

FEMSLE 05025

Stably inherited killer activity in industrial yeast strains obtained by electrotransformation

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Received 30 April 1992

Revision received 2 June 1992

Accepted 11 June 1992

Key words: Double-stranded RNA; Electroinjection; Killer stability; *Saccharomyces cerevisiae*; *Saccharomyces carlsbergensis*

1. SUMMARY

Killer-sensitive strains of *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* were transformed by electroinjection using double-stranded RNA isolated from a superkiller strain. Various recipient strains were used: both thermo-resistant and thermo-sensitive as well as mutants of industrial strains. Conversion of respiratory competent (rho^+) into respiratory deficient (rho^-) strains (mutants) resulted in a significant increase of the yield of electrotransformants and/or of longterm killer stability. Electrotransformation of rho^- mutants of distillery and brewery strains resulted in more than 100 clones, which exhibited weak or strong killer activity over some or all of the experimental period of 10 months.

2. INTRODUCTION

Yeast strains which are used in fermentation processes are normally killer-negative. The conversion of these killer-negative industrial strains to 'killer' strains would protect fermentation processes against infection by a wide range of undesirable yeasts and therefore could be of great importance in the production of wine, beer and distillates [1]. For industrial applications, the introduction of double-stranded (ds) RNA (isolated from killer strains) into cells of *S. cerevisiae* seems to be more advantageous than fusion of protoplasts [2–4]. Transmission of dsRNA can be induced by chemical means. However, studies by several authors have shown that chemically induced transformation was accompanied by fusion and the yield of stable transformants was low [5].

Satisfactory stability is generally a serious problem of recombinant strains [4] and depends closely on the genetic background of the yeasts. It is known that there are at least 30 nuclear genes needed for maintenance or replication of the

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killer plasmids. Among others these are MAK 1 through MAK 28, PET 18, KEX 1, 2, REX 1 and SPE 2 [6-8]. Defects in these genes of the acceptor strain can be bypassed by other mutations.

There is also a mounting body of evidence that mutation or deletion of mitochondrial DNA (mtDNA) plays an important role in killer plasmid maintenance [8-10] and that there exists an antagonistic relation between mitochondrial and nuclear gene products [11,12]. In particular, Gunge and Yamane [11] and Stark et al. [9] described an incompatibility of the killer plasmids pGKL (encoded by linear dsDNA) with the mtDNA of the acceptor strain. The plasmids (transmitted by cytoduction from *Kluyveromyces lactis* into cells of *S. cerevisiae*) were stably replicated and expressed in a neutral petite mutant (ρ^0) of *S. cerevisiae*.

We have recently demonstrated that electroinjection can be used to transmit killer activity and immunity into laboratory and industrial strains of *S. cerevisiae* [13]. In the light of the above data we electroinjected killer-dsRNA (isolated from a superkiller *S. cerevisiae* strain) into killer-negative wild-types and into various, respiratory-competent or -deficient mutants of industrial strains. Our efforts were directed towards the identification of possible effects of the mtDNA content on the yield of electrotransformants and also on the maintenance of killer activity.

3. MATERIALS AND METHODS

3.1. Strains

The following strains of *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* were used: *S. cerevisiae* T158 C (MAT α his 4-864 [ρ^+] [KIL-*ski*]), a superkiller strain [14]; *S. cerevisiae* 2.28/T 158 C, a killer-negative variant of strain T158 C [13]; *S. cerevisiae* S.6/1 (MAT α /a [ρ^+] [KIL-0]), a strain supersensitive for detection of killer activity; this strain was kindly made available by Prof. Dr. Palfree (Biology Department, McGill University, Montreal); *S. cerevisiae* AS.4, diploid ([ρ^+] [KIL-0]), an industrial strain used in distilleries in Poland; *S.*

