UDC 663.12:547.963.32
ISSN 1330-9862

original scientific paper

Construction of a cDNA-based $K_1/K_2/K_{28}$ Triple Killer Strain of *Saccharomyces cerevisiae*

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Received: September 10, 1997
Accepted: November 24, 1997

Summary

By transforming a natural, dsRNA-based K_1 killer strain of the wine yeast Saccharomyces cerevisiae with two multi-copy (2μ) vectors carrying cDNA copies of the K_2 and K_{28} preprotoxin/immunity genes, a triple killer strain has been constructed that (i) simultaneously secreted three different killer toxins [K1, K2, K28], (ii) expressed functional toxin immunities, and (iii) exhibited a strong and significantly broader killing spectrum than the single killer derivatives. Both plasmids were shown to be self-selective under conditions where the triple killer was cultivated in YEPD medium at pH = 4.7. It is proposed that recombinant $K_1/K_2/K_{28}$ triple killers should be able to predominate in mixed yeast cultures and therefore might be useful in industrial wine fermentations to prevent stuck fermentations and/or killer yeast contaminations.

Keywords: S. cerevisiae, killer toxin expression vectors, triple killers

Introduction

Since their first description in 1963 *(1)*, killer strains have not been found only in *Sacch. cerevisiae* but also in many other yeast genera. In all cases, the killer phenomenon is based on the secretion of a lethal protein toxin to which the corresponding killer toxin producing strain is immune. Killer strains can be found in laboratory and natural populations, and under certain conditions it has been shown that this antagonistic killer phenotype can have an increased fitness against competitive sensitive yeast strains *(2)*. Experimentally it has also been shown that killer yeast contaminations during industrial wine and beer fermentations can lead to protracted and stuck fermentations causing prolonged fermentation times and high residual fermentable sugars *(3)*. Although information and knowledge about the ecological importance of killer proteins is mostly lacking, detailed information exists on the biochemistry, genetics and molecular biology of these mycotoxins *(4,5)*. In the well-characterized killer system of *Sacch. cerevisiae*, three kinds of killers have been recognized so far (K1, K2, K28) which are all defined by a lack of cross-immunity, each of them capable of killing members of the opposite group. In each case, the killer phenotype is associated with the presence of two double-stranded RNA (dsRNA) viruses [L-A and M] that stably persist within the cytoplasm of the infected host (yeast) cell. While L-A functions as classical helper virus by providing each M-dsRNA satellite virus with the major capsid protein [Cap] and the RNA-dependent RNA polymerase [Cap-Pol], each M-dsRNA genome (M_1, M_2, M_{28}) contains the genetic information for an unprocessed precursor (preprotoxin) of the corresponding secreted killer toxin (K1, K2, K28) which is also sufficient to ensure functional toxin immunity *(6–9)*. The virally encoded killer toxins K1 and K2 cause membrane leakage in sensitive yeast cells *(4)*, while killer toxin K28 leads to cell cycle arrest at the G1/S boundary and subsequent inhibition of DNA synthesis in the yeast cell nucleus *(10)*.

Because of the antimycotic potential of killer strains, the use of commercial yeast strains harbouring a M-dsRNA »killer« virus has been suggested to prevent yeast infections and/or to eliminate stuck fermentations

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in industrial wine and beer fermentations *(11,12)*. In order to enhance the killing spectrum of such a strain, the killer yeast should produce more than just a single killer protein. In the case of the *Sacch. cerevisiae* killer viruses, the presence of all three killer virions (M_1, M_2, and M_{28}) in a single yeast strain excludes one another at the replicative level of the toxin-coding M-dsRNA genomes *(13,14)*. Recently, this limitation has been successfully bypassed by either introducing a cDNA copy of the K_1 preprotoxin gene on an episomal expression vector into a natural K_2 killer strain *(15)* or by integrating a K_1 preprotoxin expression cassette into the genome of a wild-type wine yeast of killer type K2 *(16)*. In both cases, the resulting killer strain stably produced both killer toxins, K1 and K2, but because of the striking similarity of both toxins with respect to their mode of action and killing spectra, the antimycotic activity of the double-killer towards *Saccharomyces* and non-*Saccharomyces* yeasts was not much different from that of the single killers. In order to increase the antagonistic activity of a natural *Sacch. cerevisiae* K1 killer yeast, we constructed a $K_1/K_2/K_{28}$ triple killer strain that (i) simultaneously produced three different killer toxins, (ii) expressed the corresponding immunity functions, and (iii) exhibited a significantly broader killing spectrum.

