Ustilago maydis KP6 Killer Toxin: Structure, Expression in *Saccharomyces cerevisiae*, and Relationship to Other Cellular Toxins

JIANSI TAO,¹ IDIT GINSBERG,² NANDITTA BANERJEE,¹ W. HELD,³ YIGAL KOLTIN,²
AND JEREMY A. BRUENN^{1*}

Department of Biological Sciences, State University of New York at Buffalo, Buffalo, New York 14260¹; Department of Microbiology, George Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel²; and Department of Molecular and Cellular Biology, Roswell Park Memorial Institute, Buffalo, New York 14263³

Received 18 October 1989/Accepted 27 December 1989

There are a number of yeasts that secrete killer toxins, i.e., proteins lethal to sensitive cells of the same or related species. *Ustilago maydis*, a fungal pathogen of maize, also secretes killer toxins. The best characterized of the *U. maydis* killer toxins is the KP6 toxin, which consists of two small polypeptides that are not covalently linked. In this work, we show that both are encoded by one segment of the genome of a double-stranded RNA virus. They are synthesized as a preprotoxin that is processed in a manner very similar to that of the *Saccharomyces cerevisiae* k1 killer toxin, also encoded by a double-stranded RNA virus. Active *U. maydis* KP6 toxin was secreted from *S. cerevisiae* transformants expressing the KP6 preprotoxin. The two secreted polypeptides were not glycosylated in *U. maydis*, but one was glycosylated in *S. cerevisiae*. Comparison of known and predicted cleavage sites among the five killer toxins of known sequence established a three-amino-acid specificity for a KEX2-like enzyme and predicted a new, undescribed processing enzyme in the secretory pathway in the fungi. The mature KP6 toxin polypeptides had hydrophobicity profiles similar to those of other known cellular toxins.

Killer toxins are known in eight genera of yeasts (53). Those of *Saccharomyces cerevisiae*, *Pichia kluyveri*, and *Kluyveromyces lactis* are best characterized (12, 23, 46). In *S. cerevisiae*, the k1 toxin consists of two subunits, alpha (103 amino acids) and beta (83 amino acids), joined by two disulfide bonds (5, 42, 54). These are encoded on one double-stranded RNA (dsRNA) of the *S. cerevisiae* virus (ScV) and synthesized as a prepolypeptide of 317 amino acids, which is cleaved by signal peptidase, the KEX1 and KEX2 proteases, and by at least one more unspecified protease during its maturation (5, 9, 15, 17, 22, 42, 54). Neither subunit is glycosylated, although the precursor is glycosylated (6). The k2 killer toxin of *S. cerevisiae* is also encoded on a single dsRNA, synthesized as a preprotoxin, and processed by KEX2 and other proteases, but its alpha polypeptide is glycosylated (M. Whiteway, D. Dignard, and D. Y. Thomas, personal communication). The *K. lactis* toxin, characterized by Stark and Boyd (43), consists of three polypeptides: alpha (865 amino acids), beta (252 amino acids), and gamma (229 amino acids). They are synthesized as two precursors, with alpha and beta synthesized on one transcript and gamma on another transcript of the linear double-stranded DNA k1 plasmid. The alpha polypeptide is glycosylated, but the beta and gamma polypeptides are not. Again, processing includes a KEX2-like step, and processing occurs normally in the heterologous yeast *S. cerevisiae* (43).

The *S. cerevisiae* k1 and the *P. kluyveri* toxins are thought to act by introducing new ion channels into membranes, disrupting ion transport in sensitive cells (8, 12, 23). The *K. lactis* toxin was reported to inhibit adenylate cyclase activity in *S. cerevisiae* (46), but recent reports disprove this claim without establishing any mode of action of the toxin (48, 51).

The *Ustilago maydis* killer toxins are small proteins encoded by viral dsRNAs, like those of *S. cerevisiae*. The KP1, KP4, and KP6 toxins are encoded on specific medium-size (M) segments of the *U. maydis* viruses (UmV) P1, P4, and P6, respectively (24, 26–30). Their mode of action is unknown, but like the *S. cerevisiae* k1 toxin, the KP6 toxin has cell wall receptors (44). Unlike the ScV k1 toxin, in which the two subunits are joined by disulfide bonds (5), or the *K. lactis* toxin, in which the three subunits are present in a (probably) noncovalently bonded complex (43), the KP6 toxin polypeptides interact with the cell independently, as monomers (37). Genetic and in vitro translation data did not indicate conclusively whether the two KP6 polypeptides were synthesized as one preprotoxin or as two (28, 37).

We have shown that the KP6 toxin polypeptides are synthesized as one preprotoxin of 219 amino acids with a typical signal sequence. The preprotoxin is cleaved by an enzyme with specificity similar to that of the *S. cerevisiae* KEX2 enzyme (22) and by at least one other enzyme with specificity like that of the enzyme which cleaves off the N terminus of the *S. cerevisiae* k1 preprotoxin. The result is two secreted polypeptides alpha (78 amino acids) and beta (81 amino acids), whose C termini are predicted in analogy with the k1 toxin. The KP6 preprotoxin was properly processed in *S. cerevisiae*, and active KP6 alpha and beta were secreted. KP6 alpha has some suggestive similarity to the snake venom cytotoxins. The cytotoxins are known to have a mode of action similar to that of the *Saccharomyces* k1 and *P. kluyveri* killer toxins (25). KP6 beta has possibly significant sequence similarity to the putative KP1 beta.

MATERIALS AND METHODS

Strains. All of the *U. maydis* strains used have been described elsewhere (37). These were KP6 wild type (75-1), KP6 lacking the alpha subunit (75-1NK3), KP6 lacking the

* Corresponding author. Electronic mail address: Bitnet cambruen@ubvms.bitnet.

beta subunit (75-1NK13), KP1 (27-1), and a sensitive strain (strain 18). The *S. cerevisiae* recipient strain for transformation with cDNA vectors was LL20, with the genotype *MAT α leu2-3 leu2-12 his3-11 his3-15 mal KEX1 KEX2 GAL⁺ KIL-o*.

cDNA synthesis and cloning. Synthesis of cDNAs and cloning into pUC or M13mp vectors were done as described previously (14, 35). The sequence analysis of P6 M2 was completed from four cDNA clones, occupying bases 5 to 1052, 103 to 1060, 893 to 1060, and 984 to 1233. The *S. cerevisiae* expression vector used (pMAC105) was a generous gift of M. Innis and M. Holland with the permission of Cetus Corp. and is similar to pAC1 (21), with the addition of a *GAL4* recognition sequence within the promoter.

