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Lycotoxin-1 insecticidal peptide optimized by amino acid scanning mutagenesis and expressed as a coproduct in an ethanologenic *Saccharomyces cerevisiae* strain‡

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Abstract: New methods of safe biological pest control are required as a result of evolution of insect resistance to current biopesticides. Yeast strains being developed for conversion of cellulosic biomass to ethanol are potential host systems for expression of commercially valuable peptides, such as bioinsecticides, to increase the cost-effectiveness of the process. Spider venom is one of many potential sources of novel insect-specific peptide toxins. Libraries of mutants of the small amphipathic peptide lycotoxin-1 from the wolf spider were produced in high throughput using an automated integrated plasmid-based functional proteomic platform and screened for ability to kill fall armyworms, a significant cause of damage to corn (maize) and other crops in the United States. Using amino acid scanning mutagenesis (AASM) we generated a library of mutagenized lycotoxin-1 open reading frames (ORF) in a novel small ubiquitin-like modifier (SUMO) yeast expression system. The SUMO technology enhanced expression and improved generation of active lycotoxins. The mutants were engineered to be expressed at high level inside the yeast and ingested by the insect before being cleaved to the active form (so-called Trojan horse strategy). These yeast strains expressing mutant toxin ORFs were also carrying the xylose isomerase (XI) gene and were capable of aerobic growth on xylose. Yeast cultures expressing the peptide toxins were prepared and fed to armyworm larvae to identify the mutant toxins with greatest lethality. The most lethal mutations appeared to increase the ability of the toxin α-helix to interact with insect cell membranes or to increase its pore-forming ability, leading to cell lysis. The toxin peptides have potential as value-added coproducts to increase the cost-effectiveness of fuel ethanol bioproduction. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: insecticidal peptide; amino acid scanning mutagenesis; SUMO high-level yeast expression system; fuel ethanol coproduct

INTRODUCTION

Fuel ethanol production from biomass at the industrial level shows great promise for satisfying future energy demands. However, the limited range of sugars that can be fermented efficiently using *Saccharomyces cerevisiae* remains an obstacle to cost-effective biomass-based ethanol production. Although several genetically engineered strains of *S. cerevisiae* have been developed that will ferment xylose to ethanol [1–3], further optimization is needed to achieve efficient fermentation of pentose sugars and attain an economically feasible process for the conversion of lignocellulosic biomass to ethanol. Additionally, the cost-effectiveness

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of the fuel ethanol fermentation process, especially in biomass-based (*vs* corn-derived) fuel ethanol production, could be enhanced by obtaining high-value coproducts and by-products from the process. The yeast strain used for industrial fuel ethanol production has the potential to serve as a host for expression of commercially valuable coproducts such as proteins and peptides. To utilize this capability, genes for these products can be mutagenized, placed in an expression system capable of producing high levels of functional product, and screened in high throughput for optimized function. An integrated automated platform is available to carry out the entire process required to optimize and express value-added proteins or peptides, including high-throughput production of plasmid libraries, expression of cloned genes, and functional testing of the expressed proteins or peptides [4–6].

A pesticidal protein or peptide is one such potential coproduct, which could be expressed in the industrial yeast strain. During the process, the antimicrobial properties of the compound will help prevent contamination of the fermentation process [7]. After completion of the ethanol production process, the killed yeast could be dried and used to deliver the pesticide to insects. It is estimated that insect pests destroy 20–30% of the world's crop production [8]. In many cases, natural enemies are not sufficient to control pests adequately [9]. The *Bacillus thuringiensis* (Bt) crystal protein is one example of an insecticidal protein that has been developed commercially. It is highly effective against a targeted range of species depending on the clone [10,11] and has been employed in plants through transgenic production [12–14] for complete control of some insect species, such as European corn borers [15]. With commercialization of transgenic crops expressing Bt toxins, selection pressure has increased and there is concern that target insects may develop resistance to individual Bt proteins, requiring structured refuges of non-Bt host plants where Bt hybrids are grown [15]. Even with such resistance management strategies, there is still concern that resistance will develop in insect pests receiving sublethal doses [16], and cross-resistance to different Bt proteins has been reported [17]. Combinations of insecticidal protein genes may be needed for more durable control [18]. Both synthetic materials [19] and naturally derived plant extracts [20] continue to be explored as alternatives to Bt proteins.

The peptides in spider venoms appear to have potential for insect control owing to their specificity for the insect nervous system [21,22]. Toxins from spider venoms define new insecticide targets owing to specific action to block insect voltage-gated Ca^{2+} channels [23]. These toxins show promise for development of recombinant biopesticides for control of insecticide-resistant agricultural pests [23]. Sequential alanine substitutions along the peptide chain of the insect-specific toxins of the funnel-web spiders in which each amino acid residue is separately replaced with alanine demonstrated critical features necessary for insecticidal activity of the toxins [21,24]. A more efficient method for producing large numbers of toxin variants that are potentially more effective against insects would be to substitute each amino acid in the toxin peptide with all 20 possible amino acids rather than just one.

The selective spider venom peptide, lycotoxin-1 (also known as lycotoxin-I), from wolf spider (*Lycosa carolinensis*) venom demonstrates both antimicrobial and insect neuroactive properties [7,25], which from its amphipathic nature and physiological actions appears to function as a pore former to increase membrane permeability, dissipate voltage gradients, and effect lysis of insect cells [7,25]. These properties suggest the potential use of lycotoxin-1 as a bioinsecticide. Variants of the lycotoxin-1 peptide to optimize insecticidal activity could potentially be produced by random mutagenesis (error-prone PCR), which is relatively slow, costly, and nonspecific [26], or gene shuffling, if a family of related genes with more lethal properties were available [27], but both methodologies have time-based or location-based limitations. Antimicrobial peptides for a related species of *Lycosa* indicate that some variation in peptide sequence is possible without losing biological activity [28]. A more comprehensive approach to investigating and optimizing peptide structure for biological activity is codon randomization [6,29] by which libraries are generated containing all possible amino acid substitutions for the position of interest. Because of the large number of mutants involved, this methodology requires effective automation.

We now report on the application of the multiplex method [5,6], made possible by integration of a robotic colony-picking component onto an automated workcell platform capable of high-throughput preparation of plasmid libraries expressing mutant open reading frames (ORFs), for a proof of concept improvement in activity of lycotoxin-1 against a representative insect pest, the fall armyworm (*Spodoptera frugiperda*). Coupled with this methodology, we used the small ubiquitin-like modifier (SUMO) yeast expression vectors [30,31] to produce the large amounts of properly folded mutant peptides required for screening, and performed an initial screen of multiplexed cultures from which individual variants with enhanced lethality were then identified. These mutant peptides were produced by an ethanologenic yeast strain that grew on xylose plates. An enterokinase K (EntK) site was placed in front of the lycotoxin-1 ORF, which was then inserted into the SUMO vector so that after high levels of toxin are expressed in the yeast cells, the SUMO tag is quickly cleaved by the yeast protease (Ulp1), but active toxin is not released until the EntK tag is cleaved by trypsin in the insect gut killing the insect ('Trojan horse' strategy).

