Expression of pGKL killer 28K subunit in *Saccharomyces cerevisiae*: identification of 28K subunit as a killer protein

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Received February 8, 1989; Revised and Accepted April 3, 1989 EMBL accession no. X01095

ABSTRACT

Saccharomyces cerevisiae and other yeast cells harboring the linear double stranded (ds) DNA plasmids pGKL1 and pGKL2 secrete a killer toxin consisting of 97K, 31K and 28K subunits into the culture medium (EMBO J. *5*, 1995−2002 (1986), Nucleic Acids Res., *15*, 1031−1046 (1987)). The 28K subunit of the killer toxin was successfully expressed in *S. cerevisiae* when it was cloned on a circular plasmid with its putative promoter region replaced with that of *S. cerevisiae* chromosomal genes. The expression of the 28K subunit of the killer toxin in killer-sensitive cells resulted in the death of the host cells. This killing activity by the 28K subunit was prevented by the expression of the killer immunity, indicating that the killing activity of the killer toxin complex was carried out by the 28K subunit. Although the 28K subunit was synthesized as a intact precursor protein with its own signal sequence, it was not secreted into the culture medium but remained in the host cells. This indicated that 28K subunit killed host cells from inside of the cells rather than from outside. We further suggested that 28K killer subunit without 97K and 31K subunits did not kill the killer-sensitive cells from outside.

INTRODUCTION

The plasmids, pGKL1 and pGKL2, first discovered in *Kluyveromyces lactis*, possess a number of interesting characteristics (1−4): especially, yeast cells harboring pGKL plasmids secrete a killer toxin into the culture medium which kill yeast cells from a variety of genera.

The determination of the DNA sequence of the pGKL1 plasmid has revealed the existence of four open reading frames (5−7). The pGKL killer toxin consisting of 97K , 31K and 28K subunits has been purified from the culture medium of *K. lactis* (8, 9) and *S. cerevisiae* (10), respectively. A comparison of the NH_2-terminal amino acid analysis of the purified killer toxin and the amino acid sequence deduced from the DNA sequence of pGKL1 plasmid suggests that the ORF2 encodes an 128K polypeptide which is the precursor of 97K and 31K subunits, and ORF4 encodes the precursor form of the 28K subunit (9). The three subunits of the killer toxin, 97K, 31K and 28K, must have distinct functions in the biosynthesis and maturation, and secretion of killer toxin as well as in the killing process; the precise function of each subunit and their interactions in these processes are entirely unknown.

The linear plasmids pGKL1 and pGKL2 are stably maintained in the *S. cerevisiae* ϱ^0 strain and genes encoded on these linear plasmids are successfully expressed. However, once the linear plasmid is converted to a circular form by removing terminal protein and ligation of both ends, killer toxin production cannot be detected from the cells harboring such a circular pGKL plasmid. The most probable explanation is that the putative pGKL-specific RNA polymerase only functions in recognizing pGKL promoters when they are

present on a linear structure. Indeed, an open reading frame predicted to encode protein with homology to RNA-polymerase is identified recently by the DNA-sequencing analysis of pGKL2 plasmids (11). We have two choices for subcloning and expression of genes encoded on pGKL plasmids, the construction of new linear plasmids containing a part of pGKL plasmids and the preparation of circular plasmids containing the pGKL genes whose promoter has been replaced by that of *S. cerevisiae* chromosomal genes.

In the present paper, we report the successful expression of the 28K killer toxin subunit in *S. cerevisiae* by replacing the putative promoter of the 28K gene (ORF4) with *PGK* or *GAL7* promoters of *S. cerevisiae* chromosomal genes in order to study the functions and secretion of the 28K subunit. We show that (i) the 28K subunit possesses the killing activity, (ii) this killing activity was prevented by the expression of killer immunity, (iii) the expressed 28K subunit is not secreted into the culture medium, (iv) the α-factor pro-sequence together with the signal sequence of either 28K or α-factor allows 28K protein to be secreted into the culture medium, and (v) the 28K protein kills host cells from inside of the cells but not from outside.

MATERIALS AND METHODS

Strains, plasmids and media

S. cerevisiae strains and plasmids used in the present study were listed in Table I. Plasmid pKA011 was constructed from pNW064 by introducing the TAG stop codon with end-filling and religation of 5'-protruding end of *Xba*I site located in the 28K coding region. To construct pKA009, the *Bam*HI-*Sal*I fragment of YIP32 containing *LEU2* gene was inserted at the *Aat*II site of pNW053 after making both fragments blunt-ended. Construction of all other plasmids was described in the figure legends. *E. coli* HB101 was used for the plasmid isolation.

