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The *Zygosaccharomyces bailii* antifungal virus toxin zygocin: cloning and expression in a heterologous fungal host

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Summary

Zygocin, a monomeric protein toxin secreted by a virus-infected killer strain of the osmotolerant spoilage yeast *Zygosaccharomyces bailii*, kills a broad spectrum of human and phytopathogenic yeasts and filamentous fungi by disrupting cytoplasmic membrane function. The toxin is encoded by a double-stranded (ds)RNA killer virus (ZbV-M, for *Z. bailii* virus M) that stably persists within the yeast cell cytosol. In this study, the protein toxin was purified, its N-terminal amino acid sequence was determined, and a full-length cDNA copy of the 2.1 kb viral dsRNA genome was cloned and successfully expressed in a heterologous fungal system. Sequence analysis as well as zygocin expression in *Schizosaccharomyces pombe* indicated that the toxin is *in vivo* expressed as a 238-amino-acid preprotoxin precursor (pptox) consisting of a hydrophobic N-terminal secretion signal, followed by a potentially N-glycosylated pro-region and terminating in a classical Kex2p endopeptidase cleavage site that generates the N-terminus of the mature and biologically active protein toxin in a late Golgi compartment. Matrix-assisted laser desorption mass spectrometry further indicated that the secreted toxin is a monomeric 10.4 kDa protein lacking detectable post-translational modifications. Furthermore, we present additional evidence that in contrast with other viral antifungal toxins, zygocin immunity is not mediated by the toxin precursor itself and, therefore, heterologous pptox expression in a zygocin-sensitive host results in a suicidal phenotype. Final sequence comparisons emphasize the conserved pattern of functional elements present in dsRNA killer viruses that naturally infect phylogenetically distant hosts (*Saccharomyces cerevisiae* and *Z. bailii*) and reinforce models for the sequence elements that are *in vivo* required for viral RNA packaging and replication.

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Introduction

Yeast killer toxins are secreted protein or glycoprotein toxins that can kill sensitive strains of the same or (usually) closely related species in a receptor-mediated, two-step process. In all cases studied so far, killing requires initial toxin binding to components of the outer yeast cell surface (such as cell wall β-1,6-D-glucans, α–1,3-mannoproteins or chitin) and subsequent transfer to the cytoplasmic membrane where the toxin is interacting with a secondary membrane receptor. Lethality is finally caused either by plasma membrane damage, G1 or S phase cell cycle arrest and/or by a rapid inhibition of nuclear DNA synthesis (Schmitt *et al*., 1989; Butler *et al.,* 1991; Schmitt *et al*., 1996; Gage *et al.,* 2001).

Besides chromosomally encoded killer toxins like those secreted by the yeasts *Hansenula mrakii* and *Williopsis californica* and the plasmid-driven killer proteins of *Kluyveromyces lactis* and *Pichia acaciae*, killer phenotype expression can also be associated with the presence of double-stranded M-dsRNA viruses that stably persist within the cytoplasm of the infected host cell (for recent reviews, see Wickner, 1996; Magliani *et al*., 1997; Schaffrath and Breunig, 2000; Schmitt and Breinig, 2002). In the best studied viral killer systems K1, K2, and K28 of *Saccharomyces cerevisiae*, the toxin-encoding M-dsRNA killer viruses are satellites of L-A, a cryptic 4.6 kb dsRNA helper virus that provides the M satellites with the major capsid protein Gag and the RNA dependent RNA polymerase Gag-Pol (Icho and Wickner, 1989). The viral Gag-Pol protein is *in vivo* expressed by a –1 ribosomal frameshift during translation of the L-A plus strand transcript (Dinman and Wickner, 1992; 1994). In each virion, Gag-Pol is responsible for the synthesis of the corresponding RNA plus strand [L-A or M (+)ssRNA], which is extruded from the particles into the cytosol where it is subsequently translated in a cap-independent manner. Viral (+)ssRNA encapsidation is initiated by Gag-Pol binding to a highly conserved secondary stem–loop structure (viral binding site, VBS) within the 3′-terminus of the messenger plus-strand RNA of L-A and M (Fujimura *et al*., 1990). Once the viral capsid assembly has been completed, the polymerase activity of Gag-Pol initiates minus-strand RNA synthesis on the plus-strand transcript, thereby completing the viral replication cycle (Wickner, 1996). Synthesis of RNA minus-strands depends on secondary structures at

the 3′-terminus of the (+)ssRNA transcript, which have been designated IRE (internal replication enhancer) and TRE (terminal recognition element) (Huan *et al*., 1991; Ribas *et al*., 1994a;b). Apart from the three toxin encoding M-dsRNA genomes M_1, M_2 and M_{28} in *S. cerevisiae*, complete cDNA sequences of toxin encoding dsRNAs have only been determined for the killer toxins KP1, KP4 and KP6 of the maize smut fungus *Ustilago maydis* (Tao *et al*., 1990; Park *et al*., 1994; 1996). Until now, M-dsRNA sequences in the more recently identified killer strains of the non-conventional yeasts *Hanseniaspora uvarum* and *Zygosaccharomyces bailii* have not been determined (Schmitt and Neuhausen, 1994; Schmitt *et al*., 1997).

In *Z. bailii*, killer phenotype expression is caused by the secretion of a non-glycosylated 10 kDa protein toxin that rapidly kills a broad spectrum of yeasts and filamentous fungi, including human pathogenic strains of *Candida albicans*, *Candida glabrata* and *Sporothrix schenkii*, as well as phytopathogenic strains of the fungi *Fusarium oxysporum* and *Colletotrichum graminicola* (Radler *et al*., 1993; Weiler and Schmitt, 2002). The antifungal *Z. bailii* toxin zygocin (named after its producing host) is genetically encoded by an encapsidated 2.1 kb dsRNA genome (M_{Zb}-dsRNA) persisting within the yeast cell cytosol (Schmitt and Neuhausen, 1994). In the present study, we determine the full-length cDNA sequence of the prepro-zygocin encoding M_{Zb}-dsRNA, characterize the protein toxin by N-terminal amino acid sequence analysis and matrix-assisted laser desorption mass spectrometry (MALDI-TOF), and present evidence that the mature toxin is a monomeric 10.4 kDa protein that is *in vivo* generated by Kex2p cleavage in a late Golgi compartment. Finally, we present additional evidence that in contrast with the preprotoxins in *S. cerevisiae*, toxin immunity in *Z. bailii* is not mediated by the prepro-zygocin toxin precursor itself, and therefore heterologous zygocin expression was only successful in the zygocin-resistant fungal host *Schizosaccharomyces pombe*.

