

FEMSLE 04045

Transmission of killer activity into laboratory and industrial strains of *Saccharomyces cerevisiae* by electroinjection

A. Salek *, R. Schnettler and U. Zimmermann

Institute of Biotechnology, University Würzburg, Röntgenring 11, 8700 Würzburg, F.R.G.

Received 23 February 1990

Accepted 12 March 1990

Key words: *Saccharomyces cerevisiae*; Killer activity; Double stranded RNA; Electroinjection

1. SUMMARY

The killer character was electrically introduced into protoplasts of three yeast strains. These were the killer-negative variant of the K1 killer strain *Saccharomyces cerevisiae* T 158 C (his^-); the killer-sensitive laboratory strain *S. cerevisiae* AH 215 (leu^- , his^-); and the killer-sensitive industrial strain *S. cerevisiae* AS 4/H2 (ρ^-). The killer dsRNA used for electroinjection was isolated from the super-killer strain *S. cerevisiae* T 158 C. Optimum numbers of transformed cells were obtained after regeneration and selection in appropriate media if the protoplasts were exposed to three exponentially decaying field pulses of 18.2 kV/cm strength and 40 μ s duration at 4°C. In the case of the killer-negative variant of *S. cerevisiae* T 158 C the majority of the protoplasts were transformed, whereas in the case of the two other strains the yield of transformed clones was much less. This latter result is expected if the expression of the

electroinjected dsRNA was diminished in these two strains.

Gel electrophoresis of the dsRNA of the clones of the three strains supported the conclusion that the transformed clones exhibited killer activity. The transformed clones of all three species were stable.

2. INTRODUCTION

Killer strains of yeasts secrete a polypeptide toxin (zymocyn) to which they are immune but which is lethal to sensitive strains of their own species and frequently to those of other species and genera of yeast [1,2]. Examination of the specificities of killer toxin and immunity has allowed the distinction of eleven spectra of killing activity (K_1 to K_{11}) including three, K_1 to K_3 , in *Saccharomyces* and eleven spectra of resistance (R_1 to R_{11}) [1,2]. Three different phenotypes can be classified: K^+R^+ (killer yeasts which are immune to the action of their own released toxin), K^-R^- (yeasts with no killer activity and sensitive against toxins) and K^-R^+ (neutral strains which do not produce toxin, but which are immune against the toxins of killer yeasts) [3]. In most killer strains of the genus *Saccharomyces*, the killer toxin is produced by the double stranded RNA

* On sabbatical leave from the Institute of Biotechnology, Agriculture and Food Industry, Rakowiecka 36, Warszawa, Poland.

Correspondence to: U. Zimmermann, Institute of Biotechnology, University of Würzburg, Röntgenring 11, 8700 Würzburg, F.R.G.

genome (M dsRNA) of a mycovirus. These RNA plasmids are found only in killer strains, and these contain a second genome of the mycovirus (L dsRNA). Both types of dsRNA exist in cytoplasmic virus-like particles. The L dsRNA encodes for the production of the capsid protein for both virus particles. Detailed analysis of L dsRNA isolated from *Saccharomyces* strains showed that three distinct, linear species are found (L_A, L_B, L_C) which are all similar in size (about 4.7 kb). The presence of both L and M dsRNA is necessary for the expression of killer character, whereby L_A dsRNA is necessary for the maintenance of M dsRNA. None of the brewing strains exhibits killer character. Therefore, there is a considerable potential for exploiting killer systems, e.g. in fermentation processes, and a demand for protecting yeasts of industrial interest against contamination with killer strains. The most elegant way is the transfer of the killer character into commercial yeasts by plasmid injection because this has decisive advantages over transmission of plasmids by yeast protoplast fusion [4].

Attempts to inject dsRNA by chemical means have proved unsuccessful. Therefore, incorporation of M dsRNA isolated from a killer strain was accomplished using the electroinjection technique introduced by Zimmermann et al. [5,6] and modified by Stopper et al. [7,8].

3. MATERIALS AND METHODS

3.1. Yeast protoplasts

Yeast protoplasts were prepared from the *Saccharomyces cerevisiae* laboratory mutant strains AH 215 (leu⁻, his⁻) and from an industrial haploid *S. cerevisiae* strain AS-4/H2 (rho⁻) as well as from a killer-negative variant of the super-killer *S. cerevisiae* mutant strain T 158 C (his⁻) using standard protocols [9,10]. These strains did not contain M₁ dsRNA (Fig. 1) and therefore exhibited no killer activity. The protoplasts were suspended at a density of 10⁹ cells/ml in a solution containing 1.2 M sorbitol, 30 mM KCl, 1 mM CaCl₂, 0.3 mM KH₂PO₄, 0.85 mM K₂HPO₄ and 10 µg/ml isolated M₁ and L₁ dsRNA.

