JOURNAL OF BACTERIOLOGY, Nov. 1987, p. 4991–4994
0021-9193/87/114991-04$02.00/0

Vol. 169, No. 11

Parasexual Genetics of *Torulopsis glabrata*

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Prototrophic hybrids were generated in the asexual yeast *Torulopsis glabrata* by the fusion of spheroplasts derived from parent strains which bore complementing auxotrophic markers. The DNA content (per cell) of two hybrids was essentially that predicted by summing the corresponding parental values. UV irradiation of these two hybrids resulted in the formation of sectored colonies with genetic properties consistent with their origin by either mitotic recombination or chromosomal nondisjunction.

The asexual yeast *Torulopsis glabrata* is an opportunistic pathogen (1). In a study of fungemia in immunosuppressed patients, *T. glabrata* was isolated in a significant number of episodes of fungemia (26 of 136) (9). Recently, Hickey et al. (7) described a lethal infection in an immunosuppressed patient and provided a review of the literature.

Previous basic studies with *T. glabrata* were concerned mainly with the structure of its mitochondrial genome and with the genetic basis of its killer activity against other yeasts (3, 16, 17). In a study of hybridization, Galeotti et al. (5) obtained *T. glabrata* hybrids by inducing the fusion of spheroplasts derived from parents (presumed haploid); however, those hybrids contained less DNA per cell than was predicted by summing the observed parental values of DNA per cell. Those researchers suggested, on the basis of their finding that the dose-response curves for X-ray killing of hybrids were indistinguishable from the corresponding dose-response curves for parents, that *T. glabrata* lacked a recombinational pathway for repair of DNA damage.

Development of a parasexual system based upon euploid *T. glabrata* hybrids was the object of the present study. We report here that euploid hybrids may be obtained by fusion of spheroplasts, and we provide evidence that these hybrids give rise to variants which express parental markers.

MATERIALS AND METHODS

Strains. *T. glabrata* G4 was described previously (21). The haploid *Saccharomyces cerevisiae* reference strain XP660-14D was provided by T. R. Manney. *Candida albicans* reference strain 208R1 was a derivative of clinical isolate MS24, which was described previously (18).

Culture methods. The undefined complete medium YEPD agar and the defined minimal medium MIN agar were described previously (4). MIN agar was supplemented (with one or more of the compounds L-tryptophan, L-histidine, and L-methionine, to a final concentration of 40 mg/liter) as appropriate (see below). Cultures were grown at 30°C, except when otherwise indicated.

Induction of mutation. *T. glabrata* G4 was grown on YEPD agar (2 days). A sample of this confluent culture was suspended in water, spread on YEPD agar to yield approximately 600 CFU per petri dish, and exposed for 15 s to UV light delivered by a Sylvania G8T5 germicidal lamp. The dose rate at the agar surface, 32 ergs/mm^2 per s, was estimated with a Blak-Ray UV meter (Ultra-Violet Products, San Gabriel, Calif.). Irradiated cultures and unirradiated controls were incubated for 2 days, and colonies were replica plated on MIN agar to detect auxotrophs.

Spheroplast fusion. Cultures of parent strains were grown overnight at 37°C as patches on YEPD agar. Samples of the confluent cultures were spread on fresh YEPD agar and incubated (4 h at 37°C) to obtain actively growing cultures. Cells were scraped from the agar surface and suspended in PF-1 buffer (0.5 M $MgSO_4$, 0.1 M Tris chloride, pH 7.2) at ca. 2×10^8 cells per ml. Cells in a sample (10 ml) of suspension were washed once with PF-1 (10 ml) and suspended in 10 ml of PF-2 (PF-1 plus 70 μl of 2-mercaptoethanol per 100 ml). Pairwise combinations of parent strains were then made by mixing equal volumes (0.5 ml) of suspensions; a sample (0.5 ml) of each parent strain suspension was carried through the remainder of the procedure in parallel with the hybridization mixtures. After incubation for 2 h at 37°C, the suspensions were treated with glucanase (100 μl of Zymolyase 20 T [Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.] stock solution [10 mg/ml in PF-1 buffer]) for 1 h at 37°C and were then centrifuged. The pellet was dispersed in 2.5 ml of PF-3 buffer (0.5 M $CaCl_2$, 0.1 M Tris chloride, pH 7.2). This suspension was centrifuged, the supernatant was discarded, and the pellet was gently dispersed in 1 ml of added solution PF-4 (polyethylene glycol [Sigma Chemical Co., St. Louis, Mo.; molecular weight, 20,000] dissolved in PF-3 buffer to a concentration of 250 g/liter). The suspension was left at room temperature for 15 min, when 10 ml of solution PF-5 (0.5% agar in PF-3 buffer) at 50°C was added and the mixture was poured onto the surface of MIN agar. Hybridization cultures and parallel parental cultures were examined for colony growth during incubation for 8 days at room temperature.