Results

Complete cDNA sequence of the killer virus M_{Zb}-dsRNA genome

We recently reported that *Z. bailii* killer strains contain three different encapsidated dsRNA genomes (L_{Zb}, M_{Zb}, Z) that stably persist within the cytosol of the infected host cell: In contrast with the autonomously replicating L_{Zb}- and Z-dsRNAs, the toxin-coding M_{Zb} genome can only be maintained and replicated in a cell that is simultaneously infected with ZbV-L (Schmitt and Neuhausen, 1994). In analogy to the *S. cerevisiae* dsRNA virus L-A, ZbV-L itself functions as helper virus by providing its M_{Zb}-dsRNA satellite with Gag and Gag-Pol, two essential virus proteins required for RNA packaging, replication and transcription. We also demonstrated that all three dsRNAs (L_{Zb}, M_{Zb}, Z) represent icosahedral mycoviruses that consist of a 4.6 kb L_{Zb}-dsRNA genome and a smaller (2.1 kb), toxin-coding M_{Zb}-dsRNA satellite virus (Schmitt and Neuhausen, 1994). Although both dsRNAs, L_{Zb} and M_{Zb}, are essential for killer phenotype expression, the third encapsidated dsRNA species in *Z. bailii* represents a completely unrelated cryptic mycovirus (ZbV-Z) not conferring a recognizable phenotype upon its host (Fig. 1A and B). As Z itself can stably exist in a cell, even in the absence of ZbV-L, this property clearly distinguishes Z from the zygocin encoding ZbV-M

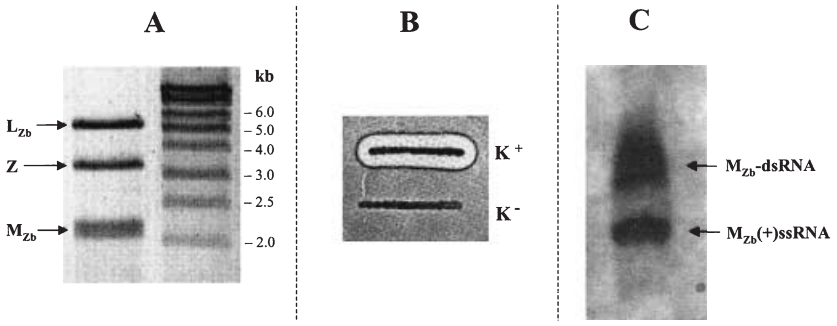

Fig. 1. dsRNA pattern and associated phenotype in a killer virus-infected *Zygosaccharomyces bailii* strain.
A. Agarose gel electrophoresis of L_{Zb}-, Z- and M_{Zb}-dsRNA isolated from the zygocin-secreting killer strain *Z. bailii* 412(positions and sizes of the corresponding dsRNA genomes determined by comparison to a dsDNA marker are indicated).
B. Killer versus non-killer phenotype of the wild-type strain *Z. bailii* 412 (K^+) and its cyclohexamide-cured, non-killer (K^-) derivative *Z. bailii* strain FN201 (Table 1) was tested by streaking the corresponding yeast onto Methylene blue agar (pH 4.0) that had been seeded with 10^5 cells of the sensitive tester strain *Candida krusei* 185. After incubating the plate for 3 days at 20°C, a cell-free growth inhibition zone around the killer streak indicates zygocin secretion.
C. Northern blot analysis of the zygocin encoding M_{Zb}-dsRNA. Total RNA from the wild-type killer *Z. bailii* 412 was subjected to electrophoresis on a 1.0% agarose-formaldehyde gel, transferred to a nylon membrane, and probed with a DIG-labelled M_{Zb}-cDNA (positions of denatured M_{Zb}-dsRNA and its plus-strand transcript [M_{Zb}(+)ssRNA] are indicated).

killer virus that depends on ZbV-L for maintenance and replication (Neuhausen and Schmitt, 1996).

To characterize the toxin-coding ZbV-M genome at the molecular level, cDNA synthesis on the purified and denatured M_{Zb}-dsRNA template was performed by using random hexanucleotides for priming first-strand cDNA synthesis. By using this strategy, most of the viral M_{Zb} sequence was obtained by simple alignment of the resulting cDNA fragments. Based on the sequences obtained, a DIG-labelled M_{Zb}-cDNA probe was used for Northern analysis of total RNA from the *Z. bailii* wild-type killer. As shown in Fig. 1C, the labelled M_{Zb} probe strongly hybridized with denatured M_{Zb}-dsRNA as well as with the single-stranded virus transcript (M_{Zb}(+)ssRNA).

Due to limitations of the Gubler and Hoffman method (Gubler and Hoffman, 1983) in obtaining a full-length M_{Zb}-cDNA, additional 5′- and 3′- rapid amplification of cDNA ends (RACE) amplifications (Frohman, 1993) became necessary to successfully clone the complete 5′- and 3′-terminal sequences of M_{Zb} (see *Experimental procedures*). The full-length M_{Zb}-cDNA sequence determined in this way has a deduced length of 2063 bp and contains a single 239 codon open reading frame (ORF; comprising bases 17–733) presumed to encode the prepro-zygocin toxin precursor (Fig. 2A and B). It is followed by an internal poly(A)-rich stretch and a residual 3′-terminal sequence of 1047 bp predicted to provide the structural elements required for RNA replication (see below).

The single ORF on M_{Zb} is located 21 bases upstream of the internal poly (A)-rich tract (bases 755–1016) and