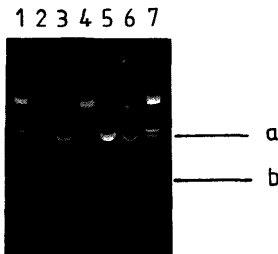


Fig. 1. Agarose-gel electrophoresis of nucleic acid extracts from yeasts. Tracks 1 and 7, standard DNA (21.7-, 5.15-, 5.0-, 4.27-, 3.48-, 1.98-, 1.9-, 1.59-, 1.37-, 0.94- and 0.83 kb); track 2, super-sensitive strain S 6-1; track 3, sensitive (laboratory) strain AH 215; track 4, sensitive (industrial) strain AS 4/H2; track 5, killer-negative variant of the K₁ killer strain T 158 C; and track 6, K₁ super-killer strain T 158 C. a, L_{1A} dsRNA; b, M₁ dsRNA. Conditions: 1% agarose, Tris-acetic acid EDTA-buffer (TAE-buffer: 40 mM Tris, 2 mM EDTA pH 8.3 adjusted by acetic acid), 80–100 V, 50–60 mA, ethidiumbromide staining.

3.2. dsRNA isolation

The dsRNA for K₁ toxin was isolated from the killer strain of *S. cerevisiae* T 158 C (his⁻) according to the procedure described by Fried and Fink [11] with the following modifications. In order to remove proteins quantitatively from the nucleic acids the crude cell extract was incubated and gently shaken for 15 min in 50 mM Tris-H₂SO₄ (pH 9.3) containing 2.5% 2-mercaptoethanol and for 1 h in solution I containing 0.1 M NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM Na₂EDTA, 0.2% sodium dodecylsulphate and an equal volume of bi-distilled phenol. After 20 min centrifugation at 5000 × g, nucleic acids were recovered from the aqueous phase by precipitation with about 2.5 volumes of 95% ethanol and stored at 4°C for 24 h. After 30 min centrifugation at 16000 × g the pellet was dissolved in solution I. After removal of the phenol phase, the aqueous phase was mixed with an equal volume of chloroform (purified by pre-treatment with TE buffer) and shaken for 10 min. 0.1 volume of 2.5 M sodium acetate (pH 6) was added to the aqueous phase and the nucleic acids precipitated by adding 2.5 volumes of 95%

ethanol. After 24 h storage at 4°C and 30 min centrifugation at 16 000 × g, the pellet was dissolved in 10 ml of a solution containing 2 M LiCl, 0.15 M NaCl and 15 mM sodium citrate. After at least 8 h incubation at 4°C, the LiCl precipitate was removed by 30 min centrifugation at 16 000 × g. The remaining nucleic acids in the supernatant were subsequently precipitated by 2.5 volumes of 95% ethanol. After drying in vacuum the pellet was dissolved in 200 to 400 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 7.8). For transformation slices of the agarose gel containing L_{1A} – and M_1 – dsRNA (Fig. 1 track 6, bands a and b) were electrolysed in TE buffer (pH 7.8) for 2 h (100 V) and then again subjected before use to the combined phenol-chloroform extraction procedure and ethanol precipitation followed by centrifugation at 175 000 × g for 90 min. Then the precipitate was dried in vacuum and dissolved in water.

From the agarose-gel electrophoresis pattern in Fig. 1 it is obvious that only the killer strain T 158 C (which produced the toxin K_1) contained M_1 and L_{1A} dsRNA. The size of the M_1 and L_{1A} dsRNA was about 1.90 kb and 4.7 kb, respectively.

3.3. Electroinjection

For electroinjection the protoplasts were exposed to three exponentially decaying field pulses of 18.2 kV/cm strength and 40 μs duration at 4°C or 20°C using an electroinjection power supply (Biojet MI manufactured by Biomed, Theres and distributed by Braun, Melsungen, F.R.G.). The pulses were applied with a time interval of 2 min. These conditions resulted in a gentle and reversible electroporation of the entire protoplast surface as demonstrated with mammalian cells [8]. After pulsing, the yeast protoplasts were kept for 10 to 20 min in the pulse medium before they were mixed with regeneration agar and poured into petri dishes.