cerevisiae AS.4/H₂-1 (MAT α [ρ^+], [KIL-0]); this strain was isolated after tetrad analysis of strain AS.4; *S. cerevisiae* AS.4/H₂-1 (MAT α [ρ^-] [KIL-0]) from this study; *S. cerevisiae* AS.4/H₂-PC (MAT α *pet 18 clo 1-1* [ρ^-] [HOK-0] [KIL-0]), isolated after tetrad analysis of strain AS.4; *S. cerevisiae* AS.4/H₂ (MAT α [HOK-0] [ρ^-] [KIL-0]), thermo-resistant strain isolated from strain AS.4/H₂-PC; *S. cerevisiae* AS.4/H₂/21 (MAT α [HOK-0] [ρ^-] [KIL-0] KRB1 *pets*), thermo-sensitive strain isolated from strain AS.4/H₂-PC; *S. carlsbergensis* 34 (aneuploid [ρ^+]), an industrial (brewery) strain which was kindly made available by Prof. Dr. S. Donhauser [15]; *S. carlsbergensis* 34 (aneuploid [ρ^-]), obtained in this study.

3.2. Mutants

The petite mitochondrial ρ^- (ρ^0) mutants were obtained by the ethidium bromide method [10].

3.3. Media

The yeast strains were grown in YPD medium. ρ^- mutants were detected by their inability to grow in RHO medium containing by weight 1% yeast extract, 2% bacto peptone, 2% glycerol as a non-fermentable carbon source, and 2% agar.

3.4. Yeast protoplasts

The protoplasts were prepared as described elsewhere [16,17].

3.5. dsRNA isolation

The procedure has been described elsewhere [13].

3.6. Electroinjection

Field conditions have been described elsewhere [13,18]. Electroinjection of the isolated dsRNA (50 μ g ml⁻¹) into protoplasts (about 10⁸ protoplasts/ml) was carried out in pulse medium (30 mM KCl, 1 mM CaCl₂, 0.3 mM KH₂PO₄, 0.85 mM K₂HPO₄, 1.2 M sorbitol) [13,18]. The subsequent regeneration of the transformed protoplasts was performed in regeneration medium rich in yeast nitrogen base [13].

Table 1

Efficiency of transformation of dsRNA into yeasts of *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*

Strains	<i>rho</i>	Number of experiments	Number of clones picked	Percentage (\pm SD) showing killer activity after		
				Initially	1 month	> 10 months
2.28/T 158 C	<i>rho</i> ⁺	5	2200	28 \pm 2.58 ^a 59 \pm 5.46 ^b	28 ^a 19 ^b	28 ^a 69 ^b
S. carl. 34	<i>rho</i> ⁺	4	1760	0.8 \pm 0.83 ^a	0.2 ^a	0
	<i>rho</i> ⁻	4	1760	6.2 \pm 3.19 ^a	3 ^a	1 ^a
AS.4/H ₂ -1	<i>rho</i> ⁺	10	4400	3 \pm 1.84 ^a	0	0
	<i>rho</i> ⁻	5	2200	3 \pm 0.65 ^a 2 \pm 1.43 ^b	3 ^a 2 ^b	3 ^a 2 ^b
AS.4/H ₂ /K ₁ ⁺ /	<i>rho</i> ⁻	2	880	3 \pm 1.82 ^b	3 ^b	3 ^b
AS.4/H ₂	<i>rho</i> ⁻	5	2200	2 \pm 0.88 ^a 1 \pm 1.62 ^b	2 ^a 1 ^b	2 ^a 1 ^b
AS.4/H ₂ /21	<i>rho</i> ⁻	5	2200	1 \pm 1.50 ^a	1 ^a	1 ^a
AS.4/H ₂ -PC	<i>rho</i> ⁻	3	1320	0	0	0

^a Phenotype /K₁⁺R⁺, weak killer activity.^b Phenotype /K₁⁺R⁻, strong killer activity.