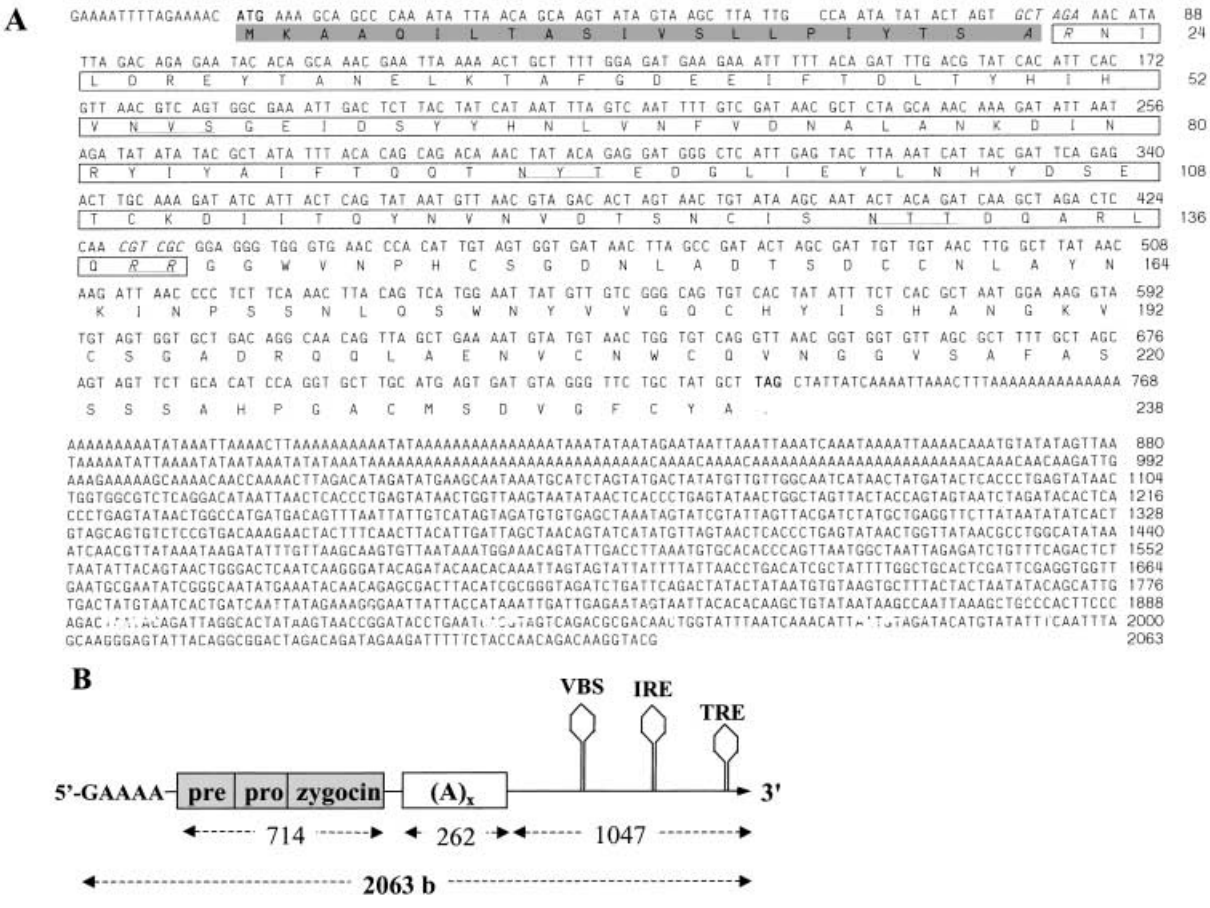

Fig. 2. Complete sequence of the prepro-zygocin encoding M_{Zb}-cDNA.
A. Sequence for the M_{Zb}-dsRNA plus-strand and deduced amino acid sequence of the encoded prepro-zygocin toxin gene. The N-terminal secretion leader sequence is shaded and the potentially N-glycosylated pro-region is boxed. Methionine start codon and the TAG stop codon are shown in bold face, the single Kex2p processing site (ArgArg[139]) and the three N-glycosylation sites (AsnValSer[56], AsnTyrThr[94], AsnThrThr[131]) are underlined. The complete M_{Zb}-cDNA sequence containing the prepro-zygocin toxin precursor (2063 bp) appears in the NCBI/GenBank under the accession no. AF515592.
B. Schematic structure and coding capacity of the prepro-zygocin encoding M_{Zb}(+)ssRNA. The prepro-zygocin open reading frame (ORF) is situated at the 5′-end, immediately downstream of the indicated 5′-GAAAA sequence that is highly conserved among yeast and fungal dsRNA viruses. The intramolecular poly (A)-rich stretch is shown [$(A)_x$] and the potential *cis*-acting 3′-sequences required for *in vivo* RNA packaging, transcription and replication are indicated (VBS, viral binding site; IRE, internal replication enhancer; TRE, 3′-terminal recognition element). Numbers indicate the size (b) of the full-length M_{Zb}(+)ssRNA virus transcript and its coding and non-coding regions.

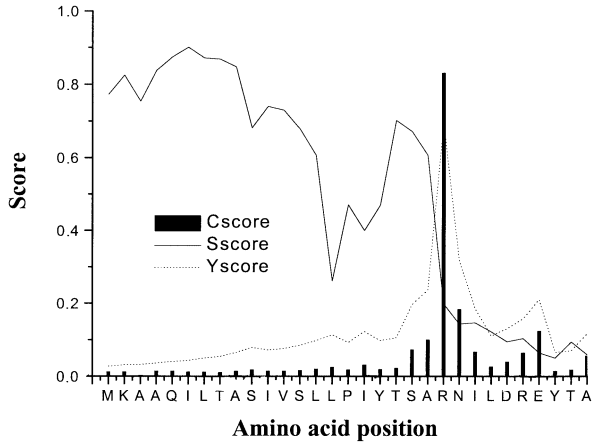

Fig. 3. Hydropathy analysis and signal peptidase cleavage probability in prepro-zygocin. The plot was made according to the rules codified by Nielsen *et al.* (1997). C-score (raw cleavage site score), the output score recognizes cleavage sites versus other sequence positions; S-score (signal peptide score), the output score recognizes signal peptide versus non-signal peptide positions; Y-score (combined cleavage site scores), the prediction of cleavage site location is optimized by observing where the C-score is high and the S-score changes from high to a low value. The Y-score formalizes this by combining the height of the C-score with the slope of the S-score.

encodes a zygocin toxin precursor with a calculated molecular weight of 26.4 kDa (Fig. 2B). The first AUG start codon downstream of the prepro-zygocin encoding ORF is found at bases 17–19. As in yeast, translation of cellular and viral mRNAs normally initiates at the first AUG codon, it is most likely that *in vivo* translation of the toxin precursor initiates at this position (see also Fig. 6).