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MATERIALS AND METHODS

Production of pYES2-DEST52 NNR Mutant Lycotoxin-1 Library in INVSc1 Yeast Strain

Two oligomers, each representing part of the sequence for lycotoxin-1 from *L. carolinensis* [7], were synthesized and annealed together. The annealed long oligomers were filled in with Taq polymerase using the ABI high fidelity kit as described previously [4]. The PCR product representing the complete wild-type lycotoxin-1 sequence was purified, TOPO-cloned into pENTR D TOPO, and moved into pYES2-DEST52 yeast expression vector, a URA-selectable plasmid having a *gal10* promoter (Invitrogen) for maximum expression in the INVSc1 yeast strain, using LR clonase according to the Invitrogen LR clonase II kit directions. The LR clonase reaction was transformed into TOP 10 competent cells and recovered on AMP 50 plates (Teknova), the resulting colonies were picked, and plasmids were prepared and transformed into INVSc1 yeast as described previously [4]. The yeast was plated onto complete minimal (CM) galactose URA-selective agar to recover transformants. In addition, a library of mutagenized lycotoxin-1 ORFs was produced using the NNR mutagenesis strategy described previously [5]. Mutant clone #59 (also called C3) that was subsequently used for further mutagenesis via amino acid scanning mutagenesis (AASM) was obtained from this library of NNR mutants, in which the codons for the last four amino acids of the wild-type Lyt-1 peptide were mutagenized [6]. Targeted mutagenesis of the last four codons of wild-type Lyt-1 was accomplished by creating a PCR primer set of synthetic oligonucleotides consisting of a long forward primer with a sequence the same as that of the wild-type Lyt-1 ORF and a reverse primer (antisense strand) differing from the wild-type ORF in the last four codons. This primer contained any nucleotide in the first and second positions and a pyrimidine in the last position (NNY) for the last four codons of the ORF. Consequently the sense strand contained the complementary NNR motif where R is a purine. This strategy generates a library of ORFs that code for all possible amino acids at each of the last four positions at the carboxy-terminus in the Lyt-1 peptide. Methods for topoisomerase ligation of mutagenized PCR products into pENTR D TOPO, transformation into TOP 10 *Escherichia coli* cells, and subsequent plasmid preparation were performed. Thirty-two colonies with sequence-verified inserts from the pENTR D TOPO library were picked in triplicate into a 96-well plate and the mutagenized clones were inserted using LR clonase into pYES2-DEST52 for transformation into INVSc1 yeast and recovery on CM galactose URA-selective medium plates. The yeast strains with the expression plasmids carrying inserts for the peptide toxins were grown on CM galactose URA-selective plates and cultured in 50 ml defined CM galactose URA-selective liquid medium for scale up. Yeast cultures were grown, pelleted, lyophilized, mixed with insect diet, and placed on a disk on agar in a Petri dish for the initial armyworm larvae assay.

Construction of pSUMOduo Two-Vector System for Simultaneous Expression of Xylose Isomerase and Value-added Product

Our objective was to express a value-added product, such as a bioinsecticide, in an ethanologenic yeast strain capable of utilizing xylose as well as glucose (Figure 1). For the initial screen that identified clone #59, the mutant lycotoxins were placed into the yeast expression vector pYES2-DEST52 having a galactose-inducible promoter. It would not be possible to use this promoter to express the mutagenized lycotoxin-1 library in a commercial yeast strain that would also need to be evaluated for growth on pentose sugars in the absence of hexose sugars. It was determined that moving the lycotoxin-1 library into an expression vector with a constitutive *ADH* promoter would produce the highest levels of xylose utilization in a yeast strain engineered for overexpression of xylose isomerase (XI) [32–34]. The pSUMOduo high-copy expression vector set, containing the protease-cleavable yeast SUMO tag (Smt3) behind an *ADH* promoter and also a T7 *in vitro* modified promoter, was selected [30].

For the novel yeast episomal pSUMOduo high-level expression vector set [30,31], the protease-cleavable yeast SUMO tag (Smt3) was placed in front of an Invitrogen Gateway cassette, producing an AMP-selectable destination vector for recombination of library inserts. The SUMO insert ORFs were expressed behind an *ADH* promoter and also a T7 *in vitro* modified promoter. Two vectors were produced, pSUMOduo/URA and pSUMOduo/TRP, each one having a different yeast selectable marker (for uracil or tryptophan prototrophy, respectively) and each used for expression of a different ORF of interest: *Piromyces* XI or lycotoxin-1 peptide. The vectors contain the yeast high-copy 2 μ origin of replication, to give a copy number of roughly 20 per yeast cell [35].

The first plasmid, pSUMOduo/URA, was constructed as follows. First, a Gateway cassette fragment (Gateway Vector Conversion System, Invitrogen) containing *att*R1-*ccd*B-Cm^R-*att*R2 was amplified by PCR with primers containing BbsI restriction sites designed to generate ACCT and GATC overhangs, then subcloned into BsaI/BamHI-digested pSUMO (aka pET24-6xHis-SUMO) [30] to construct the plasmid pSUMO-Gateway. A P_{T7}-His_6-Smt3-Gateway cassette-T_{T7} fragment was then PCR-amplified from pSUMO-Gateway, digested with EcoRV and BbsI to generate a blunt end and a GATC overhang, and subcloned into a SmaI/BglII fragment of pRS306-P_{ADH} [36] containing the *E. coli* amp^R marker, the yeast *URA3* marker, and the promoter of the yeast *ADH1* gene. The resulting plasmid was digested with SapI and ligated with a SapI fragment from pRS426 [35] containing the yeast 2 μ origin of replication. QuikChange mutagenesis [37] was then used on the resulting plasmid to delete two extraneous, tandem P_{T7} sequences present in the vector backbone from the previous construction of pRS306-P_{ADH} [36].

To replace the *URA3*-selectable marker in pSUMOduo/URA with *TRP1* and create the other vector of the 2-vector system, the *TRP1* sequence was amplified from pGBKT7 vector (Clontech) with oligos (CGCGAGCTAGCCGCGCGTTTCGGTGATGACGGTGA) and (CGCGACGCGTGATCGGCAAGTGCACAAACAATACT). Inserts were then digested with NheI and MluI and cloned into similarly digested pSUMOduo/URA vector.