Experimental

Yeast strains and culture media

The strains of *Sacharomyces cerevisiae* used in this work are shown in Table 1. Yeast growth media were YEPD-complete medium [1% yeast extract; 1% Bactopeptone; 2% glucose] and synthetic complete medium [SC] lacking various supplements (e.g. SC-Ura; SC-Leu). Methylene blue agar (MBA, which corresponds to YEPD agar plus 1.92% citric acid and 0.001% methylene blue) was prepared as previously described *(17)*. Each culture medium pH was adjusted to either pH = 4.7 or 6.8 by the addition of K_2HPO_4/citrate buffer. Yeast growth rates were determined by measuring culture turbidity at 610 nm in a spectrophotometer. Yeast strains were either grown in YEPD medium or in SC-drop/out medium and incubated at 20 °C in a rollerdrum at 35 rpm. Bacterial growth medium was LB-medium [1% Bacto-tryptone; 0.5% yeast extract; 0.5% NaCl] to which (if necessary) ampicillin was added to a final concentration of 100 μg/mL.

Assay for killer phenotype

Yeast strains that had been transformed with the K_2 and/or K_{28} killer expression vectors were tested for their killer phenotype by streaking onto MBA plates (pH = 4.7) that had been seeded with 10^5 cells of the indicated sensitive tester strain *(17)*. After incubating the plates for 3 days at 20 °C, a clear zone of growth inhibition surrounding the streak indicates toxin production. To semiquantify toxin activity in culture supernatants, samples of 100 μL were pipetted into wells (10 mm in diameter) cut into the agar, and the plates were incubated for 3 days at 20 °C. The diameter of the growth-free zone is proportional to the logarithm of the killer toxin activity.

Transformation

S. cerevisiae was transformed with dsDNA-plasmids by electroporation *(18)*; *E. coli* was either transformed by using the $CaCl_2$ or the electroporation procedure *(19)*.

Recombinant DNA techniques

For routine growth and plasmid maintenance *E. coli* strain DH5α [genotype: F⁻ *rec*A1 *end*A1 *gyr*A96 *thi hsd*R17 *sup*E44 *rel*A1 Δ(*arg*F-*lac*ZYA) U169 (ϕ80d/*lac*ZΔM15) λ⁻] was used. The multi-copy K_{28} expression vector pGES3 used in this study was constructed by cloning a K_{28} expression cassette, flanked by promotor and terminator sequences derived from the yeast *PGK* gene *(7,20)*, as a 2.0 kb *Hin*dIII/*Sal*I fragment into the *Hin*dIII/*Sal*I site of the multi-copy 2μ vector YEp351 *(21)*. The K_2 expression vector pK2-43 *(8,22)* was kindly provided by Daniel Dignard (CNRC-NRC, Montreal). Plasmid-DNA was isolated und purified by using mini columns (Qiagen). Preparative isolation and purification of vector DNA and/or restriction fragments was either done by electrophoresis through a low-melting agarose gel (Biozym) and repeated phenol/chloroform extractions or by using a DNA-QIAEX II gel extraction kit according to the recommendations of the supplier (Qiagen). All enzymes used, including restriction endonucleases and T4 DNA ligase, were used as recommended by the manufacturer (Pharmacia).

