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Mapping of functional domains within the *Saccharomyces cerevisiae* type 1 killer preprotoxin

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Strains of *Saccharomyces cerevisiae* harboring M_1-dsRNA, the determinant of type 1 killer and immunity phenotypes, secrete a dimeric 19-kd toxin that kills sensitive yeast cells by the production of cation-permeable pores in the cytoplasmic membrane. The preprotoxin, an intracellular precursor to toxin, has the domain sequence δ-α-γ-β where α and β are the 9.5- and 9.0-kd subunits of secreted toxin. Plasmids containing a partial cDNA copy of M_1, in which α, γ and β are fused to the *PHO5* promoter and signal peptide, have previously been shown to express phosphate-repressible toxin production and immunity. Here the construction of a complete DNA copy of the preprotoxin gene and its mutagenesis are described. Analysis of the expression of these mutants from the *PHO5* promoter elucidates the functions of the preprotoxin domains. δ acts as a leader peptide and efficiently mediates the secretion, glycosylation and maturation of killer toxin. Mutations within the β subunit indicate it to be essential for binding of toxin to and killing of whole cells but unnecessary for the killing of spheroplasts. Mutations within the putative active site of α prevent killing of both cells and spheroplasts. The probable role of β is therefore recognition and binding to the cell wall receptor whereas α is the active ionophore. Mutations within α causing loss of toxicity also cause loss of immunity, while the mutants described within γ and β retain partial or complete immunity. Expression of γ without α or β confers no phenotype. The immunity determinant may minimally consist of the α domain and the N-terminal portion of γ. Two new models for immunity are considered, where either protoxin or a processed form of protoxin functions as the immunity determinant.

Key words: Saccharomyces cerevisiae/killer/immunity/secretion/acid phosphatase

Introduction

The *Saccharomyces cerevisiae* type 1 killer system is characterized by secretion of a dimeric toxin consisting of two distinct 9.5- and 9.0-kd peptide subunits. These subunits are disulphide linked and unglycosylated and are termed α and β respectively (Bostian *et al.*, 1984). Toxin production and specific immunity to this toxin are encoded by a 1.9-kb dsRNA species, M_1, which is encapsidated in 40-nm icosahedral virus-like particles (VLPs). The VLP capsid is encoded by L_1-dsRNA (4.7 kb) which is separately encapsidated. The two VLP types are called ScV-M_1 and ScV-L_1 (Hopper *et al.*, 1977; Bostian *et al.*, 1980b). The toxin recognizes and binds to a 1,6-β-*D*-glucan yeast cell wall receptor in both sensitive cells (lacking M_1-dsRNA) and cells containing ScV-M_1 (immune killers). Sensitive cells are then killed by the disruption of cytoplasmic membrane functions leading to the formation of cation-permeable pores (Hutchins and Bussey, 1983; Tipper and Bostian, 1984).

Polyclonal antibody raised against secreted toxin precipitates a membrane-associated protoxin from extracts of killer cells. Protoxin is a 43-kd, glycosylated form of preprotoxin (see below) and is a direct precursor of α and β (Bostian *et al.*, 1983b; Bussey *et al.*, 1983). Comparison of the N-terminal amino-acid sequence of α and β with the nucleotide sequence of a cloned complementary DNA copy of the *in vivo* transcripts of M_1-dsRNA has localized the coding regions for these subunits within a single 316-amino acid open reading frame called preprotoxin (Figure 1; Bostian *et al.*, 1984; Lolle *et al.*, 1984). This preprotoxin is presumably identical to M_1-P1 (Bostian *et al.*, 1980a), a 35-kd protein synthesized by *in vitro* translation of denatured M_1-dsRNA or its *in vivo* transcripts (Bostian *et al.*, 1983a). The α subunit of preprotoxin is preceded by a 44-amino acid N-terminal segment called δ. α and β are separated by γ, a segment of approximately 100 amino acids, carrying all three N-glycosyl substituents of protoxin. This large, non-toxin component is an obvious candidate for the immunity determinant, as previously postulated (Bostian *et al.*, 1984). Analysis of the predicted amino acid sequence of preprotoxin (Figure 1) shows a hydrophobic membrane spanning peptide sequence in δ, between residues 12 and 27, postulated to act as a secretion leader sequence (Bostian *et al.*, 1984; Tipper and Bostian, 1984; Hanes *et al.*, 1986). The same analysis predicts the 86-amino acid α domain to possess two highly hydrophobic regions (residues 72-91 and 112-127) separated by a short, highly hydrophilic region containing many charged amino acids and all of the cysteine residues found in α. It has been postulated (Tipper and Bostian, 1984) that insertion of this region of α into a membrane could create the cation channels responsible for cell death. The C-terminal domain of preprotoxin, β (83 amino acids), lacks potential membrane spanning regions. However, by analogy to the abrin and ricin class of toxins (Olsnes and Phil, 1973), this subunit has been proposed (Bostian *et al.*, 1984) to function as a ligand for the 1,6-β-*D* glucan cell wall receptor encoded by the *KRE1* and *KRE2* genes (Al-Aidroos and Bussey, 1978). Consistent with these predictions, it has been shown (Hanes *et al.*, 1986) that the preprotoxin cDNA, in which most of δ is replaced by the *PHO5* acid phosphatase secretion signal peptide, confers both killing and immunity phenotypes when expressed from the *PHO5* promoter in an M_1-dsRNA-free yeast strain.

The processing of preprotoxin in wild-type killer cells is governed by the *SEC* and *KEX* genes and may also require the action of enzymes sensitive to the chymotrypsin inhibitor, tosyl-*L*-phenylalanylchloromethylketone (Tipper and Bostian, 1984; Hanes *et al.*, 1986). Lesions in any of these genes result in intracellular accumulation of protoxin and failure to secrete toxin (Bussey *et al.*, 1983). However, *kex1* and *kex2* mutants which carry ScV-M_1 retain immunity (Wickner and Liebowitz, 1976).