DNA and RNA sequencing. DNA sequencing on double-stranded pUC template DNAs or single-stranded M13mp template DNAs was by the technique of Sanger et al. (39). Direct RNA sequencing was done by the chemical method (36) or the technique of Sanger et al., using 5'-end-labeled synthetic oligonucleotide primers and reverse transcriptase (3).

Antibodies, Western blotting (immunoblotting), and enzyme-linked immunosorbent assays. Four synthetic peptides derived from the predicted P1 M1 prepropeptide sequence were made, and antibodies were raised to the peptides conjugated to keyhole limpet hemocyanin at Smith Kline & French Laboratories. Only one of these antibodies was used in this study: Ab22-23, which cross-reacts with the KP6 beta subunit (N. Banerjee et al., manuscript in preparation). Immunological procedures have been described elsewhere (14).

In vitro translation. Translations of denatured dsRNA or of viral transcript were performed as previously described (11, 20, 41).

Cyanogen bromide cleavage. In vitro translation products labeled with a mixture of ³H-amino acids were cut out of gels after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), electroeluted, dialyzed against distilled water, lyophilized, and cleaved with cyanogen bromide (18); products were detected by SDS-18% PAGE and fluorography.

Assay for killer activity. A 15- to 60- μ l sample of a fresh overnight culture of the sensitive strain 18 at 4 units of optical density at 660 nm per ml was suspended in 7.5 ml of YPD with 1% agar (liquefied) and poured on a plate. Concentrated toxin preparations were spotted directly on the lawn after the agar solidified. The plates were incubated at room temperature for 48 h until a clear zone was detected.

Purification of KP6 toxin subunits and amino-terminal sequence analysis. Purification was performed as previously described (37). Amino-terminal sequence analysis was performed on the affinity-purified alpha polypeptide from strain 75-1NK13 (I. Ginsberg, N. Smorodinsky, and Y. Koltin, manuscript in preparation) and on the beta polypeptide from strain 75-1NK3, since each of these strains secretes only one of the toxin polypeptides (37). Sequence analysis was performed by Sandy Kielland of the University of Victoria, British Columbia, Canada.

Expression of KP6 toxin gene in *S. cerevisiae*. Yeast transformants with the vector (pMAC105) alone (LL20::pMAC105) or the vector with a cDNA insert (YHM59-502; see above) were grown in glucose minimal medium and transferred to YPGal (1% yeast extract, 2% peptone, 2% galactose) or YPD (2% dextrose instead of galactose). Medium was harvested and dialyzed extensively against dis-

FIG. 1. Sequencing strategy and P6 M2 genome structure. DNA sequencing runs are shown as solid arrows, and RNA sequencing runs are shown as dotted arrows. □, Open reading frame; ■, P6 L1 sequence.

tilled water, and the samples were lyophilized to dryness. Final samples were concentrated 50-fold for killer tests.

Partial purification of KP6 toxin from yeast transformants. The dialyzed and lyophilized samples were dissolved in 25 mM sodium acetate (pH 5.5). Columns of carboxymethyl-Sephadex C-50 were equilibrated with the same buffer, and samples were applied. When the A_{280} of the flowthrough reached zero, 0.5 M NaCl in 25 mM sodium acetate (pH 5.5) was applied, and the eluate was collected. Fractions were dialyzed against distilled water and dried by lyophilization. Final samples for application to gels were concentrated 2,500-fold from the carboxymethyl-Sephadex fractions.

Enzymatic deglycosylation of secreted polypeptides. Samples were denatured by boiling in 0.5% SDS-1% β -mercaptoethanol for 5 min. The denatured samples were incubated in 30 mM phosphate buffer (pH 6.0) with or without 0.01 U of endoglycosidase H (endo H; Boehringer Mannheim Biochemicals) in the presence of 1.25% Nonidet P-40. The products were dialyzed against distilled water and lyophilized to dryness. Samples were redissolved in distilled water and loaded on SDS-18% polyacrylamide gels. Silver staining of gels was done as described previously (52).

RESULTS

Sequence of P6 M2. The sequence of P6 M2, the toxin-encoding dsRNA segment of the *U. maydis* virus P6, was determined on both strands by sequencing several cDNA clones and by direct RNA sequencing primed by synthetic oligonucleotides as shown in Fig. 1. There is only one long open reading frame. This is on the viral plus strand, identified as for P1 M1 (11). The sequence of P6 L1, a small noncoding dsRNA of 355 base pairs, was found to be entirely derived from the 3' end of the P6 M2 viral plus strand (Fig. 1). This is identical to the relationship between P1 M1 and P1 L1 (11, 16), with which this sequence shows a high degree of similarity (77%) over a region of 231 base pairs encompassing the border between L1 and M2 (or M1 and L1 in P1) (N. Banerjee, J. Tao, J. A. Bruenn, and Y. Koltin, manuscript in preparation).

The sequence of the plus strand is shown in Fig. 2. The open reading frame extends from bases 120 to 776, encoding a predicted polypeptide of 219 amino acids. The first ATG of the open reading frame is the first ATG of the plus strand. The predicted N terminus has a typical sequence for a signal polypeptide, with a predicted signal peptidase cleavage site after the alanine at position 19 (50). The portion of the predicted polypeptide just C terminal to the signal sequence is very rich in cysteine. There are three Lys-Arg sequences, at which KEX2-like cleavages might occur (positions 107, 138, and 141). There is only one possible N-linked glycosylation site (Asn-Met-Ser at 100). The predicted molecular size of the putative preprotoxin is 24.1 kilodaltons (kDa).