We isolated killer-resistant mutants from YNN27(pNW064) as follows. YNN27(pNW064), grown to $OD_{600}=5.0$, was washed and resuspended in 1 ml of 0.1M sodium phosphate buffer (pH 7.0). Cells were mutagenized with the addition of 30μl of ethylmethanesulfonate for 45 min at the room temperature. The survival rate of mutagenized cells under these conditions is about 40%. The mutagenized cells were plated out onto YPD plate and incubated at 24°C for 3 days. The colonies were transferred to an induction plate (0.67% yeast nitrogen base, 2% each of casamino acids, glycerol, lactate and galactose) by replica plating. The galactose-induction resistant colonies were further screened for killer toxin-resistant phenotype by the addition of 40ng per 100μl of purified killer toxin to the culture medium. One of such killer toxin-resistant mutant, strain KA26 (α, *trp1*, *ura3*, K^R), was used as the host of transformation.

Strain Pdh-1(pGKL1, pGKL2) was used for the purification of killer toxin (4, 8, 10). YPD (1% of yeast extract, 2% each of bacto-peptone and dextrose), YCD (1% of yeast extract, 2% each of casamino acids and dextrose) and SD (0.67% yeast nitrogen base and 2% dextrose) media were used.

Sample preparation for SDS polyacrylalmide gel electrophoresis

Yeast cells, harvested from a 5ml culture, were washed and resuspended in 200μl of the homogenate buffer containing 0.1M Tris-HCl buffer (pH7.5), 10% (w/v) glycerol, 1mM dithiothreitol, 1mM phenyl methyl sulfonyl fluoride and 0.3μg/ml each of other protease inhibitors (N-α-p-tosyl-L-lysine chloromethyl ketone, L-1-tosylamide-2-phenyl

Table I. Strains and plasmids

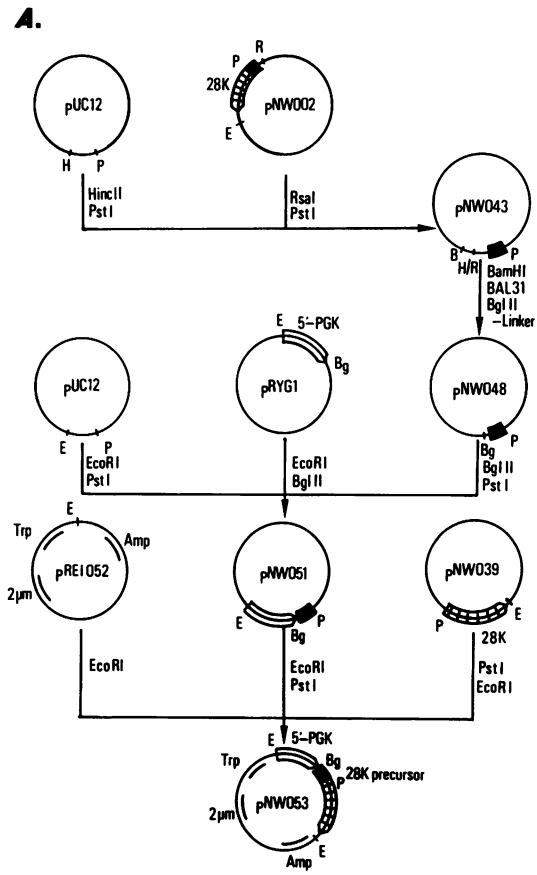
Strain or Plasmid	Characteristics	Source
AH22	*MAT*a, *his4*, *leu2*	N. G. (4)
DBY746	*MAT*α, *his3*, *leu2*, *trp1*, *ura3*	YGSC
DBY747	*MAT*a, *his3*, *leu2*, *trp1*, *ura3*	YGSC
KA26	*MAT*α, *trp1*, *ura3*, K^R 28K killer subunit and killer toxin complex resistant mutant derived from YNN27	This work
Pdh-1	*MAT*a, *his4*, *leu2*, Strain AH22 harboring pGKL1 and pGKL2	N. G. (4)
YNN27	*MAT*α, *trp1*, *ura3*	YGSC
20B-12	*MAT*α, *pep4*, *trp1*	YGSC
pGKL1	K^+, Imm^+ dsDNA linear plasmid	N. G. (1)
pGKL1D	K^-, Imm^+ 1.5kb deletion in ORF2 of pGKL1	This work
pGKL2	Maintenance of pGKL1 dsDNA linear plasmid	N. G. (1)
pKA009	*LEU2*, *TRP1* Expression vector for 28K subunit	This work
pKA011	*TRP1*, *URA3* Encoding truncated 28K subunit gene	This work
pKA014	*TRP1*, Encoding truncated 28K subunit gene	This work
pKA015	*TRP1*, Secretion vector for 28K subunit	This work
pKA026	*TRP1*, Expression vector for 28K subunit	This work
pLGSD5	*URA3* Expression vector for *CYC1* fusion gene	M. O. (19)
pMT34(+3)	*URA3*, *GAL7* promoter vector	(20)
pMT61	*URA3*, Expression vector for 28K subunit	This work
pMT68	*URA3*, Expression vector for *CYC1*-28K subunit fusion gene	This work
pNW039	amp^+, pUC13 derivative containing 28K gene	This work
pNW040	*TRP1*, Secretion vector for 28K subunit	This work
pNW053	*TRP1*, Expression vector for 28K subunit	This work
pNW054	*TRP1*, Secretion vector using 28K signal sequence	This work
pNW064	*TRP1*, *URA3* Expression vector for 28K subunit	This work
pREI052	*TRP1*, Yeast expression vector	(15)
pREI059	*TRP1*, Secretion vector using prepro α-factor sequence	(22)

