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Phaffia rhodozyma is polyploid

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The ploidy of the red yeast *Phaffia rhodozyma* was evaluated using flow cytometric analyses of propidium iodide-stained cells and mutagenic inactivation kinetics. Our findings suggest that *Phaffia rhodozyma* is not haploid. Auxotrophic strains were generated at a high frequency following treatment of mutagenized cells with a combination of benomyl and ethyl acetate. Studies of an auxotrophic mutant using flow cytometry and UV inactivation indicated possible chromosome loss to an aneuploid state.

Keywords: *Phaffia rhodozyma*; ploidy; yeast; astaxanthin

Introduction

Phaffia rhodozyma is a red yeast which produces astaxanthin as the major carotenoid pigment [3,4,15]. This organism has been considered to be a basidiomycete based on ultrastructural and chemotaxonomic characteristics [21,26]. A sexual cycle has not been identified [18], although recently the formation of holobasidia with terminal basidiospores after mother-daughter cell conjugation has been described [12]. To date, the ploidy of *P. rhodozyma* is unknown, and there are conflicting reports on this topic [6,7,27].

There is considerable interest in exploiting this organism as a source of astaxanthin for pigmentation of marine species, such as salmonids and crustacea [9,14,17], and poultry [16]. This economic interest has spurred numerous attempts to increase astaxanthin content of *Phaffia* by classical mutagenesis [1,2,18], and by protoplast fusion [6]. Knowledge of the ploidy of this organism is needed to develop selection strategies, as well as to establish the phylogeny of *Phaffia*.

In the course of performing mutagenesis experiments with this organism, we observed that recessive mutants, such as auxotrophs, are rarely isolated. Difficulty in isolating auxotrophic strains of *Phaffia* has been reported elsewhere [22,27]. This has led us to consider the possibility that *Phaffia* may not be haploid. Since auxotrophy is a recessive trait, a diploid should yield auxotrophs at a much lower frequency since mutations would have to be induced at each copy of a gene for a given locus.

Alternatively, it is possible to recover recessive phenotypes from polyploid organisms at increased frequencies by inducing an aneuploid state [5]. For example, the use of methyl benzimidazole carbamates and certain aprotic polar solvents has been reported to induce aneuploidy in a number of fungi [8,19,25,29,30]. The fungicide benomyl (MBC) has been well studied as a means of inducing aneuploidy in *Saccharomyces cerevisiae* [5,28].

The objectives of the experiments described here were to define more precisely the ploidy of *Phaffia* using, primarily, the techniques of flow cytometry and ultraviolet radiation lethality. We have also investigated the use of antitubulin compounds to increase the frequency of auxotrophic mutants of *Phaffia rhodozyma*.

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Materials and methods

Yeast strains, mutagenesis, and growth conditions

A wild-type culture of *Phaffia rhodozyma* (ATCC 24202) was obtained from the American Type Culture Collection. *Saccharomyces cerevisiae* strains of defined ploidy (X2180–1A (a) (haploid), X2180 (a/α)(diploid), and STX600 (α/α/a) (triploid)) were obtained from the Yeast Genetic Stock Center (University of California-Berkeley).

Phaffia rhodozyma was mutagenized with nitrosoguanidine to obtain a 90% kill according to the procedure described by Miller [20]. Mutant strains N/nys 2/25 (capsule$^-$) and N40 were obtained from the wild-type strain (ATCC 24202).

Cultures were maintained on YEPD agar slants (10.0 g yeast extract, 20.0 g peptone, 20.0 g glucose, 15.0 g agar per liter of deionized water) and stored at 4°C. Broth cultures were grown on a rotary shaker (200 rpm) at 25°C.

UV radiation survival

For UV survival experiments, broth cultures were monitored turbidimetrically and grown to late exponential phase in YEPD broth. Cells were washed twice in 0.85% (w/v) saline solution (PSS). Serial dilutions of the washed cells were prepared and aliquots were plated in triplicate onto YEPD agar. Plates were then exposed to UV light (254 nm at 2000 ergs mm^{-2} s^{-1}) for 0, 15, 30, 45, 60, and 90 s. Following exposure, plates were immediately incubated in the dark at 23°C. The survival curves were generated by counting the resulting colonies.