Estimation of DNA content. Total cellular DNA content was estimated by means of the diphenylamine reaction. The procedure (method B) of Riggsby et al. (13) was followed. Cellular DNA content was calculated from the mean of two determinations of DNA (on duplicate samples of the centrifuged perchloric acid extracts [13]) divided by the mean of two or more estimates of cell number obtained by hemacytometer counts of more than 400 cells. This methodology has been applied to *C. albicans* (19).

Nuclear stains. Cells from overnight cultures on YEPD agar were suspended in water, fixed with 70% ethanol, and stained with 4′,6-diamidino-2-phenylindole (Sigma) by the procedure of Williamson and Fennell (23). Stained cells were examined with an fluorescence microscope (model 110; American Optical Corp., Buffalo, N.Y.) equipped with a vertical UV source (model 3071).

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TABLE 1. DNA content of *T. glabrata* strains and reference strains[a]

Culture	DNA (fg/cell)
T. glabrata	
Parent 167	16.6
Parent 184	18.6
Hybrid THA	36.5
Parent 174	18.1
Hybrid THB	35.6
T. glabrata G4	17.9
S. cerevisiae XP660-14D	19.2
C. albicans 208R1	35.6

[a] See Materials and Methods for details.

Genetic methods. Cultures of hybrids were grown, suspended in water, spread on YEPD agar, and irradiated as described above to induce mitotic recombination, except that irradiation was continued for 20 s. Colonies grown from survivors were replica plated on MIN agar, and auxotrophic variants were detected, purified, and characterized by the methodology established previously for *C. albicans* (18, 20). The criteria used for establishing heterozygosity by analysis of sectored colonies were described previously (18, 20). When two cultures were to be compared in terms of numbers of a specified kind of auxotroph produced per survivor under standard conditions, the data for the two cultures were pooled on the (null) hypothesis that they did not differ. The null hypothesis was tested by chi-square analysis, and the hypothesis was rejected for $P < 0.01$.

RESULTS

Hybridization. Parent strains for hybridization were auxotrophic mutants derived from *T. glabrata* G4 (see Materials and Methods). Among 1,220 colonies grown from irradiated cells (480 ergs/mm^2 with 40% survival), 22 were found to be auxotrophs. No auxotropic colonies were found among 608 colonies grown from unirradiated cells. Three auxotrophs, derived from isolate G4 (see above), were chosen as parents for hybridization.

Two crosses were performed (see Materials and Methods) by inducing spheroplast fusion. Parent strain 167 which required tryptophan was crossed with parent strain 184 which required histidine to yield hybrid THA, and parent strain 167 which required tryptophan was crossed with parent strain 174 which required methionine to yield hybrid THB. Hybrids were then selected on a defined minimal medium, on the supposition that hybrids would be prototrophic as a result of complementation.

When mixtures which contained ca. 10^8 cells of each parent were subjected to the spheroplast fusion procedure and the selection procedure, prototrophic colonies were obtained at a frequency of 150 to 220 per experiment. In contrast, when ca. 10^8 cells of each of the three parent strains were subjected to fusion and selection as pure (uniparental) suspensions, no prototrophic colonies were obtained. The foregoing results suggested that prototrophs obtained from paired parents after fusion and selection were likely to be hybrids, rather than revertants derived from the parents without fusion. Two putative hybrids, THA and THB, were purified and subjected to further study.

DNA content. It was supposed that a hybrid would contain a quantity of DNA (per cell) equal to the sum of the corresponding parental values. The observed DNA content of hybrid THA was within 5% of the value predicted by summing the DNA contents determined for the parent strains; a similar result was obtained for hybrid THB (Table 1). The hybrids and parent strains were shown to be mononucleate by microscopic examination of preparations fixed in 70% ethanol and treated with the fluorescent nuclear stain 4',6-diamidino-2-phenylindole (23). These results were taken to confirm hybridization and indicated that the hybrids THA and THB were complementing monokaryons, rather than heterokaryons.

It was considered useful to obtain repeated estimates of the DNA content of *T. glabrata* isolate G4 because our result differed markedly from the value (9.02 ± 0.53 fg per cell) previously reported by Galeotti et al. (5) for a typical (presumed haploid) *T. glabrata* strain. The values obtained by us were reproducible (mean, 17.9; range, 17.0 to 18.8 fg per cell) when estimates were made on four cultures grown at different times from the same stock culture of *T. glabrata* G4. In control determinations (Table 1), values close to the established literature values were obtained for haploid *S. cerevisiae* (literature value, 18.0) and for *C. albicans* (literature value, 36.8); these literature values were reported by Riggsby et al. (13), who described the method of DNA estimation used in the present study.