Plasmid stability

Stability of the 2μ-based vectors pK2-43 and pGES3 was measured as previously described *(23)*. Briefly, a single transformed clone of *S. cerevisiae* S86 was picked and grown on selective SC-Ura/Leu medium. These cells were used to inoculate 25 mL YEPD medium (pH = 4.7 and pH = 6.8) to 5×10^3 cells per mL. A sample was spread on complete agar (SC), and the YEPD cultures were shaken at 30 °C for 24 h to reach final cell densities of 5×10^7 cells per mL. Cells were diluted into fresh YEPD medium (at either pH = 4.7 or 6.8) to the original density (5×10^3 cells/mL), aliquots were spread onto SC agar plates, and this process was repeated for up to 10 days. Yeast colonies grown on the supplemented agar plates (SC-agar) were replica plated onto SC-Ura and SC-Leu agar plates, incubated for 3 days at 30 °C and numbers of Ura^+/Leu^+ colonies were determined.

Results and Discussion

Recent studies of Bussey's group *(15,16,24,25)* have shown that K1/K2 double killer expression can be achieved by either transforming a natural K2 killer yeast with multi-copy and/or single-copy plasmids encoding the K_1 preprotoxin gene as well as by integrating a K_1 toxin/immunity expression cassette into the genome of a K_2 killer strain. To extend these findings, we tried to simultaneously express three different killer phenotypes in a single yeast by transforming a K_1 killer strain with two multi-copy expression plasmids carrying the cloned K_2 and K_{28} preprotoxin genes. As shown in Fig. 1, each vector (pK2-43, containing the K_2 preprotoxin gene *(8,22)*; pGES3, carrying the K_{28} expression cassette) rep-