3.7. Killer assay

For demonstration of killer activity in the field-treated, regenerated yeast cells the method of Salek et al. [13] was used. To Petri dishes containing a lawn of the super-sensitive strain, colonies were transferred to assay the killer activity. Superkiller clones were also qualitatively identified by the diameter of the 'methylene blue' halo and by their ability to secrete toxin at the nonpermissive temperature of 30°C (see refs. [14,19]).

3.8. Mitochondrial staining

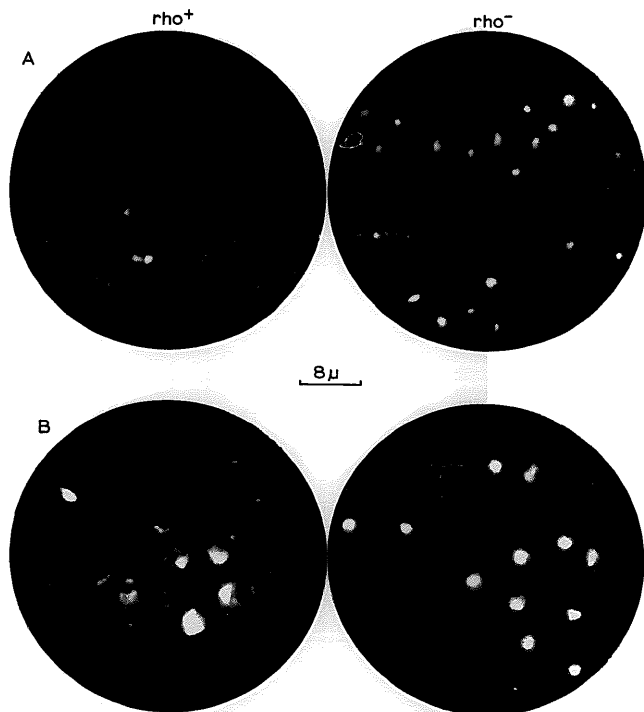
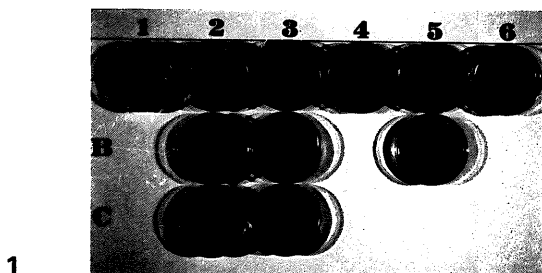
Staining of mitochondrial and nuclear DNA *in vivo* was achieved with DAPI (2,6-diamidino-phenylindole). To this end, cells synchronised by exposure to DNA killer toxin from *Kluyveromyces lactis* (arrests the cells cycle in G₁ phase) were harvested by centrifugation at 3000 \times g, for 10 min, washed and resuspended in Carnoy fixative (methanol:glacial acetic acid 3:1) at room temperature for 45 min. Then the cells were washed three times with 0.85% saline and stained with 1 μ g ml⁻¹ DAPI (dissolved in phosphate buffer,

pH 7.0, 10⁸ cells ml⁻¹, room temperature, 30 min). Alternatively (instead of using fixative), the cells were pulsed (as for electroinjection) and immediately exposed to DAPI. The two procedures gave the same results. The stained cells were viewed with a fluorescence microscope (Axiophot, Zeiss, Oberkochen, FRG).

4. RESULTS

Killer activity and stability of the transformants obtained by electrotransfection of the various *rho*⁺ and *rho*⁻ strains are given in Table 1 and Fig. 1.

Control experiments showed that relatively high numbers of stable killer clones could be obtained when the killer-negative variant of the donor strain 2.28/T 158 C was electrotransformed. In the case of the other strains, the yield and also the stability of the transformed clones were much lower. However, it is evident that prior conversion of the industrial strains *S. cerevisiae* AS.4/H₂-1 and *S. carlsbergensis* 34 from



ρ^+ to ρ^- had a beneficial effect on yield and/or stability of the electrotransformants. In the case of the strain *S. carlsbergensis* 34 the improvement in yield was very significant. After 10 months only 1% of the clones still showed killer activity. However, this result is interesting because all of the control ρ^+ clones were killer-negative after this period.