Preparation of cells for flow cytometry

Cells were prepared for flow cytometry using a modification of the method described by Olaiya and Sogin [23]. Stationary phase cells from YEPD broth were harvested and washed twice in PSS. Washed cells were fixed in 70% ethanol for 10 h at 4°C, washed again in PSS, and sus-

pended in an aqueous solution of 0.1% pancreatic RNase (Sigma, St Louis, MO, USA) for 1 h at 25°C. Pepsin (Sigma) was added to 1000 U ml^{-1} and the suspension was incubated for an additional 5 min at 25°C. The cells were harvested by centrifugation and stained twice (25°C) with aqueous solutions of propidium iodide (Sigma); once for 4 h in a 5 mg ml^{-1} solution and again in a 50 μg ml^{-1} solution for 48 h. The wavelength of the exciting argon laser was 488 nm. Cell number was recorded as the forward angle light scatter (FALS) signal, and the intensity of the fluorescence was measured by channel number using a 256 channel pulse height analyzer.

Aneuploid induction

For induction of aneuploidy, log phase cultures of *P. rhodozyma* N/nys 2/25 and N40 were mutagenized according to the procedures described above. Following outgrowth for 24 h in YEPD, benomyl and ethyl acetate were added to final concentrations of 20 μg ml^{-1} and 2.0% (v/v) respectively. Incubation was continued for 5 h at 23°C. The suspension was then incubated at 4°C for 18–20 h, followed by an additional incubation at 23°C for 5 h. Cells were removed by centrifugation and washed in PSS.

Nystatin enrichment and auxotroph selection

An enrichment for amino acid auxotrophs was performed using a modification of the method described by Gleeson *et al* [11]. A concentration of 40 μg ml^{-1} of nystatin was used for the enrichment phase just prior to plating onto YEPD agar. Following outgrowth, colonies were replicated with velvet blocks onto Difco Yeast Nitrogen Base (YNB) (1% dextrose) without amino acids. Amino acid auxotrophs were identified by lack of growth on amino acid free replica plates (YNB + 1% dextrose). Specific auxotrophic requirements were determined as described by Rose *et al* [24].

Results

Fluorescent activated cell sorting (FACS) analyses

Attempts to stain *P. rhodozyma* ATCC 24202 with propidium iodide were hampered, presumably due to the impermeability of the stain through the dense capsular layer. However, when a capsuleless strain (N/nys 2/25) was subjected to the same staining procedure, dye uptake was not impeded as revealed by fluorescence microscopy. Likewise, the haploid, diploid, and triploid tester strains of *S. cerevisiae* were readily stained. FACS analyses of the *Saccharomyces* strains indicated a nearly linear relationship between the ploidy (DNA content) and channel number (fluorescent intensity/cell). The data obtained by FACS analyses of N/nys 2/25 (Figures 1 and 2) indicate that *P. rhodozyma* shows a DNA content per cell nearest to the diploid strain of *S. cerevisiae*.

UV radiation survival

Although these results indicate that *Phaffia* cells have a DNA content which is similar to the diploid *Saccharomyces* strain, the data can also be interpreted to mean that *Phaffia* has a *ca* two-fold greater degree of DNA complexity than haploid strains of *Saccharomyces*. In order to resolve this issue, the survival of *Phaffia* and *Saccharomyces* to mutagenic agents were compared. By target theory, the probability of a lethal mutation is constant per unit length of DNA. Also, the more copies of a gene necessary for a given function, the larger the amount of time required by a given dose of a mutagen to inactivate all copies of the gene and thereby kill the cells. Results from UV inactivation experiments are summarized in Figure 3. The survival of the *Saccharomyces* strains was proportional to the ploidy. Inactivation curves for *P. rhodozyma* ATCC 24202 (wild-type) and N/nys 2/25 are similar, and both are kinetically close to the inactivation kinetics of the triploid strain of *S. cerevisiae* (STX600). Although it could be possible that the *Phaffia* strains are more resistant to the UV light, similar results were obtained when a chemical mutagen (nitrosoguanidine) was used (data not shown).