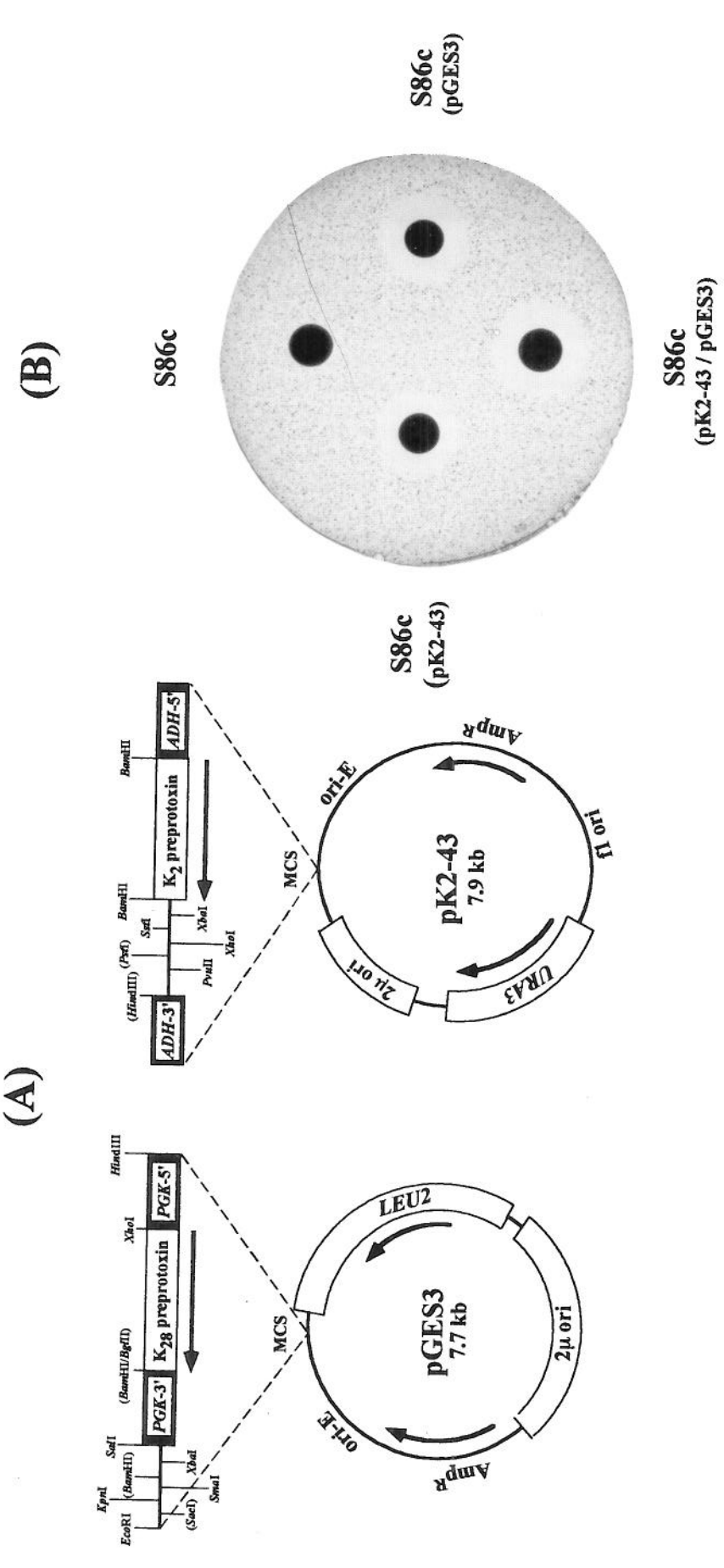

Fig. 1. Partial restriction map (A) and killer phenotype (B) of the yeast killer toxin expression vectors pK2-43 and pGES3. (A) The K_2 killer plasmid pK2-43 *(8,22)* carries the complete K_2 preprotoxin/immunity gene under transcriptional control of the yeast *ADH* promotor/terminator sequences. pGES3 represents the 2µ multi-copy vector YEp351 *(21)* carrying a 2.0 kb *HindIII/SalI PGK/*K_{28} expression cassette derived from the vector pPGK-M_{28}-I *(20)*. The *E. coli* origin (ori-E), the yeast origin (2µ ori) and the *ori* and intergenic region of the phage f1 are represented. Position and direction of transcription of the *LEU2* gene, the *URA3* gene, the β-lactamase gene (Amp^R) and of the *ADH1* and *PGK1* promotor (*ADH-5'*; *PGK-5'*) and the position of the corresponding 3'-sequences are shown. Unique restriction sites within the plasmids are shown except for some of those present twice or more (in parentheses). (B) To check plasmid-driven expression of the appropriate killer phenotype, *Sacch. cerevisiae* strain S86c, which is a heat-cured non-killer derivative of strain S86 (Table 1), was transformed with both plasmids. The untransformed strain S86c and its single and double transformants (S86c [pK2-43]; S86c [pGES3]; S86c [pK2-43/pGES3]) were tested for killer expression by pipetting 10 µL aliquots (containing 10^4 cells from a fresh overnight culture of the appropriate strain) onto MBA plates (pH = 4.7) that had been seeded with 10^5 cells of the sensitive tester strain 192.2d. Clear zones of growth inhibition were detected after the plates had been incubated at 20 °C for 3 days.

resents a 2μ-based multi-copy plasmid in which the corresponding preprotoxin gene (K_2; K_{28}) had been placed under transcriptional control of the strong and constitutive yeast *ADH* or *PGK* promotors, respectively. Upon transformation of a sensitive non-killer yeast (strain S86c), both vectors are able to create a complete killer phenotype, i.e. toxin production and functional immunity (Fig. 1B). In order to test simultaneous expression of three different killer phenotypes in just a single yeast, the natural K_1 killer strain *Sacch. cerevisiae* S86 (*ura3 leu2*; for complete genotype see Table 1) was first transformed with pK2-43 (K_2) and thereafter with pGES3 (K_{28}). The resulting Ura^+Leu^+ transformants were selected on SC--Ura/Leu plates and subsequently tested for killer phenotype expression. Selected transfomants were grown in 5 mL SC-Ura/Leu medium (pH = 4.7) at 20 °C for two days and 10 μL aliquots of the cell-free culture supernatants were spotted onto methylene blue agar plates (MBA, pH = 4.7) that had been seeded with 10^5 cells of various sensitive tester strains. As expected and summarized in Table 2, the constructed $K_1/K_2/K_{28}$ triple killer derivative of strain S86 was capable of killing all tested yeast strains and simultaneously expressed functional (i.e. protecting) immunity against the corresponding K_1, K_2, and K_{28} killer strains. Due to the unique mode of action of yeast killer toxin K28, the triple killer had a much broader killer activity than either the single K1 and K2 killer or the K1/K2 double killer, and [as already shown by Walker *et al. (26)*] exhibited lethality upon certain strains of human and plant pathogenic fungi of the genera *Candida, Phytophthera, Botrytis* and *Heterobasidium* (data not shown). Thus, the generated triple killer is not only able to kill sensitive strains, but also different killer strains, and should therefore have a competitive advantage in mixed yeast cultures as they naturally occur in commercial wine fermentation.