3.4. Regeneration and selection

The citrate-phosphate buffered regeneration agar medium contained 0.67% yeast nitrogen base without amino acids (Difco), 1.2 M sorbitol, 0.19 mM histidine, 0.23 mM leucine, 2% glucose and 2% agar as well as 1 mM of K^+ , Mg^{2+} , Ca^{2+} ,

Fe^{3+} , Mn^{2+} -chlorides, 30 mM $(NH_4)_2SO_4$, 5.2 mM citric acid, 19.4 mM Na_2HPO_4 and 565 mM glycerol. These ingredients stimulate killer toxin production (Bendova, Karl University, Prague, Czechoslovakia, personal communication). Experiments with pulsed and transformed protoplasts showed that addition of 1 mM ATP, 3.1 mM Tween 80 and 0.1 mM ergosterol to the regeneration medium enhanced cell wall regeneration and growth. In the presence of these ingredients the first colonies could be observed after 3 to 4 days in contrast to 10 to 14 days which were required when using normal yeast nitrogen base regeneration agar [9]. Regeneration of transformed protoplasts of the industrial strain was improved by using a medium containing lower concentrations of the yeast extract, glucose and peptone (0.25%, 0.5% and 0.25%, respectively).

After about 6 days regeneration, 500 colonies were manually transferred onto plates for detection of killer activity.

Killer activity in transformed clones was identified by the assay described by Young [2] using the super-sensitive strain *S. cerevisiae* S 6-1 (ade^-) which did not contain M dsRNA (Fig. 1). Strains were scored as killers when the inoculum of the yeasts to be assayed for killer activity was surrounded on the surface of the agar medium by a clear zone, in which no growth of the sensitive strains occurred, bounded by a zone of dead cells which, in the presence of methylene blue (0.03%), stained dark blue. The selection agar contained: 2% agar, 0.25% yeast extract, 0.25% peptone, 0.5% glucose, 1 mM of KCl, $MgCl_2$, $CaCl_2$, $FeCl_3$ and $MnCl_2$, 30 mM $(NH_4)_2SO_4$, 565 mM glycerol, 5.2 mM citric acid, 19.4 mM disodium phosphate, 0.03% methylene blue plus 10^7 *S. cerevisiae* S 6-1 cells/ml (Bendova, personal communication). Plates were incubated at 24°C.

4. RESULTS AND DISCUSSION

In the first set of transformation experiments the isolated and purified M_1 dsRNA from T 158 C was injected into protoplasts of the killer-negative variant of T 158 C. This variant was produced by carrying out 5-day heating in the presence of

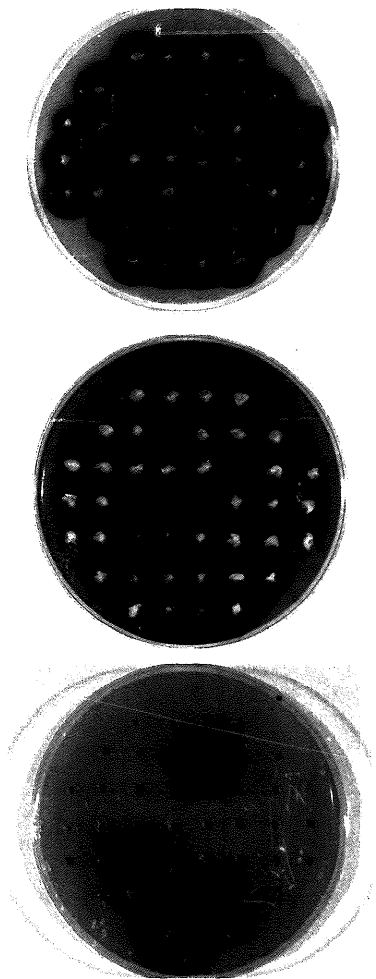


Fig. 2. Typical results of assays for killer activity in (a) transformed killer-negative variants of strain T 158 C, (b) in the laboratory (killer-sensitive) strain AH 215 and (c) in the industrial (killer-sensitive) strain AS-4/H2. Each petri dish contains the selection medium (pH 4.7) plus the super-sensitive strain S 6-1. Transformed cells (transferred with a sterile toothpick onto the surface of the indicator plate) are indicated by a zone of growth inhibition of the super-sensitive cells bounded by a ring of dead cells (visualised by using 0.03% methylene blue). The width of the rings of the inhibition zone and of the dead cells indicate the strength of killer activity.

action (0.89 μM). This treatment resulted in the removal of M dsRNA from the cells (Fig. 1) and, therefore, in the loss of the killer activity.

This model transformation system was used because it allowed conclusions about the frequency of electrically-mediated dsRNA transfer. The yield of transformed clones should not be limited by hindered expression of transmitted dsRNA in the host protoplasts.