The *Piromyces* XI wild-type ORF [32] with His tag was placed into pENTR D TOPO, cloned into vector 1, pSUMOduo/URA, of this 2-vector system (LifeSensors, Malvern, PA), and transformed as described previously [4] into INVSc1 yeast (Invitrogen), a fast growing diploid strain ideal for expression, which does not sporulate well and

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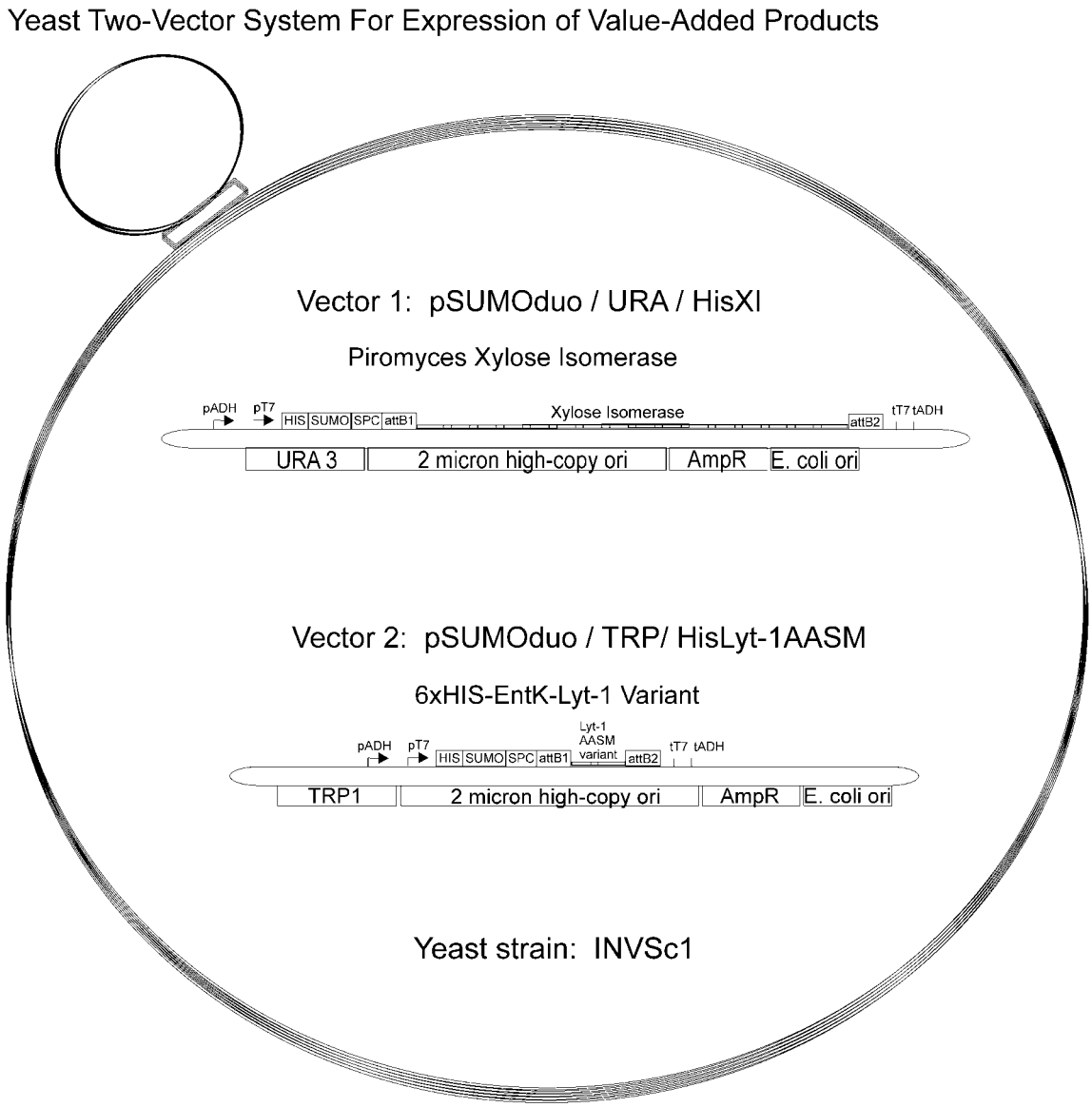

Figure 1 Schematic drawing of INVSc1 yeast strain transformed with vector 1, pSUMOduo/URA/HisXI, and with vector 2, pSUMOduo/TRP/HisLyt-1AASM, to give pSUMOduo/URA/HisXI- pSUMOduo/TRP/HisLyt-1AASM-INVSc1 (INVSc1-XI-Lyt-1).

carries the mutations *MATalpha-his3D1-leu2-trp1-289-ura3-52 MAT-his3D1-leu2-trp1-289-ura3-52*, to produce the strain pSUMOduo/URA/HisXI-INVSc1 (INVSc1-XI).

Production of pSUMOduo/TRP/Lyt-1 AASM Mutant Library in INVSc1-XI Yeast Strain

The pENTR D TOPO library of ORFs for mutant lycotoxin-1 sequences produced by AASM from clone #59 as described previously [6] was placed into vector 2, pSUMOduo/TRP, of the 2-vector system (LifeSensors, Malvern, PA). The library was designed to code for all possible 20 amino acids at each of the 25 amino acid positions in lycotoxin-1 from wolf spider *L. carolinensis* [7]. This set of multiplexed ORF inserts in pENTR D TOPO was moved by LR clonase into the pSUMOduo/TRP vector to produce the pSUMOduo/TRP/HisLyt-1 AASM library. This was mass-transformed into the INVSc1-XI strain to produce pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1, AASM-INVSc1 (INVSc1-XI-Lyt-1) strains in each of 25 multiplexed wells, one well per amino acid position mutation, with all 20 amino acid possibilities for that position in each well.

Screening of Lycotoxin-1 Mutants for Lethality

Initially, for testing the NNR mutants, the yeast cultures were pelleted, lyophilized, mixed in water used to rehydrate lyophilized pinto-bean-based insect diet [38], and placed on a Teflon disk at the center of a 32 mm Petri dish containing 3% agar. Armyworm larvae were added to the dish where they could move along the moist agar surface to the yeast culture area. The armyworms were obtained from a laboratory colony in Illinois associated with the USDA, Crop Bioprotection Research Unit, and were propagated from eggs in an insectarium at NCAUR [38]. Twenty newly hatched larvae were used per treatment [38]. Yeast carrying a GUS plasmid was used as a control. The number of dead armyworms was counted each day of the assay.