1	GAAAACTCAATAAGAACGGATTAAACCAATCACGACTAAGAAGCCTCGGTTAATTATAC	60
61	GGCAGGACTCCCTTGTAAGACTATCCTAGCAGCTCTACCTAAGGTCACTTACAAACGGAA	120
	M	1
121	TGTTGATATTACAGCTCCTTATGTACTTAGGGCTGTTGCTCGCAGGAGCTCCGCACTCC	180
2	etLeuIlePheSerValLeuMetTyrLeuGlyLeuLeuLeuAlaGlyAlaSerAlaLeuP	21
181	CAAATGGGCTCTCGCCACGCAACAATGCTTTTTGTGCTGGATTGGTCTCTCTTGCAGT	240
22	roAsnGlyLeuSerProArgAsnAsnAlaPheCysAlaGlyPheGlyLeuSerCysLysT	41
	ALPHA NH ₂	
241	GGGAATGTTGGTGCACAGCACACGGAACGGCAATGAATTACGGTATGCTACCGCAGCAG	300
42	rpGluCysTrpCysThrAlaHisGlyThrGlyAsnGluLeuArgTyrAlaThrAlaAlaG	61
301	GATCGGAGATCATCTGTCCAAGTCTTATTACGATGCTCGGGCCGGCCACTGCCTGTTCT	360
62	lyCysGlyAspHisLeuSerLysSerTyrTyrAspAlaArgAlaGlyHisCysLeuPheS	81
361	CTGACGACCTTCGCAACCAAGTTCTACAGCCATTGTTCTGCTCTTAAACAACAATATGTCGT	420
82	erAspAspLeuArgAsnGlnPheTyrSerHisCysSerSerLeuAsnAsnAsnMetSerC	101
421	GCCGGTCGTTGTCTAAACGGACTATCCAAGATAGCGCTACCGACACGGTAGACCTCGGTG	480
102	ysArgSerLeuSerLysArgThrIleGlnAspSerAlaThrAspThrValAspLeuGlyA	121
	ALPHA COOH	
481	CCGAGCTCCATAGGGATGACCCGCCCCCTACTGCTAGTGACATAGGCAAACGGGGTAAGA	540
122	laGluLeuHisArgAspAspProProProThrAlaSerAspIleGlyLysArgGlyLysA	141
	BETA NH ₂	
541	GGCCTAGACCTGTTATGTGCCAATGTGTAGACACAACGAACGGAGGGTTCGATTAGACG	600
142	rgProArgProValMetCysGlnCysValAspThrThrAsnGlyGlyValArgLeuAspA	161
601	CGGTGACTAGGGCGGCTTGCGCATAGACTCGTTTATCGACGGGTACTATACGGAAGAG	660
162	laValThrArgAlaAlaCysSerIleAspSerPheIleAspGlyTyrTyrThrGluLysA	181
661	ATGGGTTTTGTAGAGCTAAATATTCCTGGGACTTGTTTACGAGCGGCCAGTTCTACCAGG	720
182	spGlyPheCysArgAlaLysTyrSerTrpAspLeuPheThrSerGlyGlnPheTyrGlnA	201
721	CATGTTTGAGGTACTCACATGCCGGGACCAACTGCCAACCTGACCGCAGTATGAATAAG	780
202	laCysLeuArgTyrSerHisAlaGlyThrAsnCysGlnProAspProGlnTyrGlu	219
	BETA COOH	
781	TAACCAACGAGGGTTCCTTCAACCATAACTGGTGTATGTGCAACATGCTAACCAACAGA	840
841	GCTATAAAGAGCCGCCAGTATAAATAAGAGAGTTTGATCCGGCCTAAATGTGGAGTTA	900
	L1	
901	GGGCGCCGGTTGGGGTATAGTAGGTGAAGAGTACGAAGAGGAGTTGATATGAGAGTGGA	960
961	AAGTAACCAGATGACCATCCTATGTCGGTTATCCGAGGAAGCGTGGGCTGGAGAAAAATG	1020
1021	CCGTTTCGATCCGTGCGATGCTCGATTGATGCAAGTTGGGATACTATGAAGTGGGTGCT	1080
1081	TACGACGGCCCTAACTGCATATTTAGGGTGAGAGGGGACAGCTGCTACGCTGGGCTGCTC	1140
1141	CTCGCGGACTCAACAGCGGAACCGACCTGGCGTACATGAGTGAATCTGTAGGGATAGC	1200
1201	ACAAGATACGCAGGCAGGTGACTAGTAGAATGCA	1234

FIG. 2. Sequence of the P6 M2 plus-strand cDNA. The sequence of the predicted preprotoxin is indicated under the DNA sequence. Predicted or known proteolytic cleavages follow the italicized dipeptides. Underlined sequences are those of the alpha and beta N termini found by Edman degradation (Fig. 4). The known N termini of alpha and beta and the predicted C termini are indicated. The L1 sequence is shown in italics. This sequence is available as GenBank accession no. M27418.

In vitro translation. In vitro translation of the denatured P6 M2 dsRNA in the absence of cell membranes yielded a single major product, as resolved by SDS-10% PAGE (Fig. 3). Translation of undenatured dsRNA yielded no in vitro products (not shown). From this and similar gels, the estimated sizes of the P1 M1 and P6 M2 translation products were 34.5 and 27.5 kDa, respectively. Both appeared slightly larger than predicted from the sequences of their template RNAs (Banerjee et al., in preparation; Fig. 2). Translation of the P6 M2 viral transcript synthesized by viral particles in vitro gave the same result (not shown). Cyanogen bromide cleavage of the in vitro translation product of P6 M2 (not shown) gave three peptides corresponding to the three

largest peptides predicted from the positions of methionine residues: 9.8, 8.2, and 5.1 kDa.

N-terminal sequence analysis. The KP6 toxin polypeptides VP10 (now called alpha) and VP12.5 (now called beta) were purified from culture medium and subjected to N-terminal sequence analysis. The results are compared with the predicted preprotoxin sequence in Fig. 4. The N-terminal sequences of the toxin subunits correspond to positions 28 to 61 (alpha) and 139 to 166 (beta), with minor exceptions. Because of oxidation, none of the cysteine residues were so identified. One cysteine residue was not detected as an unreadable cycle in alpha (residue 39 in the preprotoxin). One histidine was identified as alanine (residue 48), and one

FIG. 3. In vitro translation of denatured P6 M2 and P1 M1 dsRNAs. An autoradiograph of the SDS-10% polyacrylamide gel of the ^{35}S -labeled products is shown. Arrows indicate the P1 M1 and P6 M2 translation products. Markers are vesicular stomatitis virus L (190 kDa), albumin (70 kDa), vesicular stomatitis virus N (50 kDa), vesicular stomatitis virus M (29 kDa), and major urinary protein (20.7 kDa).

alanine was unreadable (residue 60). In beta, there was one unreadable valine (residue 162) and one unreadable alanine (residue 165) as well as an arginine identified as aspartic acid (residue 164). The two misidentifications (Arg for His and Asp for Arg) may be due to errors in the sequencing or to allelic differences between the RNA sequenced (from the KP6 wild type) and the peptides sequenced (from mutant strains NK3 and NK13). We are now in the process of sequencing the mutant RNAs.