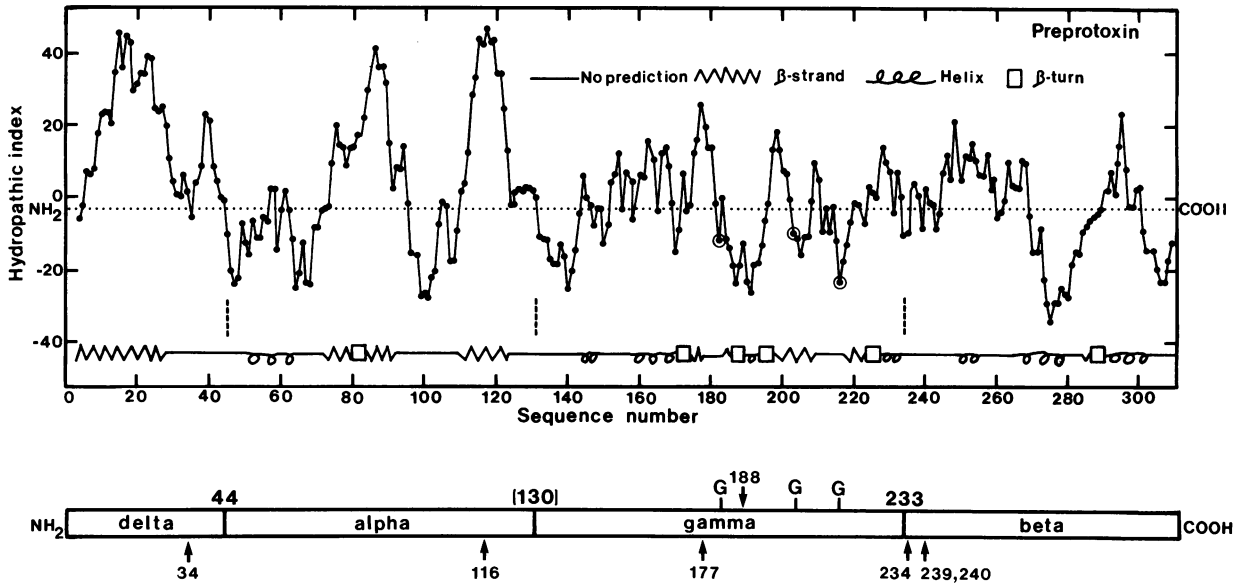

Fig. 1. Predicted hydropathicity and secondary structure of the type 1 yeast killer preprotoxin: secondary structures were predicted by the method of Chou and Fasman (1974). The hydropathic index was calculated according to Kyte and Doolittle (1982). Circled residues are the predicted points (Asn-X-Thr/Ser) of glycosylation in protoxin. Also shown, in the lower panel, is the domain structure of the type 1 killer protoxin: numbers indicate the amino acid residues of protoxin, with potential processing and glycosylation sites (G) as shown. The positions of the mutations introduced in this study are indicated by arrows.

The *KEX2* gene product is an endopeptidase with specificity for cleaving on the carboxyl side of paired basic residues, preferentially Lys-Arg (Julius *et al.*, 1984). The γ-β junction (LysArgTyr-Val) is a likely *KEX2* cleavage site. Cleavage here would be necessary for release of β, but is apparently not essential for immunity. A second potential *KEX2* cleavage site (LysArgSerAsp) occurs within the γ domain, at residue 188 (Figure 1).

To elucidate further the mechanisms involved in the maturation and function of type 1 yeast killer toxin and immunity determinant, the natural 5′ terminus of the preprotoxin cDNA (the δ sequence) was reconstructed, the now complete preprotoxin gene fused to the *PHO5* promoter and using site-directed mutagenesis and linker insertions, novel alleles of this gene were constructed. The phenotypes resulting from the phosphate-regulated expression of these mutations have confirmed the predicted functions of the α and β subunits. In addition, these analyses have allowed our theories concerning the mechanism of immunity to be refined. γ alone is clearly insufficient, although it may play a role in immunity as part of the precursor. Two new models are presented involving either differential processing or accumulation of protoxin leading to the production of an immune yeast cell.

Results

Expression of the complete preprotoxin gene from the PHO5 promoter: construction of the PTX1 allele

The complete protoxin coding sequences were constructed and fused to the *PHO5* promoter (Figure 2). Eight overlapping, phosphorylated, single-stranded oligonucleotides were annealed to form a double-stranded molecule with sequences from the ATG of protoxin to the *PstI* site at base 115. An 11-nucleotide sequence 5′ to the ATG, containing an *Eco*RI site, was designed to be ligated to an *Eco*RI site just upstream of the *PHO5* initiator ATG, created by altering 2 bp of the native sequence. The reconstructed leader was combined with the remainder of the preprotoxin gene derived from cDNA clones and the *PHO5* promoter segment in the shuttle vector p1A1-R1 to yield the pSH series of plasmids. Three of these, pSH4, 6 and 15, were shown to possess the predicted gene fusion *PTX1-316* (PHO5-ToXin 1). The 'allele' designation, 316, refers to the position of the last amino acid prior to translation termination. This nomenclature will be used for mutations in the *PHO5*-preprotoxin gene where the number indicates the position of the first alteration from the wild-type sequence. When used to transform cells of the M_1-dsRNA-free, non-killer yeast strain, GG100-14D, the *PTX1-316* constructs expressed strong, phosphate-regulated killing and immunity phenotypes. Levels were marginally higher than those obtained with strain K12-1, a wild-type killer harboring M_1-dsRNA. Killing expressed by the *PTX1-316* allele was 2- to 4-fold higher than expressed by pSH-GB1, where the majority of δ was replaced with the *PHO5* secretion leader (Hanes *et al.*, 1986). pSH-GB1 transformants expressed only ~30% of the immunity shown by the wild-type construct.

Northern blot hybridization analysis (Figure 3) of total RNA from these transformants confirmed the regulated expression of the *PTX1-316* gene. Two major transcripts of ~2.1 and 2.9 kb were present in cells grown on low-P_i (derepressing) medium. Relatively efficient transcription termination occurred at two sites within vector sequences downstream of the *PTX1-316* allele, probably at the start and end of the *URA3* gene. As in pSH-GB1 transformants, the absence of a separate transcription termination sequence did not preclude high levels of expression of preprotoxin transcripts (Hanes *et al.*, 1986).

In vitro translation (Figure 4, lane h) of total RNA from derepressed cells harboring *PTX1-316* produced a 35-kd protein immunoprecipitable with anti-toxin IgG. This had the same mobility as M_1-P1, the immunoprecipitable translation product of M_1-dsRNA transcripts isolated from strain K12-1 (Figure 4, lanes a and b). The *PHO5* leader construct (pSH-GB1) produced a 33-kd translation product due to the 14-amino acid reduction in size of its N-terminal sequence (Figure 4, lane e).

The predominant protein in extracts of membrane fractions of derepressed *PTX1-316* transformant cells, revealed by im-

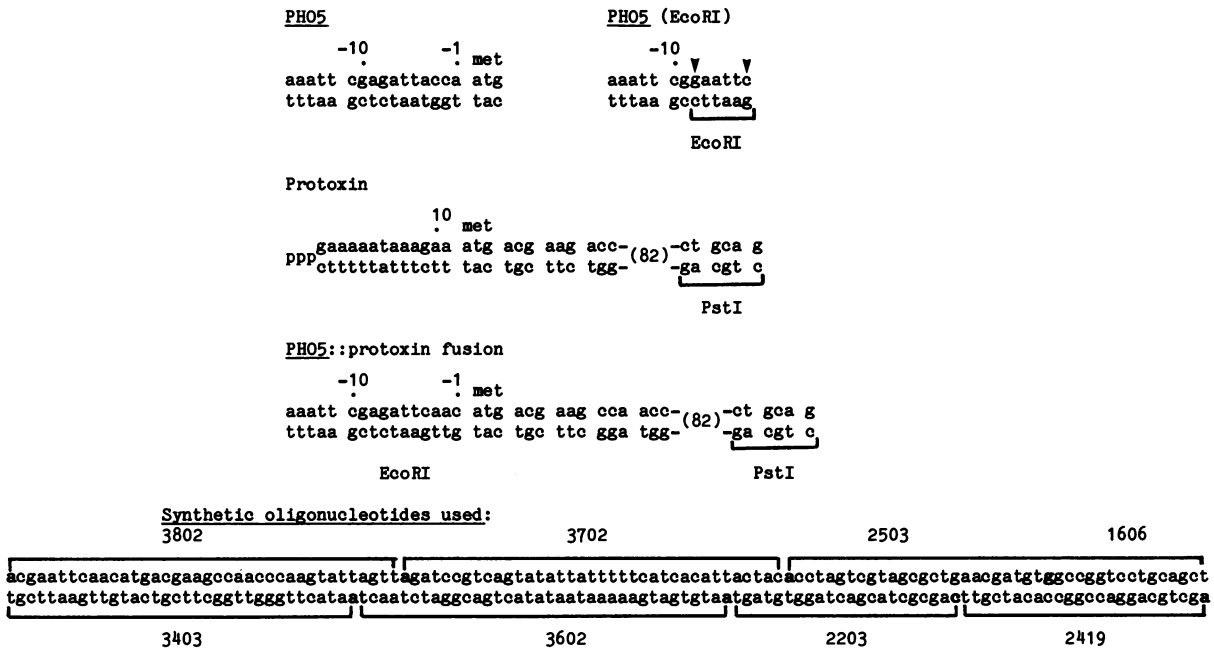

Fig. 2. Reconstruction of the δ sequence: the synthetic oligomers shown (lower panel) were phosphorylated, annealed and then digested with *Eco*RI and *Pst*I. This 115-bp complex was ligated to the *Pst*I site within the δ domain of the preprotoxin cDNA and the *Eco*RI site of the acid phosphatase (PHO5) promoter (in p1A1-R1). The *Eco*RI site was introduced into the wild-type *PHO5* sequence (from p1A1) by site-directed mutagenesis as shown.