Signal peptidase cleavage and prepro-zygocin processing

Following the rules codified by Nielsen *et al.* (1997), hydropathy analysis and signal peptidase cleavage probability of the prepro-zygocin toxin precursor strongly predict that its N-terminus represents a classical secretion leader sequence, directing the toxin precursor into the lumen of the endoplasmic reticulum (ER), where signal peptidase cleavage should occur after Ala^{21} (Fig. 3). Immediately downstream follows a 118-amino-acid pro-region that contains three potential sites for protein N-glycosylation (positions $AsnValSer^{56}$, $AsnTyrThr^{94}$ and $AsnThrThr^{131}$). The carboxy terminus of this pro-region ends in a classical Kex2p cleavage site (Park *et al.*, 1994) in which the dibasic site is preceded by a hydrophobic leucine in the P4 position (LeuGln*ArgArg*^{139}). It therefore can be predicted that endopeptidase cleavage in a late Golgi compartment generates the N-terminus of the mature and secreted zygocin toxin whose predicted N-terminal sequence (NH2-Gly-Gly-Trp-Val-Asn-Pro-His-Cys) exactly matched the sequence of the purified protein toxin determined by Edman degradation (Fig. 2A). Therefore, the mature and fully processed zygocin toxin comprises 99 amino acids, corresponding to a protein with a calculated size of 10.4 kDa. The predicted size corresponds nicely to the estimated molecular weight of 10 kDa for the purified protein toxin determined after SDS-PAGE (Radler *et al.*, 1993; Weiler and Schmitt, 2002). To address the question of post-translational toxin modifications, we additionally analysed the mass of the affinity-purified zygocin toxin by mass spectrometry as another independent method. As shown in Fig. 4, MALDI-TOF yielded a mass of 10 471 Da for the purified toxin, which is in good agreement with the calculated size of 10 421 Da deduced from its cDNA sequence, indicating that the mature virus toxin lacks significant post-translational protein modifications.

Heterologous zygocin expression, processing and secretion in Schizosaccharomyces pombe

We recently demonstrated successful transfection of a *S. cerevisiae* non-killer strain with the isolated and purified *Z. bailii* killer virus ZbV-M (Schmitt and Neuhausen, 1994). However, the resulting ZbV transfectants expressed a somehow suicidal phenotype and were killed by their own secreted toxin when cultivated under conditions in which zygocin activity is high (at pH 4.0; Neuhausen and Schmitt, 1996). Based on these observations, we speculated that zygocin immunity is either not encoded by the viral M_{Zb}-dsRNA genome or not functional in transgenic *S. cerevisiae* ZbV-M transfectants.

To verify that the obtained M_{Zb}-cDNA encodes the zygocin toxin, preprotoxin expression was achieved in the

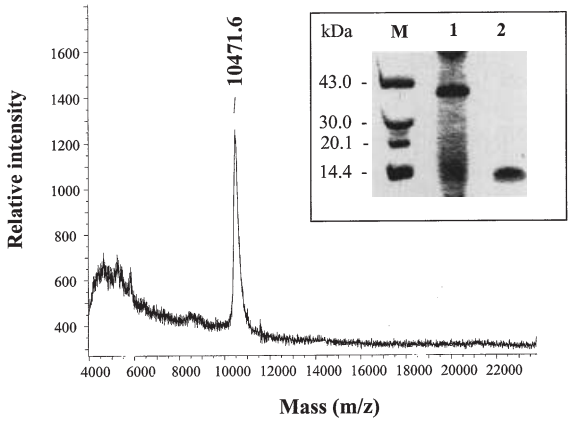

Fig. 4. Molecular weight determination of affinity-purified zygocin by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) analysis. MALDI-TOF mass spectrum of the purified zygocin toxin. The inset shows the electrophoretic analysis (SDS-PAGE) of zygocin before (lane 1) and after (lane 2) affinity-purification on a mannoprotein-coupled sepharose matrix as described previously (Weiler and Schmitt, 2002; lane M, marker proteins). The purified zygocin toxin shown in lane 2 was analysed by MALDI-TOF.

heterologous fungal host *S. pombe*. To avoid a possible suicidal phenotype after *in vivo* expression of the prepro-zygocin cDNA, we first tested intact cells and spheroplasts of fission yeast against the purified virus toxin in a standard well plate assay. Interestingly, and in contrast to *S. cerevisiae*, intact cells and spheroplasts of *S. pombe* were completely zygocin-resistant (data not shown), indicating that fission yeast should be a suitable host for the expression of a plasmid-driven *Z. bailii* killer phenotype. For this purpose, a zygocin expression vector (pREP-Zygo) was constructed in which the entire prepro-zygocin encoding cDNA was placed under transcriptional control of the tightly thiamine-regulated *S. pombe* promoter *nmt1* (Moreno *et al.,* 2000). As illustrated in Fig. 5, fission yeast cells that had been transformed with pREP-Zygo expressed a *Z. bailii* killer phenotype that was readily detectable in a standard agar diffusion assay. However, killing activity of the pREP-Zygo *S. pombe* transformants was significantly lower when compared with the toxicity of the naturally ZbV-M infected wild-type killer *Z. bailii* 412. To demonstrate that recombinant *S. pombe* cells express and secrete a correctly processed toxin indistinguishable from the homologous toxin produced and secreted by the ZbV-M infected *Z. bailii* wild-type killer, cell-free culture supernatants derived from *S. pombe* pREP-Zygo transformants as well as from the *Z. bailii* wild-type killer strain were separated by SDS-PAGE, electroblotted onto a PVDF membrane, and immediately probed with a polyclonal anti-zygocin antibody. The corresponding Western blot (Fig. 5C) confirmed that *S. pombe* transformants are perfectly capable of secreting a fully processed 10 kDa protein toxin showing the same electrophoretic mobility as the natural virus toxin. However, as the level of zygocin secretion was significantly higher in the wild-type *Z. bailii* killer, cell-free culture supernatants derived from recombinant *S. pombe* transformants had to be concentrated 35-fold compared with wild type, to give a detectable immunoreactive zygocin signal (Fig. 5C, compare lanes S and Z).