The amino-terminal sequencing results, together with the P6 M2 sequence analysis and the in vitro translation of P6 M2, demonstrate unequivocally that both of the KP6 polypeptides are derived from a single prepolypeptide. The N termini determined also identify two cleavage sites within the KP6 preprotoxin. The first is after a Pro-Arg at position 27; the second is after a Lys-Arg at position 138. These are very similar to the N-terminal processing event in the *S.*

cerevisiae k1 toxin and to the KEX2 cleavage in the same toxin, respectively.

Cleavage sites of killer preprotoxins in fungi. The known or predicted processing sites in killer toxins are compared in Table 1. The N-terminal cleavage, which is thought to follow a prior cleavage by signal peptidase in the k1 toxin (33), occurs after a Pro-Arg sequence in k1 and KP6 and is predicted to occur at the same position in KP1 as in KP6. These are the only Pro-Arg sequences in any of these prepolypeptides (5; Banerjee et al., in preparation; Fig. 2). This site must be recognized by an as yet undiscovered membrane-bound enzyme. The *K. lactis* (43) and *S. cerevisiae* k2 (Whiteway et al., personal communication) preprotoxins are missing this N-terminal cleavage site.

All of the killer toxins have KEX2-like cleavage sites. Five of these are known to be used, and another five are predicted. All show more than just the dibasic amino acid specificity determined for KEX2 (22). All of the cleavage sites have a hydrophobic aliphatic amino acid (Ala, Val, Ile, or Leu) in the position two amino acids before the cleavage site (Table 1). If the KEX2-like cleavages in the preprotoxins are limited to those that fulfill this criterion, then each known cleavage is accurately predicted. This prediction eliminates all possible KEX2-like cleavage sites that are known not to be used in these prepolypeptides: 1 possible site in KP6, 1 possible site in k1, and 12 possible sites in *K. lactis* alpha and beta polypeptides.

We have predicted the sizes of the KP6 toxin polypeptides on the basis of the known amino termini and the predicted C-terminal KEX2-like cleavage at position 108 of the preprotoxin, followed by a predicted removal of the C-terminal Lys-Arg from the alpha subunit by a KEX1-like activity (15, 54). The predicted mature polypeptides would have sizes of 78 amino acids or 8.6 kDa (alpha) and 81 amino acids or 9.1 kDa (beta). The measured sizes of the KP6 toxin polypeptides were 10 kDa (alpha) and 12.5 kDa (beta) (37). Revised estimates of the sizes of KP6 alpha and beta from SDS-PAGE (see below) are 7.5 and 9 kDa. Only alpha could have N-linked glycosylation (at position 100 of the preprotoxin). If alpha were glycosylated, its apparent size would be greater than that of beta, in contradiction to their mobilities in SDS-PAGE. Neither appeared to be glycosylated when synthesized in *U. maydis* (not shown). We conclude that the measured sizes are inaccurate because of the limitations of SDS-PAGE in this size range, although there may also be some systematic error due to peculiar structures of the toxin polypeptides, since the precursor migrated more slowly than expected (Fig. 3). The calculated isoelectric points were both near neutrality: 7.21 for alpha and 6.94 for beta. The native toxin elutes from Sephadex G-50 with a size of 9 kDa (J. Tao, unpublished data).

Secretion of KP6 toxin from *S. cerevisiae*. Given the simi-

alpha			
determined		NNAF-AGFGLS KWE-W-TAAGTGNELRYATA-G	
predicted	28	NNAFCAGFGLSCKWECWCTAHGTGNELRYATAAG	61
beta			
determined		GKRPRPVM-Q-VDTTNG-VRLDA-TD-A	
predicted	139	GKRPRPVMCQCVDTTNGGVRLDAVTRAA	166

FIG. 4. Predicted and determined N-terminal sequences of the KP6 alpha and beta polypeptides. The sequences of alpha and beta predicted from the sequence of P6 M2 (Fig. 2) are compared with those determined by N-terminal sequence analysis. Dashes indicate unreadable cycles in the Edman degradation. Numbers indicate residue positions within the KP6 preprotoxin.

TABLE 1. Cleavage sites in preprotoxins in fungi

Preprotoxin	Site ^a	Sequence ^b (reference)	
		N-terminal site	KEX2-like site
k1, <i>S. cerevisiae</i>	k 44	Leu-Leu-Pro-Arg*Glu (5)	
KP6, <i>U. maydis</i>	k 26	Leu-Ser-Pro-Arg*AsN (this work)	
KP1, <i>U. maydis</i>	p 26	Pro-Gly-Pro-Arg*Ser (Banerjee et al., in preparation)	
k1, <i>S. cerevisiae</i>	k 149		Val-Ala-Arg-Arg*Asp (5, 54)
k1, <i>S. cerevisiae</i>	k 233		Val-Ala-Lys-Arg*Tyr (5)
k2, <i>S. cerevisiae</i>	p 221		Ile-Val-Lys-Arg*Asp (D. Y. Thomas, personal communication)
k2, <i>S. cerevisiae</i>	p 268		Val-Gly-Lys-Arg*Glu (D. Y. Thomas, personal communication)
alpha, <i>K. lactis</i>	k 29		Leu-Val-Lys-Arg*Ala (43)
beta, <i>K. lactis</i>	k 894		Leu-Tyr-Lys-Arg*Gly (43)
KP6, <i>U. maydis</i>	p 108		Leu-Ser-Lys-Arg*Thr (this work)
KP6, <i>U. maydis</i>	k 137		Ile-Gly-Lys-Arg*Gly (this work)
KP1, <i>U. maydis</i>	p 108		Ala-Pro-Lys-Arg*Gly (Banerjee et al., in preparation)
KP1, <i>U. maydis</i>	p 113		Val-Val-Lys-Arg*Asp (Banerjee et al., in preparation)

^a k, Known cleavage site, determined by amino-terminal or carboxy-terminal sequence analysis; p, predicted cleavage site.

