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Transfer of DNA Killer Plasmids from *Kluyveromyces lactis* to *Kluyveromyces fragilis* and *Candida pseudotropicalis*

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Killer plasmids pGKL1 and pGKL2 of double-stranded linear DNAs were transferred from *Kluyveromyces lactis* to strains of *Kluyveromyces fragilis* and *Candida pseudotropicalis*. The resultant killer strains produced 17-fold and 6-fold larger amounts of killer toxin than *K. lactis* did, respectively. The killer toxin produced by each species appeared to be a glycoprotein.

A novel killer phenomenon has been found recently in *Kluyveromyces lactis* IFO1267 (6). This strain has two species of double-stranded linear DNA plasmids, pGKL1 (8.9 kilobases) and pGKL2 (13.4 kilobases) (6, 7, 12). pGKL1 determines the production of a killer toxin and immunity to the toxin, whereas pGKL2 is considered to play a role in the maintenance of pGKL1 (5, 11). The killer toxin has been purified (14) and has been shown to inhibit adenylate cyclase in *Saccharomyces cerevisiae* (13). The toxin is composed of 27- and >80-kilodalton subunits (14). Recently, it has been shown that plasmids pGKL1 and pGKL2 can be transferred from *K. lactis* to *S. cerevisiae* by protoplast fusion and protoplast transformation, and a resultant killer *S. cerevisiae* strain secretes double the amount of *K. lactis* killer toxin (4, 5). Therefore, we attempted to transfer the plasmids from *K. lactis* to a strain of *Kluyveromyces fragilis* which is well known for its prominent ability to secrete several exoenzymes (10) and to a strain of *Candida pseudotropicalis* which is known to be an imperfect form of *K. fragilis* (18). This paper describes the results of the transfer of the killer plasmids and the markedly increased level of production of killer toxin by the resultant strains.

K. lactis YS283 is a methionine-requiring mutant derived from *K. lactis* IFO 1267 by treatment with nitrosoguanidine. *K. fragilis* NRRL Y-610 and *C. pseudotropicalis* IAM 4829 had the same assimilation pattern and were both nonkillers. *S. cerevisiae* F102-2 was a segregant (**a** *leu his* K^+) from the fusion of *K. lactis* 2105-1D and *S. cerevisiae* AH22 (5). *K. fragilis* and *C. pseudotropicalis* are now called *K. marxianus* and *C. kefyr*, respectively (9, 17). It is known that a strain of *K. lactis* can illegitimately mate ("hybridize" [19]) with a strain of *K. fragilis*. *K. lactis* YS283 and *K. fragilis* NRRL Y-610 were cultured separately in YEPD medium containing 1% yeast extract, 1% peptone, and 2% glucose at 30°C with shaking. When the A_{550} reached 1.0, the cells were harvested, suspended in fivefold volumes of fresh YEPD medium, mixed, and incubated overnight at 30°C with gentle shaking. The cells were washed twice with water, and an appropriately diluted suspension was spread on a minimal medium plate containing 0.67% yeast nitrogen base without amino acids (Difco Laboratories), 2% agar, and 2% maltose. After 4 to 5 days of incubation at 30°C, colonies were obtained at a frequency of 10^{-4} to 10^{-3}. These colonies were purified to single colonies on YEPD agar plates, and eight strains having a higher level of killer toxin activity were chosen. All clones tested showed the same phenotype as the parent, *K. fragilis* NRRL Y-610, except for the acquired killer character (Table 1). Furthermore, *K. lactis* could not grow at 42°C, whereas *K. fragilis* could grow well at 42°C. Therefore, we consider that *K. lactis* YS283 and *K. fragilis* NRRL Y-610 mated together but that their nuclei did not fuse. Those heterokaryons might have segregated in the process of single-colony isolation, and the *K. fragilis* strains harboring the killer plasmids might have been selected. Alternatively, the mitotic segregation might have occurred on the selective plate, since *K. fragilis* NRRL Y-610 grew weakly on the maltose-containing minimal plate. We also observed that *K. fragilis* NRRL Y-610 was dimorphic (yeast form and pseudomycelium) but that *K. lactis* YS283 and the killer *K. fragilis* strain were monomorphic (yeast form only). We considered that chromosomes of the two strains might be partially recombined.

We selected one strain (4W2-1) from these segregants and used it for further studies. We grew *K. fragilis* 4W2-1 for 24 h at 30°C in fermentation medium containing 1% yeast extract, 2% peptone, 4% glucose, and 0.2% ammonium sulfate and assayed the toxin activity in the culture medium. The killer toxin assay and the definition of one unit of killer toxin have been previously described (14). This strain produced approximately 17 times the amount of killer toxin produced by *K. lactis* IFO 1267 (Table 2), even though it grew more slowly than *K. lactis* did (data not shown). *S. cerevisiae* F102-2 produced approximately double the amount of killer toxin produced by *K. lactis* IFO 1267 (Table 2).

