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KILLER TOXIN OF *SACCHAROMYCES CEREVISIAE* Y500-4L ACTIVE AGAINST FLEISCHMANN AND ITAIQUARA COMMERCIAL BRANDS OF YEAST

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ABSTRACT

The strain *Saccharomyces cerevisiae* Y500-4L, previously selected from the must of alcohol producing plants and showing high fermentative and killer capacities, was characterized according to the interactions between the yeasts and examined for curing and detection of dsRNA plasmids, which code for the killer character. The killer yeast *S. cerevisiae* Y500-4L showed considerable killer activity against the Fleischmann and Itaiquara commercial brands of yeast and also against the standard killer yeasts K2 (*S. diastaticus* NCYC 713), K4 (*Candida glabrata* NCYC 388) and K11 (*Torulopsis glabrata* ATCC 15126). However *S. cerevisiae* Y500-4L showed sensitivity to the killer toxin produced by the standard killer yeasts K8 (*Hansenula anomala* NCYC 435), K9 (*Hansenula mrakii* NCYC 500), K10 (*Kluyveromyces drosophilarum* NCYC 575) and K11 (*Torulopsis glabrata* ATCC 15126). No M-dsRNA plasmid was detected in the *S. cerevisiae* Y500-4L strain and these results suggest that the genetic basis for toxin production is encoded by chromosomal DNA. The strain *S. cerevisiae* Y500-4L was more resistant to the loss of the phenotype killer with cycloheximide and incubation at elevated temperatures (40°C) than the standard killer yeast *S. cerevisiae* K1.

Key words: Killer yeast, *Saccharomyces cerevisiae*, killer toxin

INTRODUCTION

The killer system in yeasts has been extensively investigated since it was first described in *Saccharomyces cerevisiae* by Bevan and Makower (1). Killer strains secrete a protein toxin which is lethal to sensitive strains of the same genus and, less frequently, strains of different genera (10). Among the yeasts, killer, sensitive, and neutral strains have been described. Eleven distinct patterns of the range of killer activity against killer yeast have been found (K1-K11) according to the interaction between the killer yeasts (12, 15).

Genetic studies have shown that the killer phenotype of *S. cerevisiae* is inherited cytoplasmically and has been linked to the presence of a double stranded RNA (dsRNA) associated with virus-like particles within the cytoplasm of the killer cells. However, dsRNA need not always be the determinant. In other genera, like *Kluyveromyces lactis*, the information for the killer phenotype is carried by linear dsDNA (6, 11). The killer character

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of *Candida* sp. (13, 14) and *Hansenula anomala* (7) is encoded by chromosomal genes, not by extrachromosomal ones.

In *Saccharomyces cerevisiae*, two distinct dsRNA species exist: L-dsRNA (4.6-4.8 Kb) and M-dsRNA (1.0-1.8 Kb) (12). It has been established that L-dsRNA encodes the major virus-like particle capsid protein, and M-dsRNA encodes the killer toxin synthesis (2, 3). L-dsRNA encodes a protein for encapsidation of M-dsRNA and plays an essential role in maintenance or expression of the killer phenotype (3). Kitano *et al*. (8) furthermore observed two new killer types belonging to *Saccharomyces*. Using tetrad analysis, their killer genes were found to be encoded on chromosomal DNA. One gene, designated KHR, was on chromosome IX and another, designated as KHS, was on chromosome V (4).

The purpose of this investigation was to characterize the previously isolated killer strain *S. cerevisiae* Y500-4L, which shows high fermentative capacity.

MATERIALS AND METHODS

Yeast strains. *Saccharomyces cerevisiae* Y500-4L and *Hansenula* sp. Y66-1 killer yeasts, previously isolated from the must of alcohol producing plants (9). Two commercial brands of yeast (Fleischmann e Itaiquara) were used as sensitive strains. The standard killer yeasts are listed in Table 1.

Table 1- Standard killer yeast strains used

Strains	Killer type
Saccharomyces cerevisiae KL88	K1
Saccharomyces diastaticus NCYC 713	K2
Saccharomyces capensis NCYC 761	K3
Candida glabrata NCYC 388	K4
Debaryomyces vanrij NCYC 577	K5
Kluyveromyces marxianus NCYC 587	K6
Pichia membranaefaciens NCYC 333	K7
Hansenula anomala NCYC 435	K8
Hansenula mrakii NCYC 500	K9
Kluyveromyces drosophilarum NCYC 575	K10
Torulopsis glabrata ATCC 15126	K11

Media. YEPD medium (1.0% yeast extract, 2.0% peptone, 2.0% glucose) and YM medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1.0% glucose) were used for yeast culturing. YEPD-MB agar (YEPD containing 0.1M citrate-phosphate buffer pH 4.5, 0.01% methylene blue and 2.0% agar) was used for the determination of killer phenotype.