^b Asterisks indicate cleavage sites.

larities in processing of the KP6 preprotoxin and the *S. cerevisiae* k1 preprotoxin (Table 1), we thought that the KP6 preprotoxin might be properly processed in *S. cerevisiae*. The KP6 preprotoxin may not (unlike the k1 preprotoxin) confer immunity (T. Peery and Y. Koltin, unpublished data), but this should not cause a problem, since *S. cerevisiae* is not susceptible to the effects of the KP6 toxin (data not shown). The KP6 toxin gene, from bases 103 to 1060 of P6 M2 (Fig. 1), was inserted into pMAC105 downstream of a *GAL/ENO* promoter in the correct orientation for expression of the plus strand, and a *KIL-o* strain of *S. cerevisiae* was transformed with the resultant plasmid (strain YHM59-502; see Materials and Methods). As expected, transcription occurred only in the presence of galactose and absence of glucose, and a transcript of about 1.5 kilobases resulted (not shown). Active KP6 toxin was secreted by the *S. cerevisiae* transformants, although not as copiously as from the P6 *Ustilago* strain (Fig. 5, well 6). The toxin was made only when the *GAL/ENO* promoter was induced by galactose, as expected (Fig. 5; compare wells 1 and 2 with wells 4 and 5). The toxin had the same specificity as authentic KP6 toxin: it

killed sensitive cells and P1 cells but not P6 cells (Fig. 5; compare the 18 and KP1 lawns with the KP6 lawn). Complementation with the KP6 alpha subunit, from NK13, was more effective than complementation with the beta subunit, from NK3 (Fig. 5; compare well 5 with well 4). This result may be due to the glycosylation of alpha in *S. cerevisiae* but not in *U. maydis* (see below). Comparison with the control (NK13 plus NK3; wells 3 of 18 and KP1 lawns) shows that limiting amounts of both alpha and beta were secreted from the *S. cerevisiae* transformant YHM59-502, which is consistent with the results of SDS-PAGE (see below).

The secreted polypeptides of YHM59-502 were examined by SDS-PAGE (Fig. 6). There were a number of secreted polypeptides present in the medium from YHM59-502 grown in galactose (Fig. 6, lane 4) that were absent in the medium from the same strain grown in glucose (lane 3) or from the medium of a control strain with the vector alone grown in galactose (lane 2). Among these were a prominent polypeptide with the mobility of authentic KP6 beta (9 kDa; marked beta) and a polypeptide with the size expected (2) of alpha N glycosylated with a single carbohydrate core (8.6 kDa + 2.6

FIG. 5. Killer test with recombinant KP6 polypeptides. Small amounts of medium containing various killer toxins were placed on lawns of sensitive cells (strain 18) or of KP1 or KP6 cells, and the plates were incubated at room temperature for 2 days. Positive controls were NK13 plus NK3 media on the strain 18 and KP1 lawns or KP1 medium on the KP6 lawn (spot 3). On the strain 18 and KP1 lawns were placed the following: 1, 4 μ l of NK3 medium concentrated 10-fold plus 8 μ l of YHM59-502 YPD medium concentrated 50-fold; 2, 4 μ l of NK13 medium concentrated 10-fold plus 8 μ l of YHM59-502 YPD medium concentrated 50-fold; 3, 2 μ l of NK3 medium concentrated 10-fold plus 2 μ l of NK13 medium concentrated 10-fold; 4, 4 μ l of NK3 medium concentrated 10-fold plus 8 μ l of YHM59-502 YPGal medium concentrated 50-fold; 5, 4 μ l of NK13 medium concentrated 10-fold plus 8 μ l of YHM59-502 YPGal medium concentrated 50-fold; and 6, 8 μ l of YHM59-502 YPGal medium concentrated 50-fold. Applications to the KP6 lawn were the same except that the positive control (spot 3) contained 1 μ l of KP1 medium concentrated 25-fold.

FIG. 6. Secretion of KP6 alpha and beta in *S. cerevisiae*. Media from the growth of various strains were concentrated, partially purified (see Materials and Methods), and applied to an SDS-18% polyacrylamide gel. The gel was silver stained. The samples applied were as follows: lane 1, *U. maydis* KP6; lane 2, LL20::pMAC105 grown in YPGal; lane 3, YHM59-502 grown in YPD; lane 4, YHM59-502 grown in YPGal; lane 5, YHM59-502 grown in YPGal and treated with endo H; and lane 6, YHM59-502 grown in YPGal and mock treated. Lane 1 contains 1 μ l of 20-fold-concentrated KP6 medium; lanes 2 to 6 contain 1 μ l of medium concentrated about 1,200-fold. Positions of alpha and beta subunits are indicated to the left of each relevant lane. Size markers were the low-molecular-weight standards from Bethesda Research Laboratories; their sizes are indicated in kilodaltons on the right.

kDa = 11.2 kDa; marked alpha). This latter polypeptide ran more slowly than KP6 beta, as expected, with an apparent size of 11.8 kDa. After deglycosylation with endo H, this band disappeared and a new band with a mobility slightly less than that of authentic KP6 alpha appeared (lane 5). This band had a mobility corresponding to 7.8 kDa, as opposed to an apparent size of 7.5 kDa for authentic alpha. This result is probably due to the residual *N*-acetylglucosamine left by endo H digestion, which would increase the molecular size of alpha by 221 D. We conclude that, as expected from the presence of KP6 toxin activity in the YHM59-502 medium, RNA transcribed from the P6 M2 cDNA is correctly translated in *S. cerevisiae*, the preprotoxin is correctly processed, and functional alpha and beta polypeptides are secreted. Unlike the secreted product of *U. maydis*, the alpha subunit secreted in *S. cerevisiae* is *N* glycosylated, presumably at the one possible site (residue 100 of the preprotoxin). This may account for the lower specific activity of the alpha subunit secreted from the heterologous host (Fig. 5; compare wells 4 and 5). As expected from the killer toxin assays, much less KP6 toxin was secreted from YHM59-502 than from the *Ustilago* KP6 strain (Fig. 6; compare lanes 1 and 4).

The presence of the authentic KP6 beta polypeptide in medium of YHM59-502 was confirmed by probing Western transfers with antibody Ab22-23, specific for the KP6 beta subunit (Fig. 7). This antibody was made against a peptide encompassing residues 41 to 55 of the KP1 beta sequence of Fig. 8. It recognized a number of glycosylated species of KP1 beta, the most prominent of which was about 21 kDa (Banerjee et al., in preparation; Fig. 7, lane 1), as well as cross-reacted with KP6 beta (lane 2). YHM59-502 grown in galactose medium secreted a polypeptide the same size as authentic KP6 beta that was recognized by Ab22-23 (lane 5). This band was rather diffuse because of overloading of the

FIG. 7. Secretion of KP6 beta in *S. cerevisiae*. Secreted proteins were partially purified (see Materials and Methods), concentrated and separated on an SDS-18% polyacrylamide gel, transferred to nitrocellulose, and probed with Ab22-23 (see Materials and Methods). Lanes: 1, KP1 strain (5 μ l of 20-fold-concentrated medium); 2, KP6 strain (20 μ l of 20-fold-concentrated medium); 3, *S. cerevisiae* strain carrying only the expression vector; 4, *S. cerevisiae* strain (YHM59-502) expressing the KP6 preprotoxin grown in glucose medium (uninduced); 5, YHM59-502 grown in galactose medium (induced). Lanes 3 to 5 contain 40 μ l of about 2,500-fold-concentrated medium. The major glycosylated species of P1 M1 beta (21 kDa), the KP6 beta polypeptide, and the major secreted species of KP6 beta produced in yeast cells are indicated. Size markers were as for Fig. 6.

gel, and it was not as dark as expected because of interfering substances present in the YHM59-502 medium not as concentrated in the *Ustilago* medium. This species was not present in the medium of YHM59-502 grown in glucose (lane 4). No cross-reacting species were present in the medium of the control transformant harboring only the vector without the P6 M2 cDNA and grown in galactose (lane 3). We conclude that YHM59-502 secretes authentic KP6 beta.

