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Possible chromosomal location for the killer determinant in *Torulopsis glabrata*

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Summary. We have used cytoduction and protoplast fusion to characterise the killer trait in *Torulopsis glabrata*. Killer trait could not be transferred by cytoduction to a non-killer strain of *T. glabrata* suggesting that the determinant(s) is/are not present in the cytoplasm. Consistent with this interpretation is the observation that all fusion products, where chromosomes of both the parents are represented, are killers. Non-killer segregants could be isolated from fusion products after treatment with benlate suggesting that loss of the trait is associated with non-disjunction and loss of a chromosome.

Key words: Killer-*Torulopsis* yeast – Cytoduction

Introduction

"Killer" phenotype is widespread among yeasts whereby toxins are secreted that kill other yeasts. In *Saccharomyces cerevisiae* this trait is associated with virus-like particles containing double stranded RNA (dsRNA) (Bevan et al. 1973; Sweeney et al. 1976). On the other hand in *Kluyveromyces lactis* the killer trait is associated with linear DNA plasmid (Gunge et al. 1981; Wesolowski et al. 1982). Killer phenotype also exists in *Torulopsis glabrata* (Bussey and Skipper 1975), yet there has been no report of an association between this phenotype and extranuclear dsRNA or linear DNA. Conventional methods used to cure killer phenotype such as heat shock (Wickner 1974) and cycloheximide (Fink and Styles 1972) did not produce killer sensitive *T. glabrata* (Young and Yagiu 1978; and this study) suggesting that the killer determinant is different from the two characterised examples. In this report we describe results obtained from protoplast fusion and cytoduction between a killer and a sensitive strain that are consistent with chromosomal location for the killer trait in *T. glabrata*.

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Materials and methods

Strains. T. glabrata strains CBS138 and 71-91 were described earlier (Galeotti et al. 1981). Strains 72-33, 72-28, 75-1 were obtained from Dr. Phaff, and YB4025, Y2242, Y7337 were from the U.S. Department of Agriculture. *S. cerevisiae* strain 188S, a killer, was obtained from the NCYC and strain D6, a sensitive, from D. Wilkie. Auxotrophic mutants, antibiotic resistant mutants and cytoplasmic respiratory deficient mutants of *T. glabrata* were described earlier (Galeotti et al. 1981; Sriprakash and Batum 1981).

Media. MMK medium contained 0.67% Difco yeast nitrogen base without amino acids, 2% glucose, 0.6M KCl and 2% agar. GlyMM medium contained 0.67% Difco yeast nitrogen base without amino acids, 4% glycerol and 1.5% agar. Methylene blue agar plates were as described by Fink and Styles (1972). GYP, GlyYP and MM media were described earlier (Galeotti et al. 1981; Oakley and Clark-Walker 1978).

Restriction endonuclease. Digestions with AvaII were performed in Tris/Acetate buffer (O'Farrell et al. 1980) followed with EcoR1 in 50 mM Tris pH 7.5, 100 mM NaCl and 10 mM $MgCl_2$.

Killer assay and curing killer trait. Strains were tested for sensitivity by spreading them as a lawn of approximately 2×10^6 cells on methylene blue agar plates and patching onto this lawn, a killer strain. Strains to be tested for killing ability were patched onto a sensitive lawn. A clear zone appeared around a killer strain patched onto a sensitive lawn. Curing by cycloheximide was performed essentially according to Fink and Styles (1972).

Cytoduction. Cytoduction was performed between respiratory sufficient and cytoplasmic respiratory deficient mutants with complementing growth requirements. Cytoductants were distinguished on the basis of restoration of mitochondrial function to the respiratory deficient parent. The method used for cytoduc-

Table 1. Killer properties of *T. glabrata* strains

Tester \ Lawn	138	71-91	72-33	D6	188S
138		+	–	+	+
71-91	–		–	–	–
72-33	–	–		–	–

The experimental procedure for determining killer property is detailed in the methods. *T. glabrata* strains 72-28, 75-1, YB4025, Y2242, Y7337 were like 72-33 – i.e. – they neither killed nor were sensitive. The symbols + and – denote killing or not killing respectively

Table 2. Effect of cycloheximide on killer trait in *S. cerevisiae* and *T. glabrata*

Yeast	Total number of colonies	Number of non-killers	% Efficiency of curing
S. cerevisiae 188S	281	269	95.7
T. glabrata 138	276	0	0