Aneuploid induction

Our early attempts to generate auxotrophic strains of *P. rhodozyma* from either strain ATCC 24202 or N/nys 2/25 consisted of subjecting the organisms to NTG mutagenesis (90–99% kill) and then plating the mutagenized culture onto YNB without amino acids from YEPD plates. Under these circumstances, the frequency of auxotroph isolation was significantly lower (<1 in 10^4) than the frequency expected for a haploid yeast, despite antibiotic enrichment with nystatin prior to plating.

The experiments described above, therefore, clearly substantiated the use of alternative methods to obtain strains showing recessive phenotypes (ie auxotrophs). To accomplish this, we studied the influence of antitubulin agents (benomyl; MBC) in conjunction with an aprotic polar solvent (ethyl acetate) on *P. rhodozyma*.

Morphological changes as a result of exposure to antitubulin agents such as benomyl have been reported for a variety of fungi [5,10]. Treatment of the wild-type *Phaffia* with benomyl (20 μg ml^{-1}) for as long as 48 h did not result in any gross morphological changes or in a significant loss of viability. In contrast, exposure of the capsuleless strain (N/nys 2/25) to 20 μg ml^{-1} benomyl resulted in the formation of pseudomycelia within 4 h after exposure as well as a proportional decline in viability with increasing time of exposure.

The use of benomyl alone, at a concentration of 20 μg ml^{-1} resulted in very low frequencies of auxotrophs ($<0.01\%$). The synergistic effect of combining nocodazole, a methyl benzimidazole compound, with aprotic polar solvents has been reported to greatly increase the efficiency of aneuploid induction [19,29]. Therefore, we chose to use the combination of 20 μg ml^{-1} benomyl with 1.0% (v/v) ethyl acetate. Using this procedure in conjunction with a nystatin enrichment, a frequency of 0.27% auxotrophs representing four auxotrophic phenotypes (ala^-, asp^-, $glu^-ser^-ala^-asp^-$, and arg^- were isolated from the plated viable population.

A representative of the above isolates, strain N/nys 2/25–56, was selected for further evaluation in order to assess the loss of genetic material due to this treatment. The UV inactivation curve for this mutant was shifted towards that of the haploid strain of *S. cerevisiae* (Figure 3) with inactivation occurring much more rapidly in comparison to the parental strain (N/nys 2/25) and wild-type (ATCC 24202).