DISCUSSION

Similarity of the KP6 killer toxin alpha polypeptide to the cytotoxins. The sequences of KP6 toxin polypeptides were compared with all sequences in the NBRF data bank, using the Genetics Computer Group program WORDSEARCH (13) and the FASTP program (32), and with sequences of other known killer toxins, using the programs COMPARE and GAP (13). The only similarity detected with possible biological significance was a rather tenuous primary sequence similarity of KP6 alpha to the scorpion neurotoxins. These proteins are related to the snake venom neurotoxins (1, 34, 47, 49) and cytotoxins (34, 38, 47), which are known to affect ion channels in sensitive cells (25, 38). This similarity with the cytotoxins is not scored as significant by the RDF program (32), but the hydrophobicity profiles of the cytotoxins and KP6 alpha are rather similar (Fig. 9). The elapid neurotoxins and cytotoxins are basic proteins of some 60 to 80 amino acids in length, with eight cysteines and four intramolecular disulfides, similar primary sequences, and essentially identical three-dimensional structure (25, 38, 47, 49). The KP6 alpha polypeptide is of similar length (78 amino acids), also has eight cysteines, and is known to require intramolecular disulfides for activity (37).

The role of the alpha polypeptide in *S. cerevisiae* k1 toxin (4, 19, 45) is the interaction with the cell membrane that kills the cell by disrupting ion transport (8, 12, 23). This is similar to the mode of action of the cytotoxins (25, 38). The KP6 and

```

7  GPVPDGGQGD.TYGYTADGNP.TNVNENDVH.SAATEIDNOIDDYIYGROTLT. 56
   |. | . .: : |.:|   |. :. | .||. ||. ||:|. :.:.:
1  GKRPRPVMCQCVDTTNGG...VRLDAVTRAACSIDSFIDGYYTEKDGF.C 46

57 PVITANIEDPYLGRVGTLEADFTFDHNFQSDDPDD 92
   . |... .: :.: | : :. |. .: :.:
47 R...AKYSWDLFTSGQFYQACLRYSHAGTNCQPD.Q 79

```

FIG. 8. Sequence comparison of KP6 beta (lower line) and the putative KP1 beta (upper line). Alignment was performed with the program GAP (13). The P1 M1 beta peptide used to generate antibody Ab22-23 is underlined. The putative KP1 beta sequence is from Banerjee et al. (in preparation). Symbols: |, identity; :, similarity.

FIG. 9. Hydrophobicity of KP6 alpha and cytotoxins and the beta polypeptides of the ScV and UmV killer toxins. The program PEPLOT was used to plot hydrophobicity, using the Kyte-Doolittle algorithm, with a window of five (alpha) or nine (beta) amino acids. Hydrophobic sequences are above the axis and hydrophilic sequences are below, with a maximum value of 3 indicated on the scale. The hydrophobicity of the cytotoxin consensus sequence (see text) is plotted in the upper panel; this is superimposed on the hydrophobicity of the KP6 alpha polypeptide in the lower panel. Hydrophobicities of the four beta subunits are shown: the hydrophobicities of UmV KP6 beta and ScV k1 or UmV KP1 beta and ScV k2 beta are superimposed in the lower two panels for comparison. The hydrophobicity of each polypeptide is plotted in its entirety except for KP1 beta, for which only the first 86 amino acids are shown. Sequences were from the following sources: P1 M1 beta (Banerjee et al., in preparation); ScV k2 beta (Whiteway et al., personal communication); and ScV k1 beta (5, 42).

KP1 alpha polypeptides might also have this function. Highly purified KP6 toxin does not have the nuclease activities previously reported (31; I. Ginsberg and Y. Koltin, unpublished data), and we are currently trying to ascertain its mode of action. The ScV k1 alpha subunit is also known to have a role in providing immunity prior to secretion, possibly in the form of a precursor or alternatively processed precursor (4, 19, 45). The KP1 preprotoxin may have a similar function (Banerjee et al., in preparation). Some of the killer toxin alpha polypeptides are normally glycosylated (UmV KP1 and ScV k2), whereas others are not (UmV KP6 and ScV k1). The abnormal glycosylation of KP6 alpha in *S. cerevisiae* does not appear to abolish its activity, since no unglycosylated alpha is detectable.

Similarities among the killer toxin beta polypeptides. The KP6 beta polypeptide has detectable sequence similarities only to the putative KP1 beta polypeptide. This similarity is scored as possibly significant ($z = 4.0$) by the RDF program. The primary sequence similarity is most evident in the region of residues 26 to 45 of KP6 beta (Fig. 8). An antibody to a synthetic peptide from this region recognized both KP1 beta and KP6 beta (Banerjee et al., in preparation; Fig. 7). KP6 and KP1 beta are identical at five and similar at two additional positions within the 14 amino acids of this synthetic peptide.

Despite the lack of primary structure similarity, the hydrophobicity profiles of KP6 beta and ScV k1 beta are very similar, whereas the hydrophobicity profiles of KP1 beta and ScV k2 beta are nearly identical (Fig. 9). This suggests a relatedness of the killer toxin beta polypeptides. The k1 beta polypeptide is known to be responsible for interaction with the *S. cerevisiae* cell wall, facilitating interaction of the alpha polypeptide with the cell membrane (45). Therefore, this may also be the role of the KP6 and KP1 beta polypeptides. In *S. cerevisiae* k1, the beta subunit is joined by disulfide bonds to the alpha subunit; in UmV KP6 and in UmV KP1, it acts as a monomer. In some cases this region is glycosylated (UmV KP1), and in others it is not (ScV k1 and UmV KP6).