munoblotting with anti-toxin IgG, was a 43-kd species identical in mobility to protoxin from wild-type strain K12-1 killer cells (Figure 5A, lanes d and f respectively). In addition, a less abundant 35-kd species was discernible, presumably equivalent to the preprotoxin, M_1-P1. Two minor protein species of intermediate mobility, ~38 and 40 kd, were also visible. Following endoglycosidase H treatment, all four species comigrated at 35 kd, indicating that the 43-kd species was normal protoxin, and the minor species were glycosylation intermediates containing one and two core units (data not shown). Four other immunoreactive proteins were also consistently present. Two species, of mol. wt 9.5 and 9.0 kd, represent either intracellular forms of the secreted α and β toxin subunits or, more probably, toxin eluted from cell wall fragments which are present in the membrane pellet. α was much less abundant than β in these preparations. Since the same disparity was not seen in immunoprecipitates of the secreted subunits (Figure 5B, lane d), it did not reflect differing affinities of the toxin antibody for α and β. Rather, it probably reflects selective binding of β to the cell wall. Two previously uncharacterized antigenic species are called p22 and p14, based on their respective mobilities. The possible origins of these proteins are discussed below.

The replacement of δ with the *PHO5* leader sequence in pSH-GB1 decreased processing efficiency. As previously reported (Hanes *et al.*, 1986), intracellular accumulation of the unglycosylated hybrid preprotoxin (Pre_{hy}) predominated over accumulation of the glycosylated form (Pro_{hy}; Figure 5, lane b). Accordingly, the processed forms of preprotoxin were present at correspondingly lower levels.

The *PTX1-316* constructs directed the secretion of α and β toxin subunits at high levels in derepressed cells. In contrast, approximately 10 times the equivalent concentration of supernatant derived from pSH-GB1 was necessary to detect the secreted toxin subunits (Figure 5B, lanes b and d). This is consistent with the absence of abundant β in the 'membrane' fractions from these cells (Figure 5A, lane b), if this β component was derived from

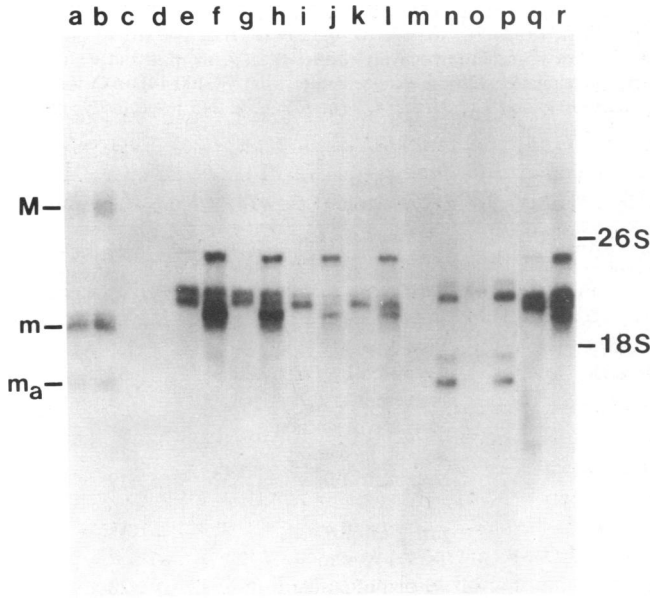

Fig. 3. Northern hybridization of RNA from mutant *PTX1* transformants: total yeast nucleic acid from strains K12-1 (**a**, **b**) and GG100-14D transformed with YEp24 (**c**, **d**), pSH-GB1 (**e**, **f**) or the *PTX1* alleles, *316* (**g**, **h**), *116* (**i**, **j**), *177* (**k**, **l**), *239* (**m**, **n**) *240* (**o**, **p**) and *234* (**q**, **r**) was extracted from cells grown on high-P_i (**lanes a**, **c**, **e**, etc.) and low-P_i (**lanes b**, **d**, **f**, etc.) media. Following electrophoresis in 1.5% agarose, 2.2 M formaldehyde gels and transfer to nitrocellulose, the RNAs were hybridized with the 944-bp *Eco*RI to *Bgl*I preprotoxin fragment of pSH4 comprising the entire preprotoxin gene. Mol. wt markers were the 25S and 18S ribosomal RNAs (1.5 and 3.0 kb respectively) and transcripts derived from the M_1-dsRNA plasmid (m and m_a, 1.9 and 1.2 kb respectively). M is the undenatured M_1-dsRNA plasmid present in strain K12-1.

secreted toxin bound to cell walls in these preparations. The smaller difference detected in relative toxin activity levels (Table I) probably represents non-linearity in the toxin assay at low concentrations.

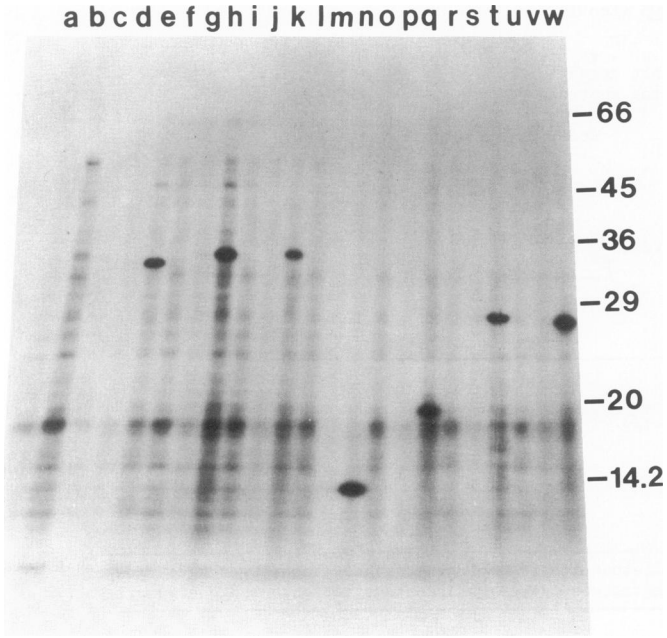

Fig. 4. Translation of preprotoxin mRNAs from *PTX1* transformants: total RNA was prepared, translated in a wheat-germ system using [^{3}H]leucine as label, the products precipitated with anti-toxin IgG and analysed by SDS–PAGE and autoradiography. RNA was from strains K12-1 (**a**, **b**) or GG100-14D harboring the pSH-GB1 plasmid (**c**, **e**) or the *PTX1*$^-$ alleles *316* (**f**, **h**), *234* (**i**, **k**), *116* (**l**, **n**), *177* (**o**, **q**), *239* (**r**, **t**) or *240* (**u**, **w**) grown on high- and low-P_i media respectively. **Lanes d**, **g**, **j**, **m**, **p**, **s** and **v** show the proteins precipitated with preimmune serum from GG100-14D transformed with pSH-GB1, or *PTX1-316*, *234*, *116*, *177*, *239*, *240* respectively, grown in low-P_i medium.