The industrial strain *S. cerevisiae* AS.4/H₂-1 showed a different behaviour, only a slight increase in yield was observed after ρ^+ to ρ^- conversion, but the transformants were stable over 10 months. One of the ρ^- clones (AS.4/H₂/K₁⁺) with weak killer activity generated in the above experiments was given a further round of electroinjection with the isolated plasmid. This did not result in a further increase of yield or stability, but all of the clones resulting from the double transformation exhibited strong killer activity (Table 1, and Fig. 1).

Other haploid thermo-resistant ρ^- mutants (e.g. AS.4/H₂) and thermo-sensitive ρ^- mutants (e.g. AS.4/H₂/21) which were derived from a *pet 18* mutant (AS.4/H₂-PC), showed no increase in the yield of transformants. However, all the generated clones were killer-positive over 10 months. Electrotransfection of the *pet 18* mutant AS.4/H₂-PC (derived from the diploid industrial strain AS.4) did not lead to any stable clone (Table 1).

The results described above support the view that deletion of mitochondrial genes significantly improves the yield and/or the stability of electrotransformants. However, considerable differences were also observed in the response of the various strains and mutants. The reason for this may have been differences in the amount of mtDNA in the ρ^+ cells and a different degree of deletions of

the wild-type mtDNA in the ρ^- cells (data not shown). This conclusion was confirmed by mtDNA-staining of the ρ^+ and ρ^- *S. cerevisiae* strain of AS.4/H₂-1 and of the strain *S. carlsbergensis* 34 with the fluorescent dye DAPI (Fig. 2). It is evident that the ρ^- mutants contained considerable fewer mitochondria than the ρ^+ wild-type. This can be seen in the case of the strain *S. carlsbergensis* 34 in particular, which contained a large number of mitochondria. After ethidium bromide treatment of the cells the deletion of the mitochondrial genomes seemed to be nearly complete (ρ^0). As shown in Fig. 2A the ρ^- fermenter strain AS.4/H₂-1 had fewer mitochondria. This is expected: strains which are efficient in alcohol production generally contain fewer mitochondria than other strains [20,21]. Due to the reduced number of mitochondria in the ρ^+ strain, the deletion of mitochondrial genomes in response to ρ^- conversion is not as pronounced as in the other case (Fig. 2B). However, careful inspection of the figure shows that a reduction of mitochondria had also occurred in the ρ^- mutant of this strain. Staining of the mitochondria of the other ρ^+ and ρ^- strains of Table 1 yielded analogous results.

5. DISCUSSION

The results reported here demonstrate that non-killer strains of *S. cerevisiae* and *S. carlsbergensis* can be converted to killer strains by electroinjection of dsRNA plasmids. Clones were obtained from all strains and mutants, with the exception of the *pet 18* mutant AS.4/H₂-PC. The reason for this exception might be the fact that yeast cells containing the *pet 18* mutation

Fig. 1. Petri dishes carrying assays for killer activity of single colonies of the following strains: (1A) T 158 C, superkiller clone; (2A) AS.4/H₂, strain before transformation [K⁻]; (2B) AS.4/H₂-1, strain before transformation [K⁻]; (2C) *S. carlsbergensis* 34, [ρ^-], strain before transformation, [K⁻]; (3A) AS.4/H₂/21, transformant, [K⁻]; (3B) AS.4/H₂-1, transformant, [K⁺]; (3C) *S. carlsbergensis* 34 [ρ^-], transformant, [K⁺]; (4A) AS.4/H₂, transformant, [K⁺]; (5A) AS.4/H₂, transformant, [K⁺]; (5B) AS.4/H₂-1, transformant, [K⁺]; (6A) AS.4/H₂/K₁⁺, double transformant, [K⁺].