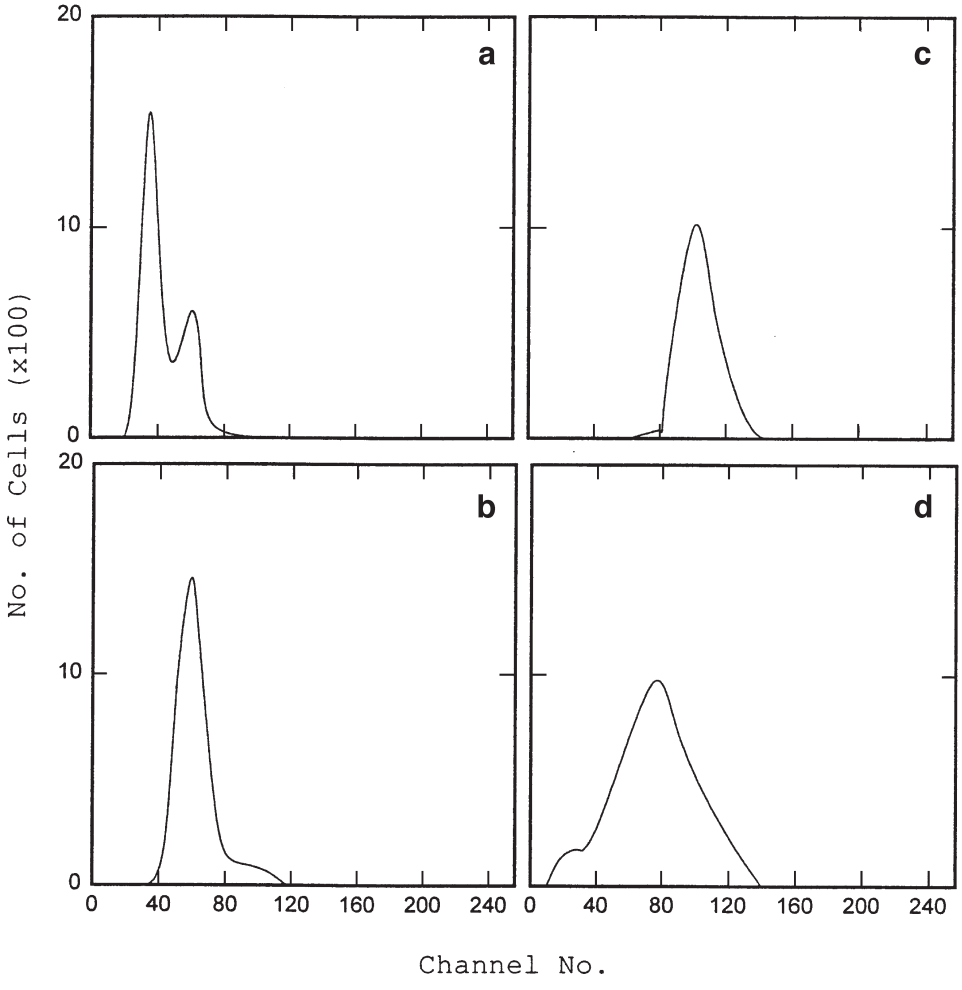

Figure 1 Results of FACS analysis of strains of *S. cerevisiae* differing in ploidy as compared with *Phaffia rhodozyma* strain N/nys 2/25. FACS histograms were derived from stationary-phase cells stained with propidium iodide and subjected to FACS analysis as described in the text. The numbers on the abscissa represent channel numbers which are proportional to the amount of fluorescent dye contained by the cells. Therefore, the greater the channel number, the greater the DNA content. (a) haploid *S. cerevisiae*; (b) diploid *S. cerevisiae*; (c) triploid *S. cerevisiae*; (d) *Phaffia rhodozyma* strain N/nys 2/25.

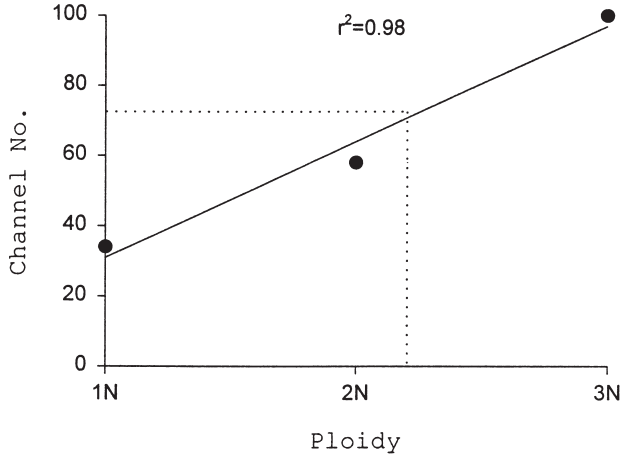

Figure 2 Relationship between ploidy of haploid, diploid, and triploid standard strains of *S. cerevisiae* and channel number (relative fluorescence) as determined by FACS analysis. Data for channel numbers were extrapolated from the peaks of individual FACS histograms for each strain as reported in Figure 1. Extrapolation of ploidy of *P. rhodozyma* strain N/nys 2/25 is derived via the dotted line.

