Classic techniques for improvement of industrial yeast strains: Part II – Transmission of killer activity into laboratory and industrial strains of *Saccharomyces cerevisiae* by electrotransformation

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Summary

Killer-sensitive strains of *Saccharamocyces cerevisiae* and *Saccharomyces uvarum* var. *carlsbergensis* were transformed by electroinjection using dsRNA 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 in the yield of electrotransformants and/or of long-term 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.

Key words:
Saccharomyces cerevisiae, industrial strains, electrotransformation/transfection, yeast killer mutants.

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1. Introduction

Yeast strains which are used in fermentation processes (distillery, brewery) 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, they could be of great importance in the production of wine, beer and distillates (1).

For industrial applications, the introduction of dsRNA (isolated from killer strains) into non-killer cells of *S. cerevisiae* seems to be more advantageous than fusion of protoplasts (2-4). Transmission of dsRNA can also 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 very low (5).

Satisfactory stability is generally a serious problem of recombinant strains (4) and it depends on the genetic background of the yeasts. It has been shown that more than 30 nuclear genes are necessary for the expression and stable maintenance of the killer genome. These genes include PET 18, MAK 1 to MAK 28, KEX 1, and SPE 2 (6-8). The presence of mutations in some of these genes can be bypassed by certain other mutations.

It is known that some mitochondrial products (nucleases, e.g. active RNase and DNase) inhibit killer plasmid replication. Therefore, mutants in mtDNA (respiratory-deficient strains **rho**$^-$/***rho***°) have been made. As a result, overproduction of dsRNA in strains with the genotype of ***mak 10-1 rho***° is possible (but not in the ***pet 18 rho***° mutants) (8,9). In addition, four recessive ***ski*** genes (super-killer, ***ski 1*** through ***ski 4***) remove down-regulation of the expression of the killer genome. In this case, production of mitochondrial nucleases is inhibited. These mutations have also the ability to bypass the ***mak*** mutation. The ***ski 1-1*** mutation supresses ***spe2*** and all ***mak*** mutations, except ***mak 16-1***. Moreover, the ***ski 2***, ***ski 3*** and ***ski 4*** mutations bypass the ***spe 2*** mutation, but they are unable to supress ***mak 3***, ***mak 10*** or ***pet 18*** mutations (10).

As mentioned above, there is a vast body of evidence that mutation or deletion of mitochondrial DNA (mtDNA) plays an important role in killer plasmid maintenance (8.9,11). There exist an antagonistic relation between mitochondrial and nuclear gene products (12,13). In particular, Gunge & Yamane (12) and Stark et al. (11) described the 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 the cells of *S. cerevisiae*) were stably replicated and expressed in a neutral petite mutant (***rho***°) of *Saccharomyces cerevisiae*.

It was recently demonstrated that electroinjection can be used to transmit killer activity and immunity into laboratory and industrial strains of *Saccharomyces cerevisiae* (14).

In light of the above data and in order to obtain stable strains of *Saccharomyces* sp. with elevated killer activity, special methods were used to preselect and mutate the transformed recipients. The killer dsRNA (isolated from a superkiller *S. cerevisiae* strain) was electroinjected into killer-negative wild-types and into various, respiratory- competent or -deficient mutants of industrial strains. In the final step, electro-

injection was used to transform dsRNA (M_1 dsRNA and L_{1A} dsRNA together) into ***rho***$^-$ or ***rho***° clones.

The 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.