YGSC, Yeast Genetic Stock Center; N. G., Norio Gunge; M. O., Masayuki Ohoba

ethylchloromethyl ketone, antipain, nystatin and chymostatin), and frozen at −70°C with 200μl of glass beads (o. d. 0.3mm) in a pyrex test tube. The frozen sample was then vigorously vortexed for 2 min at the room temperature, and repeated once more. Glass beads and most of the cell debris were removed by centrifugation at 80×g for 2 min. The supernatant fraction was then centrifuged at 435,000×g for 1 hr at 4°C (Beckman TL-100).

Preparation of antiserum against 28K killer subunit

A *Hin*dIII-*Eco*RI fragment of pNW039 encoding the 28K protein filled at both ends to adjust reading frame was inserted into the *Sal*I-digested and end-filled protein A fusion vector pRIT2T (Pharmacia; 12). In this construction, the synthesis of the protein A-28K fusion protein (58Kd) was under the control of λ P_R promoter. *E. coli* N4830 containing *ts* λ repressor was used as the host, and the synthesis of the fusion protein was induced by the temperature shift from 30°C to 42°C. Harvested cells were disrupted by sonication, and centrifuged at 31,000×g for 30 min at 4°C. The supernatant was applied onto IgG-Sepharose fast flow (Pharmacia) and the adsorbed fusion protein was eluted with 1M acetic acid (pH2.7). The fusion protein was further purified by C_{18} reverse phase HPLC column