Interaction between killer yeasts. Killing ability and resistance were determined by inoculating killer strains onto YEPD-MB agar and after incubation for 2 days at 25°C the sensitive yeast suspension, previously incubated on YEPD medium for 2 days at 25°C, was sprayed onto YEPD-MB agar. The plates were incubated for 2 more days at 25°C and the strains with killer activity appeared surrounded by a clear zone.

Extraction and electrophoresis of double-stranded RNA plasmids. Extraction was performed by a modification of the method of Goto *et al*. (5). Yeast cells grown in YEPD medium were harvested and washed with 50 mM EDTA (pH 8.0). After centrifugation at 5000 rpm for 10 minutes, the cells were incubated for 1 hour at 60°C in 600µl of a solution containing 25 mM EDTA, 200 mM Tris-HCl (pH 8.0), 25 mM NaCl and 1.0% sodium dodecyl sulfate. The cell suspension was then centrifuged and the aqueous phase treated twice with an equal volume of phenol, phenol-chloroform (1:1) and chloroform for extraction of the plasmids. The upper phase was transferred and precipitated with isopropanol (1:1). The precipitate was dissolved in buffer and analyzed by 1% agarose gel electrophoresis (75V, 0.7A for 2 hours).

Curing test. Killer yeast cells grown in YM medium were suspended in sterilized water (10^6 cells/ml) and 10 µl streaked onto YM agar with or without 0.2 ppm cycloheximide. The plates were incubated at temperatures of 25°C (control), 37°C, 38.5°C or 40°C for 3 days. Colonies of the yeast which grew in each treatment were inoculated onto YM agar, and after incubation at 25°C for 36 hours, were replica-plated on YEPD-MB agar plates and then the sensitive yeast sprayed. After 2 days, the cured strains, which had lost their killer activity, were detected.

RESULTS AND DISCUSSION

Interaction between yeasts. Killing ability and resistance of *S. cerevisiae* Y500-4L, previously selected (9) as being a strain showing high fermentative capacity, were determined by interaction between yeasts. The results are shown in Table 2. The

killer yeast *S. cerevisiae* Y500-4L showed considerable killer activity against the Fleischmann and Itaiquara commercial brands of yeast, suggesting that this strain could be a potential competitor in alcoholic fermentations using these commercial brands as starter cultures. It also showed killer activity against the standard killer yeasts K2, K4 and K11. This killer character was similar to that of the K1 type killer. However, the strain Y500-4L showed sensitivity to the killer toxin produced by the standard killer yeasts K8, K9, K10 and K11 and was immune to the action of the K2, K3 and K5 killer toxins.

Extraction and electrophoresis of double-stranded RNA plasmids. Double-stranded RNA plasmids of killer strains were analyzed by agarose gel electrophoresis (Fig. 1). Standard killer type strains K1, K2 and K3 contain the two species of plasmid previously reported (15). The larger species (L-dsRNA) had a molecular weight of 5.0 Kb; the killer type strain K1 showed the presence of M1-dsRNA with 1.8 Kb and the strains K2 and K3 showed M2 and M3-dsRNA with 1.4 Kb. The molecular weights of each species of dsRNA were similar to those described by Wickner (12).

The killer strain *S. cerevisiae* Y500-4L showed the presence of L-dsRNA with 5.0 Kb but no M-dsRNA plasmid was detected. This result was the same as that obtained for another strain of *S. cerevisiae*, Y-9, described by Kitano (8), suggesting that the genetic basis for toxin is encoded by chromosomal DNA in these strains.

Fig. 1 also shows the result of the electrophoretic analysis of plasmid samples from *Hansenula* sp. Y66-1. No plasmids were detected. According to Kagiyama (7), the killer character of this genus is encoded by chromosomal genes.

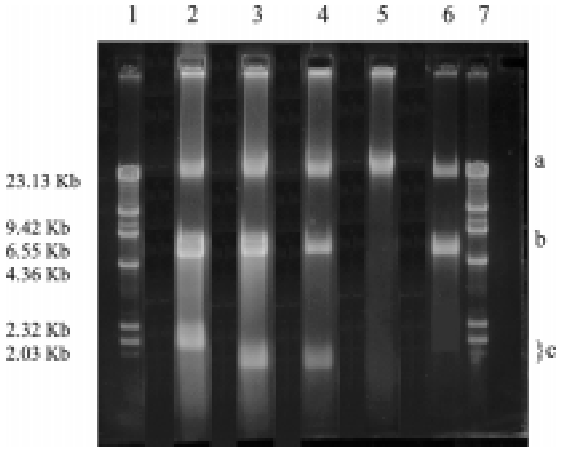

Figure 1-Agarose gel electrophoresis of dsRNA from killer yeasts. **a-** DNA; **b**- L-dsRNA; **c**- M-dsRNA;
1 and **7**- λ HindIII (molecular weight marker); **2**- standard killer yeast type K1; **3**- standard killer yeast type K2; **4**- standard killer yeast type K3; **5**- strain Y66-1; **6**- strain Y500-4L.

