended in 50 µl of YEPD broth containing 1.2 M sorbitol, and allowed to incubate for 20 min at 30°C. The mixture was diluted with 5 ml of 0.6 M KCl. Fusion products were recovered by adding 1 ml of the mixture to 10 ml of regenerating agar and plating. Regenerating agar contained 0.67% (wt/vol) yeast nitrogen base without amino acids, 2% dextrose, 2% (vol/vol) YEPD broth, 0.6 M KCl, and 3% agarose. Regeneration of the final plating mixture averaged 0.4%.

**Preparation of spores.** To free spores for isolation, asci were treated in a zymolysate solution (4 mg/ml) for 10 min.

**Chemicals.** *N*-Methyl-*N'*-nitro-*N'*-nitrosoguanidine was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis.; zymolysate 60000 was obtained from Miles Laboratories, Ruxdale, Ontario, Canada; polyethylene glycol 4000 was from J. T. Baker Chemical Co., Phillipsburg, N.J.; agarose (Seakem TM [LE]) was from Marine Colloids Div., FMC Corp., Rockland, Maine; sorbitol was from Fisher Scientific Co., Fair Lawn, N.J.; amino acids were from Sigma Chemical Co., St. Louis, Mo.; and yeast nitrogen base was from Difco Laboratories, Detroit, Mich.

* Corresponding author.
base without amino acids was from Difco Laboratories, Detroit, Mich.

RESULTS

Sporulation of fusion products and evidence of genetic heterogeneity. Four double auxotrophs were used to produce three different fusion hybrids. The hybrid strains so obtained were A3 (met− arg− × lys− his−), A4 (met− ade− × lys− his−), and A5 (met− ade− × lys− ser−).

Ten independent prototrophic isolates of each strain were compared. All were alike in that they had increased cell sizes. However, they differed widely in their response to sporulation conditions. The frequency of ascus varied between 4 and 40%, and the frequency of two-spored ascus varied between 15 and 80%. It is evident from this variability that fusion hybrids that are supposedly of the same constitution need not be genetically identical. An explanation may lie in chromosome losses at the time of cell fusion or immediately afterward which lead to various degrees of aneuploidy within the hybrids.

In haploids, the frequency of two-spored ascus is less than 1%. The increase in frequency of two-spored ascus in hybrid cells is thus dramatic. The question of whether there is any intrinsic difference between the monads of one-spored ascus and the diads of two-spored ascus was answered by comparing monads and diads of the same strain for spor viability and marker segregation. The results showed that spore viability was higher among diads (χ², 5.6; df, 1; 0.010 < P < 0.025) than among monads. However, segregation ratios (see below) were apparently the same for the two ascus types (χ², 1.5; df, 1; 0.10 < P < 0.25). It was concluded that two-spored ascus reflect only an increase in probability of survival among the four products of meiosis. This was reinforced by the fact that three-spored ascus were occasionally noted in the strains which sporulated more profusely. On the assumption that diad analysis would be more infor-
mative than monad analysis, all subsequent studies were carried out with two-spored asci.

Reduced spore viability and abnormal meiosis. A drastic reduction in the ability of spores to form colonies is apparently an inevitable consequence of hybridization by protoplast fusion. Such a reduction was found in each of more than 12 different fusion products and is exemplified by the data in Table 1. Here, the highest frequency of colony-forming spores was 36%, whereas for haploid strains the frequency was about 95%. We note that the statistically significant differences among the various isolates of strain A3 in this regard support the previous evidence of genetic heterogeneity of fusion products.

Among the spores of hybrids, a complete failure to germinate is the more common expression of reduced colony-forming ability. However in 10 to 20% of the cases, affected spores actually germinate to produce a few cells or even abortive microcolonies.

With two-spored asci, the inability of spores to produce colonies is not distributed at random. Instead, the frequencies with which diads are composed of one viable spore and one nonviable spore are always lower than expected with random death, often to an extent that is statistically significant. Assuming a genetic component in the death process, this pattern of behavior suggests that the lethal events responsible for spore death are associated with the first meiotic division.