The KP6 alpha subunit is known to recognize some cellular receptor before interaction of the complex with KP6 beta, precipitating cell death (37). This function may be consistent with the roles for alpha and beta postulated above if the role of beta is not simply facilitating access of alpha to the cell membrane. Since, in contrast to the situation with the ScV k1 toxin, spheroplasts of *U. maydis* do not appear to be sensitive to the toxin (44), it is possible that some interaction of KP6 alpha and beta must occur within the cell wall or at the juncture of cell wall and cell membrane before interaction of KP6 alpha with the cell membrane.

Processing of preprotoxins. The processing of the KP6 preprotoxin and secretion of mature toxin polypeptides in *S.*

cerevisiae follow the precedents established for other fungal prepropeptides (21, 43), at least one of which is known to require the KEX2 product (21). Our results imply that the KEX2 product or associated receptors in the Golgi in *S. cerevisiae* (5, 7, 9, 10, 40) have their counterparts in *U. maydis* and that these receptors that normally interact with glycosylated precursors in *S. cerevisiae* are accessible to nonglycosylated precursors in *U. maydis*. At least among the killer toxins, the specificity of the KEX2-like enzyme includes an aliphatic amino acid at the -2 position. The existence of the Pro-Arg cleavage in two of the killer toxins (KP6 and k1) and its apparent existence in KP1 as well argue for the presence of another, undescribed processing enzyme in the secretory pathway in the fungi.

ACKNOWLEDGMENTS

We thank Michael Innis, Michael Holland, and Cetus Corp. for cDNA vectors and recipient strains; A. Constantinescu of Smith Kline & French Laboratories for synthetic peptides; Sandy Kielland for amino-terminal sequence analysis; and Dave Thomas for communication of results prior to publication.

This work was supported by U.S. Department of Agriculture grants 87CRCR12368 and 89372634716 (to J.A.B.), U.S.-Israel BNSF grant 86-00035 (to Y.K.), and Public Health Service grant GM22200 from the National Institutes of Health (to J.A.B.).

LITERATURE CITED

- Babin, D. R., D. D. Watt, S. M. Goos, and R. V. Mlejnek. 1974. Amino acid sequences of neurotoxic protein variants from the venom of *Centruroides sculpturatus* Ewing. Arch. Biochem. Biophys. **164**:694-706.
- Balou, C. E. 1982. Yeast cell wall and cell surface, p. 335-360. In J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces cerevisiae*: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Bassel-Duby, R., D. R. Spriggs, K. L. Tyler, and B. N. Fields. 1986. Identification of attenuating mutations on the reovirus type 3 S1 double-stranded RNA segment with a rapid sequencing technique. J. Virol. **60**:64-70.
- Boone, C., H. Bussey, D. Greene, D. Y. Thomas, and T. Vernet. 1986. Yeast killer toxin: site-directed mutations implicate the precursor protein as the immunity component. Cell **46**:105-113.
- Bostian, K. A., Q. Elliott, H. Bussey, V. Burn, A. Smith, and D. J. Tipper. 1984. Sequence of the preprotoxin dsRNA gene of type 1 killer yeast: multiple processing events produce a two-component toxin. Cell **36**:741-751.
- Bostian, K. A., S. Jayachandran, and D. J. Tipper. 1983. A glycosylated protoxin in killer yeast: models for its structure and maturation. Cell **32**:169-180.
- Bruenn, J. A. 1986. The killer systems of *Saccharomyces cerevisiae* and other yeasts, p. 85-108. In K. W. Buck (ed.), Fungal virology. CRC Press, Inc., Boca Raton, Fla.
- Bussey, H. 1981. Physiology of killer factor in yeast. Adv. Microb. Physiol. **22**:93-122.
- Bussey, H. 1988. Proteases and the processing of precursors to secreted proteins in yeast. Yeast **4**:17-26.
- Bussey, H., D. Saville, D. Greene, D. J. Tipper, and K. A. Bostian. 1983. Secretion of *Saccharomyces cerevisiae* killer toxin: processing of the glycosylated precursor. Mol. Cell. Biol. **3**:1362-70.
- Chang, T. S., N. Banerjee, J. A. Bruenn, W. Held, T. Peery, and Y. Koltin. 1988. A very small viral dsRNA. Virus Genes **2**:195-206.
- de la Pena, P., F. Barros, S. Gascon, P. S. Lazo, and S. Ramos. 1981. Effect of yeast killer toxin on sensitive cells of *Saccharomyces cerevisiae*. J. Biol. Chem. **256**:10420-10425.
- Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387-395.
- Diamond, M. E., J. J. Dowhanick, M. E. Nemeroff, D. F. Pietras, C.-L. Tu, and J. A. Bruenn. 1989. Overlapping genes in a yeast dsRNA virus. J. Virol. **63**:3983-3990.
- Dmochowska, A., D. Dignard, D. Henning, D. Y. Thomas, and H. Bussey. 1987. Yeast KEX1 gene encodes a putative protease with a carboxypeptidase B-like function involved in killer toxin and alpha-factor precursor processing. Cell **50**:573-584.
- Field, L., J. Bruenn, T. Chang, O. Pinhasi, and Y. Koltin. 1983. Two *Ustilago maydis* viral dsRNAs of different size code for the same product. Nucleic Acids Res. **11**:2765-2778.
- Fuller, R. S., R. E. Sterne, and J. Thorner. 1988. Enzymes required for yeast prohormone processing. Annu. Rev. Physiol. **50**:345-362.
- Gross, E. 1967. The cyanogen bromide reaction. Methods Enzymol. **11**:238-255.
- Hanes, S. D., V. E. Burn, S. L. Sturley, D. J. Tipper, and K. A. Bostian. 1986. Expression of a cDNA derived from the yeast killer preprotoxin gene: implications for processing and immunity. Proc. Natl. Acad. Sci. USA **83**:1675-1679.
- Held, W. A., K. West, and J. F. Gallagher. 1977. Importance of initiation factor preparations in the translation of reovirus and globin mRNAs lacking a 5'-terminal 7-methylguanosine. J. Biol. Chem. **252**:8489-8497.
- Innis, M. A., M. J. Holland, P. C. McCabe, G. E. Cole, V. P. Wittman, R. Tal, K. W. K. Watt, D. H. Gelfand, J. P. Holland, and J. H. Meade. 1985. Expression, glycosylation, and secretion of an *Aspergillus* glucoamylase by *Saccharomyces cerevisiae*. Science **228**:21-26.
- Julius, D., A. Brake, L. Blair, R. Kunisawa, and J. Thorner. 1984. Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-alpha-factor. Cell **37**:1075-1089.
- Kagan, B. 1983. Mode of action of yeast killer toxins: channel formation in lipid bilayer membranes. Nature (London) **302**:709-711.
- Kandel, J., and Y. Koltin. 1978. Killer phenomenon in *Ustilago maydis*: comparison of the killer proteins. Exp. Mycol. **2**:270-278.
- Karlsson, E. 1979. Chemistry of protein toxins in snake venoms. Handb. Exp. Pharmacol. **52**:159-204.
- Koltin, Y. 1977. Virus-like particles in *Ustilago maydis*: mutants with partial genomes. Genetics **86**:527-543.
- Koltin, Y. 1988. The killer system of *Ustilago maydis*: secreted polypeptides encoded by viruses, p. 209-243. In Y. Koltin and M. Leibowitz (ed.), Viruses of fungi and simple eukaryotes. Marcel Dekker, Inc., New York.
- Koltin, Y., and J. S. Kandel. 1978. Killer phenomenon in *Ustilago maydis*: the organization of the viral genome. Genetics **88**:267-276.
- Koltin, Y., R. Levine, and T. Peery. 1980. Assignment of functions to segments of the dsRNA genome of the *Ustilago* virus. Mol. Gen. Genet. **178**:173-178.
- Koltin, Y., I. Mayer, and R. Steinlauf. 1978. Killer phenomenon in *Ustilago maydis*: mapping of viral functions. Mol. Gen. Genet. **166**:181-186.
- Levine, R., Y. Koltin, and J. S. Kandel. 1979. Nuclease activity associated with the *Ustilago maydis* virus induced killer proteins. Nucleic Acids Res. **6**:3717-3731.
- Lipman, D. J., and W. R. Pearson. 1985. Rapid and sensitive protein similarity searches. Science **227**:1435-1441.
- Lolle, S. J., and H. Bussey. 1986. In vivo evidence for posttranslational translocation and signal cleavage of the killer preprotoxin of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6**:4274-4280.
- Miranda, F., C. Kupeyan, B. Rochat, C. Rochat, and S. Lissitzky. 1970. Purification of animal neurotoxins. Isolation and characterization of eleven neurotoxins from the venoms of the scorpions *Androctonus australis Hector*, *Buthus occitanus tuncetanus*, and *Leirus quinquestriatus quinquestriatus*. Eur. J. Biochem. **16**:514-523.