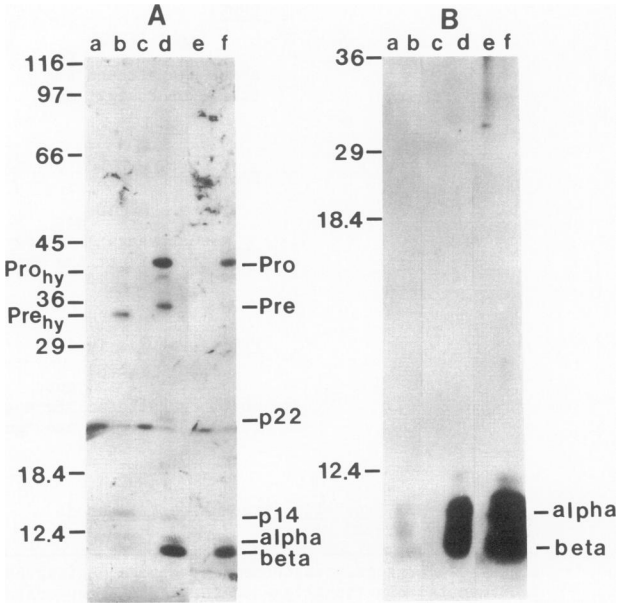

Fig. 5. *In vivo* synthesized proteins from *PTX1* transformants: proteins were fractionated by SDS-PAGE, transferred to nitrocellulose and incubated with anti-toxin IgG. Antigen–antibody complexes were envisaged as described in Materials and methods. **A**: Triton soluble, intracellular proteins were extracted from strain GG100-14D transformed with pSH-GB1 (**a**, **b**), *PTX1-316* (**c**, **d**) or YEp24 (**lane e**) and strain K12-1 (**lane f**). Cells were grown in high-P_i medium (**lanes a** and **c**) or low-P_i medium (**lanes b**, and **d-f**). **B:** Total secreted yeast proteins were concentrated by ultrafiltration and lyophilization from cell-free culture supernatants of strain GG100-14D transformed with pSH-GB1 (**a**, **b**), *PTX1-316* (**c**, **d**) or strain K12-1 (**e**, **f**) grown in high- or low-P_i media respectively. Pro, Pro$_{hy}$, Pre, Pre$_{hy}$, p22, p14, α and β are discussed in the text.

Table I. Phenotypes of mutant alleles of the *PTX1-316* protoxin gene

Preprotoxin mutant	Description	*In vitro* translation product	*In vivo* protein		Phenotype		Effect on spheroplasts
			Intracellular	Secreted	Kill. (rel)	Imm. (rel)	
PTX1-316	Wild-type	35	43[1]	9.5, 9.0	100	100	killed
pSH-GB1	δ replaced by *PHO5* leader	33	40[1]	9.5, 9.0	25	30	killed
PTX1-34	Frameshift at *Pst*I Stop at δ/α junction	5	ND	ND	0	0	ND
PTX1-116	Stop at *Spe*I in α	13	13	–	0	0	none
PTX1-177	Stop at *Acc*I in γ	19	19	–	0	15	partial killing
PTX1-234	first 2 aa of β TyrVal-AspPro	35	43[1]	9.5, 9.0	0	100	killed
PTX1-239	deletion of β after *Stu*1, Stop 16 aa into vector	28	36[1]	–	0	60	killed
PTX1-240	Stop at *Xho*I in β	26	34[1]	–	0	50	killed
SUP/SH6	*PTX1-316* in *SUP7* background	35	43.5[1]	9.5, 9.0	100	100	killed
SUP/SS26	*PTX1-116* in *SUP7*, In-frame	13	44	–	0	0	none
SUP/SA10	*PTX1-177* in *SUP7*, Out-of-frame	19	–	–	0	20	partial killing
SUP/SX1	*PTX1-240* in *SUP7*, Read-through into vector	29.5	38[1]	–	0	90	killed

Observed mol. wts of *in vitro* and *in vivo* synthesized proteins are in kd. Observed proteins were as predicted assuming core glycosylation at each of the asparagine glycosylation sites present (indicated [1] as shown by endoglycosidase H treatment of the same protein preparation). Killing and immunity phenotypes are expressed as % of the wild-type *PTX1-316* levels. The effect of the secreted proteins from these strains, grown under derepressed conditions, on spheroplasts of a sensitive strain was determined relative to the effect of culture supernatants from the same strains grown under repressed conditions. ND indicates not determined. – indicates no immunoreative protein detected.

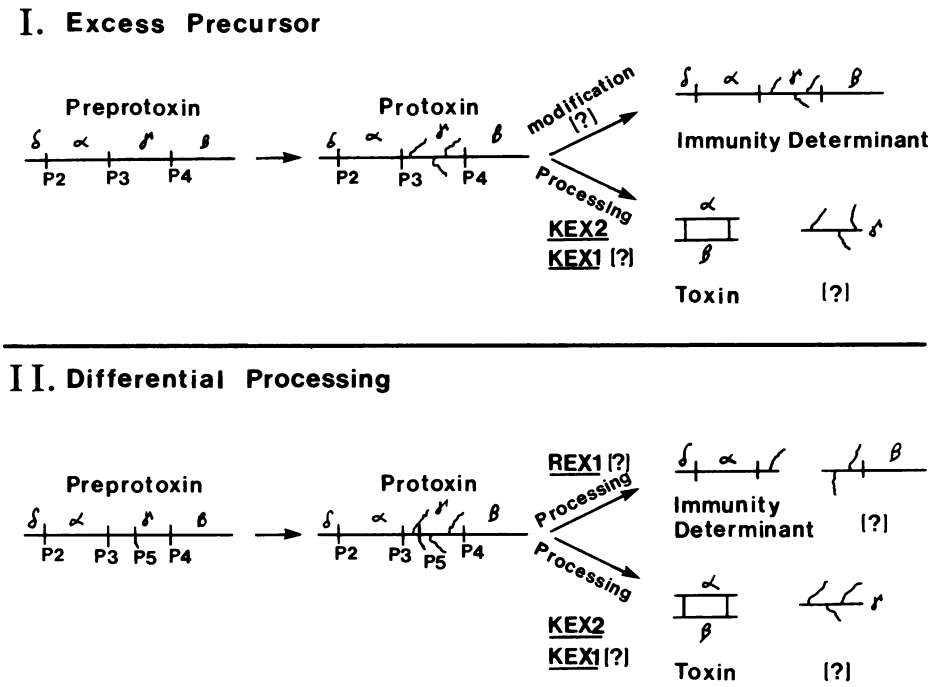

Fig. 6. Possible mechanism leading to immunity to killer toxin: two models are proposed; both suggest the binding via a common region of α to a membrane receptor (*KRE3*?). Thus, the receptor is altered so that it can no longer interact with α delivered from outside of the now immune cell. Model I (excess precursor) requires the initial binding of protoxin to the receptor to confer immunity, with the subsequent processing of the remaining protoxin to produce active α and β. Model II (differential processing) predicts two fates for an intracellular pool of protoxin, cleavage to give an immunity determinant (p22? See text) and an alternate series of proteolytic events to produce the secreted toxin subunits. Likely processing sites are indicated as P2−P5 using the nomenclature of Bostian *et al.*, 1984. P1 (not shown) is the predicted leader peptidase recognition site within δ. P5 is the alternate cleavage site at residue 188, suggested by this study. Glycosylation is denoted by the appendages to the γ domain. Likely host genes involved in these maturation processes are also shown and are discussed in the text.