UV-induced segregation. Hybrid THA was subjected to UV irradiation in an attempt to induce mitotic recombination and resultant homozygosity of the marked genes (*TRP/trp* and *HIS/his*). On the basis of previous studies with *S. cerevisiae* and *C. albicans* (10, 12, 18, 20, 22), it was supposed that mitotic recombination in the interval between the marked gene and the centromere would result in formation of a mixed (sectored) colony derived from the homozygous products of segregation (e.g., *TRP/trp* → *trp/trp* + *TRP/TRP*).

Consistent with the proposed genotype (*TRP/trp HIS/his*), irradiation of hybrid THA resulted in the appearance of Trp auxotrophs and His auxotrophs as the predominant classes (Table 2). Auxotroph 426 (Trp) was found as a sector in a colony that contained a prototrophic sector (426P). Irradiation of a clone derived from sector 426P did not result in the appearance of Trp auxotrophs, and in this respect sector 426P differed significantly from hybrid THA ($P < 0.01$). That result was consistent with homozygosity (*TRP/TRP*) and therefore with the postulated origin of colony 426 (Trp:P) by mitotic recombination, rather than by mutation. It should be noted that our experiments do not exclude the possibility that colony 426 originated in a nondisjunction event which generated a monosomic auxotroph and a trisomic prototroph (+/−→−:+/+/−). This consideration, which applies to all sectored colonies discussed in the present paper, was dis-

TABLE 2. Analysis of hybrid THA[a]

Colony[b]	Auxotrophs isolated					
	Trp	His	(Trp His)	Other	n[c]	Survival frequency
THA	13	32	0	2	8,995	0.59
426 Trp:P	0	20	0	1	7,805	0.60
483 His:P	0	0	0	1	4,600	0.60

[a] The genotype inferred from these data was *TRP/trp HIS/his*.

[b] Hybrid THA yielded Trp auxotrophs and His auxotrophs after irradiation. 426 Trp:P was colony 426 that contained a tryptophan-requiring sector and a prototrophic sector. Irradiation of a clone from that prototrophic sector (426P) yielded no Trp auxotrophs but did yield His auxotrophs, among which was the colony 483 His:P. Irradiation of subclone 483P yielded neither Trp auxotrophs nor His auxotrophs.

[c] Number of colonies examined for auxotrophs.

cussed previously with reference to similar experiments with *C. albicans* (20).

Irradiation of strain 426P yielded His auxotrophs as expected. Auxotroph 483 (His) was found as a sector in a colony which also contained prototrophic sector 483P. Irradiation of a clone from sector 483P did not result in the appearance of His auxotrophs, and in that respect sector 483P differed significantly from its immediate parent (sector 426P) and from hybrid THA ($P < 0.01$). Taken together, the foregoing results are consistent with sequential homozygosis of the marked genes *TRP/trp* and *HIS/his*.

Irradiation of hybrid THB yielded Trp auxotrophs and Met auxotrophs as the predominant classes, consistent with the proposed genotype *TRP/trp MET/met* (Table 3). Two sectored colonies, each derived from hybrid THB, were studied. A clone isolated from the prototrophic sector of sectored colony 347 (Met:P) yielded no Met auxotrophs when irradiated and, in that respect, differed significantly from hybrid THB and prototrophic sector 492P (see below) taken together ($P < 0.01$). Irradiation of a clone isolated from the prototrophic sector of sectored colony 492 (Trp:P) yielded no Trp auxotrophs; this prototrophic sector (492P) differed significantly from hybrid THB and prototrophic sector 347P taken together ($P < 0.01$).

The data presented in Tables 2 and 3 are internally consistent. For example, the prototrophic sector from colony 426 (Trp:P) was expected to yield His auxotrophs at the same frequency as did hybrid THA; the observed frequencies did not differ significantly ($0.3 > P > 0.2$). As for other examples, the prototrophic sector from colony 347 (Met:P) was not expected to differ significantly from THB in the frequency of Trp auxotrophs produced, and the prototrophic sector from colony 492 (Trp:P) was not expected to differ from THB in terms of the frequency of Met auxotrophs produced. These predictions were satisfied ($P > 0.8$ and $0.2 > P > 0.1$, respectively). Finally, hybrids THA and THB share a common parent (mutant strain 167 Trp) and were expected to yield Trp auxotrophs with equal probabilities under standard conditions; similar frequencies were obtained ($P > 0.9$).