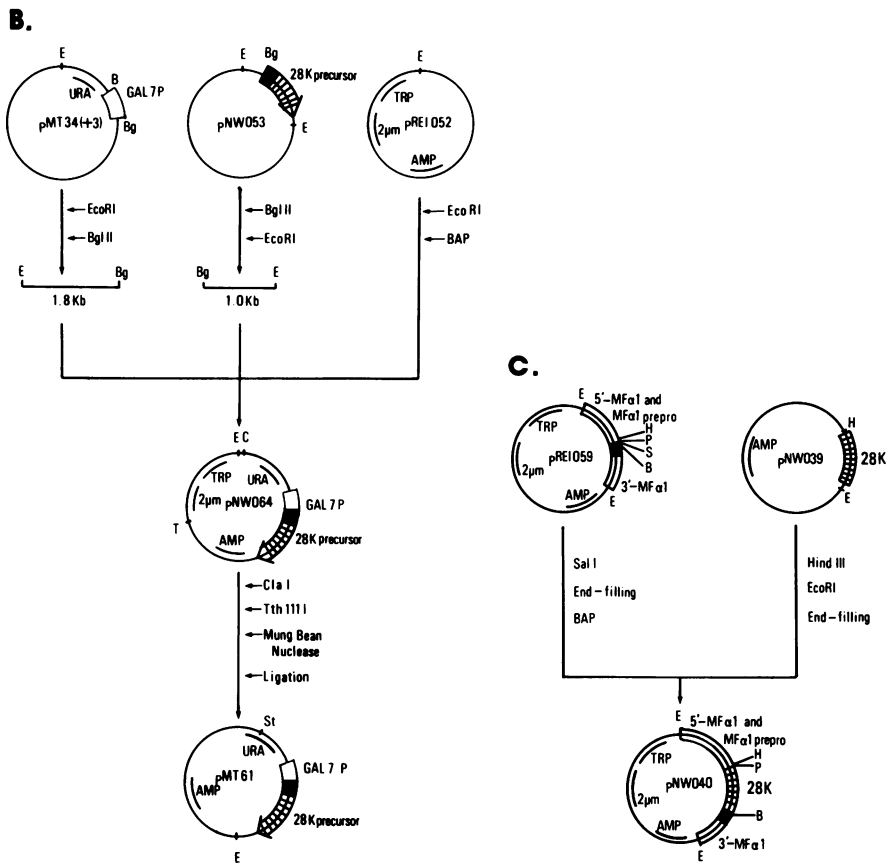


Fig. 1: Construction of 28K protein expression vectors, pNW053, pNW064, pMT61 and pNW040.
The construction of pNW051 was described previously (21). The *Eco*RI-*Pst*I fragment of pNW051 containing phosphoglycerate kinase promoter and the 28K signal sequence, and the *Pst*I-*Eco*RI fragment of pNW039 containing the gene for mature part of 28K protein were inserted into the *Eco*RI site of *E. coli*-yeast shuttle vector pREI052 (15) to obtain pNW053. Plasmid pNW039 was constructed by subcloning the *Pst*I-*Eco*RI fragment encoding mature part of 28K subunit into *Pst*I-*Eco*RI digested pUC13. The *Eco*RI-*Bgl*II fragment containing *GAL7* promoter region of pMT34(+3), and the *Bgl*II-*Eco*RI fragment containing the gene for 28K precursor protein were inserted into the *Eco*RI site of pREI052 to obtain pNW064. For the construction of chromosome integration vector, pMT61, pNW064 was digested with *Cla*I and *Tth*111I, and religated after Mung bean nuclease digestion. To obtain plasmid pNW040, the *Hin*dIII-*Eco*RI fragment of pNW039 encoding the mature part of the 28K subunit was inserted at the *Sal*I site of secretion vector pREI059 (22) after end-filling of both ends.
Fig. 1A: ■, 28K signal sequence; ▥, 28K mature protein; □, phosphoglycerate kinase promoter; Fig. 1B, □, GAL7 promoter; ■, 28K signal sequence; ▥, 28K mature protein; Fig. 1C, □, *MFα*1 promoter and prepro-sequence; ▥, 28K mature protein

(Waters, micro Bonda sphere) to homogeneity with the fusion protein being eluted at the 50% Acetonitril-0.1% TFA fraction. The purified fusion protein was injected into rabbit with complete Freund adjuvant. Two weeks after the 2nd booster, rabbit was bled and the serum was used for immuno-blotting without further purification.

Other methods

DNA manipulation was as described (13). Yeast transformation was carried out by the methods of Ito et al.(14). Immuno-blotting was carried out as described previously (15).