The yeasts were grown in GYP medium. Diluted cultures were plated on GYP + 2 μg/ml cycloheximide. After 7 days, the cells were replicated on to methylene blue agar plates pre-spread with a lawn of D6 for *S. cerevisiae* and 188S for *T. glabrata*. The plates were scored for non-killer colonies after 72 h

tion was essentially as described previously for protoplast fusion (Sriprakash and Batum 1981), with the following modifications. After protoplast formation and incubation with polyethylene glycol and $CaCl_2$ the cells were plated on MMK medium, supplemented with the appropriate nutrients to allow growth of the respiratory deficient mutant. After growth on this medium for 48 h, allowing regeneration of the cell wall, the resulting lawn of cells was replica plated onto GlyMM and GlyMM supplemented with the requirements of the respiratory deficient parent. Colonies growing only on the supplemented GlyMM were respiratory sufficient, but still had the auxotrophic requirements of the respiratory deficient parent and were scored as cytoductants. Those colonies growing on both media were prototrophic fusion products.

Isolation of mitotic segregants from fusion products. Benlate treatment was carried out as described previously (Galeotti et al. 1981), except the concentration of benlate was increased to 600 μg/ml as *T. glabrata* is more resistant than *S. cerevisiae* to this compound. Induction of mutations was never observed even at this increased concentration. After 5 days incubation at 30 °C, colonies growing on benlate containing medium were replica plated onto GYP agar medium, MM agar medium and killer assay plates containing a lawn of sensitive cells. Colonies growing on GYP, but not on MM plates were tested as described earlier (Galeotti et al. 1981) to identify mitotic segregants. Colonies not killing the sensitive lawn were scored as non-killer segregants.

Isolation of total cellular DNA. Whole cell DNA was isolated essentially as described by Davis et al. (1980). 5 ml of cells grown to stationary phase in GYP medium were harvested, resuspended in 1 ml of 1 M Sorbitol, transferred to a 1.5 ml Eppendorf tube and resedimented. Cells were resuspended in 0.5 ml of protoplasting buffer (1 M Sorbitol, 50 mM EDTA pH 8.5, 14 mM β-Mercaptoethanol and 40 μg/ml zymolyase) and incubated at 30 °C for 1 h. Protoplasts were sedimented and lysed by resuspension in 0.5 ml 0.2% SDS in 50 mM EDTA pH 8.5. 1 μl of diethylpyrocarbonate was added at room temperature, and the suspension was incubated at 70 °C for 15 min. SDS was precipitated by the addition of 50 μl of 5 M potassium acetate and cooling the suspension on ice for at least 30 min. The precipitate was sedimented by centrifugation for 15 min. The clear supernatant was decanted into a new Eppendorf tube and the DNA precipitated by filling the tube with ethanol at room temperature. After centrifugation for 15 s the precipated DNA was dried and dissolved in 50–100 μl of 10 mM Tris/HCl pH 7.5, 1 mM EDTA pH 7.5 and 10 μg/ml RNase A.

Other methods. Agarose gel electrophoresis was carried out as described previously (Clark-Walker et al. 1980). Bidirectional transfer of DNA from the gel to nitrocellulose filters was achieved as described by Smith and Summers (1980). Labelling of DNA, hybridization and autoradiography were described earlier (Clark-Walker et al. 1980; Sriprakash and Batum 1981).

Results

Killer phenotype in T. glabrata strains

We examined several strains of *T. glabrata* for their ability to kill other yeasts. Only the strain 138 killed the two *S. cerevisiae* strains and *T. glabrata* 71-91 as judged by a zone of clearance in the killer assay (Table 1). On this basis, we classify 138 as killer and 71-91 as sensitive strains. Since the remaining *T. glabrata* strains were insensitive to 138, and were incapable of killing we regard them as neutral. Although 188S is by itself a killer, it is sensitive to 138 killing, thus confirming an earlier observation that *T. glabrata* killer immunity system is different to that of *S. cerevisiae* (Bussey and Skipper 1975). Furthermore we observed that 188S is more sensitive to 138 than is 71-91 or D6. Therefore we used a 188S lawn in further experiments as this allowed scoring of the plates within 72h.

Cycloheximide at 2 μg/ml cured the killer trait in 188S at 95.7% efficiency whereas no curing was observed in *T. glabrata* under similar conditions (Table 2). The concentration of cycloheximide chosen was sublethal to both the yeasts.