2. Materials and methods

2.1. Strains

The following strains of *S. cerevisiae* and *S. uvarum* var. *carlsbergensis* were used: *S. cerevisiae* T158C (MATα ***his4C-864*** [*rho*$^+$] [KIL-***ski***$_1$]), a superkiller strain (15); *S. cerevisiae* 2·28/T158C, a killer negative variant of strain T158C (14); *S. cerevisiae* S.6/1 (MATα/a [*rho*$^+$] [KIL-0]), a strain super-sensitive for detection of killer activity; this strain was kindly made available by Palfree (Biology Department, McGill University, Montreal); *S. cerevisiae* AH 215 (MAT a, ***leu***$^-$, *his*$^-$), *S. cerevisiae* AS·4, diploid ([*rho*$^+$] [KIL-0]), an industrial strain used in distilleries in Poland; *S. cerevisiae* AS·4/H_2-1 (MATα [*rho*$^+$], [KIL-0]); this strain was isolated after tetrad analysis of strain AS·4; *S. cerevisiae* AS·4/H_2-1 (MATα [***rho***$^-$] [KIL-0]) from this study; *S. cerevisiae* AS·4/H_2-PC (MATα ***pet18 clo1-1*** [***rho***$^-$] [HOK-0] [KIL-0]), isolated after tetrad analysis of strain AS·4; *S. cerevisiae* AS·4/H_2 (MATα [HOK-0] [***rho***$^-$] [KIL-0]), thermo-resistant strain isolated from strain AS·4/H_2-PC; *S. cerevisiae* AS·4/H_2/21 (MATα [HOK-0] [***rho***$^-$] [KIL-0] KRB1 ***pets***), thermo-sensitive strain isolated from strain AS·4/H_2-PC; *S. uvarum* var. *carlsbergensis* 34 (aneuploid [*rho*$^+$]), an industrial (brewery) strain which was kindly made available by S. Donhauser (16); *S. uvarum* var. *carlsbergensis* 34 (aneuploid [***rho***$^-$]), obtained in this study.

2.2. Mutants

The petite mitochondrial ***rho***$^-$ (***rho***°) mutants were obtained by the ethidium bromide method (100 μg/ml for 24 hours in growing medium) (9).

2.3. Media

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

2.4. Yeast protoplasts

The protoplasts were prepared as described elsewhere (17,18).

2.5. dsRNA isolation

The procedure was described elsewhere (14).

2.6. Electroinjection

Field conditions are described elsewhere (14,19). Electroinjection of the isolated dsRNA (50 μg ml^{-1}) into protoplasts (about 10^8 protoplasts/ml) was carried out in pulse medium (30 mM KCl, 1 mM $CaCl_2$, 0.3 mM KH_2PO_4, 0.85 mM K_2HPO_4, 1.2 M sorbitol) (13,18). The subsequent regeneration of the transformed protoplasts was performed in regeneration medium, rich in yeast nitrogen base (14).

2.7. Killer assay

For demonstration of killer activity in the field-treated, regenerated yeast cells the method of Salek et al. (14) was used. Transformants to assay the killer activity were transferred to petri dishes containing a lawn of the super-sensitive strain colonies. Killer clones were qualitatively identified by the diameter of the "methylene blue" halo (or as units of killer protein activity/ml of supernatant or ng protein), i.e. by their ability to secrete toxin at the nonpermissive temperature of 30°C (Fig. 1) (1,15).

2.8. Nuclear and 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 dsDNA killer toxin from *Kluyveromyces lactis* (arrests the cell cycle in G_1 phase) were harvested by centrifugation at 3000 × 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 (10^8 cells ml^{-1}) were washed three times with 0.85% saline and stained with 1 μg ml^{-1} DAPI (dissolved in phosphate buffer pH 7.0) at room temperature for 30 min. Alternatively (instead of using fixative), the cells were pulsed (as for electroinjection) and immediately exposed to DAPI. These two procedures gave similar staining results. The stained cells were viewed with a fluorescence microscope (Axiophot, Zeiß, Oberkochen, Germany).