RESULTS

Expression of the pGKL killer 28K subunit in S. cerevisiae

S. cerevisiae cells harboring linear plasmids pGKL1 and pGKL2 express killer and immunity characters. However, despite our extensive efforts (10, 16, M. Tokunaga, unpublished results) and those of others (11, 17, 18), the killer genes were not expressed where they were cloned on the circular autonomously replicating plasmids in *S. cerevisiae*. We, therefore, constructed circular plasmids pNW053 encoding the precursor form of the 28K subunit in which the putative promoter for 28K gene had been replaced with that of *S. cerevisiae* phosphoglycerate kinase gene (Fig. 1A). When the wild type *S. cerevisiae* YNN27 was transformed with pNW053, unusually tiny colonies were obtained with a very low frequency of transformation (below 0.1% of the normal efficiency). We assumed that the low efficiency of transformation of YNN27(pNW053) might be due to the expression of the killer toxin activity. In order to analyze the expression of 28K killer subunit in detail, a clone with the 28K subunit under the control of the *GAL7* promoter, YNN27(pNW064), was obtained (Fig. 1B). When the synthesis of the 28K subunit was induced by the addition of galactose to the culture medium, the growth of YNN27(pNW064) was completely inhibited within 24 hrs, resulting in eventual cell death. YNN27(*ura3*::pMT61, *URA3*) cells, which contains a single copy of integrated pMT61 at the *ura3* position of YNN27 chromosome, showed essentially the same results. As a control, YNN27(pKA011) cells producing a truncated 28K protein (NH_2-terminal to 46th Ser) grew normally. These data clearly indicate that the expression of the 28K protein is lethal for the host cells. In order to ascertain the specificity of the killing activity associated with the expression of the 28K subunit, we isolated a spontaneous variant form of pGKL1 plasmid, called pGKL1D, which contained an 1.5 kb deletion within the ORF2 region.(Kitada, K. unpublished results). AH22(pGKL1D, pGKL2) showed killer$^-$ and immunity$^+$ phenotype, and was used as the host to test whether the killing activity of the 28K protein could be prevented by the expression of the killer immunity protein. Plasmid pKA009, a derivative of pNW053 with an additional *LEU2* marker, was used to transform AH22(pGKL1D, pGKL2)(K$^-$, Imm$^+$) and AH22(pGKL2)(K$^-$, Imm$^-$), and transformants were selected for *LEU2*. AH22(pGKL1D, pGKL2, pKA009) transformants were obtained with a normal efficiency, but no AH22(pGKL2, pKA009) transformant was obtained. AH22(pGKL1D, pGKL2) and AH22(pGKL2) have a same efficiency of transformation with vector plasmid. These results clearly indicate that the killing activity of 28K could be prevented by the killer immunity protein and, therefore, this killing activity by the 28K protein is a reflection of the native killer activity of the pGKL killer toxin complex. We conclude, therefore, that the killing activity of the killer toxin complex resides in the 28K subunit.

We selected many galactose-induction resistant mutants from mutagenized YNN27(pNW064), and screened for killer toxin-resistant mutants by the addition of purified killer toxin complex to the culture medium. More than 60% of galactose-induction resistant mutants showed killer toxin resistant phenotype. This high frequency of linkage of galactose-induction resistance with killer toxin resistance supports the view that the killing activity of 28K subunit represents the native killer activity of the killer toxin complex.

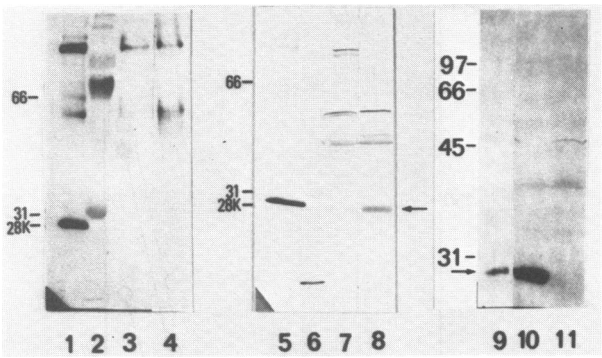


Fig. 2: Identification of 28K protein by SDS-polyacrylamide gel electrophoresis and immuno-blotting
KA26(pNW064) was grown up to an early log phase in 0.67% YNB, supplemented with 2% each of casamino acids, glycerol and lactate, and then the synthesis of the 28K protein was induced by the addition of 2% galactose for an additional culture of 12 hr at 24°C (final OD_{600}=5.9). Proteins secreted in the culture medium (5 ml) were precipitated with 10% TCA (final conc.). Cells were disrupted and fractionated as described in the *Materials and Methods*. The supernatant fraction (435,000×g, 1 hr) including the fluffy material at the bottom of the tube was collected. To obtain plasmid pMT68, the *Hin*dIII-*Eco*RI fragment of pNW039 encoding the mature part of the 28K protein was inserted at the *Bam*HI site of pLGSD5 after end-filling treatment of both ends. KA26(pMT68) cells were grown in the medium containing 0.67% yeast nitrogen base, 20 mg/l of tryptophan, 2% each of casamino acids, glycerol and lactate with (lane 10) or without (lane 11) galactose. After 3 days growth at 30°C, the supernatant fraction of cell homogenates (435,000×g centrifugation for 1 hr) were analyzed by immuno-blotting. The colorization of peroxidase reactions were carried out for 15 min for lanes 5 to 11, and for overnight for lanes 1 to 4 (see text). Lanes 1, 5 and 9, standard 28K protein; lanes 2 and 6, Rainbow marker (Amersham RPN756); lanes 3 and 4, medium fractions of KA26 and KA26(pNW064), respectively; lanes 7 and 8, 435,000×g supernatant fractions of KA26 and KA26(pNW064), respectively.