Mutagenic analysis of the PTX1-316 preprotoxin allele

To characterize further the functional domains of the preprotoxin molecule, a series of mutations were introduced into *PTX1-316* and the phenotypes studied in pSH6 derived plasmids under derepressed conditions in strain GG100-14D. A combination of site-directed mutagenesis and linker insertion was used to create the mutant *PTX1* alleles indicated in Figure 1 and described in Table I.

Mutations were of several types. Insertion of the SH-TERM linker, CTAGTCTAGACTAG, introduced amber termination codons in all reading frames (Perlman and Halvorson, 1986) producing truncated protoxins (*PTX1-116, 177* and *240*). Use of a host strain carrying the tyrosine inserting *SUP7* mutation allowed analysis of expression of the 'read-through' products of these mutants. C-terminal deletions of the β region were also constructed by conventional means (*PTX1-239*) and termination within δ (*PTX1-34*) occurred as a result of a construction error. Finally, oligonucleotide mutagenesis was used to create a missense mutation (*PTX1-234*). The authenticity of these constructions was confirmed by DNA sequence analysis.

In each case, RNA blot hybridization demonstrated the phosphate-repressible production of transcripts whose sizes and concentrations were consistent with those seen in pSH6 (Figure 3). *In vitro* translation showed these transcripts to be functional, producing proteins precipitable by anti-toxin IgG of the predicted length (Figure 4). Immunoblotting of *in vivo* synthesized proteins (Table I) revealed the presence of modified forms of these proteins. Endoglycosidase H hydrolysis prior to immunoblotting demonstrated that these products probably differed from those synthesized *in vitro* only by *N*-glycosylation (data not shown).

The phosphate-regulated killer and immunity phenotypes of these mutants are summarized in Table I. In addition to assaying killer toxin against intact cells, the sensitivity of spheroplasts to proteins secreted by various transformants was also tested. Killing should be independent of the ability of toxin to bind to cell walls. As previously shown for strains secreting wild-type killer toxin (Bussey *et al.*, 1973), the concentrated supernatants from pSH6 or pSH-GB1 transformants were lethal to the spheroplasts of a sensitive non-killer strain while the spheroplasts of an isogenic M_1-dsRNA killer strain (GGMS1) were immune. Killer toxin is readily inactivated and boiled preparations had no effect on either sensitive or resistant spheroplasts.

The effect of amber suppression was studied by crossing GG100-14D transformants harboring the terminator constructs to *SUP7* strain SSY7-C. Suppression of the homozygous *trp1-1* amber mutation in these diploids was used to confirm the presence of the *SUP7* allele. The preprotoxin specific transcripts and their *in vitro* translation products were identical to those observed in the unsuppressed parental strain (data not shown). Translation termination of the wild-type *PTX1-316* allele should occur at an opal UGA codon, six residues downstream of normal termination. This C-terminal extension had no effects on the killer or immunity phenotypes. Similar opal terminators should occur on suppression of the mutant alleles studied here, at the positions indicated in Table I. The degree of suppression of the nonsense mutations was ~10−15%, as indicated by the intensity of the appropriate protein in immunoblots of membrane preparations from these strains (data not shown). This degree of suppression should produce levels of read-through preprotoxin products comparable to those seen in a wild-type killer (compare Figure 5A, lanes d and f). Their functions should, therefore, be readily detectable.

Mutations in α prevent expression of both active toxin and immunity

The *PTX1-116* allele (on pXTSS26) resulted from the insertion of SH-TERM within α at the unique *Spe*I site. The peptide AlaSerLeuAsp is inserted after residue 115 followed by a stop codon. The primary translation product of RNA from derepressed cells was a 13-kd protein consistent with the predicted point of truncation. Antitoxin detected a protein of the same size *in vivo* but at very low levels. No immunoreactive proteins were detected in culture supernatants. Transformants with this construct expressed neither killing nor immunity phenotypes. Furthermore sensitive spheroplasts were unaffected by the proteins secreted by these strains grown on low-P_i medium.

Suppression of the *PTX1-116* allele should result in the in-frame insertion of the peptide AlaSerLeuAspTyrLeu after residue 115 of the wild-type sequence of preprotoxin. This would be expected to disrupt the second hydrophobic region of α, producing a 44.1-kd full length protoxin. Such a species was observed by immunoblotting but only at ~10% of the level of wild-type protoxin produced by the *PTX1-316* allele in the same background (data not shown). This strain expressed neither killing nor immunity under derepressed conditions and no toxin-like peptides were detectable in culture supernatants. Sensitive spheroplasts were also resistant to the secreted proteins from this strain.

Mutations within λ and β prevent active toxin production but preserve immunity

The *PTX1-177* allele (on pXTSA10) was produced by the insertion of SH-TERM at the *Acc*I site within γ. Translation terminates after residue 177 of the wild-type molecule producing an *in vitro* translation product of 19.1 kd (Figure 4). An *in vivo* gene product of the same mobility was found in derepressed cell extracts but immunoreactive secreted proteins were not detectable. The *PTX1-177* allele had no killing phenotype against intact cells but did produce a low, but consistent level of immunity (~15% of *PTX1-316*). Secreted proteins killed sensitive spheroplasts, but much less efficiently than wild-type killer toxin.

Suppression of the *PTX1-177* allele should produce a frameshift after the insertion point. A protein of 28.7 kd should occur, consisting of 72 missense codons after residue 177 of preprotoxin. In a *SUP7* background, *PTX1-177* expressed slightly higher immunity (20% of *PTX1-316*), but as expected, failed to produce toxin with activity against intact cells. The intracellular product of suppression of this allele could not be detected, possibly due to changes in antigenic properties resulting from the altered C-terminus. No secreted toxin-like proteins were detected and killing of spheroplasts was not enhanced by suppression.

In the *PTX1-234* allele (on pDT5A), a *Bam*HI site at position 711 of the cDNA changes the Tyr-Val residues (234, 235) immediately downstream of the *KEX2* processing site in wild-type preprotoxin to Asp-Pro in the mutant. Transformants produced transcripts which, upon *in vitro* translation, gave a 35-kd product with the same mobility as the wild-type preprotoxin molecule (Figure 4). Similarly, the *in vivo* proteins detected in derepressed cell extracts by immunoblotting were identical to those found in wild-type *PTX1-316* cell extracts although a slightly enhanced accumulation of the protoxin molecule apparently occurred. α and β toxin components detected in culture supernatants were of normal size, but reduced to ~50% of the levels seen in *PTX1-316* transformants. The transformants were completely immune on low-P_i medium but lacked the ability to kill sensitive cells. However, the secreted proteins retained the ability to efficiently kill spheroplasts of a sensitive strain.