Meiosis and the occurrence of disomic segregation. Meiotic segregation of the three hybrid strains A3, A4, and A5 are included in Table 1. It is evident that these are not the separations expected of normal heterozygous diploids. For diads, that expectation is phenotype ratios of 1+:+4+:−:1− for any one locus. Only the meager data of strain A5 conform to this ratio; otherwise, there is a great preponderance of + phenotypes. A bias imposed by the selective death of auxotrophs can account for this deviation.

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Isolate no.</th>
<th>No. of asci with the following characteristics</th>
<th>Spore viability</th>
<th>No. of isolates that are:</th>
<th>Diad analysis</th>
<th>Random spore analysisa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>met</td>
<td>arg</td>
<td>lys</td>
<td>his</td>
</tr>
<tr>
<td>A3+</td>
<td>1</td>
<td>93</td>
<td>0.35</td>
<td>20</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>62</td>
<td>0.36</td>
<td>14</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3−6a</td>
<td>109</td>
<td>0.23</td>
<td>14</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>A4b</td>
<td>1</td>
<td>35</td>
<td>0.28</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>19</td>
<td>0.25</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>A5c</td>
<td>1</td>
<td>19</td>
<td>0.30</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

a Accumulated data from nongerminating or abortive spore and colony-forming spore ascii.

b x, Nongerminating or abortive spores; 1, colony-forming spores.

c Based on an expected ratio of 0.63+:+0.34+:−:0.03− (ref. 2).
d Based on an expected ratio of 4+:1−(ref. 1).

e met− arg− × lys− his−.

f Statistically significant at the 5% level.
g Isolates 3 to 6 sporulated poorly, and the few data are combined.
h met− ade− × lys− his−.
i met− ade− × lys− ser−.

Statistically significant at the 1% level.
but such an explanation is unattractive for two reasons. First, the discrepancy of negative phenotypes is almost identical for each of the four loci being tested, suggesting that each of the auxotrophic phenotypes suffers the same risk of death, a rather unlikely possibility. The second reason is provided by the frequencies of double auxotrophs, whether recombinant or parental. These data are not included in Table 1 because they are remarkable only for their uniformity. For every combination the frequency of double auxotrophs was almost exactly that expected of random assortment, as calculated from the existing frequency of each auxotroph. The data imply, then, that double auxotrophs are in no greater jeopardy than single auxotrophs.

A more acceptable explanation of the data can be based on the assumption that these represent the meiotic segregation of tetraploids. The frequencies of + +, + –, and – – phenotypes expected among the segregants of a tetraploid depend on the relative frequencies of first- and second-division segregation and on the relative frequencies of tetravalent and bivalent chromosome pairing. Tetrad analysis of tetrasomic or tetraploid *Schwanniomyces cer Spread* have usually indicated that the frequency of second division segregation is 66.7% and that chromosome pairing is mainly tetravalent in nature. The expected frequencies of phenotype ratios (+ +: – –) of 4:0, 3:1, and 2:2 under these assumptions are 0.395, 0.395, and 0.21, respectively. For diad analysis, the corresponding frequencies of + +, + –, and – – phenotypes are 0.63, 0.34, and 0.03, respectively. The fit of the *Schwanniomyces alluvius* data to these latter values is included in Table 1, in which χ² tests demonstrate a good fit in three of five instances and a particularly poor fit in one (A5).

The rather plentiful data from ascii containing one moribund and one colony-forming spore are included in Table 1, and these are available for random spore analysis. For a tetraploid under the meiotic chromosomal conditions stipulated above, the expected ratio of + +:– = segregation is 4:1. The fit of the data to these expectations (Table 1) is extremely good for every strain, including A5.

**Meiosis in successive generations and the perpetuation of diosity.** Confirmation that spores produced by fusion products of *Schwanniomyces alluvius* are typically disomic, if not diploid, was obtained by genetic analyses of segregants through successive generations.