The protoplasts were subjected to three consecutive field pulses of 18.2 kV/cm strength and 40 μs duration. After pulsing of the protoplasts of the killer-negative variant of *S. cerevisiae* T 158 C at either 4°C or at 20°C about 10^3 to 10^4 cells could be regenerated. Screening for killer activity by using the selection medium plus cells of the super-sensitive strain *S. cerevisiae* S 6-1 revealed that the majority of these protoplasts were transformed (Fig. 2a). On average 14% of the clones exhibited weak killer activity and 82% super-killer activity when electroinjection was conducted at 4°C. Since the killer activity apparently depends on the number of plasmid copies (about 10 to 12) [1,2], the occurrence of transformed cells with weak killer activity indicates either that these cells had received only one or a few plasmids due to electroporability, that replication of electrically-transmitted plasmids was disturbed or that the structure of the dsRNA was modified during uptake.

Similar yields were obtained when the dsRNA was added immediately after pulsing, indicating that the field pulses had no adverse side effects on the plasmids.

Variations of the field strength and the number as well as of the duration of the field pulses resulted in a significant decrease of transformed clones. Pulsing at room temperature also generally resulted in a reduction of transformed clones (about 2% showing weak killer activity and about 25% exhibiting super-killer activity). This temperature-dependence of the yield is consistent with previous results and can be explained by the temperature-dependence of the resealing process of the field induced perturbations within the membrane [12].

In the second set of experiments we investigated the electric-field mediated injection and ex-

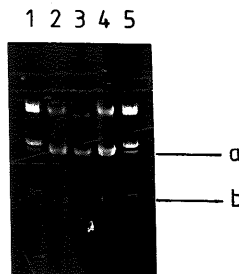


Fig. 3. Agarose-gel electrophoresis of dsRNA of yeast cells transformed with M1 dsRNA isolated from the super-killer strain T 158 C. Tracks 1 and 5, standard DNA; track 2, transformed, killer-negative variant of T 158 C; track 3, transformed, killer-negative variant AH 215; and track 4, transformed strain AS 4/H2.

pression of the M₁ dsRNA in the sensitive laboratory and industrial *Saccharomyces* strains mentioned above under the same conditions.

As shown in Figs. 2b and c transformants were also obtained from both strains (including super-killer clones of the industrial strain). However, the yield of transformed clones was much less than in the model transformation experiments, particularly at 4°C. This result is expected if the expression of the electroinjected dsRNA was diminished. This conclusion was supported by preliminary experiments using dsRNA preparations from killer types K₂ and K₃. It was found that some of these dsRNA preparations did not lead to any transformants.

Gel electrophoresis of the dsRNA of some of the obtained transformed clones shown in Figs. 2a–c supported the conclusion that the transformed clones exhibited killer activity (Fig. 3).

The transformed clones of the model transformation system and of the laboratory and industrial strains were stable. After about 4 weeks they showed no significant change in killer character.

The results reported here demonstrate for the first time that the killer character can be transferred directly into laboratory and industrial yeast strains when the electroinjection method is used.

This opens new avenues for the control and expression of eukaryotic viruses, for study of the mechanisms of protein-processing and secretion as well as for fermentation and possibly medicine.

ACKNOWLEDGEMENTS

This work was supported by grants of the BMFT (03188261) and the Fund of Chemical Industry to U.Z. and of Projekt Pol. 87/008 of UNDP/FAO to A.S.

REFERENCES

- [1] Tipper, D.J. and Bostian, K.A. (1984) *Microbiol. Rev.* 48, 125-156.
- [2] Young, T.W. (1987) in *The Yeasts* Vol. 2 (Rose, A.H. and Harrison, J.S., eds.) pp. 131-164, Academic Press, London.
- [3] Röcken, W. (1984) *Brauwiss.* 9, 384-389.
- [4] Bendova, O., Kupcova, L., Janderova, B., Vondreys, V. and Vernerova, J. (1983) *Brauwiss.* 4, 167-171.
- [5] Zimmermann, U., Pilwat, G. and Riemann, F. (1974) *Z. Naturforsch.* 29c, 304-310.
- [6] Zimmermann, U., Riemann, F. and Pilwat, G. (1976) *Biochim. Biophys. Acta* 436, 460-474.
- [7] Stopper, H., Jones, H. and Zimmermann, U. (1987) *Biochim. Biophys. Acta* 900, 38-44.
- [8] Stopper, H., Zimmermann, U. and Neil, G. (1988) *J. Immunol. Methods* 109, 145-151.
- [9] Schnettler, R., Zimmermann, U. and Emeis, C.C. (1984) *FEMS Microbiol. Lett.* 24, 81-85.
- [10] Broda, H.G., Schnettler, R. and Zimmermann, U. (1987) *Biochim. Biophys. Acta* 899, 25-34.
- [11] Fried, H.M. and Fink, G.R. (1978) *Proc. Natl. Acad. Sci. (U.S.A.)* 75, 4224-4228.
- [12] Zimmermann, U. (1986) *Rev. Physiol. Biochem. Pharmacol.* 105, 175-256.