35. Nemeroff, M. E., D. F. Pietras, and J. A. Bruenn. 1988. Construction of full-length cDNA copies of viral double-stranded RNAs. *Virus Genes* 1:243-253.
36. Peattie, D. A. 1979. Direct chemical method for sequencing RNA. *Proc. Natl. Acad. Sci. USA* 76:1760-1764.
37. Peery, T., T. Shabat-Brand, R. Steinlauf, Y. Koltin, and J. Bruenn. 1987. The virus encoded toxin of *Ustilago maydis*—two polypeptides are essential for activity. *Mol. Cell. Biol.* 7: 470-477.
38. Rees, B., J. P. Samma, J. C. Thierry, M. Gilbert, J. Fischer, H. Schweitz, M. Lazdunski, and D. Moras. 1987. Crystal structure of a snake venom cardiotoxin. *Proc. Natl. Acad. Sci. USA* 84:3132-3136.
39. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. Sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
40. Schekman, R. 1985. Protein localization and membrane traffic in yeast. *Annu. Rev. Cell Biol.* 1:115-143.
41. Schreier, M. H., and T. Staehelin. 1973. Initiation of mammalian protein synthesis: the importance of ribosome and initiation factor quality for the efficiency of in vitro systems. *J. Mol. Biol.* 73:329-349.
42. Skipper, N., D. Y. Thomas, and P. C. Lau. 1984. Cloning and sequencing of the preprotoxin-coding region of the yeast M1 double-stranded RNA. *EMBO J.* 3:107-111.
43. Stark, M. J. R., and A. Boyd. 1986. The killer toxin of *Kluyveromyces lactis*: characterization of the toxin subunits and identification of the genes which encode them. *EMBO J.* 5: 1995-2002.
44. Steinlauf, R., T. Peery, Y. Koltin, and J. Bruenn. 1988. The *Ustilago maydis* virus encoded toxin—effect of KP6 on cells and spheroplasts. *Exp. Mycol.* 12:264-274.
45. Sturley, S. L., Q. Elliot, J. LeVitre, D. J. Tipper, and K. A. Bostian. 1986. Mapping of functional domains within the *Saccharomyces cerevisiae* type 1 killer preprotoxin. *EMBO J.* 5:3381-3389.
46. Sugisaki, Y., H. Gunge, K. Sakaguchi, M. Yamasaki, and G. Tamura. 1983. *Kluyveromyces lactis* killer toxin inhibits adenylate cyclase of sensitive yeast cells. *Nature (London)* 304: 464-466.
47. Tamiya, N., and T. Yagi. 1985. Non-divergence theory of evolution: sequence comparison of some proteins from snakes and bacteria. *J. Biochem.* 98:289-303.
48. Tokunaga, M., A. Kawamura, and F. Hishinuma. 1989. Expression of pGKL killer 28K subunit in *Saccharomyces cerevisiae*: identification of 28K subunit as a killer protein. *Nucleic Acids Res.* 17:3435-3446.
49. Tsernoglou, S., and G. A. Petsko. 1977. Three-dimensional structure of neurotoxin a from venom of Philippines sea snake. *Proc. Natl. Acad. Sci. USA* 74:971-974.
50. von Heijne, G. 1985. Signal sequences. The limits of variation. *J. Mol. Biol.* 184:99-105.
51. White, J. H., A. R. Butler, and M. J. R. Stark. 1989. *Kluyveromyces lactis* toxin does not inhibit yeast adenylate cyclase. *Nature (London)* 341:666-668.
52. Wray, W., T. Bouliskas, V. P. Wray, and R. Hancock. 1981. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* 118:197-203.
53. Young, T. 1987. Killer yeasts, p. 131-164. In A. H. Rose and J. S. Harrison (ed.), *The yeasts*, vol. 2. Yeasts and the environment. Academic Press, Inc., Orlando, Fla.
54. Zhu, H., H. Bussey, D. Y. Thomas, J. Gagnon, and A. W. Bell. 1987. Determination of the carboxyl termini of the alpha and beta subunits of yeast K1 killer toxin. Requirement of a carboxypeptidase B-like activity for maturation. *J. Biol. Chem.* 262: 10728-10732.