Engineered *Saccharomyces cerevisiae* strain for improved xylose utilization with a three-plasmid SUMO yeast expression system


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**A B S T R A C T**

A three-plasmid yeast expression system utilizing the portable small ubiquitin-like modifier (SUMO) vector set combined with the efficient endogenous yeast protease Ulp1 was developed for production of large amounts of soluble functional protein in *Saccharomyces cerevisiae*. Each vector has a different selectable marker (URA, TRP, or LEU), and the system provides high expression levels of three different proteins simultaneously. This system was integrated into the protocols on a fully automated plasmid-based robotic platform to screen engineered strains of *S. cerevisiae* for improved growth on xylose. First, a novel PCR assembly strategy was used to clone a xylose isomerase (XI) gene into the URA-selectable SUMO vector and the plasmid was placed into the *S. cerevisiae* INVSc1 strain to give the strain designated INVSc1-XI. Second, amino acid scanning mutagenesis was used to generate a library of mutagenized genes encoding the bioinsecticidal peptide lycotoxin-1 (Lyt-1) and the library was cloned into the TRP-selectable SUMO vector and placed into INVSc1-XI to give the strain designated INVSc1-XI-Lyt-1. Third, the *Yersinia pestis* xylulokinase gene was cloned into the LEU-selectable SUMO vector and placed into INVSc1-XI-Lyt-1 yeast. Yeast strains expressing XI and xylulokinase with or without Lyt-1 showed improved growth on xylose compared to INVSc1-XI yeast.

1. **Introduction**

Fuel ethanol production from biomass at the industrial level using *Saccharomyces cerevisiae* shows great promise for satisfying future energy demands, but the limited range of materials that can be fermented remains an obstacle to cost-effective bioethanol production (Farrell et al., 2006; Saha, 2003). Although several genetically engineered
strains of *S. cerevisiae* have been developed that will ferment xylose to ethanol (Karhumaa et al., 2007; Sedlak and Ho, 2004; Wisselink et al., 2007), further optimization is needed. It will require the simultaneous expression, at sufficiently high level, of all the enzymes and proteins needed to allow industrial yeast strains to grow efficiently on pentose as well as hexose sugars anaerobically. In addition, for cost-effective industrial ethanol production from biomass it will be necessary to express the enzymes required to saccharify the lignocellulosic feedstocks that are the source of hexose and pentose sugars. Genes considered necessary for complete fermentation of xylose and arabinose, the two major pentose sugar constituents of lignocellulosic biomass, include those encoding xylose isomerase (XI), xylulokinase (XKS), arabinose A, arabinose B, and arabinose D (Karhumaa et al., 2007; Wisselink et al., 2007), which may be obtained from a microorganism naturally capable of fermenting these sugars. Saccharification of lignocellulosic feedstocks also requires utilization of hydrolytic enzymes including cellulases and hemicellulases after initial chemical pre-treatment (Rudolf et al., 2007; Saha et al., 2005). The cost-effectiveness of the fuel ethanol fermentation process could be further enhanced by obtaining high-value co-products and by-products from the process, such as monomers for polymer production and commercially important proteins and peptides. Genes for these proteins and peptides can be mutagenized, placed in an expression system capable of producing high levels of functional proteins or peptides, and screened in high throughput to optimize desired characteristics. An integrated automated platform is available to carry out all the processes for gene assembly, plasmid library production, expression of proteins, enzymes, and value-added proteins or peptides, and growth testing of the transformed yeast strains (Hughes et al., 2005, 2006, 2007). In addition, a system to express numerous genes simultaneously is essential. A yeast plasmid system was designed to accomplish this using three small ubiquitin-like modifier (SUMO) yeast expression vectors (Butt et al., 2005; Malakhov et al., 2004). We integrated this three-plasmid vector system into the automated protocols on the plasmid-based robotic workcell to engineer and screen improved ethanologenic yeast strains expressing (1) xylose isomerase, (2) a library of mutagenized peptides that are putative bioseisitcides, and (3) an enzyme of the pentose phosphate pathway to enhance xylose metabolism. This set of plasmids used on the automated platform offers the possibility of expressing pentose-utilization enzymes and commercially important peptides in yeast, and screening the resulting yeast strains in high throughput for those that grow rapidly anaerobically and produce ethanol at sufficiently high levels for industrial application.

2. Materials and methods

2.1. Construction of pSUMOduo three-plasmid vector system designed for use on an automated workcell

For the novel yeast episomal pSUMOduo high-level expression vector set, the protease-cleavable yeast small ubiquitin-related modifier (SUMO) tag (Sm3) was placed in front of an Invitrogen Gateway cassette, producing an AMP-selectable destination vector for recombination of library inserts. The SUMO insert open reading frames (ORF) were expressed behind an *ADH1* promoter and also a T7 modified promoter for *in vitro* transcription/translation. The vectors contain the yeast high-copy 2 μm origin of replication, to give a copy number of roughly 20 per yeast cell (Christianson et al., 1992).