K. lactis IFO 1267, *K. fragilis* 4W2-1, or *S. cerevisiae* F102-2 was grown in 10 ml of fermentation medium at 30°C to the late logarithmic phase. The cells were sedimented, washed twice with C-P buffer (0.1 M citric acid, 0.2 M Na_2HPO_4 [pH 6.0]), and suspended in 1 ml of C-P buffer containing 1 mM diisopropylfluorophosphate. The cells were sedimented and ground with an equal volume of alumina powder at 0°C until the viability of the cells became less than 10%. The mixture was centrifuged at 8,000 × *g* for 10 min, and the supernatant was collected. The precipitate was suspended in an equal volume of C-P buffer containing 1 mM diisopropylfluorophosphate and centrifuged. The resultant supernatant was combined with that from the first centrifu-

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TABLE 1. Assimilation patterns of parent strains and their clones

Strain	Assimilation[a] of:					Methionine requirement	Killer activity[b]
	Maltose	Trehalose	Inulin	L-Arabinose	Citrate		
K. lactis YS283	+	+	−	−	−	Yes	+
K. fragilis NRRL Y-610	±	−	+	+	+	No	−
C. pseudotropicalis IAM 4829	±	−	+	+	+	No	−
K. fragilis 4W2-1	±	−	+	+	+	No	+
Other *C. pseudotropicalis*							
Class a (n = 15)	±	−	+	+	+	No	+
Class b (n = 9)	±	−	+	+	−	No	+
Class c (n = 7)	±	−	+	−	+	No	+
Class d (n = 3)	±	−	+	−	−	No	+
Class e (n = 3) (B315)	±	+	+	+	−	No	+

[a] +, Good assimilation; −, no assimilation; ±, poor assimilation.
[b] +, Positive; −, negative.

gation. The toxin activity of the combined solution was regarded as the intracellular toxin activity. The intracellular toxin activity of the tested strains was negligible (Table 2).

We unsuccessfully attempted to transfer pGKL plasmids from *K. lactis* to *C. pseudotropicalis* IAM 4829 by the mating method. Subsequently, we tried to transfer them by protoplast fusion of *K. lactis* YS283 and *C. pseudotropicalis* IAM 4829. Protoplast fusion was performed as previously described (5), except that we used 50 mM potassium phosphate buffer (pH 7.5) and 1.2 M sorbitol instead of 0.1 M citrate-phosphate buffer (pH 6.1) and 0.6 M KCl, respectively. *C. pseudotropicalis* IAM 4829 had the same carbohydrate assimilation pattern as *K. fragilis* NRRL Y-610, so we tried to select colonies on the maltose-containing minimal medium after protoplast fusion. After 6 to 8 days of incubation, colonies were obtained at a frequency of 10^{-5} to 10^{-6} per regenerated *C. pseudotropicalis* protoplast. Single colonies were isolated on YEPD agar plates. Thirty-seven strains which showed the killer character were picked and grouped into five classes according to their assimilation patterns (Table 1). The strain designated B315 had the highest killer activity of the 37 strains tested. An approximately sixfold larger amount of killer toxin was produced by B315 grown in fermentation medium than by *K. lactis* IFO 1267 grown in fermentation medium (Table 2). However, the maintenance of the killer phenotype was relatively unstable in strain B315 (data not shown).

Plasmids were prepared from *S. cerevisiae* F102-2, *K. fragilis* 4W2-1, and *C. pseudotropicalis* B315 and electrophoresed in an agarose gel as previously described (4, 6). The three killer strains each had two species of plasmids (about 13 and 9 kilobases), whereas the nonkiller strains *K. fragilis* NRRL Y-610 and *C. pseudotropicalis* IAM 4829 had no plasmids (Fig. 1). *S. cerevisiae* F102-2 and *K. fragilis* 4W2-1 had nearly equal numbers of plasmids, but *C. pseudotropicalis* B315 had a smaller number of plasmids. As the killer character was not stably maintained from generation to generation in this strain (unpublished data), the proportion of cells with plasmids might have gradually decreased during cultivation for the preparation of plasmids.