Table 2- Killing reaction of killer yeast against various yeasts

Seeded strains / Killer strains	K1	K2	K4	K6	K7	K8	K9	K10	K11	Y66-1	Y500-4L	Fleisch.	Itaiquara
K1	-	+	+	-	-	-	-	-	+	-	-	+	+
K2	+	-	-	-	-	-	-	-	-	-	-	-	+
K3	+	-	+	-	-	-	-	-	-	-	-	-	++
K4	-	+	-	-	-	-	-	-	-	+	-	ND	ND
K5	+	-	-	-	-	-	-	-	+	-	-	ND	ND
K6	-	-	-	-	-	-	-	-	-	-	-	ND	ND
K7	-	-	+	-	-	-	-	-	+	+	-	ND	ND
K8	+	+	++	-	-	-	-	-	++	+	++	ND	ND
K9	++	++	++	-	-	-	-	-	++	+	++	ND	ND
K10	+	+	++	-	-	-	-	-	+	+	+	ND	ND
K11	+	++	-	-	-	-	-	-	-	++	+	++	++
Y66-1	-	-	++	-	-	-	-	-	++	-	-	-	-
Y500-4L	-	++	+	-	-	-	-	-	+	-	-	+++	++++

- = No killing activity
+ = Killing activity. Each + = 3 mm f (clear zone)
ND indicates that the test was not carried out.

Curing test. The results are shown in Table 3. The curing of the phenotype means the loss of killer toxin production by cycloheximide treatment or temperature.

Table 3- Curing of killer yeasts

	Methods of curing			
	Cycloheximide	Temp. 37°C	Temp. 38.5°C	Temp. 40°C
Strains				
K1	-	75 %	95 %	-
Y500-4L	55 %	0 %	0 %	40 %
Y66-1	0 %	-	-	-

The standard killer yeast K1 *S. cerevisiae* was cured easily by incubation at elevated temperature. At 37°C and 38.5°C, 75% and 95% respectively of curing were obtained. It did not grow in 0.2 ppm cycloheximide or at 40°C.

The strain *S. cerevisiae* Y500-4L was shown to be more resistant to curing than killer standard K1. Only 40% of curing was obtained when incubated at 40°C or 55% when grown in 0.2 ppm cycloheximide. These results indicated that this strain is more resistant to the loss of killer capacity.

The strain *Hansenula* sp. Y66-1 was not cured by cycloheximide treatment and did not grow at elevated temperature (37-40°C). These results were expected because the killer character in this genus is encoded by chromosomal genes.

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CONCLUSIONS

The killer strain *S. cerevisiae* Y500-4L, which have high fermentative capacity, was characterized. This yeast showed considerable killer activity against the Fleischmann and Itaiquara commercial brands of yeast and also against other genera like *Candida*, conferring a selective advantage over sensitive strains competing to grow in the same environment.

No M-dsRNA plasmid was detected in the strain Y500-4L, suggesting that the genetic basis for toxin production is encoded by chromosomal DNA. This result was in accordance with the greater resistance to treatments for the loss of the capacity to produce killer toxin. These results showed a potential use of this competitive yeast in alcoholic fermentation.

RESUMO

Toxina "killer" de *Saccharomyces cerevisiae* Y500-4L ativa contra leveduras comerciais Fleischmann e Itaiquara

A linhagem de *Saccharomyces cerevisiae* Y500-4L com alta capacidade fermentativa e atividade "killer", previamente selecionada de mosto de fermentação de usina de álcool, foi caracterizada quanto ao espectro de atividade e quanto à perda do caracter "killer". A linhagem "killer" de *S. cerevisiae* Y500-4L, mostrou alta atividade "killer" contra as leveduras comerciais Fleischmann e Itaiquara, e também contra as linhagens "killer" padrões K2 (*S. diastaticus* NCYC 713), K4 (*Candida glabrata* NCYC 388) e K11 (*Torulopsis glabrata* ATCC 15126) e mostrou ser sensível às toxinas produzidas pelas leveduras padrões "killer" K8 (*Hansenula anomala* NCYC 435), K9 (*Hansenula mrakii* NCYC 500), K10 (*Kluyveromyces drosophilarum* NCYC 575) e K11 (*Torulopsis glabrata* ATCC 15126). A linhagem de *S. cerevisiae* Y500-4L não apresentou plasmídio M-dsRNA e, provavelmente, o caracter genético responsável pelo fenótipo "killer" é codificado por genes cromossomais. Em ensaios para a perda do fenótipo, a linhagem *S. cerevisiae* Y500-4L apresentou maior resistência ao tratamento com cicloheximida e a temperatura elevada (40°C) do que a levedura *S. cerevisiae* padrão "killer" K1.

Palavras-chave: Levedura "killer", *Saccharomyces cerevisiae*, toxina "killer"

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