Other auxotrophs. In addition to the auxotrophs which expressed parental markers (*trp his met*), other auxotrophs were obtained from hybrids THA and THB (Tables 2 and 3). The overall frequency of these auxotrophs was low (3×10^{-4} per survivor), and their origin is uncertain. With one exception, the phenotypes of these auxotrophs were not determined. The exceptional auxotroph (strain 758, isolated from THA) required adenine for growth and accumulated red pigment under conditions of adenine limitation. Phenotypically similar auxotrophs are known in *S. cerevisiae*, *C. albicans*, and other species (8, 11, 14, 15). Phenotypically similar auxotrophs were isolated from typical (presumed haploid) *T. glabrata* previously (21).

TABLE 3. Analysis of Hybrid THB[a]

Colony[b]	Auxotrophs isolated					
	Trp	Met	(Met Trp)	Other	n[c]	Survival frequency
THB	6	5	1	0	3,693	0.58
347 Met:P	21	0	0	6	6,561	0.44
492 Trp:P	0	7	0	2	4,255	0.55

[a] The genotype inferred from these data was *TRP/trp MET/met*.
[b] Sectored colonies 347 Met:P and 492 Trp:P were derived from hybrid THB independently. Clones from prototrophic sectors 347P and 492P were subjected to irradiation and yielded the indicated auxotrophs.
[c] Number of colonies examined for auxotrophs.

TABLE 4. Segregation of pigmented adenine auxotrophs[a]

Colony[a]	Red auxotrophs		
	No. isolated	n[b]	Survival frequency
THA	1	8,995[c]	0.59[c]
758 (Ade)			
758R	14	2,354	0.64
875 (Ade:P)	0	2,470	0.61
886 (Ade:P)	0	2,822	0.54

[a] Hybrid THA gave rise to strain 758 (Ade) under standard conditions of irradiation. A spontaneous prototrophic derivative, 758R, was isolated from strain 758 (Ade). Sectored colonies 875 (Ade:P) and 886 (Ade:P) were obtained from strain 758R independently, and prototrophic clones 875P and 886P were subjected to irradiation.
[b] Number of colonies examined for auxotrophs.
[c] These data appear also in Table 2.

Segregation of pigmented adenine auxotrophs. It was supposed that adenine auxotroph 758 would give rise to prototrophic derivatives by mutations within the mutant *ade* gene (intragenic reversion) or by mutations in other genes (suppressor mutations). It was not possible to distinguish between these alternatives. However, it seemed likely that a prototroph generated by reversion or by suppressor mutation would be heterozygous (*ADE/ade* or *SUP/sup*) and would provide a readily observed genetic marker. A prototroph (758R) was obtained by selection on MIN medium and was shown to be heterozygous at a gene (*ADE/ade* or *SUP/sup*) which determined the red-pigmented adenine-requiring phenotype (Table 4). Prototrophic sectors 875P and 886P, derived independently from prototroph 758R, satisfied the criterion for *ADE/ADE* homozygosity.

DISCUSSION

In a previous study, we found that a wide variety of auxotrophic derivatives could be obtained readily from typical *T. glabrata* isolates including the isolate, G4, used in the present study (21). That result was consistent with the hypothesis that this asexual species was haploid (5, 21), although it was noted that certain aneuploid states ($n+1$) would not be detected by the methods employed (21).

In the present study, we found that hybrid *T. glabrata* strains may be generated from appropriate parents by application of a spheroplast fusion method used previously to hybridize *C. albicans* (11, 19). *T. glabrata* hybrids THA and THB were chosen for detailed study because their DNA content (per cell) was close to twice the average DNA content of the parents. We did not study in detail the possibility that spheroplast fusion may result in aneuploidy or polyploidy. Only two other hybrids were subjected to DNA determination (data not shown); one contained slightly less than twice the average parental amount of DNA, whereas the other contained nearly three times the average parental amount. Galeotti et al. generated hybrid *T. glabrata* by fusion of spheroplasts but found that each of the six hybrids they examined contained significantly less than the expected DNA value (twice the parental value) (5).

We supposed that our hybrids bore parental markers which were masked in the heterozygous state, and we found that derivatives which expressed parental markers could be found in sectored colonies after UV irradiation. UV is known to stimulate mutation, recombination, and aneuploi-

dization (2, 6, 10, 12). The occurrence of sectored colonies was consistent with either mitotic recombination or aneuploidization; further studies, using linked markers, are necessary to distinguish between these alternatives.

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