In order to perform stability studies, the triple killer strain was grown at 20 °C in YEPD liquid medium under non-selective conditions (pH = 6.8) and under self--selective conditions (pH = 4.7), and plasmid loss as well as concomitant loss of killer expression was determined by plating culture aliquots onto (i) YEPD, (ii) SC-Ura/Leu, and (iii) MBA plates (pH = 4.7). As shown in Fig. 2, the triple killer transformants remained fully stable for up to 61 generations when cultivated at pH = 4.7. Under these culture conditions both, stability and toxicity of the secreted killer toxins are in the optimal range and therefore, plasmid-carrying triple killers have to maintain their killer plasmids in order to express functional toxin

Table 1. *Saccharomyces cerevisiae* strains used

Strain	Genotype	Killer class	Source/ reference
MS300C (ATCC 201204)	MATα *leu2 ura3-52 ski2-2*	K28	*(17)*
S86	MATα *ura3-52 leu2 his pra1* prb2 *prc1 cps1*	K1	*(4)*
S86c	heat-cured non-killer derivative of strain S86	Non-killer	This work
M471	MATa/MATα *HO/HO*	K2	*(4)*
192.2d	MATα *ura3 leu2*	Non-killer	This work

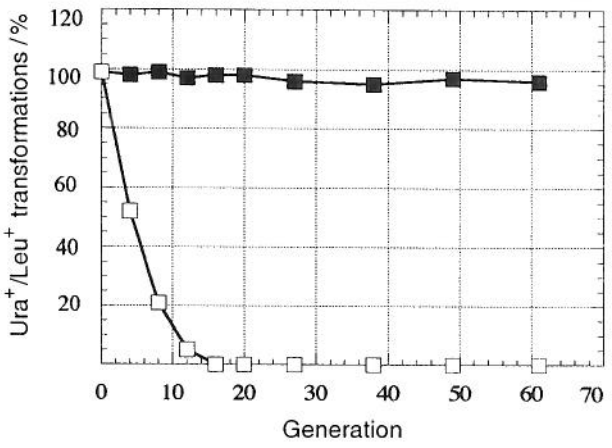

Fig. 2. Stability of the $K_1/K_2/K_{28}$ triple killer derivative of *Sacch. cerevisiae* S86 in YEPD medium at pH 4.7/6.8 and 20 °C.
The $K_1/K_2/K_{28}$ triple killer S86 [pK2-43, pGES3] was serially subcultured in YEPD medium of the appropriate pH. The percentage of $K_1/K_2/K_{28}$ triple killers (measured indirectly as the amount of Ura^+Leu^+ transformants grown on selective SC-Ura/Leu agar plates) was determined as described in Material and Methods
■, subcultures at pH = 4.7; □, subcultures at pH = 6.8.