The segregant strains derived from viable spores of hybrid fusion cells were even more variable with regard to frequency of ascii, relative frequency of diads, and spore viability than were the original hybrid strains. Seven segregants of A3 (selected for higher spore and diad frequencies) were subjected to genetic analysis. The second-generation data generated by these strains are presented in Table 2, in which it can be seen that a dominant phenotype masked a heterozygous genotype in the first-generation segregation in 14 of 21 instances.

The accumulated frequencies of + and – segregants were tested for conformity to the 4:1 ratio expected of random spore analysis of a tetraploid. For each of the seven strains the fit was very good (Table 2). The results of diad analyses are not included in the table; data of each strain provided a satisfactory fit to the expected 0.63× +, 0.34× –, 0.03× – values. Accumulated data from all strains yielded a χ² value of 0.47 (df, 2; 0.75 < P < 0.90).

Persistence of heterozygosity into the third generation is demonstrated in the lower portion of Table 2, in which the data produced by three segregants of A3-6B are presented. Once again, heterozygosity of spores in the previous generation is evident. The frequency of negative segregants was 18.4%, a close approximation to the expected value of 20%.

**Higher ploidy levels.** Is it possible to construct strains of higher ploidy? This question was answered affirmatively in the following manner. The two segregants A3-56A, auxotrophic for lysine, and A3-6A, auxotrophic for methionine (Table 2), were subjected to protoplast fusion. A single prototrophic colony (A6) appeared on the plates. Spore viability of this strain was exceptionally low; of 128 isolated spores, only 8 germinated to produce colonies. However, among these, the segregation ratios (+ +:–) were 7:1 for methionine, 4:4 for arginine, 7:1 for lysine, and 8:0 for histidine. These results provide good evidence that hybrid strain A6 was tetrasomic if not tetraploid.

**DISCUSSION**

The finding that heterozygosity introduced by protoplast fusion persists through successive spore generations in *Schwanniomyces alluvius* is of fundamental, as well as practical, significance. This yeast is haploid in nature (3). What is known or suspected about the events leading to spore production in this homothallic organism is derived from studies relating to its distinctive ascus: inevitably attached to the ascus is a wide-necked bud apparently devoid of nuclear content. It is generally considered that the
transient diploid phase, essential to meiosis, occurs when the nucleus of this bud fuses with that or the mother cell to produce a zygote. The tetraploidy detected in this study can be explained by the supposition that the acquisition of diploidy at the time of protoplast fusion is simply not recognized by this organism. As a consequence, sporulation, when induced, proceeds as for a haploid. In effect, transient diploidy is replaced by transient tetraploidy.

It seems likely that the meiotic behavior of fusion products as described here is not restricted to Schwanniomyces alluvius. For instance, results of a study of meiotic segregation of fused cells of like mating types in the yeast Saccharomyces lipolytica have been included in a recent publication (9), and although limited in amount, they are remarkably similar to those presented in this report. On the other hand, with the strongly homothallic haploid yeast Pachysolen tannophilus, the rare diploidy achieved through prototrophic selection among mixtures of auxotrophic cells does not persist through meiosis (5).

The results of this study indicate that hybridization by protoplast fusion does not provide a simple alternative to the normal mating processes common to heterothallic yeasts. Certainly the construction of new strains by procedures that utilize such routine techniques as backcrossing would be hampered by the failure of meiosis to restore the haploid phase. Perhaps some of these difficulties can be mitigated by taking advantage of the evident chromosomal instability that is introduced at the time of fusion or meiosis. Indeed, two segregants in this series of analyses had the cell size expected of haploids. For some purposes, the complications imposed by persistent diploidy are of minor importance. Thus, to determine the genetic constitution of a fusion product, genetic analysis by meiotic segregation is a completely feasible procedure.

The clarification of the meiotic process in fused cells of Schwanniomyces alluvius obtained here was greatly facilitated by the techniques of micromanipulation and diad analysis. However, for future studies it seems likely that the plating techniques associated with random spore analysis may be adequate or even preferable. This is a welcome finding because, at least in our hands, the micromanipulation of spores of this yeast is an arduous undertaking, despite, or perhaps because of, large spore sizes.

ACKNOWLEDGMENTS

We thank Eric Stephen for preparing the double auxotrophs used in this study.

LITERATURE CITED