Similar site-directed mutagenesis was used to construct the *PTX1-239* allele (in pSHSX1). An *Xho*I site was introduced at position 728 of the cDNA, changing a cysteine residue (239) in wild-type preprotoxin to a leucine residue in the mutant. Due to a cloning artifact, the preprotoxin sequence downstream of the *Stu*I site (base pair 749, residue 244) was lost, resulting in the deletion of the C-terminus of β. Translation termination occurs 16 amino acids into the neighbouring vector sequence (derived from pUC18). This would predict a 28-kd protein to be the primary translation product of RNA from derepressed cells (Figure 4). Transcripts of approximately 2.2 and 1.4 kb were observed upon blot hybridization analysis of this construct (Figure 3), consistent with the 700-bp deletion of β and intervening pBR322 sequences. The *in vivo* product of this allele had a mobility of ~36 kd, reduced to 28 kd by endoglycosidase H treatment, indicating that the primary *PTX1-239* translation product was glycosylated to the same extent as wild-type protoxin. Normal processing apparently resulted in the low level production of the intracellular form of α, although this was undetectable outside the cell. Transformants grown on low-P_i media were immune, but at 50% of the level of wild-type *PTX1-316* transformants, although they lacked the ability to kill intact cells, proteins secreted by these strains were toxic to spheroplasts of sensitive strains.

Insertion of the termination linker at the *Xho*I site of *PTX1-239* produced the *PTX1-240* allele (in pXTSX1). It results in the addition of an asparagine residue and stop codon after Leu_{239} of *PTX1-239*. *PTX1-240* displayed similar attributes to its progenitor. The regulated production of a primary translation product of ~26 kd indicated accurate expression of this allele. This protein was completely glycosylated to yield a 34-kd protein *in vivo* and was also processed to intracellular α. *PTX1-240* conferred an immune non-killer phenotype and the proteins secreted by strains transformed with this gene were lethal to sensitive spheroplasts.

Read-through of the *PTX1-240* terminator by *SUP7* should produce a protoxin lacking the C-terminal sequences of β with the insertion, AspTyrSerArgLeuVal between Leu_{239} and Glu_{240} of the *PTX1-239* encoded protein. It displayed no phenotypic characteristics that distinguished it from the unsuppressed allele.

Expression of γ without α or β fails to confer immunity

The portion of preprotoxin cDNA from the *Spe*1 site in α to the *Stu*I site in β, comprising all of γ plus 45 bp of α and 30 bp of β, was fused in-frame to the *PHO5* promoter and leader. A synthetic oligonucleotide linker, used to insert this fragment into p1A1 (Parent *et al.*, 1985), inserts the sequence GlyThrIle between the leader peptidase cleavage site of acid phosphatase and the leucine residue (110) of preprotoxin. The nucleotide sequence of the hybrid gene was confirmed and efficient phosphate-regulated expression of the γ domain cDNA segment demonstrated by Northern blot analysis in transformants of strain GG100-14D. Entry into the endoplasmic reticulum and the subsequent co-translational cleavage of the *PHO5* leader peptide is expected to occur, based on the ability of a similar construct (pSH-GB1; Table I) to express protoxin. However, no immunity was detectable on either high or low phosphate medium.

Discussion

α is the lethal toxin subunit while β probably recognizes the cell wall receptor

Cells harboring the *PTX1-239* or *PTX1-240* alleles, deleted for most of the β sequence, encoded normal levels of preprotoxin

transcripts of the expected reduced length which were translated *in vitro* to produce truncated polypeptides of the predicted size presumably lacking all of β except the first six residues. A fully glycosylated truncated protoxin was seen *in vivo* and normal processing produced the α toxin component although this may have been inefficiently secreted or labile after secretion since it was only detected in intracellular protein fractions. Although intact cells were not killed, culture supernatants did kill spheroplasts of a sensitive strain (Table I). It was previously suggested (Tipper and Bostian, 1984) that α attacks the membrane and that its delivery to this site in intact cells is dependent on the interaction of the β component of the α−β toxin dimer with the cell wall glucan receptor. Our data is consistent with this hypothesis, as is the demonstration (unpublished observations) that detergent treatment of intact cells or cell walls of toxin-producing strains elutes large quantities of β but very little α. It was also suggested (Tipper and Bostian, 1984) that β might play a second role by masking the active site of α through disulfide bonding, contributing to immunity by preventing membrane insertion of α during secretion and protecting the producing cell. Since these transformants were also immune, a requirement of β in immunity is ruled out. Nevertheless, immunity was only 50−60% of wild-type and these cells grew slowly with reduced viability especially at lower temperatures favouring toxin activity (unpublished observations). Thus production of α without β appears to be deleterious. Amounts of secreted α were low, implying that the α−β interaction may be important in allowing export of this toxin subunit, a relatively hydrophobic protein.

Cells harboring *PTX1-234* were fully viable. They accumulated normal levels of a fully glycosylated protoxin of normal size, and processed it with secretion of near normal quantities of α and β subunits, identified by size and immunogenicity. The only difference from wild-type should be the replacement of Tyr-Val at the N-terminus of β by AspPro; apparently this was compatible with processing by the *KEX2* and other proteases and with facilitation of toxin export, but not with toxin activity on intact cells. However, secreted proteins killed spheroplasts as effectively as wild-type toxin. Moreover, very little of the modified β can be eluted from the cell walls of *PTX1-234* transformants, suggesting that the modified β fails to bind to the cell wall receptor (unpublished observations). The proposed functions of α and β in toxin action are substantiated by the phenotypes of these mutants.

The function of δ

δ has many of the features of a conventional secretion signal peptide, including a potential signal peptidase cleavage site (Tipper and Bostian, 1984) although cleavage may not occur *in vivo* (Hanes *et al.*, 1986). Replacement of most of δ by the *PHO5* signal peptide in pSH-GB1 clearly limited toxin secretion and led to marked accumulation of intracellular preprotoxin, presumably because of inefficient entry into the endoplasmic reticulum. Over-expression of the native *PHO5* gene results in a similarly delayed secretion (Haguenaur-Tsapis and Hinnen, 1984). Clearly δ provides a much more efficient signal for protoxin secretion. If δ persists in protoxin, it may also be of importance in providing a membrane anchor, possibly facilitating endopeptidase processing by the membrane-bound *KEX2* gene product (Julius *et al.*, 1984) in the Golgi.

The immunity determinant includes the active site of α

The immunity conferred by pSH-GB1 eliminates a role for all but the short residual C-terminal fragment of δ in immunity determination. This is consistent with the inability of the *PTX1-34* allele (in which translation terminates close to the δ−α boundary) to confer immunity (Table I). The γ domain is presumably a processing product of protoxin and is a rational candidate for the immunity conferred by expression of M_1-cDNAs (Lolle *et al.*, 1984; Hanes *et al.*, 1986). The fate of this fragment has not been determined. It would not be detected with antitoxin and anti-γ sera produced so far have been of low affinity and fail to detect any additional product (unpublished data). Although it was anticipated that expression of γ might confer immunity, an in-frame fusion of this entire region, together with 16 preceding residues of α and 11 succeeding residues of β, to the *PHO5* promoter-leader construct used in pSH-GB1 conferred no immunity or other phenotype. Our new data now demonstrate that mutations within the C-terminal half of α, encompassing the proposed toxin active site, eliminate production of both active toxin (assayed on whole cells or spheroplasts) and immunity.

Termination at residue 75 of α in *PTX1-116* had this phenotype. Suppression gave a normal sized read-through product but did not change the phenotype or lead to detectable secretion of toxin components. Insertion of the dipeptides ProGly or LeuGlu after residue 102 (58 of α, in the proposed cation channel determinant), produced the same result (unpublished observations). Substitutions in this region of α seem to prevent expression of immunity and apparently prevent efficient endopeptidase processing but not glycosylation of protoxin.