The expressed 28K subunit was not secreted but accumulated inside the cells.
For the identification of 28K protein, we prepared anti-28K antiserum in rabbit using protein A-28K fusion protein as an antigen. This antiserum specifically reacts 28K subunit produced in the culture medium of cells harboring pGKL1 and pGKL2 (Fig. 2, lane 5). *S. cerevisiae* KA26, the 28K and killer toxin resistant mutant, was used as the host for pNW064 transformation. KA26 has no defect in its secretory pathway (Fig. 4). KA26(pGKL1, pGKL2) cells normally secrete killer toxin into the culture medium (unpublished results). As shown in Fig. 2, the 28K band was not detected at all in the culture medium (lane 4) but detected in the cell homogenate of KA26(pNW064)(lane 8, shown by arrow). To make sure that the 28K polypeptide was not present in the culture media, the peroxidase colorization of nitrocellulose membrane in the left panel of Fig. 2 was carried out overnight; because of this overstaining, the bands in lanes 1 and 2 were non-specifically stained. Even under these conditions, no 28K band was seen (Fig. 2, lane 4). These data clearly showed that the 28K subunit expressed with its own signal sequence was not secreted into the medium, but was retained inside the cells. *K. lactis* cells harboring the derivative plasmids of pGKL1, which contained short deletion in ORF2 region, have been reported to fail to secrete killer toxin including 28K subunit (9). These data suggest that interaction between the 28K protein and other subunits is required for the secretion of killer toxin, and that the 28K polypeptide expressed in YNN27(pNW064) kills the host cells from within rather than without.

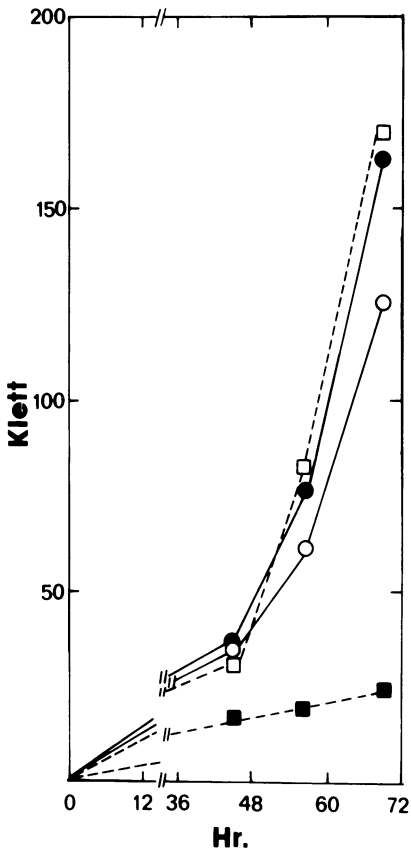


Fig. 3: Lethal action of cytosolically expressed 28K protein. Transformants were grown in the same medium described in the legend to Fig. 2, and cell growth was monitored and expressed in klett unit.
■—■, YNN27(pMT68), + galactose; □—□, YNN27(pMT68), −galactose; ●—●, YNN27(pMT68, pGKL1, pGKL2), + galactose; ○—○, YNN27(pMT68, pGKL1, pGKL2), −galactose.

Expression of the 28K subunit without a signal sequence is also lethal for the host cells. The mature part of the 28K protein was fused to the NH_2-terminal tripeptide M-T-G of *S. cerevisiae CYC1* protein encoded on plasmid pLGSD5 (19) to obtain plasmid pMT68. Due to the lack of a secretion-directing signal at the NH_2-terminus of the 28K protein, it was synthesized and accumulated in the cytoplasm of KA26 cells by the addition of galactose to the culture medium to induce the *GAL1−10* promoter activity (Fig. 2, lane 10). As shown in Fig. 3, YNN27(pMT68) grew normally without induction of 28K synthesis in the absence of galactose in the medium. On the other hand, cell growth was almost completely inhibited in the presence of galactose in the medium, indicating 28K subunit expressed in the cytoplasm showed killer activity. The slight growth of YNN27(pMT68) in the presence of galactose was due to the instability of plasmid pMT68, because 28K protein was not detected in these growing cells (data not shown). YNN27(pMT68, pGKL1, pGKL2) cells grew normally in the medium containing galactose, indicating that the killing

Table II. Killing assay of KA26(pNW064) and KA26(pMT68)

Addition		Cell Growth (% control)
No addition		100
Homogenate (50ng 28K)	pNW064	97.5
	pMT68	94.2
Homogenate (50ng 28K) + Purified killer toxin complex (10ng 28K)	pNW064	0.1
	pMT68	0.1

Killing assay was carried out as described (10).

activity of 28K subunit expressed in the cytoplasm was again prevented by the killer immunity encoded on pGKL1 (Fig. 3).