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Because it was found that assays using growing yeast appeared to give more sensitive bioassay results, the testing of the AASM mutants was performed by applying the armyworm larvae directly to plates containing areas of growing yeast. Samples of the transformation reactions from each of the multiplex wells were recovered on 86 × 128 mm rectangular CM glucose URA/TRP-selective plates for 2 days at 30 °C and diluted 10 000-fold in sterile water; then 200 µl was spotted onto the respective CM xylose URA/TRP-selective plates and allowed to grow for 3 days to generate a yeast growth area (spot) expressing toxins. The size of the spot was recorded and used to normalize the percent killed, since the percent is a function of the amount of yeast encountered. Twenty newly hatched armyworm larvae were added to the growth spot on the plate, the plate was covered with a lid having five small 0.4 mm openings in the center protected with cotton organdy cloth, and the lid was sealed to the plate with Microseal 'A' film (MJ Research, Waltham, MA) cut so that a gasket 5-mm wide could seal the edges. The plates were scored for the number of dead armyworms daily after adding the larvae until the assay was ended. Results from days 1 and 2 were of the greatest importance for a commercially viable insecticide. The multiplexed wells having the mutants showing greatest lethality (positions 8, 9, and 10) were selected and the multiplexed wells containing the lycotoxin-1 inserts in pENTR D TOPO corresponding to those positions were transformed into TOP 10 cells, single colonies picked from spread plates, and a plasmid preparation performed. The sequences of 90 AASM variant single isolates picked from the three most lethal multiplexed wells were determined. Forty-five of these had inserts with complete sequences in the correct orientation. Nine were C3, while 36 of them were unique variants and these were selected for the second lethality assay [6]. They were moved into pSUMOduo/TRP via LR clonase, retransformed into INVSc1-XI yeast, and recovered on CM glucose URA/TRP-selective plates. Single colonies with sequence-verified inserts were picked into CM glucose URA/TRP-selective liquid medium, grown, and diluted 10 000-fold. A 100 µl sample was spotted onto CM xylose URA/TRP-selective plates and analyzed for lethality, along with samples containing C3, in quadruplicate, with 20 newly hatched armyworm larvae added to each plate. The variants were scored for dead armyworms and the percent killed was calculated.

Polyacrylamide Gel Electrophoresis and Western Blot Procedure

Transformed yeast strains carrying the pSUMOduo plasmids, each with a different selectable marker, were inoculated into 1.0 ml of the respective selective medium and grown for 2 days at 30 °C in deepwell plates. The resulting cultures were spun down at 4000 rpm for 20 min in a swinging bucket centrifuge with plate carriers, the supernatant was discarded, and the pellet in the first well of the first column of the plate was resuspended by vortexing with 250 µl Y-PER lysis buffer. This solution was used to resuspend the pellets in each successive well of the first column. The solution was transferred to the first well of the next column and vortexed to resuspend the pellet. The process was repeated until all the pellets were combined and resuspended in the last well of the last column. This 250-µl sample was pipetted into a 1.5-ml tube and incubated at 4 °C with rotating for 3–4 h. A 30-µl aliquot from this tube was pipetted into the wells in an MJ plate and 30 µl of 2× tris-glycine SDS loading buffer (Invitrogen) with β-mercaptoethanol (BME) was added to each well. To the remaining 220 µl of sample in the 1.5-ml tube was added 100 µl of Super Flow Ni beads (Qiagen), and the sample was incubated at 4 °C with rotating for approximately 3 h. The sample was centrifuged at 13 000 rpm for 2 min and 30 µl of the supernatant was pipetted into a new 1.5-ml tube. Thirty microliters of 2× tris-glycine SDS loading buffer with BME was added. These samples and the samples in the MJ plates were heated to 95 °C for 10 min, loaded onto 1.5-mm thick 16% tris-glycine 15-well SDS-PAGE gels (Invitrogen), and run at 110 V for 150 min. The gel was assembled into an X-Cell Novex box and run in 1× tris glycine running buffer. It was removed and transferred to polyvinylidene difluoride (PVDF) cut sheets (Invitrogen). The transfer took place in the Novex transfer apparatus with 1× transfer buffer (Invitrogen) for 10 h at a constant current of 200 mA. Westerns were chromogenic and performed with the Western Breeze kit (Invitrogen) according to the manufacturer's directions using 30 µl of a Qiagen mouse anti-penta-His antibody resuspended in 1 ml of sterile 1× phosphate buffered saline (PBS) with magnesium and calcium (secondary antibody was anti-mouse antibody conjugated to alkaline phosphatase). Western blot images were captured using the Alpha Innotech 3400.

Scanning Electron Microscopy

Yeast cells from the plate were suspended in saline (0.85% NaCl) and centrifuged to remove the residual medium. Following a modified procedure of Bang and Pazirandeh [39], the cell pellet was suspended and fixed in 2.5% glutaraldehyde prepared in 100 mM cacodylate buffer, pH 7.2, for 1 h on ice. To remove remaining glutaraldehyde, the cells were rinsed with the buffer twice and then with distilled water once, allowing several minutes for each step. The cells were dehydrated in solutions containing 50, 70, 80 and 100% ethanol successively for 15 min for each treatment. Cells were mounted on the aluminum stub and placed in the desiccator to dry overnight or until needed. The samples were subjected to scanning electron microscopy and analysis (Zeiss Supra 40 VP).

RESULTS AND DISCUSSION

Lethality to Armyworms of NNR Mutant Lycotoxin-1 Library in INVSc1 Yeast Strain (Initial Screen)

The lyophilized yeast cultures expressing wild-type lycotoxin-1 and the library of mutagenized lycotoxin-1 ORFs that was produced using the NNR mutagenesis strategy described previously [5] were each mixed with rehydrated lyophilized insect diet and the mixture was placed on the Teflon feeding disk at the center of a Petri dish [38]. When the armyworm larvae were added, they tracked the material off the disk onto the agar in the dish. The yeast became rehydrated and began to grow and express toxin. The armyworms not only ingested the yeast but also carried it across the agar producing numerous yeast colonies. One of the yeast cultures was

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highly lethal to the armyworms [5]. This yeast strain was expressing a lycotoxin-1 variant, identified as clone #59 (designated C3 in this paper), with the sequence H_2NHHHHHH(6XHis)DDDK(EntK)IWLTALKFLGKHAAK HLAKQQLSPWCOOH, produced by the mutagenesis strategy described previously [5], in which the codons for the last four amino acids in the peptide were randomized for NNR. This C3 variant differed from wild-type lycotoxin-1 in the two amino acids at the carboxy terminus, with proline and tryptophan residues replacing the lysine and leucine residues at positions 24 and 25, respectively, in the wild-type lycotoxin-1. These initial screening results demonstrated that the solid agar plate would support the growth of the yeast colonies and indicated that the fall armyworm larvae would consume growing yeast cultures expressing peptide toxins. The lycotoxin-1 variant (C3) that was highly lethal to the armyworms in this initial evaluation was selected for further optimization by AASM [6]. The wild-type lycotoxin-1 peptide was not found to be lethal in this initial screening. It is possible that wild-type Lyt-1 did not demonstrate lethality in this setting because the wild-type Lyt-1 form does not have the proper pharmacodynamic properties to reach the target site when administered orally. Prior work demonstrating activity of this peptide used injections or cell assays [7].