We also examined the cell extracts of *T. glabrata* 138 for double stranded RNA and linear DNA plasmids using the procedures described by Fried and Fink (1978) and Gunge and Sakaguchi (1981). We have not detected such molecules in this yeast (data not shown). Earlier

Table 3. Phenotype of cytoductants and their parents

Strain	Phenotype		
	Nuclear	Mitochondrial	Killer
Parents			
138-11-1	ade$^-$ pro$^-$	D^S A^S O^S	+
138-11-1 e$\rho^0$3	ade$^-$ pro$^-$	Respiratory deficient	+
7191-DAO-1	arg$^-$ his$^-$	D^R A^R O^R	–
7191-DAO-1 e$\rho^0$1	arg$^-$ his$^-$	Respiratory deficient	–
Cytoductants			
1. 138-11-1 x 7191-DAO-1 e$\rho^0$1	arg$^-$ his$^-$	D^S A^S O^S	–
2. 7191-DAO-1 x 138-11-1 e$\rho^0$3	ade$^-$ pro$^-$	D^R A^R O^R	+

The details of cytoduction are described in "methods". The parental strains were described earlier. The suffix eρ^0 is added to the respiratory deficient parent to denote complete loss of mitochondrial DNA as judged by hybridization to the labelled DNA probe. D^R, A^R and O^R represent resistance to Diuron, Antimycin and Oligomycin, D^S, A^S and O^S correspond to sensitivity to these three drugs. The symbols + and – denote killer and non-killer phenotype respectively

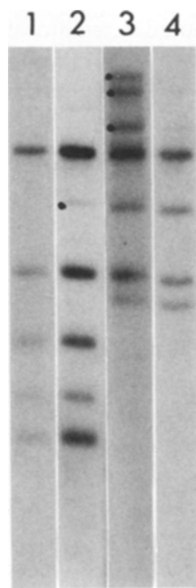

Fig. 1. Restriction endonuclease patterns of mtDNAs from cytoductants and their parents. The whole cell DNA was digested with EcoRI + AvaII and run on a 1% agarose gel. DNA was then blotted onto nitrocellulose. The mtDNA fragments were visualised after hybridization to labelled mtDNA probe and autoradiography. Channels 1 and 4 are mtDNAs from parental strains 138 and 71-91 respectively. Channels 2 and 3 are mtDNAs from two cytoductants one each from experiment 1 and experiment 2 in Table 3 respectively. The bands marked (.) are due to partial digestions

work in this laboratory revealed closed circular DNA of only two size classes in *T. glabrata* – one a 6μ size class identified as mitochondrial DNA (O'Connor et al. 1976) and the other a 3 μ size class identified as DNA containing ribosomal RNA genes (Clark-Walker and Azad 1980). Together, these experiments suggest that the killer trait in *T. glabrata* is not associated with extranuclear elements as in *S. cerevisiae* and *K. lactis*.

Cytoduction in T. glabrata

Cytoduction is a useful method by which cytoplasmic inheritance of a trait can be established. In cytoductants the cytoplasm of both the parents mix but not the nuclei, thus the buds issued from such fusants are heteroplasmic. Cytoduction in *T. glabrata* was achieved by transfer of mtDNA from a respiratory sufficient strain to a cytoplasmic respiratory deficient mutant without altering the growth requirements of the latter. Cytoductants will have mtDNA from one parent and nuclear background of the other.

In Table 3 we present data on reciprocal cytoduction between mutant strains of 138 and 71-91. The cytoductants were characterised for: a) mitochondrial antibiotic resistance if the respiratory competent parent was appropriately marked and b) differences in restriction pattern of their mtDNA. Polymorphism of mtDNAs in *T. glabrata* strains (71-91 and 138) was described earlier (Clark-Walker et al. 1981 and Sriprakash and Batum 1981). As evident from Table 3, the cytoductants issued from experiment 1 are sensitive to diuron, antimycin and oligomycin, like the 138-11-1 parent. On the other hand all cytoductants from experiment 2 are resistant to these three antibiotics like the 7191-DAO-1 parent. Furthermore the restriction patterns of mtDNAs from the cytoductants are similar to those of the corresponding respiratory competent parent in each experiment (Fig. 1). From both these criteria we conclude

Table 4. Phenotype of fusion products and their parents

Strain	Phenotype		
	Nuclear	Mitochondrial	Killer
Parents			
7191-C-5	$arg^- his^-$	O^S	–
7191-C-5 $e\rho^0 6$	$arg^- his^-$	Respiratory deficient	–
138-0-1	$ade^- pro^-$	O^R	+
138-0-1 $e\rho^0 1$	$ade^- pro^-$	Respiratory deficient	+
Fusion products			
1. 7191-C-5 x 138-0-1	Prototrophic	O^R/O^{S}[a]	+
2. 7191-C-5 x 138-0-1 $e\rho^0 1$	Prototrophic	O^S	+
3. 7191-C-5 $e\rho^0 6$ x 138-0-1	Prototrophic	O^R	+

The protoplast fusion was carried out as described earlier (Galeotti et al. 1981). Explanation of the symbols are the same as in the legend to Table 3