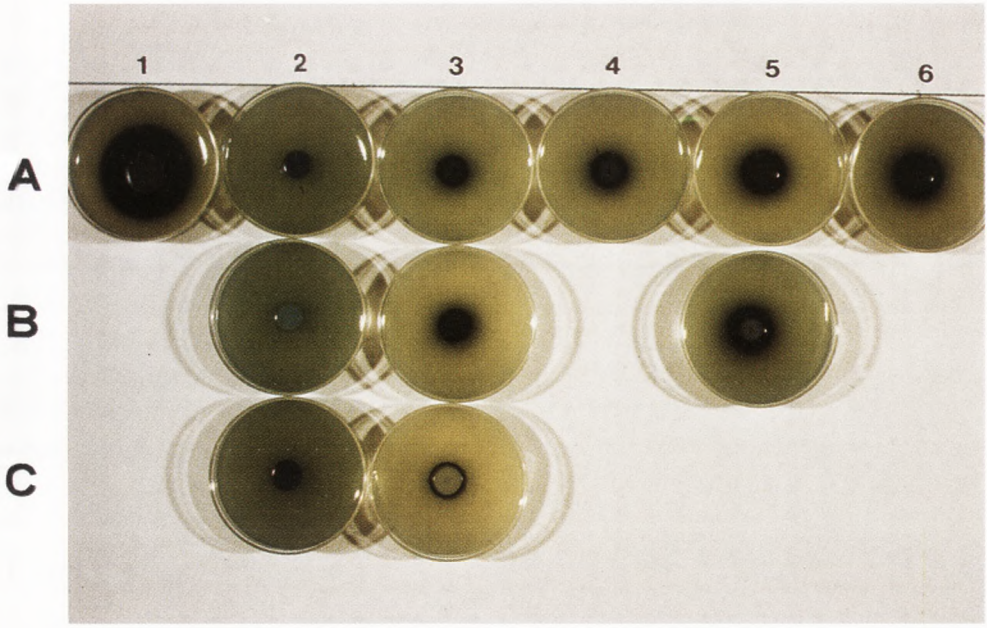


Fig. 1. Petri dishes carrying assays for killer activity of single colonies of different strains, e.g.: 1A – *Saccharomyces cerevisiae* T158C, super-killer strains; 5A – *Saccharomyces cerevisiae* 2 · 28/T158C, transformant of killer-negative strains T158C with killer activity; 5B – *Saccharomyces cerevisiae* AS · 4/H_2-1, transformants with killer activity; 3C – *Saccharomyces uvarum* var. *carlsbergensis* 34, transformant with killer activity.

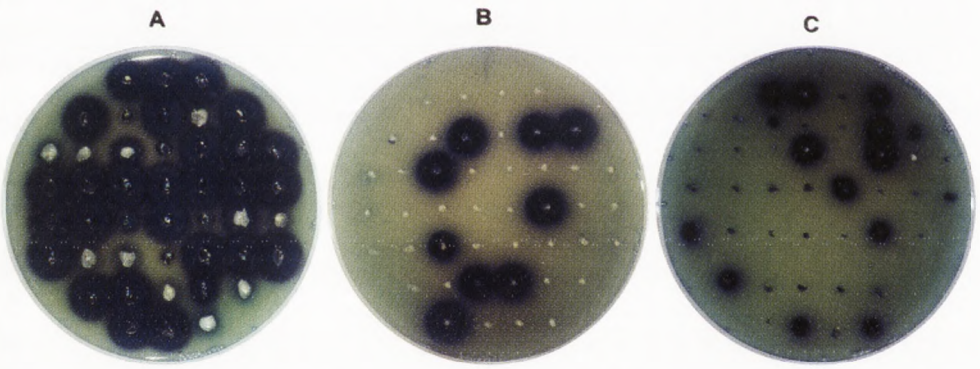


Fig. 2. Results of assay for killer activity in: A – transformed killer-negative variants of strain T158C; B – in the laboratory (killer-sensitive) strain AH 215; C – in the industrial (killer-sensitive) strain AS · 4/H_2-1. Each petri dish contains the selection medium (pH 4,7) plus the super-sensitive strain S·6-1. Colonies of transformed cells were transferred with a sterile toothpick onto the surface of the plate with S·6-1 strains and indicated by a zone of growth inhibition of the super-sensitive cells (S·6-1), bounded by a ring of dead cells (visualised by using 0,003% methylene blue). The width of the rings of the inhibition zone and of the dead cells indicate the strength of killer activity.

3. Results

Results of killer activity (Fig. 2) and stability of the transformants obtained by electrotransfection of the various *rho*$^+$ and ***rho***$^-$ strains (listed in Tab. 1) are given in Table 2. Control experiments showed that a relatively high number of stable killer clones could be obtained when the killer-negative variant of the donor strain 2·28/T158C was electrotransformed. For the other strains, the yield and also the stability of the transformed clones were much lower. It is evident that the prior *rho*$^+$ – to – ***rho***$^-$ conversion of the industrial strains *S. cerevisiae* AS·4/H_2-1 and *S. uvarum* var. *carlsbergensis* 34 had a beneficial effect on the yield and/or stability of the electrotransformants. In the case of the strain *S. uvarum* var. *carlsbergensis* 34 the improvement in yield was very significant. After 10 months only 1% of the clones still showed killer activity. This result is interesting, because all of the *rho*$^+$ clones were killer-negative after this period.