Table 2. Killer and immunity phenotype of *Sacch. cerevisiae* S86 before and after transformation with the K_2 and K_{28} expression vectors pK2-43 and pGES3

Test strain (phenotype)[1]	*Sacch. cerevisiae* killer strains (killer class)[2]			
	S86 (K1)	S86 [pK2-43] (K1/K2)	S86 [pGES3] (K1/K28)	S86 [pK2-43/pGES3] (K1/K2/K28)
192.2d (NK)	++	++	+++	+++
S86 (K_1)	–	++	+++	+++
M471 (K_2)	++	++	+++	+++
MS300C (K_{28})	+	++	+	++
S86 [pK2-43] (K1/K2)	–	–	++	++
S86 [pK2-43/pGES3] ($K_1/K_2/K_{28}$)	–	–	–	–

[1] Lawns of 10^5 cells of the indicated tester strains were overlaid with 10 μL aliquots of cell-free culture supernatants from the K_1 killer strain S86 and its K1/K2 and K1/K2/K28 killer derivatives obtained after transformation with pK2-43 and pGES3. Killing zones were determined on MBA plates (pH = 4.7) that had been incubated for 3 days at 20 °C (NK, non-killer; K1, K2, K28 represent the 3 known killer classes).

[2] Killer activity is expressed as the diameter of growth inhibition: +++, inhibition zone 20–25 mm; ++, inhibition zone 15–20 mm; +, inhibition zone <15 mm; –, no inhibition (functional toxin immunity).

immunity and to survive attack of their own toxins. In contrast to the strain behaviour at low pH, cultivation in YEPD at pH = 6.8 (i.e. under non-selective conditions where the killer toxins are biologically inactive) made the triple killers highly unstable, resulting in a complete loss of both killer expression plasmids after 16 generations. These data nicely confirm the published data on K_1/K_2 double killers *(15)* and indicate that low pH and more acidic culture conditions are sufficient to confer a self-selectivity upon the K_2 and K_{28} expression plasmids.

Since virtually the same was true for triple killers that had been constructed by transforming the K_1 killer strain *Sacch. cerevisiae* S86 with single-copy centromeric K_2 and K_{28} expression plasmids (data not shown), it can be asumed that simultaneous co-expression of two additional killer phenotypes in a natural K_1 killer strain should also be possible by directly integrating the corresponding K_2 and K_{28} expression cassettes into the yeast genome. Such experiments are planned for the near future in order to construct wine yeasts that simultaneously express three different killer phenotypes without loosing their fermentation and vinification qualities.

Conclusions

By using recombinant DNA technology and yeast/*E. coli* multi-copy shuttle vectors carrying episomal expression cassettes of the viral K_2 and K_{28} preprotoxin/immunity genes, triple killer strains of *Saccharomyces cerevisiae* have been constructed that stably expressed three different killer phenotypes and simultaneously secreted three different killer toxins (K1, K2, K28).

Due to the pronounced killer activity and the significantly broader killing spectrum of such a yeast, recombinant triple killer derivatives of commercially important yeast strains might be useful in wine fermentations to prevent killer yeast contaminations and/or frequently occuring stuck fermentations.

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Konstrukcija trostrukog ubilačkog soja *Saccharomyces cerevisiae* na osnovi cDNA

Sažetak

Transformiranjem prirodnog soja, koji je sadržavao na dsRNA zasnovani K_1 ubilački soj vinskog kvasca Sacch. cerevisiae s dva vektora (2μ) što se pojavljuju u većem broju i nose kopije cDNA za K_2 i K_{28} preprotoksin/imunitet gena, konstruiran je trostruki ubilački soj. Dobiveni je soj izlučivao istodobno tri različita ubilačka toksina (K_1, K_2, K_{28}), pokazivao funkcionalnu toksičnu imunost te imao jaki i puno širi spektar ubijanja od jednostrukog ubilačkog soja. Oba su plazmida samoselektivna kada se trostruki ubilački soj uzgaja u podlozi YEPD pri pH = 4,7. Ako bi rekombinantni $K_1/K_2/K_{28}$ trostruki ubilački soj prevladavao u miješanim kulturama kvasaca, mogao bi biti koristan u industrijskoj proizvodnji vina, sprječavajući zastoj fermentacije i/ili onečišćenja s ubilačkim kvascima.