Fig. 2. Fluorescence micrographs of DAPI-stained cells of ρ^+ and ρ^- strains of *S. cerevisiae* AS.4/H₂-1 (A) and *S. carlsbergensis* 34 (B). It is evident that large differences in mitochondrial content existed between these strains, and that mtDNA was significantly attenuated after ρ^- conversion.

apparently fail to express and maintain killer plasmid activity [8]. The isolated, thermo-resistant mutant, AS.4/H₂ derived from the mutant in *pet 18* (AS.4/H₂-PC), could be transformed at yields of a few percent. This is presumably because the *pet 18* mutation is eliminated in temperature-resistant clones of such mutants [7]. However, we have also shown that temperature-sensitive mutants (*pets* KRB1) could be isolated (such as AS.4/H₂/21) which yielded a few stable, transformed, killer strains.

The effect of mitochondrial DNA (and proteins) on the yield of killer clones and on the maintenance of killer activity is evident from the transformation experiments in which a *rho*⁻ mutation was introduced into the *rho*⁺ strains by ethidium bromide pretreatment. This finding is consistent with results of Gunge and Yamane [11] and of Stark et al. [9].

rho⁻ mutant strains of *S. cerevisiae* and *S. carlsbergensis* showed a significant increase in the yield of transformants and/or stability of killer activity after electrotransformation. The strain-dependent changes could be qualitatively correlated with the mitochondrial DNA and protein content of the parental *rho*⁺ strains and with the changes in mtDNA induced by *rho*⁻ conversion. The *rho*⁺ strain *S. carlsbergensis* 34 possessed many more mitochondria than the corresponding strains derived from the industrial diploid strain AS.4 (e.g. AS.4/H₂-1). Thus, it is understandable why *rho*⁻ conversion resulted in a dramatic increase in the yield of killer clones in the case of the former strain, but not in the case of the latter strain. This increase in killer clones, however, was associated with a relatively poor maintenance of killer activity in part of the clones of *S. carlsbergensis* 34. The reasons for this are unknown. They may be related to the genome pattern of these strains.

Another interesting finding was that most of the transformed strains showed normal killer activity, even in the case of strain 2.28 T 158 C (the killer-negative variant of the donor strain T 158 C, see ref. [13]). This superkiller *rho*⁺ strain has a recessive mutation in one of the *ski* nuclear genes. Mutation of these genes normally leads to the inhibition of the production of mitochondrial

nucleases [6]. Despite the lack of *ski* mutations in the other strains investigated here, about half of the transformants in the case of the *S. cerevisiae* strains AS.4/H₂-1 and AS.4/H₂ showed high killer activity, similar to that of superkillers. As with true superkiller mutants, these transformed killer strains gave large halos and an area of dead cells of the super-sensitive strain used in the killer assay (see Fig. 1). A possible reason for this may be the changes in part of the M-dsRNA induced during the field-mediated uptake into the host cells (in the presence of cell nucleases). This explanation is supported by the finding that an electrically produced clone with weak killer activity (AS.4/H₂/K⁺/) showed, after a second round of electrotransformation, a very stable phenotype /K⁺/ (Fig. 1).

The data show that, independent of the chromosomal loci of the mutations in the various strains, industrial killer strains with long-term stability can be obtained by single or double electrotransformations of *rho*⁻ mutants. The various killer transformed mutants derived from the parental diploid strain AS.4 seem to be good candidates for industrial alcohol production [16,22]. These strains produce 15% alcohol and are osmophilic, ethanol-resistant and thermo-resistant. The latter feature allows fermentation at 35°C. On the other hand, *S. carlsbergensis* 34 is a good brewery strain [15].

Finally we would like to point out that the electric-field induced introduction of killer activity into brewery and distillery yeast strains may be also of biotechnological interest and a useful tool to generate stable markers in yeast strains.

ACKNOWLEDGEMENTS

This work was supported by grants of the BMFT (0318826A) to Prof. Hannig and the Deutsche Forschungsgemeinschaft (SFB 176, B5) to U.Z.

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