Table 1

Genetic characteristic of *Saccharomyces cerevisiae* strains

Designation	Killer phenotype	Genotype	Source of information
T158C, super killer	K_1^{++} R^+	MATα *his 4C-864* [*rho*$^+$] [KIL-*ski*$_1$]	Vodkin M., Fink G., Katterman F. (35)
2·28/T 158C, cycloheximide nonkiller "cured"	K^- R^-	MATα *his 4C-864* [*rho*$^+$] [KIL-*ski*$_1$]	This work
S·6/1, super-sensitive for killer strain	K^- R^-	MATα/a [*rho*$^+$] [KIL-0]	Palfree G. E.
AH 215, laboratory strain	K^- R^-	MATα *his 3-11 his 3-15 leu 2-3 Leu 2-112* [*rho*$^+$] [KIL-0]	Hinnen A. J.
AH 215, laboratory strain	K^- R^-	MATα *his 3-11 his 3-15 leu 2-3 Leu 2-112* [*rho*$^-$] [KIL-0]	This work
AS·4 industry strain	K^- R^-	diploid [*rho*$^+$] [KIL-0]	Salek A.
AS·4/H_2-1	K^- R^-	MATα [*rho*$^+$] [KIL-0]	Isolation after tetrad analysis of strain AS-4
AS·4/H_2-1	K^- R^-	MATα [*rho*$^-$] [KIL-0]	This work
AS·4/H_2-PC	K^- R^-	MATα *pet 18 clo 1-1* [*rho*$^-$] [HOK-0] [KIL-0]	Isolation after tetrad analysis of strain AS-4
AS·4/H_2	K^- R^-	MATα [HOK-0] [*rho*$^-$] [KIL-0]	Thermo-resistant strain isolated from strain AS·4/H_2-PC
AS·4/H_2/21	K^- R^-	MATα [HOK-0] [*rho*$^-$] [KIL-0] KRB1 *pets*	Thermo-resistant strain isolated from strain AS·4/H_2-PC
S. carlsbergensis 34, industry strain (brewery)	K^- R^-	Aneuploid [*rho*$^+$]	Donhauser S. (44)
S. carlsbergensis 34	K^- R^-	Aneuploid [*rho*$^-$]	This work