Similar results were obtained with strain N40–2 which is another auxotroph obtained by the procedure described above. Likewise, the FACS analysis of propidium iodide stained cells shifted towards a lower channel number in comparison to the parental strain, suggesting chromosomal loss (Figure 4). Microscopic examination of the auxotrophic strains also indicated a significant reduction in cell size when compared to the parent strain and wild-type.

These experiments indicate that an aneuploid (or haploid) state may be induced in *Phaffia* following treatment with agents affecting microtubule assembly, such as a combination of a methyl benzyl carbamate compound (benomyl) with an aprotic polar solvent (ethyl acetate). This synergistic combination resulted in a significant increase in the frequency of recovered auxotrophs (0.01% *vs* 0.27%).

Discussion

Although the approaches which are taken can vary, the ultimate goal of an industrial strain improvement/mutagenesis program is to improve the overall productivity or fermentation characteristics of the target organism. While random

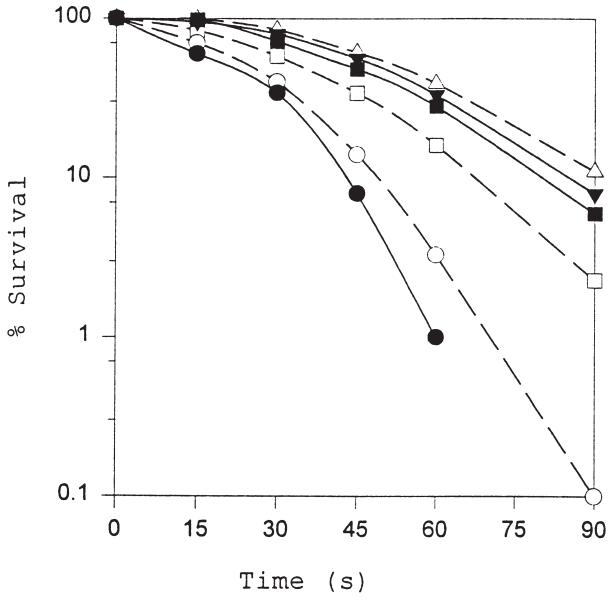

Figure 3 Survival curves for *Phaffia rhodozyma* and *Saccharomyces cerevisiae* exposed to UV light. Data points represent the averages of triplicate samples. Symbols: ○, haploid *S. cerevisiae*; □, diploid *S. cerevisiae*; △, triploid *S. cerevisiae*; ■, wild-type *P. rhodozyma*; ▼, *P. rhodozyma* N/nys 2/25; •, *P. rhodozyma* N/nys 2/25–56.