As inefficient zygocin secretion in *S. pombe* might have been caused by the heterologous prepro-zygocin secretion signal, we replaced the N-terminal secretion sequence and the complete pro-region of the zygocin precursor by a secretion and processing signal derived from the viral *S. cerevisiae* K28 pptox gene (Riffer *et al.,* 2002). This sequence was recently demonstrated to be fully functional in *S. pombe* ensuring efficient protein secretion in this host (Heintel *et al.,* 2001). Although expression of the constructed K28/zygocin fusion protein was driven by the same promoter (*nmt1*) and from the same episomal vector (pREP-K28/Zygo), the resulting *S. pombe* transformants did not show a stronger *Z. bailii* killer phenotype (data not shown). It therefore can be predicted that prepro-zygocin import into the ER lumen is probably

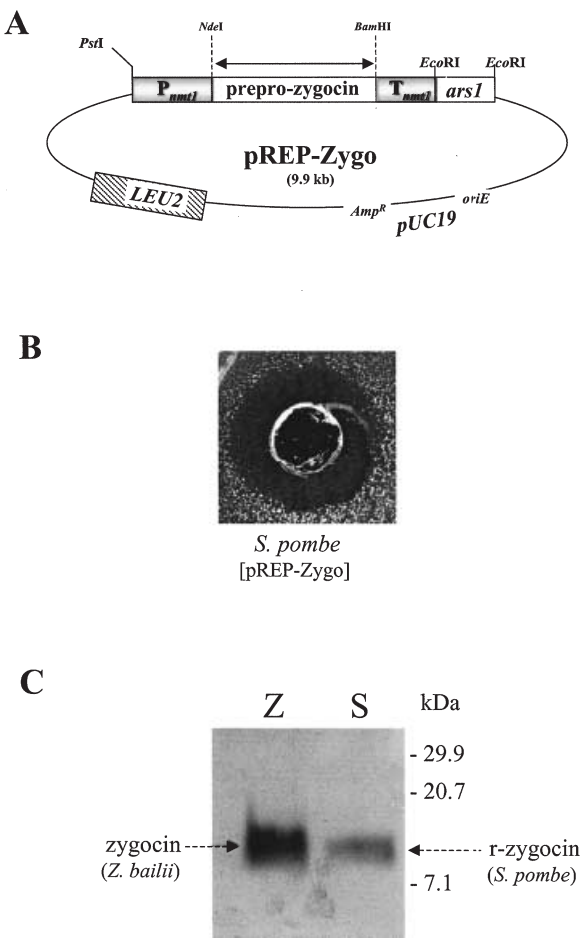

Fig. 5. Killer activity and toxin secretion in recombinant *Schizosaccharomyces pombe* after heterologous expression of the prepro-zygocin encoding M_{Zb}-cDNA.
A. Schematic structure and partial restriction map of the thiamine-repressible prepro-zygocin expression vector pREP-Zygo containing the entire M_{Zb}-cDNA under transcriptional control of the fission yeast *nmt1* promoter. Sites for transcription initiation within the *nmt1* promoter (P_{nmt1}) and transcription termination (T_{nmt1}) are indicated. Yeast sequences are shown by thick lines; *Escherichia coli* sequences derived from pUC19 are shown in thin lines (oriE, *E. coli* origin of replication; Amp^R, β-lactamase gene; *ars1*, *S. pombe* autonomously replicating sequence; *LEU2*, *Saccharomyces cerevisiae LEU2* gene).
B. Thiamine-regulated killer phenotype expression in *S. pombe* after transformation with the zygocin expression vector pREP-Zygo. Yeast transformants were grown under derepressed culture conditions (in the absence of 25 μM thiamine), and toxin activity in the cell-free culture supernatant was determined on a Methylene blue agar plate (pH 4.0) against the sensitive strain *S. cerevisiae* 192.2d.
C. Western analysis of zygocin secretion in recombinant *S. pombe* after transformation with pREP-Zygo and comparison with the homologous toxin secreted by the ZbV-M infected *Z. bailii* killer strain 412. Cell-free culture supernatants of a *S. pombe* pREP-Zygo transformant and of the *Z. bailii* wild-type killer were concentrated by ultrafiltration, separated by SDS-PAGE under reducing conditions, and subsequently probed with a polyclonal antizygocin antibody (lanes S and Z, secreted toxin of *S. pombe* [pREP-Zygo] and *Z. bailii* 412 respectively; positions of the homologous and the recombinant 10.4 kDa zygocin toxin are indicated).

not the limiting step in heterologous zygocin expression. Nevertheless, these data indicate that zygocin-resistant cells of *S. pombe* represent a suitable host for the heterologous expression of a broad-spectrum antifungal toxin that is naturally produced by a virus-infected strain of the osmotolerant spoilage yeast *Z. bailii*.

Secondary structure predictions within the zygocin-encoding M_{Zb} plus-strand

Within the codogenic plus-strand RNAs of the *S. cerevisiae* dsRNA viruses L-A and M, at least three *cis*-active sequence elements have been recognized that are required for viral RNA packaging, replication and transcription (Wickner, 1996). For correct transcription initiation of the viral plus-strand from the parent dsRNA template within the intact virion, the RNA polymerase Gag-Pol recognizes the 5′-terminal sequence 5′-GAAAAA. Interestingly (Fig. 6), the 5′-end of the preprozygocin encoding M_{Zb} plus-strand initiates with such a sequence (5′-GAAAA) that represents a highly conserved consensus sequence present in many yeast dsRNA viruses, presumed to be important for transcription initiation from the viral dsRNA template (Bruenn, 1988; Fujimura and Wickner, 1988a;b). As there is no other additional homology immediately downstream, this 5′-terminal recognition element (5′-TRE) might be all that is required for transcription initiation of M_{Zb}.

The next stage within the conservative replication cycle is encapsidation of the viral (+)ssRNA transcript and subsequent minus-strand RNA replication, thereby completing the viral replication cycle (Wickner, 1993; 1996). The packaging process is mediated by the specific binding of the viral RNA polymerase Gag-Pol to unique secondary stem–loop structures (viral binding sites, VBS) near the 3′-terminus of the viral transcript. For L-A, it has been shown that two sequence elements on the viral (+)ssRNA transcript are essential for L-A replication (Fujimura *et al*., 1990): (i) an internal replication enhancer (IRE) virtually indistinguishable from the VBS element and (ii) a small stem–loop structure near the 3′-terminal end (3′-TRE, for 3′-terminal recognition element). As illustrated in Fig. 6, the M_{Zb}(+)ssRNA contains a single VBS element at base position –429 and an IRE element at base position –356 (i.e. 429 and 356 bases from the 3′-terminus respectively). Both have 5 bp loops that are identical to those present in the transcripts of the *S. cerevisiae* killer viruses M_1 and M_{28} (GAUUC), and each is separated from a bulged (i.e. unpaired) A by a 3 bp stem (Fig. 6). In addition to the VBS and IRE elements, the 3′-terminus of the M_{Zb}(+)ssRNA also contains a stem–loop structure at –26, which closely matches the 3′-TRE in L-A and M_1 of *S. cerevisiae* and therefore might function as a 3′-terminal recognition element *in vivo* (Fig. 6).