Table 2

Efficiency of transformation of ds RNA into yeasts

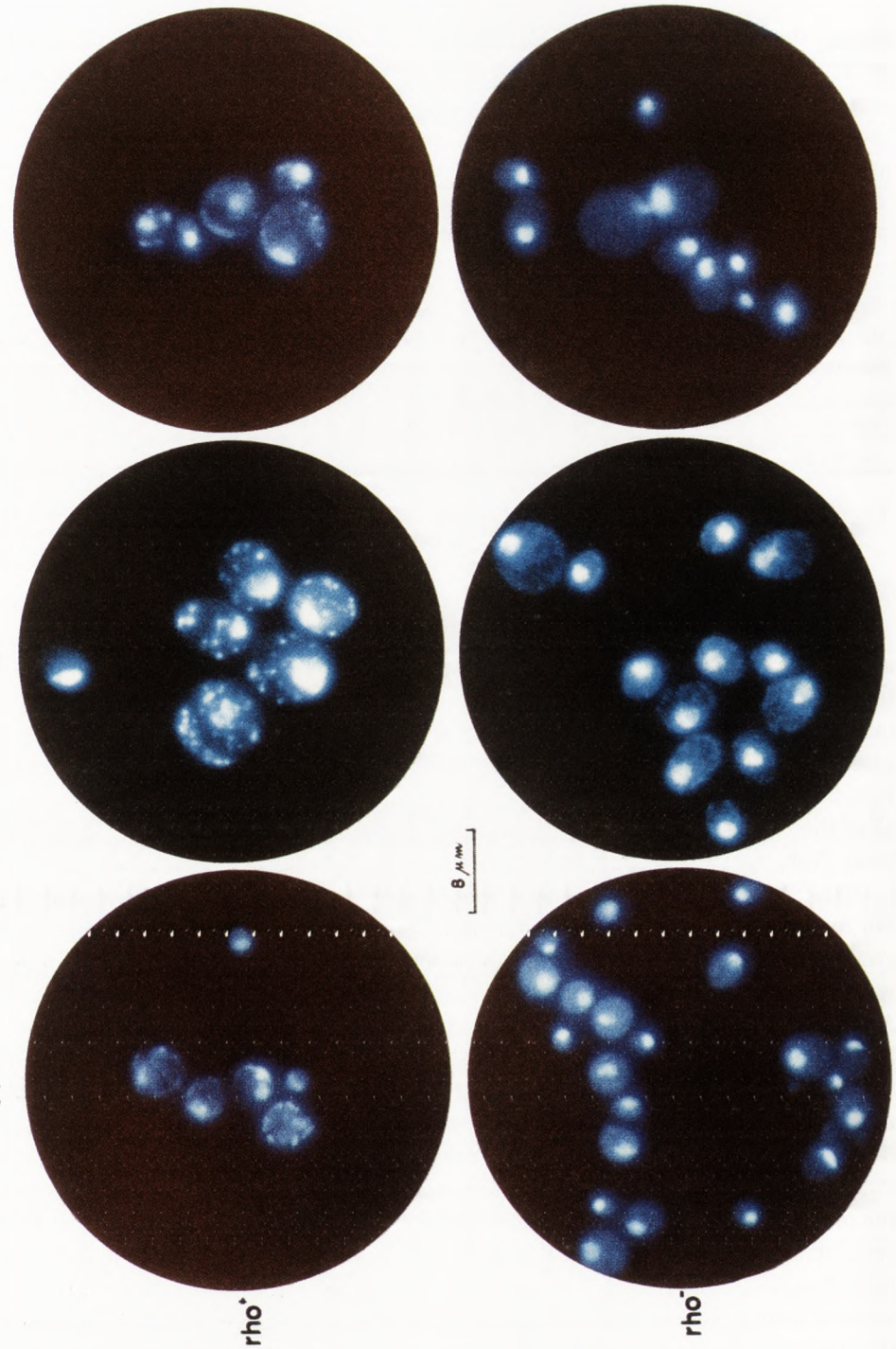
Strains	*rho*	Killer active clones after transformation (%)		Killer active clones after six months (%)	
		K_1^+	K_1^{++}	K_1^+	K_1^{++}
2·28/T158C	*rho*$^+$	28	69	28	69
AH 215	*rho*$^+$	3	0	0	0
	rho$^-$	69	0	15*	0
AS·4/H_2-1	*rho*$^+$	3	0	0	0
	rho$^-$	3	2	3	2
Sc. 34	*rho*$^+$	0.8	0	0	0
	rho$^-$	6.2	0	1**	0
AS·4/H_2-PC	*rho*$^-$ *pet 18*	0	0	0	0
AS·4/H_2	*rho*$^-$	2	1	2	1
AS·4/H_2/21	*rho*$^-$	1	0	1	0

* Stable only two weeks! ** Stable more than four month!

The industrial strain *S. cerevisiae* AS·4/H_2-1 showed a different behaviour, i.e. only a slight increase in yield was observed after *rho*$^+$ – to – ***rho***$^-$ conversion, but the transformants were active and stable over 10 months. One of the ***rho***$^-$ clone (AS·4/H_2/K_1^+) with weak killer activity, generated in the reported experiments, was given a further round of electroinjection with the isolated dsRNA (VLPs, Virus like Particles). However, that 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 (Tab. 1).

Other haploid, thermo-resistant ***rho***$^-$ mutants (e.g. AS·4/H_2) and thermo-sensitive ***rho***$^-$ mutants (e.g. AS·4/H_2/21), which were derived from a ***pet 18*** mutant (AS·4/H_2-PC), showed no increase in the yield of transformants. However, all the generated clones were strong killer-positive over 10 months. Electrotransfection of the ***pet 18*** mutant AS·4/H_2-PC (derived from the diploid industrial strain AS · 4) did not lead to any stable clone (Tab. 2).