Strategy Using pSUMOduo Vector System

For the initial screen that identified C3, the NNR mutant lycotoxins were placed into the yeast expression vector pYES2-DEST52 having a galactose-inducible promoter. This promoter could not be used to express the mutagenized lycotoxin-1 library in a commercial yeast strain that would also need to be evaluated for growth on pentose sugars in the absence of hexose sugars. The pSUMOduo high-copy expression vector set, containing the protease-cleavable yeast SUMO tag (Smt3) behind an *ADH* promoter and also a T7 *in vitro* modified promoter [30], was selected to produce high levels of expression in the INVSc1 yeast strain engineered for xylose utilization (Figure 1).

In nature, the SUMO modulates protein structure and function by covalently binding to the lysine side chains of the target proteins [30]. In genetic and functional proteomic studies, the SUMO gene fusion system can be used to improve expression and chaperone correctly folded proteins and peptides [31]. Yeast cells contain a protease (Ulp1 or SUMO protease 1) that can efficiently cleave a variety of SUMO fusions robustly and with great specificity [30]. The enzyme is active over a broad range of pH and temperature conditions. Attachment of SUMO to the *N*-terminus of proteins has been found to greatly enhance their expression [30]. Vector 1 of the SUMO system was used to express the PCR-assembled XI ORF. The SUMO protease 1 (Ulp1) in yeast is highly efficient and cleaves the SUMO tag to completion giving large amounts of functional XI. Vector 2 was used for expression of the lycotoxin-1 clone library produced by AASM to allow automated high-throughput plasmid production and expression of this insecticidal peptide. This vector can be used for other optimized ORFs, such as cellulases, to produce improved yeast strains for industrial use in conjunction with the XI gene to enable xylose utilization. A third SUMO vector (LEU selectable) is available that would make possible introduction of additional clones of interest. These could be obtained from traditionally produced cDNA libraries using superscript reverse transcriptase or the FLEXGene collections [40] of expression-ready fully annotated inserts for whole library introductions or ordered grid transformations of select FLEXGene sets. This vector could allow expression of enzymes to improve tolerance, permit anaerobic growth on xylose, and increase ethanol output in industrial yeast strains.

Lethality to Armyworms of AASM Mutant Lycotoxin-1 Library in INVSc1-XI Yeast Strain

Lethality testing on fall armyworm larvae of the multiplexed cultures of INVSc1-XI-Lyt-1 yeast strains grown on CM xylose URA/TRP-selective plates indicated that variants with mutations at positions 8, 9, or 10 gave the greatest effect at days 1 and 2, while those having mutations at positions 5, 6, 7, or 11 showed the next most lethal effect. Mutations at positions 14, 15, 19, 20, 21, or 23 were less effective and mutations at positions 4, 12, 13, 16, 17, 18, or 22 showed no effect on the fall armyworms (Table 1). All variants contained the mutations in the parent clone #59 (designated C3 in this paper), which had proline and tryptophan at positions 24 and 25, respectively, adjacent to the carboxy terminus. Wild-type lycotoxin-1 has a lysine residue at 24 and a leucine at 25.

Single colonies were picked from the multiplexed wells for the mutation positions showing greatest lethality. Growing yeast spots containing the individual variants were tested for lethality to fall armyworm larvae. The results for the parent variant (C3) and for the three most lethal mutations derived from C3 are presented in Table 2. Percent dead on day 2 for C3, B9, A6, and C6 were 71, 97, 74, and 100%, respectively (Table 2). The two AASM mutants B9 and C6 were more lethal than the NNR mutant C3. The numbers are normalized for diameter of the colony on the xylose plates. A test for normality, performed using SAS Proc Univariate (SAS Institute 1999), indicated that the mortality data did not follow a normal distribution, and therefore analysis of variance was not appropriate. Frequency analysis was performed using SAS Proc Freq (SAS Institute 1999) with data adjusted for colony size and assuming a conservative 20-insect sample for each treatment. Under these conditions, relative mortality caused by mutants B9 and C6 was significantly greater

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Table 1 Lethality to fall armyworms of multiplexed cultures of INVSc1-XI-Lyt-1 yeast strains with libraries of Lyt-1 variants grown for 3 days on CM xylose URA/TRP selective plates. Each multiplexed culture contained lycotoxin-1 variants with all possible amino acids at one of the 25 positions in the amino acid sequence. Twenty fall armyworm larvae were fed the culture and the number of dead worms was scored on day 1 and day 2. The percent killed was adjusted for the diameter of the yeast colony on the medium

Multiplex yeast cultures expressing HIS-tagged Lyt-1 toxin variants grown on xylose plates kill fall armyworms		
Strain carrying toxin with mutation at aa position	Armyworms killed day 1 (%)	Armyworms killed day 2 (%)
aa position 4	0.0	0.0
aa position 5	0.0	47.3
aa position 6	18.3	55.7
aa position 7	19.5	83.3
aa position 8	43.0	83.3
aa position 9	44.3	92.7
aa position 10	0.0	100.0
aa position 11	0.0	47.0
aa position 12	5.5	0.0
aa position 13	4.9	5.6
aa position 14	19.7	27.7
aa position 15	16.5	17.8
aa position 16	0.0	0.0
aa position 17	0.0	0.0
aa position 18	0.0	0.0
aa position 19	0.0	27.8
aa position 20	0.0	15.4
aa position 21	0.0	37.9
aa position 22	0.0	0.0
aa position 23	0.0	27.8

than mortality caused by mutants C3 and A6 ($P < 0.05$). Mortality caused by mutant C6 compared to B9 and mortality caused by A6 compared to C3 were not significantly different in this analysis.

The function of lytic peptides such as lycotoxin-1 is to cause cell lysis by disrupting the cell membrane. Common features of these peptides include an overall basic charge, a small size (23–39 amino acid residues), and the ability to form amphipathic α-helices. They appear to disrupt the membrane lipid bilayer either by association with the amphipathic α-helix portion or by ion channel formation. In either case, an ordered secondary conformation such as an amphipathic α-helix and positive charge appear to participate in the lytic function [41–43]. The net positive charge promotes interaction with negatively charged prokaryotic membranes (normal mammalian cell membranes have more positive-charge character).

The sequence of the mutant peptides was designed to allow expression of high levels of appropriately folded, soluble peptides after ingestion by the insect before release of active toxin by trypsin in the insect gut. The automated robotic platform allowed rapid mutagenesis and multiplex evaluation of mutations to identify the positions where optimization produced variants that were more deadly to fall armyworms. Out of all possible theoretical mutations created, the critical positions were found to be positions 8, 9, and 10. It is possible that the strains with the optimized clones had already been selected to some extent by lack of viability on xylose or toxicity to yeast. The screening process identified the yeast strains that tolerated the presence of the toxin and also grew aerobically on xylose. The fall armyworms willingly consumed the yeast cultures that were expressing the optimized variants and died.