[a] In experiment 1 both O^R and O^S phenotypes were recovered among fusion products

Table 5. Mitotic segregation of *T. glabrata* fusion product F-7

	Number of colonies	
	Killers	Non-killers
Auxotrophic segregants		
ade^-	8	0
his^-	0	1
$arg^- his^-$	1	0
$arg^- ade^-$	1	2
$ade^- his^-$	1	1
$arg^- his^- ade^-$	2	2
$ade^- pro^- his^-$	0	1
Prototrophic colonies	227	1

Fusion product F-7 was obtained by fusion of 138-0-1 with 7191-C-5 $e\rho^0 6$ (experiment 3 in Table 4). It has the genotype $ade^- pro^- arg^+ his^+/ade^+ pro^+ arg^- his^-$. Benlate treatment is explained in the methods. A total of 248 colonies were analysed of which 20 were auxotrophic segregants

that a) polyethylene glycol mediated cytoductants are indeed heteroplasmons, b) the mtDNA of either strain is compatible with heterologous nuclear background and c) the mitochondrial mutant phenotype is unaltered in heteroplasmic state.

Killer phenotype is not cytoplasmically inherited in T. glabrata

Having established cytoduction in *T. glabrata* as described above we obtained further evidence that the killer trait is not cytoplasmically inherited in this organism by using this technique. Killer activity was measured in reciprocal cytoductants obtained by fusing mutants of 138 and 71-91 (Table 3). Unlike the mitochondrial function the killer trait could not be transferred by cytoduction, thus clearly suggesting that the trait is not associated with an extrachromosomal element.

Killer trait segregates like a nuclear gene in fusion products

Earlier we successfully used polyethylene glycol induced protoplast fusion to study segregation of mitochondrial mutations (Sriprakash and Batum 1981). In these fusants, chromosomes of both the parents are represented at least in part (Galeotti et al. 1981). Protoplast fusion between complementing auxotrophic mutants of killer (derived from 138) and sensitive (derived from 71-91) strains resulted in prototrophic cells which are killers (Table 4). These results establish dominance of the trait. If the killer trait is nuclearly encoded we would expect the trait to segregate like other nuclear genes from fusion products when treated with haploidising agents. Benlate which induces chromosomal nondisjunction in fungi (Upshall et al. 1977) is one such agent we used successfully for this purpose. This agent did not cure killer phenotype from 138. From data presented in Table 5 it is evident that the killer trait segregated independently of the other nuclear markers; a third (7/20) of the auxotrophic segregants were non-killers. A non-killer segregant was also recovered among prototrophs. Although linkage between killer trait and other markers could not be deduced, these experiments strongly favour the notion that the killer phenomenon in *T. glabrata* is a nuclearly encoded trait.

Discussion

Our attempts to find dsRNA or linear DNA in a killer strain of *T. glabrata* were unsuccessful. Curing of killer trait by heat (Wickner 1974) or cycloheximide (this study) was also not possible. It was therefore thought that the trait in *T. glabrata* was not extrachromosomal. In support of this, we were unable to transfer killer trait in this organism from killer to non-killer strain by cytoduction. Since the killer trait is dominant (see results) inability to recover killer phenotype among heteroplasmic cytoductants is not because of the dominance/recessive characteristic of the trait. Furthermore the demonstration of segregation of the killer trait in fusion products in response to benlate suggests a nuclear location for this trait in *T. glabrata*.

Nuclear genes involved in maintenance (MAK) and expression (KEX) of the killer plasmid in *S. cerevisiae* were described (Wickner 1976). Likewise mutants of *K. lactis* defective in the expression of the killer plasmid were isolated (Wesolowski et al. 1982). Interestingly the linear killer plasmid DNA from *K. lactis* could be transferred to *S. cerevisiae* by intergeneric fusion (Gunge and Sakaguchi 1981). The plasmid DNA is maintained and expressed in such fusion products. These intergeneric fusion products are phenotypically *S. cerevisiae* like. On the contrary, Galeotti and Clark-Walker (1983) described fusion between *K. lactis* and *S. cerevisiae* and the resulting fusion products were predominantly *K. lactis* like. The differences could be due to different selection procedures applied by the two groups of investigators. Whereas Gunge and Sakaguchi were essentially performing cytoduction, Galeotti and Clark-Walker were selecting for fusion products by complementation of nuclear markers. The maintenance and expression of *K. lactis* killer plasmid in *S. cerevisiae* suggests that all the necessary nuclear genes for normal function of this plasmid are present in *S. cerevisiae*.

We are currently investigating the possibility of cloning the killer gene(s) of *T. glabrata*. These studies will elucidate the control and expression of the killer trait in this yeast.

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