The results described above support the view that deletion of mitochondrial genes significantly improves the yield and/or the stability of electrotransformants. Nevertheless, considerable differences were observed in the response of the various strains and mutants. This may be due to the differences in the amount of mtDNA in the *rho*$^+$ cells and a different degree of deletions of the wild-type mtDNA in the ***rho***$^-$ cells (data not shown). This conclusion was confirmed by Figure 3, which shows mtDNA-staining of the *rho*$^+$ – and ***rho***$^-$ *S. cerevisiae* strain of AS·4/H_2-1 and of the strain *S. uvarum* var. *carlsbergensis* 34 with the fluorescent dye DAPI. It is evident that the ***rho***$^-$ mutants contained considerably less mitochondria than the *rho*$^+$ wild-type.


A
B
C
8 µm
rho+
rho-

This can be seen in the case of the *S. uvarum* var. *carlsbergensis* 34 strain (in particular), which contained a large number of mitochondria. After the treatment of the cells with ethidium bromide, the deletion of the mitochondrial genomes seemed to be nearly complete (***rho***°). As shown in Figure 3A the *rho*$^+$ fermenter strain AS·4/H_2-1 had less mitochondria. This is expected: strains which are efficient in alcohol production generally contain less mitochondria than other strains (20,21).

Due to the reduced number of mitochondria in the *S. cerevisiae* AS·4/H_2-1 *rho*$^+$ strain the deletion of mitochondrial genomes in response to ***rho***$^-$ conversion is, therefore, not so pronounced as in the case of *S. uvarum* var. *carlsbergensis* 34 strains (Fig. 3B). However, careful inspection of the figure shows that a reduction of mitochondria had also occurred in the ***rho***$^-$ mutant of this strain. Staining of the mitochondria of the other *rho*$^+$ and ***rho***$^-$ strains (listed in Tab. 1) yielded analogous results.

4. Discussion

In order to obtain *Saccharomyces* spp. strains with stable, elevated killer activity, the laboratory and industrial strains presented in Table 1 were electroinjected with dsRNA. The transformation results (Tab. 2) varied with the strain, and particularly with the genotype. Therefore, a few genetic changes in transformed yeasts were introduced.

Results of electroinjection of dsRNA show that the greatest yield of transformants and stability of the killer activity is obtained by electroinjection of dsRNA into a cycloheximide "cured" strain (Fig. 2A). This strain, *S. cerevisiae* 2·28/T158C, was described previously (14). However, some clones show weak killer activity. The possibilty that the yield of transformants from strains lacking an expression of the ***ski*** mutation is very low led us to first optimize the uptake of dsRNA in a ***ski*** mutant strain. It is also clear from literature that super killer activity [$K_1{}^{++}$] could only be found if the strain is ***ski*** mutant. Therefore, the strain *S. cerevisiae* 2·28/T158C (***ski***) was used as a model system. On the other hand, strain 2·28/T158C was very stable over more than 40 generations, and a complete loss of killer activity was never observed.

Previously described results show (14) that the efficiency of transformation of the laboratory strain AH 215 (Fig. 2B) *rho*$^+$ was much smaller than that of *S. cerevisiae* 2·28/T158C. The activity and stability of the killer character was also very low:

Fig. 3. Fluorescence micrographs of DAPI-stained cells of *rho*$^+$ and ***rho***$^-$ strains of: A – *Saccharomyces cerevisiae* AS·4/H_2-1; B – *Saccharomyces uvarum* var. *carlsbergensis* 34; C – *Saccharomyces cerevisiae* AH 215. It is evident that significant differences in the mitochondrial content existed between these strains, and that mtDNA was significantly attenuated after ***rho***$^-$ conversion.

after about 10 generations most of the killer clones from strain AH 215 *rho*$^+$ had lost their activity completely.

In order to decrease the production of mitochondrial nucleases (including RNase), which usually degrade the stability and maintenance of dsRNA, mitochondrial mutants ***rho***$^-$***/rho***$^\circ$ were generated. These clones have some mitochondrial DNA deletions (Fig. 3) and less mitochondrial proteins (date not shown). Using these ***rho***$^-$ mutants as recipients for dsRNA in transformation experiments more stable killer clones and higher yields were obtained. For example, the most significant increases in the number of killer clones were observed when ***rho***$^-$ strain AH 215 and *S. uvarum* var. *carlsbergensis* 34 was used (Tab. 2).