It can be seen from Figure 2 that the armyworms on the control plate, containing INVScI-XI yeast culture, are alive, have grown quite large, and are very active as seen by the trail of yeast colonies. The fact that the INVSc1-XI strain does not kill the armyworm larvae excludes the possibility that the alcohol has insecticidal

Table 2 Lethality to fall armyworms of single colonies from the three most lethal multiplexed position mutations with sequence-verified lycotoxin-1 inserts grown on CM xylose URA/TRP-selective plates for 3 days compared with the parent variant (clone #59 (C3))

Fall armyworm mortality from single INVSc1-XI-Lyt-1 yeast strain isolates expressing Lyt-1 toxin variants grown on xylose plates		
Yeast strain	Day 1 (% killed)	Day 2 (% killed)
XI-yeast control	0.0	0.0
XI Lyt-1 C3 (template ORF #59, has mutations K24P and L25W)	3.4	71.0
XI Lyt-1 A6 (variant has mutations F8H, K24P, L25W)	13.2	74.1
XI Lyt-1 B9[a] (variant has mutations G10Q, Q20H, Q21S, K24P, L25W)	0.0	97.2
XI Lyt-1 C6[a] (variant has mutations L9S, K24P, L25W)	3.7	100.0

Percent fall armyworms killed adjusted for colony size (4 plates averaged, each with 20 larvae added/plate)
[a] Mortality significantly greater than C3 and A6 ($P < 0.05$). C6 not significantly greater than B9. A6 not significantly greater than C3.

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activity since this strain would produce a quantity of ethanol similar to that produced by the INVSc1-XI-Lyt-1. Beyond 2 days some cannibalism occurred and it was difficult to observe unharmed worms as they first ate the yeast and then each other. On the other hand, the armyworms on the plates containing cultures expressing variants designated C3 (clone #59), A6, B9, and C6 are much smaller and mostly or all dead (Figure 2). An enlargement of one larva from each plate provides increased detail of the dead armyworms against the plate background (Figure 2 insets). These cultures may be lethal because the expressed toxin is not immediately cleaved at the EntK site, giving so-called Trojan horse peptides. The Lyt-1 peptide with the uncleaved EntK site is seen in the Western blot analysis (Figure 3).

The SUMO fusion technology used here in the second round of lycotoxin-1 peptide mutagenesis provides high levels of expression of the mutant toxins in the yeast strains and generates toxins that are lethal to the pests. When the sequence for the HisEntKLyt-1 is used with the SUMO vector, it is possible to express large amounts of engineered peptide for cleavage by endogenous yeast SUMO protease 1, which removes the SUMO tag after proper folding and increased solubility are accomplished [30]. The high levels of peptide with the uncleaved EntK site produced in the yeast cells are taken in by the armyworms, followed by release of active toxin by cleavage at the EntK site, DDDK, which is less active for insect gut trypsin than trypsin sites, permitting large amounts of toxin to get inside the armyworm before it is affected and stops ingesting the yeast. By consuming yeast expressing high levels of the peptide, the armyworms rapidly accumulate a lethal amount of toxin in their system before they are aware they have ingested it. Expression of the lycotoxin-1 variants did not affect the doubling time of the yeast cultures on glucose (1.9 h; SD 0.3; $n = 5$; data not shown), indicating the toxin was not lethal to the yeast.

Western Blot Analysis of Variant Yeast Strains

Western blot analysis of the INVSc1-XI-Lyt-1 yeast strains using mouse anti-penta-His antibody is shown in Figure 3. The labels at the top of the lanes identify the variant carried on SUMO vector 2. Lysates prepared after growth on CM glucose URA/TRP-selective plates and run directly on a 16% polyacrylamide gel show three major bands for all four strains: 53.7 kD for the attB1HisXI recombinant protein; 16.6 kD for the HisSUMO tag removed from both HisLyt-1 and HisXI; and 10.7 kD for the attB1HisEntKLyt-1 fusion toxin (Figure 3). The band for the HisSUMO leader is twice as intense as either of the other two bands since it is released from both the XI protein and the lycotoxin-1 variants in those samples.

Lysates prepared after growth on CM glucose URA/TRP-selective plates and additionally subjected

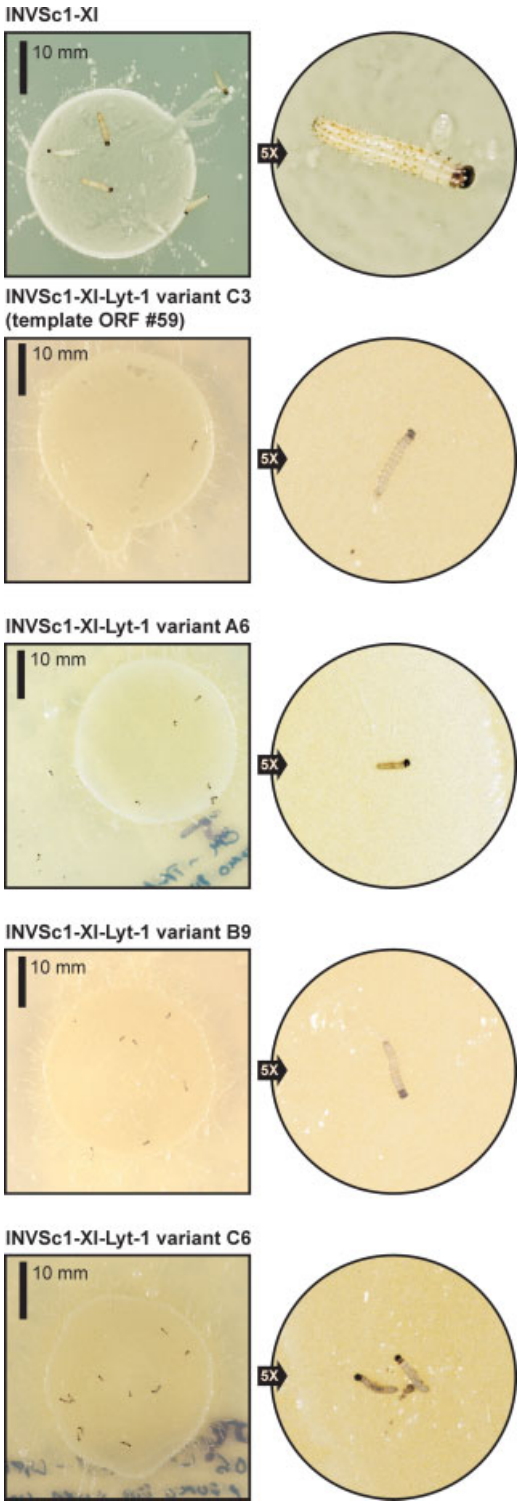

Figure 2 INVSc1-XI yeast, transformed with variants from the single isolates and grown on CM xylose URA/TRP-selective plates, 4 days after 20 fall armyworm larvae were added. The armyworms on the control plate, containing INVSc1-XI yeast culture, are all alive and large. The armyworms on plates with cultures expressing variants designated C3 (clone #59), A6, B9, and C6 are much smaller and mostly or all dead. The inset picture in each section is an enlargement of a part of the plate containing one larva.