The 28K killer protein without other subunits did not kill the killer-sensitive cells from outside of the cells.

We examined whether 28K subunit could kill the killer-sensitive cells from outside of the cells or not. Because, so far, the isolation of individual killer-active subunit from killer toxin complex secreted into the culture medium has not been achieved, we measured killer activity of cell homogenate prepared from KA26(pNW064) or KA26(pMT68), or we looked

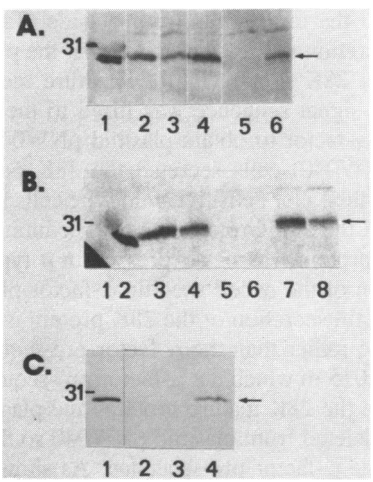


Fig. 4: Immuno-blotting of secreted 28K proteins

All samples were prepared from culture medium (5 ml) precipitated with 10% TCA (final conc.).

A. Lane 1, Rainbow marker and 28K standard; lane2, 20B12(pNW040); lane 3, YNN27(pNW040); lane 4, DBY746(pNW040); lane 5, DBY747(pNW040); lane 6, KA26(pNW040)

B. Lane 1, Rainbow marker; lane 2, 28K standard; lane 3, YNN27(pNW040); lane 4, KA26(pNW040); lane 5, YNN27(pKA014); lane 6, KA26(pKA014); lane 7, YNN27(pKA015); lane 8, KA26(pKA015); pKA014 was constructed from pNW040 by introducing the TAG stop codon at the *XbaI* site in the 28K coding region.

C. Lane 1, 28K standard; lane 2, KA26(pKA026); lane 3, KA26; lane 4, KA26(pNW040).

Construction of plasmids are described in legend of Fig. 5.

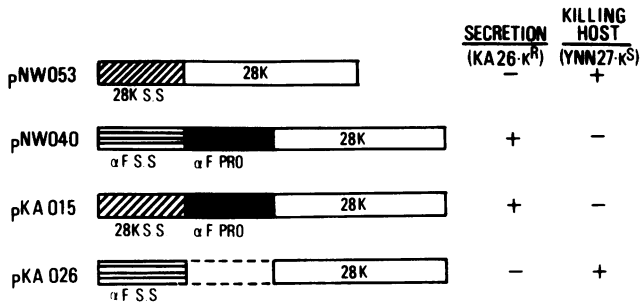


Fig. 5: Schematic illustration of the 28K expression and secretion vectors
Secretion and killing were examined in the hosts of KA26(K^R) and YNN27(K^S), respectively. In order to obtain plasmid pKA015, the *Hin*cII-*Hin*cII fragment of pNW040 encoding the α-factor pro-sequence fused with the 28K mature protein was inserted at the *Sal*I site of the secretion vector pNW054, which contains the phosphoglycerate kinase promoter-28K secretion signal at upstream of the *Sal*I cloning site. The *Hin*cII-*Sal*I fragment encoding the α-factor pro-sequence was deleted from pREI059, and the *Hin*dIII-*Eco*RI fragment of pNW039 was inserted at the regenerated *Sal*I site after end-filling of both ends to obtain pKA026. 28K S. S., 28K signal sequence; αF S. S., α-factor signal sequence (pre-sequence); αF pro, α-factor pro-sequence.

for the secretion signal which could direct secretion of 28K subunit solely into the culture medium without other (97K and 31K) subunits.

Cell homogenates containing the killer-active subunit prepared from KA26(pNW064) or KA26(pMT68) were added to the culture medium of tester cells for the killing assay: the 28K subunit did not kill the tester cells from outside (Table II).