The *kex1* and *kex2* mutations (Wickner and Liebowitz, 1976) allow normal expression of immunity while preventing secretion of active toxin. Protoxin is accumulated in normal or increased amounts but no subsequent processing is detectable (Bussey *et al.*, 1983). Since the *KEX2* product cleaves at the γ−β junction, this step is not essential for immunity expression. The role of *KEX1* is unknown, but its product may cleave at the δ−α or α−γ junctions. At least some steps in protoxin processing are thus unnecessary for immunity, suggesting a role for the entire precursor in this process. Termination within γ (*PTX1-177*) led to accumulation of truncated protoxin of the expected size and secretion of weak killing activity against spheroplasts, presumably indicating marginal production of secreted α. Immunity was expressed, but also weakly. This was only slightly enhanced in a *SUP7* strain that should elongate the protoxin by a 77-residue out-of-frame peptide (Table I). Thus while the C-terminal half of γ is dispensable for immunity expression, comparison with *PTX1-234* and *PTX1-239* indicates that a complete γ domain enhances immunity expression to normal levels, even in the absence of β. Its role in combination with α may be direct or through effects on stability, secretion efficiency or processing.

While the precise requirements for cleavage by the *KEX2* product are unknown (Julius *et al.*, 1984), a potential alternate processing site exists at residue 188 (LysArgSer), falling in one of the most hydrophilic regions of protoxin. In contrast, the γ−β junction is moderately hydrophobic (Figure 1). Overproduction of protoxin by expression of *PTX1-316* has allowed visualization of two intracellular immunoreactive components, p22 (22 kd) and p14 (14 kd), not seen in wild-type extracts. The mobility of p22 coincides approximately with the size expected from cleavage of protoxin at residue 188, leaving a single core N-glycosylation unit attached. If p22 does indeed result from processing at this site, it may be that this product is the natural determinant. The *PTX1-177* product would lack a few C-terminal residues and the glycosylation site, possibly crucial for efficient secretion or function of the immunity determinant. The antigenic p14 fragment may correspond to δ + α (14.3 kd) or some other product of partial processing.

Models for immunity

The existence of a cytoplasmic membrane receptor for toxin, (presumably the α component) is implied by the phenotype of *kre3* mutants (Bussey, 1981) which yield resistant spheroplasts. It is also consistent with the immunity of spheroplasts of killer cells themselves, in which such a receptor may be absent or masked. γ was previously invoked as the masking agent, and presumed to interact with the receptor during secretion. However, the absence of apparent peptide homology between α and γ was disappointingly inconsistent with this model (Tipper and Bostian, 1984). An alternative model in which the 'active site' region of α can interact with this hypothetical receptor in either of two modes, through the same domain but with different consequences, is much more satisfactory (Figure 6). Mature α, delivered from outside the cell, kills the cells as a consequence of this interaction. In contrast, α as a component of protoxin, or in partially or differentially processed protoxin, would interact with the receptor during secretion, either leading to its loss, for example by shunting of the complex to the vacuole, or to masking as a consequence of tight binding. Occupancy of the receptor could be maintained if the membrane retained a substantial excess of the immunity ligand, as a consequence of binding via the δ domain. Uncleaved protoxin could perform this function early in the secretion pathway, before access to the processing enzymes, presumed to act in a Golgi compartment (Tipper and Bostian, 1984). Alternatively, *KEX2* processing at residue 188 could pre-empt processing at residue 234. The product (p22?) may no longer be a substrate for cleavage between α and γ. The *REX1* gene product is a second candidate for an enzyme cleaving protoxin at 188, or elsewhere in γ, since *rex1* mutants express immunity poorly (Wickner and Leibowitz, 1976). It could modify *KEX2* function or be a protease itself. We hope to test these models by investigating the effect of *kex* and *rex* mutations on the formation of p22, by further characterization of the structure and location of this component and by determining the effects on immunity of mutations of the LysArg sequence at 188.

Materials and methods

Strains, media, DNA manipulations and oligonucleotide synthesis

Yeast strains S6 (a/α) and GG100-14D (α *his3 ura3-50 trp1-1 pho5 pho3*) lack M_1-dsRNA and are sensitive to toxin. GGMS1 is an isogenic derivative of the latter strain infected with the M_1-dsRNA virus (El-Sherbeini and Bostian, in preparation). Strain K12-1 (α *ade arg*) contains M_1-dsRNA and is the standard type K1 killer used in previous studies (Bostian *et al.*, 1980a). Strain SSY7 (*a ura3-52 SUP7-a trp1-1 met8-1 aro7-1 leu2-1 his5-2 cyc1-76 lys1-1 ade3-26 ilv1-1*) was derived from strains kindly donated by S.Liebman. Strain SSY7-C was a non-killer, non-immune, heat-cured (Fink and Styles, 1972) derivative of SSY7 and was shown to be free of M_1-dsRNA by published procedures (El-Sherbeini *et al.*, 1983). Low phosphate (low-P_i) and high phosphate (high-P_i) uracil minus media (Thill *et al.*, 1983; Bostian *et al.*, 1983c) were employed for growth of yeast strains under repressed (high-P_i) or derepressed (low-P_i) conditions.

Escherichia coli strains MC1061 (Casadaban *et al.*, 1983) and JM103 (Messing *et al.*, 1981) were used as host strains for the cloning procedures described here. Cloning manipulations involving ligation, restriction digestion, phosphorylation, radioactive labeling and Klenow treatment of DNA were performed essentially as described by Maniatis *et al.* (1982). DNA sequencing was performed by the Sanger dideoxy chain termination procedure (Sanger *et al.*, 1977). The oligonucleotides used in this study were manufactured using a Biosearch SAM ONE automated DNA synthesizer by the phosphate triester method and purified by extraction from polyacrylamide gels.

Transformation and killer assays

S. cerevisiae and *E. coli* transformations were performed as described by Hinnen *et al.* (1978) and Maniatis *et al.* (1982) respectively. Yeast transformants were initially tested for their killing and immunity phenotypes by patch assays on low- or high-P_i media lacking uracil and containing 0.003% methylene blue (Hanes *et al.*, 1986). Relative levels of immunity were ascertained by a well assay. The strain of interest was grown in high- or low-P_i media to a density of 2.5×10^7 cells/ml and added to molten assay medium at a concentration of 5×10^5 cells/ml. Wells of 0.9 mm diameter were cut in the agar and a constant amount of concentrated killer toxin (from strain K12-1) was applied. After 48 h incubation at 23°C the size of killing zone around the well was estimated. By comparison to the size of the killing zone obtained with strain K12-1 and the sensitive recipient strain (GG100-14D), the relative level of immunity of the transformant strain was calculated. To ensure the reproducibility of these results, assays were replicated over three plates with four applications of toxin per plate.

The effect of mutant and wild-type killer toxins on yeast spheroplasts was determined by incubating concentrated cell-free culture supernatants with spheroplasts (strain GG100-14D or GGMS1) transformed with a *URA3*-containing plasmid (YEp24, Botstein *et al.*, 1979) for 30 min at room temperature. The spheroplasts were then regenerated in uracil minus media by the standard procedure. The supernatant concentrates were osmotically stabilized and buffered to a final concentration of 1.2 M sorbitol and 0.05 M citrate buffer, pH 4.7. Supernatants were applied at a constant total protein concentration equivalent to the amount of wild-type toxin which produced a distinct killing zone on whole cells of the recipient strain. Toxin corresponds to ~5% of total secreted protein in strain K12-1.