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to Ni bead purification of the His-tagged proteins and then run on a 16% polyacrylamide gel show the same three bands (with a decrease in intensity of the 10.7 kD attB1HisEntKLyt-1 band) and an additional band at 21.4 kD, attributed to a dimer of the attB1HisEntKLyt-1 protein for all four strains (Figure 3). Dimer formation is postulated because in the Ni bead purification the beads concentrate the peptide on their surface and highly favor aggregation. Numerous amphipathic peptides are commonly thought to form multimeric pores in cell membranes [25]. In a model for pore formation in a membrane by magainin II, the molecules are arranged in dimers of α-helices aligned to form antiparallel amphipathic units. The amphipathic peptide, amyloid β-protein, associated with senile plaques in Alzheimer's disease, forms stable dimers [44] as shown by fluorescence resonance energy transfer and gel filtration chromatography. Aggregate formation by amyloid β-protein has been studied using SDS-PAGE [45,46]. The attB1HisEntKLyt-1 monomer band on the Western blot gel is dramatically decreased in intensity as would be expected if it were being used to form dimer. The gel also shows that a large amount of toxin is still tagged with attB1HisEntK and so was being

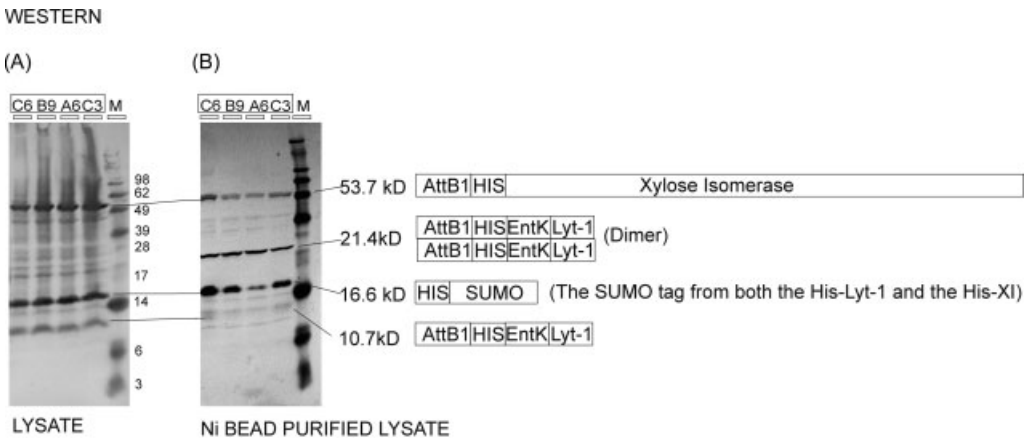

Figure 3 Western analysis of INVSc1-XI-Lyt-1 strains using penta-His antibody. The labels above the lanes identify the variant carried on SUMO vector 2. (A) Lysates prepared after growth on CM glucose URA/TRP-selective plates and run directly on a 16% polyacrylamide gel show three major bands for all four strains, 53.7 kD for attBlHisXI recombinant protein; 16.6 kD for His SUMO tag removed from both HisLyt-1 and HisXI by yeast SUMO protease; and 10.7 kD for attB1HisEntKLyt-1 fusion toxin band. (B) Lysates prepared after growth on CM glucose URA/TRP-selective plates and subjected to Ni bead purification of the His-tagged proteins, then run on a 16% polyacrylamide gel show the same three bands (with a decrease in intensity of the 10.7 kD attB1HisEntKLyt-1 band) and an additional band at 21.4 kD, attributed to a dimer of the attB1HisEntKLyt-1 protein for all four strains.

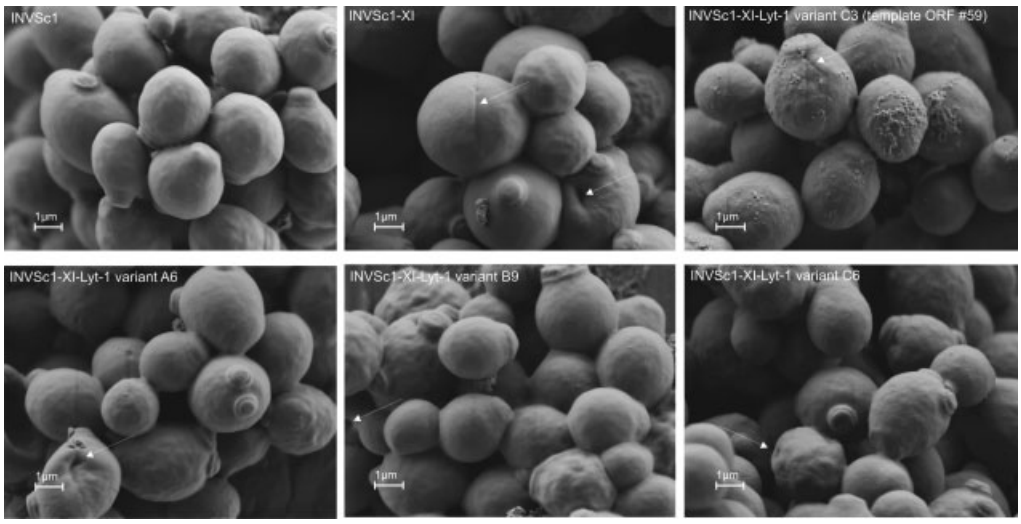

Figure 4 Scanning electron micrographs of yeast strains. INVSc1 yeast cells (top left) have the normal appearance of *S. cerevisiae*. Cells of INVSc1-XI (top center) are larger than INVSc1 cells and have a squared shape and uneven surface with a center dent. Cells of INVSc1-XI-Lyt-1 variants C3, A6, B9, and C6 show numerous bulges and irregularities on the surface in addition to the dents (indicated by arrows).