We used several yeast secretion signals and found that the prepro-sequence of α-factor could direct the secretion of 28K subunit into the culture medium. The gene encoding the 28K protein without its signal sequence was fused to the 3′ end of DNA encoding the prepro-sequence of the α-factor to obtain plasmid pNW040 (Fig. 1C). As shown in Fig. 4A, lane 6, KA26(pNW040) cells secreted the 28K polypeptide into the culture medium. Surprisingly, both the killer-sensitive wild type cells harboring pNW040 as well as KA26(pNW040) secreted the 28K protein into the medium without being killed (Fig. 4A, lanes 2−4). The 28K protein was not expressed in *a* type cell DBY747(pNW040) due to the lack of expression of the α-cell specific α-factor promoter (Fig. 4A, lane 5). To ascertain that the successful secretion of the 28K protein is solely due to the presence of the α-factor pro-sequence rather than the α-factor pre-sequence, we constructed two new plasmids, plasmid pKA015 in which the α-factor pro-sequence was inserted between the 28K signal sequence and the 28K mature protein, and plasmid pKA026 in which the α-factor pro-sequence was deleted from plasmid pNW040 so that the 28K mature protein was fused directly behind the α-factor pre-sequence. As shown in Fig. 4B, lanes 7 and 8, the 28K protein expressed as a chimeric protein consisting of the 28K signal sequence plus α-factor pro-sequence followed by 28K mature protein was successfully processed and secreted into the culture medium. The 28K protein thus expressed again did not kill the killer-sensitive host cells. The deletion of the α-factor pro-sequence from plasmid pNW040 resulted in the failure of the 28K protein to be secreted into the culture medium (Fig. 4C, lane 2), and at the same time, the killer sensitive host cells were killed by the 28K protein expressed inside of the cells. These data clearly indicate that the α-factor pro-sequence together with the signal sequence of either 28K or α-factor allows 28K protein to be secreted into the culture medium, and that the secretion of the 28K protein spares

the host cells from its killing activity (Fig. 5). These lines of evidence shown in Table II and Fig. 4 suggested that 28K killer subunit could not kill the killer-sensitive cells from outside of the cells without aids of the 97K and/or 31K subunits.

DISCUSSION

We have demonstrated that the killing activity, the central function of the killer toxin complex, is associated with the 28K subunit. This is the first demonstration that a pGKL killer subunit is expressed in *S. cerevisiae*.

In our system, the 28K subunit is assumed to attack its target via an unidentified intracellular pathway instead of its normal pathway involving the interaction of the killing toxin complex with the cell surface of the sensitive cells. The 28K subunit expressed solely without its natural partners (97K and 31K) might assume to have an unfolded conformation, and is retained in the secretory pathway. On the other hand, at least some of the 28K subunit expressed in our system exhibits its native killing activity; this observation indicates that 28K subunit reaches its target site and is functionally active. If a putative endocytosis of the 28K subunit is involved in the killing processes of intact cells by the killer toxin complex from outside the cells, the 28K protein might contain a specific signal to be targeted to its killing site. One possibility is that the 28K subunit expressed from within is localized to its target site by this putative targeting signal rather than exported to the outside. In this respect, the function of the α-factor pro-sequence is very interesting. It might function as a specific sorting signal along the post-ER transport pathway, or it might mask the putative targeting signal in 28K protein. It is also possible that glycosylation of the α-factor pro-region may facilitate the proper folding of 28K fusion protein, and thereby promotes its exit from the cell.

While the 28K protein can kill the host cell from within, it cannot kill sensitive cells from without. Thus 97K and/or 31K subunits may be essential for both the secretion of the 28K subunit and the killing action of the killer toxin on intact cells.

ACKNOWLEDGEMENTS

We are most grateful to Dr. Henry C. Wu (USUHS, MD, U. S. A.) for the critical reading of the manuscript and the helpful discussions. We also thank Dr. Akira Sakai for helpful discussion, Dr. Kunio Kitada for yeast strains and helpful discussions, Drs. Toshio Fukasawa, S. M. Kingsman and Masayuki Ohoba for plasmids, Miss Naomi Wada for the construction of plasmids. We would like to acknowledge especially Miss Reiko Matsuura for her excellent secretarial assistance. This work was supported by the Research and Development Project of Basic Technologies for Future Industries from MITI of Japan.

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