Isolation, Northern blotting and in vitro translation of total cellular RNAs

Total RNA was isolated from cells grown in low- or high-P_i uracil minus media, electrophoresed through 1.5% agarose formaldehyde gels and transferred to nitrocellulose by published procedures (Bostian *et al.*, 1983a, b). Hybridization and autoradiography of the filters was performed as described previously (Koren *et al.*, 1986) using a preprotoxin specific, nick-translated probe of the 944-bp *Eco*RI–*Bgl*I fragment of pSH4.

Undenatured RNAs were translated in a wheat germ system, with *L*-[3, 4, 5-^{3}H(N)]leucine and the radiolabeled translation products analysed by immunoprecipitation with anti-toxin IgG, fractionation by SDS-polyacrylamide (7.5–17.5% gradient) gel electrophoresis, and fluorography, as previously described (Bostian *et al.*, 1980b, 1983c, 1984).

Preparation of intracellular and secreted proteins

Transformant strains were grown on low- or high-P_i, uracil minus media to a cell density of 2.5×10^7 cells/ml, and protein extracts were prepared in the presence of Triton X-100, as previously described (Bostian *et al.*, 1983b). Total secreted yeast proteins were prepared by concentration of cell free culture supernatants derived from strains grown at room temperature in the appropriate media to a density of 2.0×10^7 cells/ml. Proteins were concentrated ~100-fold in an Amicon stirred ultrafiltration cell using a PM10 membrane. Further concentration was achieved by lyophilization following dialysis of the concentrate against 0.1 M ammonium acetate (pH 4.7). Intracellular and secreted proteins were fractionated as before by SDS-PAGE and analysed by Western blotting.

Immunoblotting of in vivo proteins

Following electrophoresis, yeast proteins were transferred to nitrocellulose by electroblotting for 16 h at 150 mA in a Biorad 'trans-blot cell' with minor modifications from the procedure of Burnette (1981). The nitrocellulose was then immersed in 0.9% NaCl, 10 mM Tris-HCl, pH 7.4, (Tris-saline) and 5% fraction V bovine serum albumin for 1 h at room temperature followed by overnight incubation with an aliquot of the same solution containing 50 μg/ml of antitoxin rabbit IgG. After three washes in Tris-saline containing 0.1% Triton-X-100 and two washes in Tris-saline, the antigen–antibody complex was detected by a chromogenic assay using alkaline phosphatase conjugated sheep anti-rabbit IgG (Blake *et al.*, 1984).

Reconstruction of the 5′ end of the preprotoxin gene

Fusion of the complete protoxin coding sequences to the *PHO5* promoter was accomplished using the oligonucleotides shown in Figure 2. These were phosphorylated and annealed to form a double-stranded 115-bp complex bounded by *Pst*I and *Eco*RI sites and cloned (*Eco*RI–*Pst*I) into the preprotoxin fragment of pH4-G1 (Bostian *et al.*, 1984). The *Eco*RI–*Bgl*II preprotoxin fragment from the resulting plasmid, pCTC11, was ligated with the *Bgl*II–*Bam*HI preprotoxin fragment of pSH-GB1 (*Bam*HI site adjacent to *Bgl*I, Hanes *et al.*, 1986), into the *Eco*RI, *Bgl*II-digested *PHO5* expression vector p1A1R1-15 (Parent *et al.*, 1985). Three resulting plasmids, pSH4, 6 and 15 were sequenced by the rapid collapsed supercoiled plasmid method (Hattori and Sakaki, 1986) using a primer that hybridized to the *PHO5* promoter.

Site-directed mutagenesis of the preprotoxin gene

The 1.03-kb *Bam*HI fragment from pSH-GB1 was cloned into the *Bam*HI site of M13mp18 so that the viral strand contained the minus strand of the M_1-cDNA fragment. This was then used to direct the *in vitro* synthesis of an RF using one of two mutagenic oligomers by published procedures (Zoller and Smith, 1983). The oligomer 3007 (5′ TATGTTTATCCTATGCTCGAGCATGGTATC) introduced an *Xho*I site at position 728 of the cDNA of wild-type preprotoxin. The oligomer DT1 (5′-CCAAGCGGGATCCTTATCCTATG) was similarly used to introduce a *Bam*HI site at position 711 of the cDNA. In both cases, transformants of *E. coli* (JM103) were identified by colony hybridization with the

radiolabeled mutagenic oligomer. Clones with the correct restriction map were sequenced using a primer hybridizing within γ and found to be as predicted. The 635-bp *Hin*dIII, *Stu*I fragment from a positive *Xho*I mutant, p3007-G was ligated to pUC18 digested with *Hin*dIII and *Hin*cII to yield pX-12. The *Bgl*II, *Sma*I fragment from this plasmid was then ligated to *Bgl*II, *Nru*I-digested pSH6 to produce a *PHO5*, preprotoxin expression vector (pSHX10) with a unique *Xho*I site but lacking the protoxin sequences downstream of the *Stu*I site. Similarly, a *Hin*dIII site was introduced at the *Nru*I site of pSH6 by linker insertion and the *Bgl*II–*Hin*dIII fragment of a positive *Bam*HI mutant plasmid DT4HB was ligated into *Bgl*II, *Hin*dIII-digested pSH6 and used to transform *E. coli* MC1061. One such clone, pDT5A, exhibited the predicted restriction map and sequence and was chosen for further study.

Insertional mutagenesis of the preprotoxin gene

The 1500-bp *Eco*RI, *Nru*I preprotoxin fragment of pSH6 was cloned into pUC18 at the *Eco*RI, *Hin*cII sites within the polylinker region. This plasmid, pSH6/pUC18, was digested with *Spe*I or *Acc*I which are unique to preprotoxin and made blunt-ended with the Klenow fragment of DNA polymerase 1. A single SH-TERM oligomer (CTAGTCTAGACTAG) was inserted at these points by the method of linker-tailing (Lathe *et al.*, 1984). The unphosphorylated, de-annealed oligomer was ligated to the plasmids, at a 100-fold molar excess, to yield DNA termini with covalently linked single stranded tails reassociated due to their self-complementarity. The residual nicks were then repaired *in vivo* by transformation of *E. coli* (MC1061). DNA from two positive clones, distinguished by the presence of an additional *Xba*I site and loss of the *Spe*I (pS-3) or *Acc*I (pA-13) sites was subcloned into the expression vector. The terminator plasmids were digested with *Hin*dIII, treated with Klenow fragment and digested with *Bgl*II. The resulting 1350-bp fragment was ligated to pSH6 that had been previously digested with *Bgl*II and *Nru*I. Two clones, pXTSS26 (termination at *Spe*I) and pXTSA1 (termination at *Acc*I), showed the expected restriction maps and were chosen for further study. Similarly, the same SH-TERM linker was inserted at the *Xho*I site of pSHX10 by linker-tailing. *Xho*I-digested pSHX10 was Klenow-treated and ligated to the SH-TERM linker as described above. *E. coli* transformants harboring the correct construct were identified by the loss of the *Xho*I site and the acquisition of a novel *Spe*I site formed at the junction between the linker and the filled-in *Xho*I site. One such clone, pXTSX1, was identified and used in future studies.

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Note added in proof

Since submission of this manuscript, Boone *et al.* (*Cell*, **46**, 105–113) have conducted mutagenesis of the preprotoxin gene, with similar findings.