Discussion

The antifungal protein toxin produced and secreted by a ZbV-M infected killer strain of *Z. bailii* represents a unique protein toxin with respect to its rapid *in vivo* killing kinetics and its broad-spectrum activity against a great variety of pathogenic and non-pathogenic yeasts and filamentous fungi (Weiler and Schmitt, 2002). Previous studies indicated that killer phenotype expression in this host is associated with the presence of a M_{Zb}-dsRNA-harbouring killer virus that stably persists within the cytosol of the infected cell (Schmitt and Neuhausen, 1994). For the first time, we now describe the complete cDNA sequence of the viral zygocin encoding dsRNA genome of *Z. bailii*. The length of the obtained M_{Zb}-cDNA (2063 bp) is consistent with the 2.1 kb size determined for the dsRNA, and the predicted zygocin-encoding ORF includes the exact N-terminal sequence of the mature and secreted protein toxin. Furthermore, zygocin cDNA expression in a heterologous fungal host conferred the naturally ZbV-M associated *Z.*

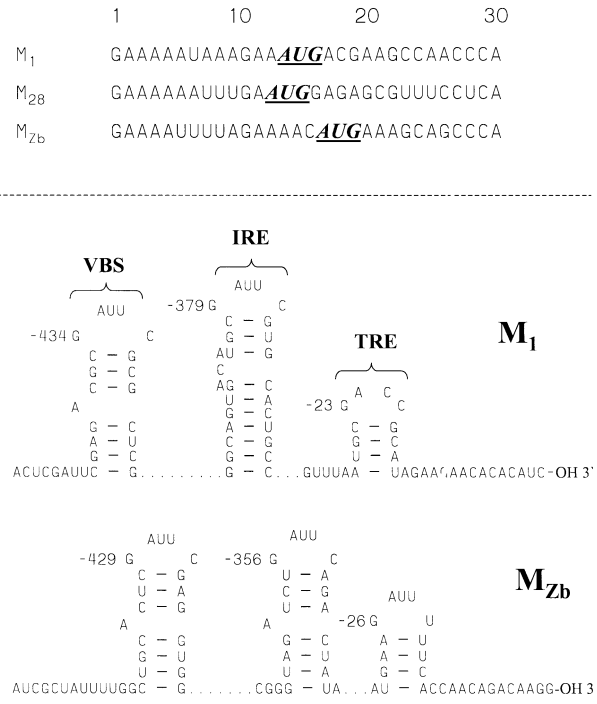

Fig. 6. Comparison of the 5′- and 3′-RNA sequences in the toxin-coding transcripts of the *S. cerevisiae* killer viruses ScV-M1 and ScV-M28 with the corresponding sequences in the *Z. bailii* killer virus ZbV-M. The underlined AUG codons represent the initiation codons in the preprotoxins. Potential *cis*-active sequences within the 3′-terminus of the killer virus transcripts of ScV-M1 (M1) and ZbV-M (M_{Zb}) are indicated (VBS, viral binding site; IRE, internal replication enhancer; TRE, 3′-terminal recognition element). Numbers are shown for distance from the 3′-terminus of each M(+)ssRNA. The 5′- and 3′-sequences of M_1 and M_{28} were taken from Hannig *et al*. (1986) and Schmitt and Tipper (1995).

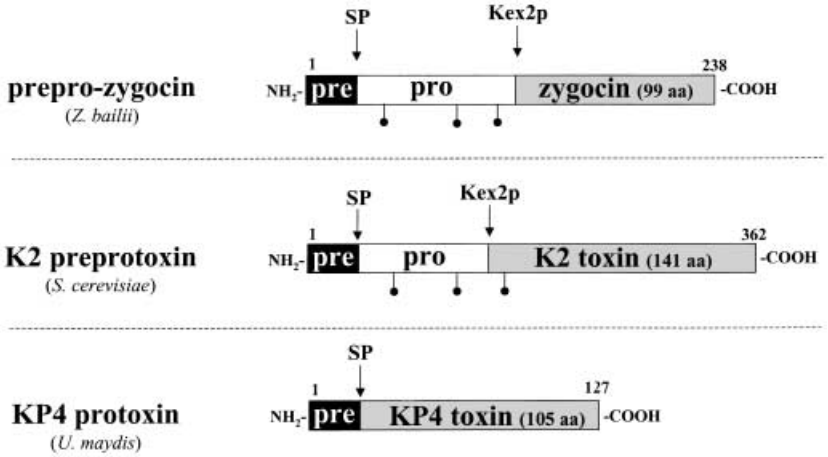

Fig. 7. Analogy of zygocin precursor processing in *Z. bailii* and preprotoxin maturation in *S. cerevisiae* and *Ustilago maydis*. Schematic structures of the toxin precursors of zygocin (*Z. bailii*), K2 (*S. cerevisiae*) and KP4 (*U. maydis*) are shown and signal peptidase (SP) and Kex2p endopeptidase cleavage sites are indicated. Potential N-glycosylation sites are indicated with black circles. Data for the *S. cerevisiae* K2 toxin and the *U. maydis* KP4 toxin were taken from the published data by Dignard *et al*. (1991) and Park *et al*. (1994) respectively.

bailii killer phenotype on *S. pombe*. Comparison of the VBS, IRE, and TRE elements identified on the zygocin-coding M_{Zb} plus-strand showed a striking homology to the corresponding *cis*-sequences present in the *S. cerevisiae* killer viruses ScV-M1, ScV-M2 and ScV-M28 (Hannig *et al*., 1986; Dignard *et al*., 1991; Schmitt, 1995). Although ZbV-M naturally infects a phylogenetically distant host (*Z. bailii*), the observed homologies strongly suggest that even in ZbV-M, *cis*-active 3′-sequences might function as structural elements that are *in vivo* required for RNA packaging, transcription and replication (Shen and Bruenn, 1993; Wickner, 1993; Schmitt and Tipper, 1995). This is further confirmed by the competence of L-A (the *S. cerevisiae* helper virus) for stable maintenance of ZbV-M in a yeast strain that had been infected with the *Z. bailii* killer virus ZbV-M (Schmitt and Neuhausen, 1994).