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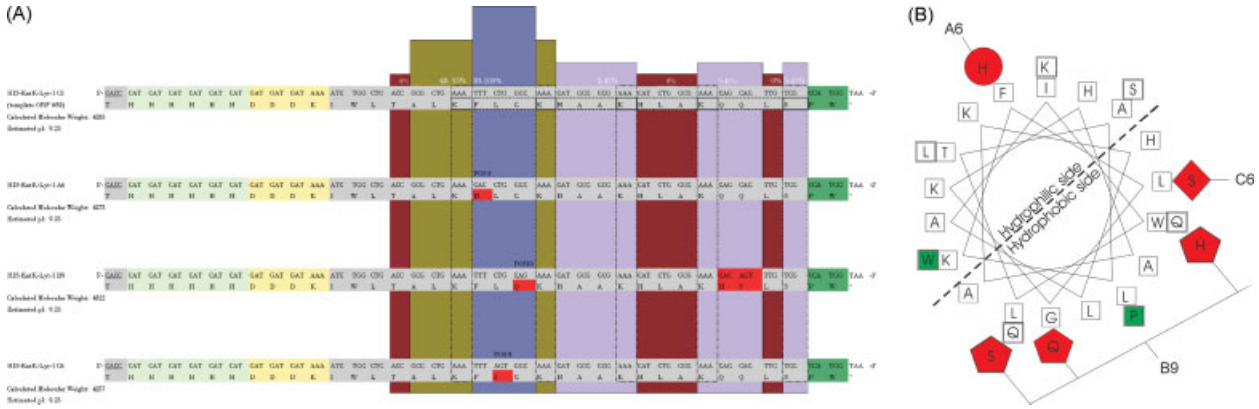

Figure 5 (A) Sequences of the most lethal lycotoxin-1 mutants, C3 (clone #59), A6, B9, and C6. (B) Helical-wheel projection of the amphipathic peptide and the position of the lycotoxin-1 mutations in the hydrophobic and hydrophilic portions of the three-dimensional structure. The lethality for the mutations at each position in the lycotoxin-1 sequence is indicated by the percents and heights of the bars above the first sequence at the various positions along the sequence.

ingested by worms in the uncleaved form, supporting the 'Trojan horse' hypothesis.

Morphology of Yeast Strains

In scanning electron micrographs (Figure 4), INVSc1 yeast cells have the typical rounded appearance of the *S. cerevisiae* fast-growing diploid-type strain with limited sporulation and some bud scars visible. Cells of the INVSc1-XI yeast strain are larger and have a somewhat squared shape and uneven surface with dents, some quite deep, in the center, as well as slight linear indentations along the surface of the cell. These effects could be the result of interactions of the SUMO vector with the membrane. The structure of Smt3 consists of two α-helices and one β-sheet. The α1 helix is strongly amphipathic, with hydrophobic residues pointing inward and hydrophilic residues pointing into the solvent [47]. Interactions of amphipathic peptides with cell membranes have been shown to produce significant perturbations in the cell structure [48]. These perturbations include membrane adhesions, vesicle aggregation, membrane tubulation, and invaginations. The change in the cells of the INVSc1-XI-Lyt-1 variants is even more striking (Figure 4). Cells of the INVSc1-XI-Lyt-1 yeast strain, expressing variants C3, A6, B9, and C6, have numerous bulges and irregularities on the surface suggesting that large amounts of material are present inside the cells. Cells of these four INVSc1-XI-Lyt-1 strains also show dents similar to the INVSc1-XI strain. No dents, bulges, or irregularities are evident in the pictures of the INVSc1 strain (Figure 4). Localization of the peptide is strongly dependent on the charge and hydrophobic nature of the peptide and the charge on the cell membranes [48–50]. Cells containing variant C3 have large amounts of matted material on the outside of the cells. Since all cells were prepared in the same way, it is unlikely to be an artifact of the preparation, but it is not clear if it affects lethality.

Mutations in Lycotoxin-1 Variants Showing Greatest Lethality

Sequence analysis of the most lethal toxin positions indicated mutations at position 8 in A6 from phenylalanine to histidine, at position 10 in B9 from glycine to glutamine, and at position 9 in C6 from leucine to serine. Additionally, in clone B9 there are PCR mutations in position 20 and 21, putting histidine and serine, respectively, in place of two glutamine residues. All three of these lethal clones also have mutations in positions 24 and 25 from lysine and leucine to proline and tryptophan, respectively, that were present in clone #59 (C3) (Figure 5). A helical-wheel projection of the amphipathic peptide illustrates the lycotoxin-1 mutations relative to the hydrophobic and hydrophilic sections of the three-dimensional structure (Figure 5). All variants have mutations Lys24Pro and Leu25Trp indicated by the green squares (wild-type amino acid residues are not shown at those positions in wheel). Red symbols indicate additional mutations in A6 (Phe8His, circle), B9 (Gly10Gln, Gln20His, Gln21Ser, pentagons), and C6 (Leu9Ser, diamond). None has mutations in the lysine residues in the hydrophilic section. The lethality for the mutations at each position in the lycotoxin-1 sequence is indicated by the percents and heights of the bars above the sequences at the various positions along the sequence. The greatest lethality, 83–100% (blue bars) is found for variants having mutations in positions 8, 9, or 10.

The mutations in the lycotoxin-1 variants showing greatest lethality affect the amphipathic properties of the peptide structure by altering charge, steric effects, and polarity changes. The substitutions of proline and tryptophan replacing lysine and leucine at positions 24 and 25, respectively, have possible steric and charge effects. The change at position 24 increases the hydrophobic nature of the hydrophobic side of the helix (Figure 5) and ensures two hydrophobic residues at the

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C-terminal, which have been found to be important for activity [51], although the exact functional significance is unknown. Tryptophan residues in conjunction with lysines have also been shown to contribute to favorable interaction with negatively charged cell membranes [51]. The substitution of a polar Ser residue for a neutral Leu residue at position 9 may increase the interaction with the interior of the cell membrane. The positively charged lysine core, with Lys residues at positions 7, 11, 15, and 19, is unchanged in the individual variants that showed the greatest lethality, and this region is increased in positive charge in optimized mutant A6, which has a phenylalanine to histidine mutation at position 8. This positively charged region is crucial for binding to negatively charged insect cell membranes [51].

CONCLUSIONS

These bioinsecticidal peptides have potential as commercially valuable coproducts that can be expressed in yeast strains capable of converting cellulosic biomass into fuel ethanol in order to improve the cost-effectiveness of the process. Toxins from spider venoms are promising from the perspective of pest resistance since they define new insecticide targets owing to specific actions to block insect voltage-gated Ca^{2+} channels. The optimized toxin peptides are expressed and accumulate in large amounts in the ethanologenic yeast strains. The test insects are killed upon ingesting the yeast. More work remains to be done before any commercial implementation of pesticidal uses of bioactive coproducts of fuel ethanol production. Issues such as the effect of coproduct expression on the yield of ethanol, separation of the bioactive coproduct (if desired), a suitable encapsulation method if the bioactive coproduct is to be kept inside the yeast, efficacy in the field, and appropriate regulatory testing and tolerance establishment must be adequately addressed.

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