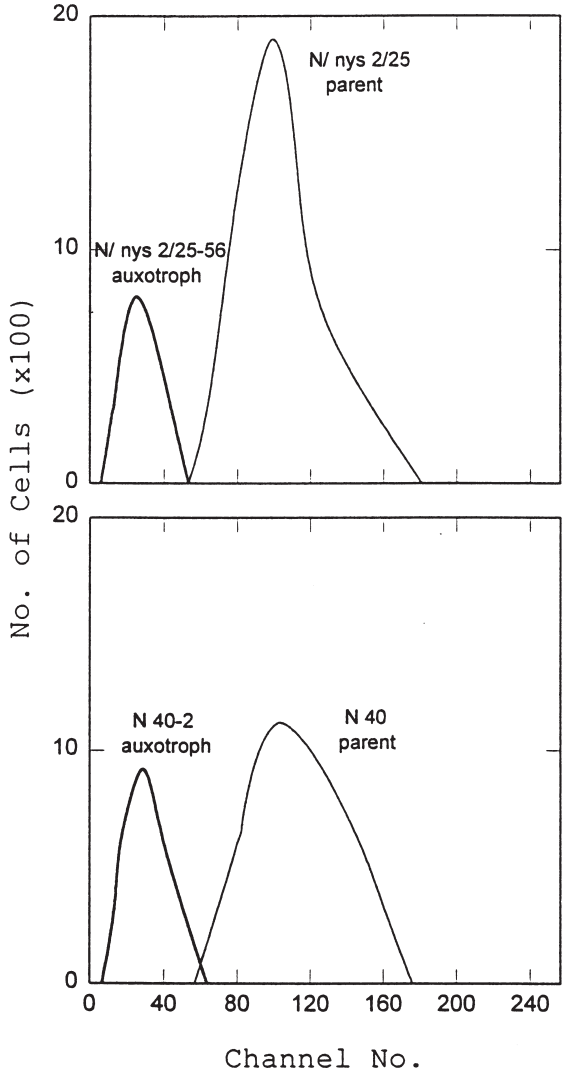

Figure 4 Comparison of parental strains of *P. rhodozyma* and auxotrophic progeny generated using benomyl/ethyl acetate following NTG mutagenesis. FACS analyses of propidium iodide-stained cells and benomyl/ethyl acetate treatment were performed as described in the text.

mutagenesis can offer a substantial amount of improvement, the use of rational screens and auxotrophs can often be more successful. The latter approach, however, requires some basic knowledge of the biosynthetic pathways involved in product formation as well as some fundamental understanding of the genetic characteristics of the target organism. The inability to isolate auxotrophs of *Phaffia* caused us to reassess our overall selection strategies and to develop alternative methods. The rationale used to gather critical data regarding the ploidy of *Phaffia* was similar to that used by Olaiya and Sogin [23] and Janatova [13] in establishing the ploidy of *Candida*. Our overall objectives, however, were not to precisely determine the ploidy of *Phaffia*, but instead to answer the question as to whether the organism is haploid or polyploid, and then to develop appropriate strategies to obtain auxotrophs.

The studies described here have collectively suggested that *P. rhodozyma* is not haploid. This conclusion is based on several types of data.

We had consistently been unable to isolate auxotrophs (recessive mutations) from mutagenized populations of *Phaffia* at haploid frequencies. Three independent efforts involving over 40 000 screened colonies failed to yield a single amino acid auxotroph. These data are consistent with the findings of other investigators [22,27] which indicate difficulty in obtaining *Phaffia* auxotrophs.

FACS analyses of propidium iodide-stained cells indicated a DNA content per cell slightly greater than the diploid strain of *Saccharomyces*. There are two possible interpretations of these results: (1) *Phaffia* is polyploid; or (2) the *Phaffia* yeast has a much greater DNA complexity for each cell than *Saccharomyces*. Mutagen inactivation kinetics for a physical agent (UV radiation), and a chemical agent (nitrosoguanidine) do not support the latter. If *Phaffia* were assumed to be haploid with only one copy of a given gene and twice the DNA content per cell (twice the amount of potential sites at which a lethal event might occur), *Phaffia* should be even more susceptible to physical and chemical mutagens than are *Saccharomyces* haploids. These data are consistent with the results of Cifuentes *et al* [7] who suggested that *Phaffia* is diploid based upon electrophoretic karyotyping and cellular DNA content derived by chemical determination. The FACS data generated in the current study further estimate the genomic size of the *Phaffia* wild-type ATCC 24202 to approximate the estimated size of a *Saccharomyces cerevisiae* diploid strain, which Genbank indicates at *ca* 24 Mb. These data are also consistent with the estimated genome size of other *Phaffia* wild-types (25 Mb for ATCC 24230; 19.3 for ATCC 24229; 22.2 for ATCC 24203) [7,22].

The success in obtaining a high frequency of auxotrophs through the use of benomyl in conjunction with ethyl acetate is in agreement with the results of Zimmerman *et al* [29,30] who reported a greater induction of aneuploids in

Saccharomyces with the synergistic effect of aprotic solvents and nocodazole. Our data suggest that the auxotrophic strains of *Phaffia* generated by this procedure show aneuploid characteristics, ie reduced cell size, DNA content, and increased susceptibility to UV irradiation.

Although the assignment of an exact ploidy of *Phaffia* cannot be determined conclusively from these studies, the recognition that the organism is not haploid should provide more insight to investigators seeking to conduct successful strain improvement programs.

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