The cDNA-encoded prepro-zygocin sequence does not show any significant homology to other known killer toxins or other proteins in the currently available databases. However, *in vivo* processing of the toxin precursor shows striking homology to the preprotoxin processing in killer strains of *S. cerevisiae* and *U. maydis* (Skipper *et al*., 1984; Park *et al*., 1996). All these killer viral proteins are *in vivo* expressed as a preprotoxin requiring multiple processing events by the late Golgi Kex2p endopeptidase to be secreted in the biologically active form (Fig. 7). Amino-terminal sequence analysis of the purified zygocin toxin confirmed that zygocin processing is mediated by Kex2p cleavage in a late Golgi compartment, resulting in the final secretion of a monomeric, non-glycosylated and not otherwise post-translationally modified 10.4 kDa protein toxin. In this respect, zygocin is similar to the likewise monomeric, non-glycosylated KP4 toxin of *U. maydis* and the *S. cerevisiae* K2 toxin (Pfeiffer and Radler, 1984; Dignard *et al*., 1991; Park *et al*., 1994; Gage *et al.,* 2001), however, unlike KP4 but in analogy to K2, prepro-zygocin processing requires Kex2p cleavage for correct maturation (Fig. 7). This conserved processing pattern extends to the zygocin pro-region and to the intervening γ sequences in the *S. cerevisiae* K1, K2 and K28 preprotoxins: all these toxin precursors contain three similarly located N-glycosylation sites, and it has been speculated that this intermediate protein N-glycosylation might have a chaperone function for correct protoxin folding in the ER (Hanes *et al*., 1986; Dignard *et al*., 1991; Riffer *et al.,* 2002). In addition to the chaperone function, γ has also been presumed to be involved in the expression of functional toxin immunity in K1 and K28 killer strains of *S. cerevisiae* (Zhu *et al*., 1993). In contrast to *S. cerevisiae* killer strains, but in analogy to toxin-secreting strains of the maize smut fungus *U. maydis*, intact cells and spheroplasts of *Z. bailii* are naturally zygocin-resistant and toxin immunity therefore is dispensable in this yeast (M. J. Schmitt and F. Neuhausen, unpublished). However, this situation dramatically changes when prepro-zygocin expression in a heterologous host is desired. We recently demonstrated successful transfection of a *S. cerevisiae* non-killer strain with the isolated and purified zygocin encoding M_{Zb}-dsRNA virus ZbV-M from *Z. bailii* (Schmitt and Neuhausen, 1994). Interestingly, the resulting ZbV-M transfectants were capable to secrete a correctly processed and biologically active *Z. bailii* toxin, but the cells showed a somehow suicidal phenotype when cultivated under conditions where zygocin activity was high (pH 4.0; 20°C). In fact, *S. cerevisiae* transfection with the ZbV-M killer virus was only successful in a single virus recipient strain (*S. cerevisiae* GG100–14D); in all other strains tested virus uptake and concomitant zygocin expression resulted in a suicidal phenotype and rapid cell death (Neuhausen and Schmitt, 1996). However, ZbV-M transfectants were fully viable in the genetic background of a Δ*kex2* null mutation, confirming the predicted importance of the late Golgi endopeptidase Kex2p in prepro-zygocin maturation and processing (F. Neuhausen and M. J. Schmitt, unpublished data).

Interestingly, a suicidal phenotype could also be observed when zygocin-sensitive *S. cerevisiae* strains were transformed with a vector containing the prepro-zygocin encoding M_{Zb}-cDNA that was cloned in this study (data not shown), strongly indicating that transformation and heterologous zygocin expression is only successful in a zygocin-resistant host. Taken together, all these data indicate that zygocin immunity is probably not mediated by the preprotoxin precursor itself. As ZbV-M harbouring killer strains of *Z. bailii* are naturally toxin-resistant, expression of a zygocin-specific immunity component is dispensable in this host. This limitation, however, can be bypassed by expressing the prepro-zygocin gene in the few yeast genera that are known to be naturally zygocin-resistant. In this respect, we could demonstrate that fission yeast is such a host, suitable for heterologous zygocin expression; although toxin secretion in recombinant *S. pombe* was significantly lower than in the natural ZbV-M infected *Z. bailii* killer, successful expression of the full-length M_{Zb}-cDNA in *S. pombe* will nevertheless allow a more detailed analysis of the molecular mode of action of this unique antifungal virus toxin; such experiments are planned for the near future.

Experimental procedures

Strains and culture conditions

All yeast strains used in this work are listed in Table 1. *Escherichia coli* DH5α [F^- *rec*A1 *end*A1 *gyr*A96 *thi hsd*R17 *sup*E44 *rel*A Δ(*arg*F-*lac*ZYA) U169 (Φ_{80}d*lac*ZΔM15) λ] was used as a general host for the amplification and propagation of all vector constructs. Yeast cultures were grown at 30°C either in complex yeast extract peptone dextrose (YEPD) medium or in synthetic medium (YNB) supplemented with the appropriate amino acid/base requirements of each strain (Moreno *et al*., 1991).

Killer and immunity tests

Zygocin-specific killing was determined by the agar diffusion method on Methylene blue agar plates (MBA, pH 4.0) as described previously (Schmitt and Tipper, 1990). Toxin-containing, cell-free culture supernatants of the *Zygosaccharomyces bailii* wild-type killer strain 412 and of the *Schizosaccharomyces pombe* transformants were concentrated by ultrafiltration using omega membranes with a 8 kDa molecular weight cut-off (Pall Filtron). Aliquots of 100 µl were pipetted into wells cut into the agar, which had been seeded with the indicated sensitive tester strain. After incubating the plates for 4 days at 20°C, zones of growth inhibition surrounding the wells were measured. To determine toxin sensitivity/resistance patterns in *S. pombe* spheroplasts, zymolyase-treated cells of *S. pombe* strain PW260 (Table 1) were embedded in osmotically stabilized MBA (pH 4.0, 1.2 M sorbitol) and incubated for 6 days at 20°C as described previously (Heintel *et al.,* 2001).