The clones from ***rho***$^-$ strains were more stable, particularly strain AS·4/H_2-1 ***rho***$^-$ which maintained its killer activity for more than 10 months. After transformation, strain AS·4/H_2-1 ***rho***$^-$ showed an increase in the killing effect (2% of total isolated clones), but with activity like super-killer (Tab. 2 and Fig. 2C). Very weak killer clones of AH 215 ***rho***$^-$ were stable for only about 2 weeks (14).

Transformation of dsRNA into AS·4/H_2-PC (originally ***rho***$^-$) was also carried out. Because this strain was a ***pet 18 clo1-1*** [HOK-0] mutant, we did not obtain killer clones. After the supression of this ***pet 18 clo1-1*** mutation, a thermo-resistant clone (called AS·4/H_2) was obtained. It was used in industry.

A clone which is temperature-sensitive for growth at 37°C, called AS·4/H_2/21, was also selected (with locus KRB1, which bypasses ***pet*** mutation). This clone showed some diminution of the AS·4/H_2-PC killer plasmid replication defect at a temperature of 24°C (permissive). It presented petite character, but transformation by killer dsRNA was possible.

Experiments with double transformation of the weak killer clone of AS·4/H_2 ***rho***$^-$ strain were interesting. An extract of dsRNA from T158C (super-killer strain) was transformed into the weak killer strain AS·4/H_2 [K^+]. We obtained an increase in the yield of the killer active transformants: 3% of the clones showed the [K^{++}] phenotype. Although these killer clones showed very good secretion of killer toxin, they were not ***ski*** mutants.

It was found for transformed clones from all strains and mutants (presented in Tab. 1), with the exception of the ***pet 18*** mutant AS·4/H_2-PC. The reason for this exception might be the fact that yeast cells containing the ***pet 18*** mutation apparently fail to express and maintain killer plasmid activity (8). However, the isolated thermo-resistant mutant AS·4/H_2, derived from the mutant in ***pet 18*** (AS·4/H_2-PC), can be transformed at yields of a few percent. This is presumably due to the fact that ***pet 18*** mutation is eliminated in temperature-resistant clone of AS·4/H_2 mutants (7). Moreover, it was also shown that temperature-sensitive mutants (***pets*** KRB 1), called AS·4/H_2/21, could be isolated. This yielded a few stable, transformed killer strains.

The effect of mitochondrial DNA 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.

rho$^-$ mutant strains of *S. cerevisiae* and *S. uvarum* var. *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. This finding is consistent with the results of Gunge & Yamane (12) and Stark et al. (11).

The *rho*$^+$ strain *S. uvarum* var. *carlsbergensis* 34 possessed many more mitochondria than the corresponding strains AS·4/H_2-1 (Fig. 3). Thus, it is understandable why ***rho***$^-$ conversion resulted in a dramatic increase in the yield of killer clones in the case of the *S. uvarum* var. *carlsbergensis* 34 strain, but not in the case of the *S. cerevisiae* AS·4/H_2-1 strain. This increase in killer clones, however, was associated with a relativly poor maintenance of killer activity in some of the clones of *S. uvarum* var. *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 T158C which is the killer-negative variant of the donor strain *S. cerevisiae* T158C (14). This super-killer *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 ***ski*** mutations, the other strains investigated here (about half of the transformants in the case of the *S. cerevisiae* strains AS·4/H_2-1 and AS·4/H_2) showed quite high killer activity, similar to that of super-killers. As with true super-killer mutants, these transformed killer strains gave large halos and an area of dead cells of the super-sensitive strain used in the killer assay (Fig. 1 or 2A). 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/H_2/$K_1{}^+$) showed a very stable phenotype ($K_1{}^{++}$) after the second round of electrotransformation.

The data show that 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 (17,22). These strains produced 15% alcohol and were osmophilic, ethanol-resistant and thermo-resistant. The latter feature allows fermentation at 35°C. On the other hand, *S. uvarum* var. *carlsbergensis* 34 is a good brewery strain (16).

It was found that the frequency and the stability of killer activity (at a permissive temperature) depends on the general genetic background of the strain used. Strains without any mutation in the genes necessary for expression and maintenance of killer activity showed more effective dsRNA transformation and better stability of the killer character if they were ***rho***$^-$.

The results reported here demonstrate that non-killer strains of *S. cerevisiae* and *S. uvarum* var. *carlsbergensis* can be converted to killer strains by electroinjection of dsRNA plasmids.

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

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