The killer toxin produced by *K. lactis* IFO 1267 is considered to be a mannoprotein because of its adsorption to concanavalin A-Sepharose and its elution with methyl α-D-mannoside (14). The toxin produced by *K. fragilis* 4W2-1 also adsorbed to a concanavalin A-Sepharose column and eluted with a linear gradient of 0 to 0.4 M methyl α-D-mannoside (data not shown). Tamura et al. found that tunicamycin specifically inhibits the biosynthesis of *N*-glycoside glycoproteins (15). We therefore treated *K. lactis* IFO 1267 or *K. fragilis* 4W2-1 with tunicamycin. *K. lactis* IFO 1267 or killer *K. fragilis* 4W2-1 was grown in 10 ml of

TABLE 2. Intra- and extracellular killer toxin activity

Strain	Killer activity (units/ml of culture) in:	
	Culture filtrate[a]	Cell lysate
K. lactis IFO 1267	4.07 (1)	0.272
S. cerevisiae F102-2	9.09 (2.23)	0.183
K. fragilis 4W2-1	68.0 (16.7)	0.182
C. pseudotropicalis B315	22.9 (5.63)	Not tested

[a] Numbers in parentheses represent the amount of toxin produced relative to that produced by *K. lactis* IFO 1267.

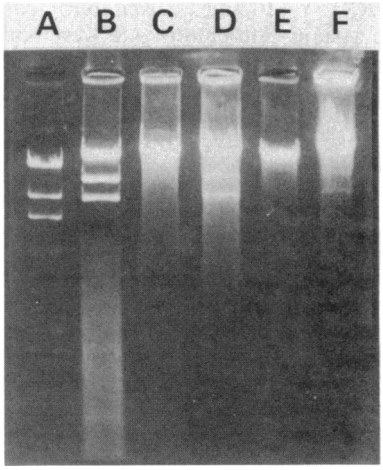

FIG. 1. Agarose gel electrophoresis of yeast plasmids. Lane A, Bacteriophage lambda DNA digested with *Hin*dIII. Lanes B, C, D, E, and F, DNAs extracted from killer *S. cerevisiae* F102-2, nonkiller *K. fragilis* NRRL Y-610, killer *K. fragilis* 4W2-1, nonkiller *C. pseudotropicalis* IAM 4829, and killer *C. pseudotropicalis* B315, respectively.

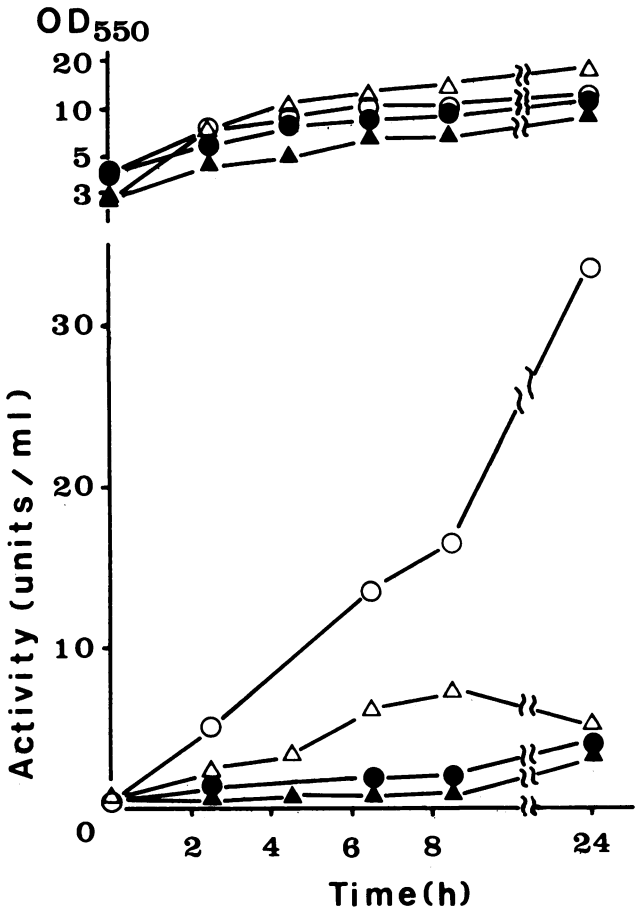

FIG. 2. Inhibition of toxin production by tunicamycin. Symbols: ▲ and △, *K. lactis* IFO 1267 treated and not treated with tunicamycin, respectively; ● and ○, *K. fragilis* 4W2-1 treated and not treated with tunicamycin, respectively. OD_{550}, Optical density at 550 nm.

fermentation medium at 30°C to the late logarithmic phase with shaking. The cells were washed twice with fermentation medium, suspended in 10 ml of fermentation medium containing 1.0 μg of tunicamycin per ml, and incubated at 30°C with shaking. In each case, toxin production was markedly inhibited by tunicamycin, which hardly affected the growth of the two strains (Fig. 2). These results suggest that the killer toxin is a mannoprotein. On the other hand, K1 killer toxin (1–3, 16) and α-factor (8) are known to be derived from unsecreted precursors of mannoprotein.

This study suggested that *K. fragilis* is proficient in secreting mannoproteins of higher molecular weights. It was shown that killer plasmids can also propagate in *C. pseudotropicalis*. It is hoped that these killer plasmids will serve as potential vectors in some *Candida* species.

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