DsRNA preparation, cDNA synthesis and cloning

Total RNA from the wild-type *Z. bailii* killer was isolated by the hot-phenol method as described previously (Kohrer and Domdey, 1991). dsRNA preparations that were used as template for cDNA synthesis and 5′- and 3′-RACE (rapid amplification of cDNA ends) were purified by preparative electrophoresis through a low-melting agarose gel (0.8%) as described previously (Schmitt, 1995). A partial cDNA copy of the zygocin encoding M_{Zb}-dsRNA was synthesized according to the method of Gubler and Hoffman (1983), using gel-purified dsRNA as template. Before first-strand synthesis, the dsRNA was denatured using methyl-mercury hydroxide (Esteban *et al*., 1988). First- and second-strand cDNA synthesis were performed using AMV reverse transcriptase, DNA polymerase I, random hexanucleotide primers [$p(dN)_6$] and RNase H. The generated double-stranded cDNA was blunt-ended with T4-DNA polymerase, and size-fractionated cDNAs (> 0.1 kb) were pooled, ligated into the *Sma*I-restricted and dephosphorylated cloning vector pUC18 (Pharmacia), and electroporated into competent cells of *E. coli* DH5α. A great proportion of the total mycoviral sequence could be deduced after simple sequence alignment of the obtained cDNA fragments. The obtained sequences enabled us to construct specific primers for the rapid amplification of cDNA ends (RACE) to obtain the complete 5′- and 3′-termini of the toxin-coding M-dsRNA. All RACE amplifications were performed according to the instructions of the manufacturer (Roche Diagnostics, Mannheim). Denatured dsRNA was used as template for cloning of the codogenic 3′-terminus of M_{Zb}, whereas total RNA derived from the *Z. bailii* wild-type killer (containing the viral M_{Zb}(+)ssRNA transcript) served as template for the amplification of its complete 5′-terminus.

Table 1. Yeast strains used in the present study.

Strain	Genotype	Viral dsRNA(s)	Killer phenotype	Reference/source
Z. bailii 412	Unknown, wild-type	L_{Zb}, Z, M_{Zb}	Killer	Radler *et al.* (1993)
Z. bailii FN201	Unknown, wild-type	L_{Zb}, Z	Non-killer derivative of *Z. bailii* 412	Schmitt and Neuhausen (1994)
S. cerevisiae 192.2d	MATα *ura*3 *leu*2	L-A	Non-killer	Schmitt *et al.* (1996)
S. pombe PW260	h^- l*eu*1.32 *ura*4 *ura*4 *dl*18 *ade*6.210	None	Non-killer	Heintel *et al.* (2001)
C. krusei 185	Unknown, wild-type	None	Non-killer	This study

Heterologous zygocin expression in fission yeast

The preprotoxin encoding open reading frame (ORF) was constructed based on the largest obtained cDNA fragment encompassing most of the coding sequence. Missing bases were introduced by using specific primers that contained 5′- and 3′-flanking *Nde*I–*Bam*HI restriction sites. For heterologous zygocin expression, the complete pptox encoding ORF was cloned into the episomal fission yeast vector pREP1 (Maundrell, 1990; 1993), resulting in the zygocin expression vector pREP-Zygo. Vector pREP-K28/Zygo was constructed by in-frame insertion of a *Bgl*II–*Kpn*I fragment containing the cDNA sequence of the mature zygocin toxin downstream of a secretion and processing signal derived from the viral *S. cerevisiae* K28 pptox gene (Heintel *et al.,* 2001). In all constructs, expression of the prepro-zygocin gene is under transcriptional control of the thiamine-repressible fission yeast promoter *nmt*1 (*nmt* for 'no message in thiamine'; Moreno *et al.,* 2000). *S. pombe* was transformed by the lithium acetate method as described by Okazaki *et al*. (1990), and the resulting fission yeast transformants were selected on minimal agar lacking leucine. Cell-free culture supernatants of the *S. pombe* pREP-Zygo transformants were tested for zygocin activity in a modified agar diffusion assay as described previously (Heintel *et al.,* 2001). *E. coli* DH5α was electroporated using the gene pulser II system (Bio-Rad). All DNA constructs were routinely sequenced by fluorescent cycle sequencing on an automated DNA sequencer (Li-Cor 4200, MWG-Biotech).

Western analysis

To estimate the amount of killer toxin secreted by zygocin-expressing *S. pombe* transformants, cultures of the appropriate yeasts were grown in synthetic minimal medium (MM) in the absence of thiamine (derepressed culture conditions) for 2 days at 30°C until cell numbers of about 5×10^7 cells ml^{-1} were reached. The cell-free culture supernatant was concentrated by ultrafiltration and electrophoretically separated in tricine/sodium dodecyl sulphate polyacrylamide gels according to the procedure described by Schagger and von Jagow (1987). After electrotransfer of the proteins onto a PVDF membrane in transblot buffer (25 mM Tris, 192 mM glycine, 20% methanol, 0.1% SDS), Western analysis of zygocin was carried out using affinity-purified polyclonal antipeptide antibodies that were directed against a 10-amino-acid epitope at the C-terminus of the toxin. An alkaline phosphatase-conjugated goat anti-rabbit-IgG antiserum was used as secondary antibody, and colorimetric signal detection of the immunoprecipitate was performed using a NBT/BCIP stock solution (Roche), diluted 1:500 in staining buffer as described previously (Eisfeld *et al.,* 2000).

Northern analysis

For detection of the toxin encoding M_{Zb}-dsRNA and its *in vivo* transcript [M_{Zb}(+)ssRNA], total RNA was isolated from exponentially growing cells of the wild-type killer strain *Z. bailii* 412 and subsequently analysed by electrophoresis in denaturing formaldehyde-agarose gels as described previously (Schmitt *et al*., 1997). After RNA transfer onto a positively charged nylon membrane (Roche), zygocin-encoding RNA species were detected by Northern hybridization using a polymerase chain reaction (PCR) amplified M_{Zb}-cDNA probe, which encompassed the entire 717 bases of the toxin-coding ORF. Digoxigenin-labelling of the PCR fragment and chemoluminescent signal detection of the immunoprecipitate were performed with a PCR DIG Probe Synthesis Kit and a DIG Easy Hyb Kit as recommended by the manufacturer (Roche).

Protein sequencing and MALDI-TOF analysis

Affinity-purified zygocin toxin was fractionated by SDS-PAGE, electroblotted onto a PVDF membrane and subsequently used for N-terminal sequence analysis by Edman degradation as described previously (Schmitt and Tipper, 1995). For molecular mass determination of the purified toxin by matrix-assisted laser desorption mass spectrometry (MALDI-TOF), zygocin was purified by receptor-mediated affinity chromatography on a mannoprotein-coupled sepharose matrix (Weiler and Schmitt, 2002). MALDI-TOF analysis was performed in the positive mode on a Bruker Reflex III unit with delayed extraction technology. Samples were diluted 1:10 with matrix, and 0.5 μl of the resulting mix was deposited onto a smooth plate. Acceleration voltage was set at 25 kV and 10–50 laser shots were summed. Sinapinic acid (D13, 460–0; Sigma-Aldrich) was used as matrix. The mass spectrometer was calibrated with cytochrome *c*.

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