The first plasmid pSUMOduo/URA was constructed as follows. First, a Gateway cassette fragment (Gateway Vector Conversion System, Invitrogen) containing attR1-cccB-CmR-attR2 was amplified by PCR with primers SUMO-GW-F (5′-GATCGAGAAGACTCGTACAGTTTTGTAACAAAAGACG-3′) and SUMO-GW-R (5′-GATCGAGAAGACTCGTACAGTTTTGTAACAAAAGACG-3′) containing BbsI restriction sites designed to generate ACCT and GATC overhangs, then subcloned into BsaI/BamHI-digested pSUMO (aka pET24-6×His-SUMO) (Malakhov et al., 2004) to construct the plasmid pSUMO-Gateway. A P<sub>T7</sub>-His<sub>6</sub>-SUMO-Gatecasse cassette-T<sub>T7</sub> fragment was then PCR-amplified from pSUMO-Gateway using primers PT7EcoRV-F (5′-GAATTCGATATCTAATACGACTCACTATAGGGAGACCACAACGGTTTCCTCTAGAATAATTTGGTTTAAC-3′) and T7BbsI-R (5′-GATCGAGAAGACTCGTACAGTTTTGTAACAAAAGACG-3′), digested with EcoRV and BbsI to generate a blunt end and a GATC overhang, and subcloned into a Smal/BglII fragment of pRS306-P<sub>ADH</sub> (Sterner et al., 1999), containing the *Escherichia coli* amp<sup>α</sup> 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 (Christianson et al., 1992) containing the yeast 2 μm origin of replication. QuikChange mutagenesis (Wang and Malcolm, 1999) was then used on the resulting plasmid to delete two extraneous, tandem *P<sub>T7</sub>* sequences present in the vector backbone from the previous construction of pRS306-P<sub>ADH</sub> (Sterner et al., 1999).

The second and third SUMO vectors of the three-plasmid system were created by replacing the *URA3* selectable marker in pSUMOduo/URA with either *TRP1* or *LEU2*. The *TRP1* sequence was amplified from pGBK7 vector (Clontech) with oligos 82 (5′-GGCGAGATCTAGCCGCTTCCCTGTGATGACGGTG-3′) and 83 (5′-GGCGAGATCTAGCCGCTTCCCTGTGATGACGGTG-3′), and 84 (5′-GGCGAGATCTAGCCGCTTCCCTGTAACAAAACAGTTTT-3′) and 85 (5′-GGCGAGATCTAGCCGCTTCCCTGTAACAAAACAGTTTT-3′). The *LEU2* sequence was amplified from pGADT7 vector (Clontech) with oligos 86 (5′-GGCGAGATCTAGCCGCTTCCCTGTAACAAAACAGTTTT-3′) and 87 (5′-GGCGAGATCTAGCCGCTTCCCTGTAACAAAACAGTTTT-3′). Inserts were then digested with NheI and MluI and cloned into similarly digested pSUMOduo/URA vector.

2.2. Assembly of xylose isomerase ORF and production of pSUMOduo/URA/HisXl-INVSc1 yeast strain (INVSc1-XI)

The *Piromyces* sp. E2 xylose isomerase (XI) gene sequence (GenBank Accession No. AJ248909) (Kuyper et al., 2003) was used to synthesize oligonucleotides for assembly into the XI ORF that was placed directionally into the expression vector pSUMOduo/URA (Fig. 1, Step 1). Oligonucleotides A-R and EE used in the gene assembly protocol adapted for the automated robotic platform are listed in Table 1. In the first step of the XI ORF assembly, section 1
forward oligo (B) was annealed to section 2 reverse oligo (C) and filled in with dNTP using the ABI high-fidelity AmpLiTaq/C210 PCR kit (Applied Biosystems), as described previously (Hughes et al., 2005). PCRs were carried out using...
and the XI section3 long reverse oligo (D) (Fig. 1, Step 1, ABI high-fidelity PCR using the short forward primer (A) and XI section 1–2 plasmid preparation was then used as a template. The PCR amplicons were purified as before and TOPO cloned into pCR8/GW/TOPO vector (Invitrogen). The resulting plasmids were digested with BsrGI, and the XI section 1-2-3 insert was verified at 298 bp. The resulting pENTR//D-TOPO XI section 1-2-3 plasmid preparation was used as a template for the next PCR assembly step with the same short forward primer (A) and the XI section 4 long reverse oligo (E) (Fig. 1, Step 1, right column). The resulting PCR product was purified, TOPO cloned into pENTR//D-TOPO vector, and transformed into TOP10 cells that were recovered and selected on LB SPEC 100 plates (Teknova) (Fig. 1, Step 1, center column). The plasmids were digested with BsrGI, and the XI section 1-2-3-4 insert was verified at 250 bp. The resulting plasmids were digested with BsrGI, and the XI section 1-2-3-4 insert was verified at 250 bp. The resulting pENTR//D-TOPO XI section 1-2-3-4 plasmid was then used as a template in the next PCR gene assembly step with short forward primer (A) and the XI section 4 long reverse oligo (E) (Fig. 1, Step 1, right column). The resulting PCR product was purified, TOPO cloned into pENTR//D-TOPO vector, and transformed into TOP10 cells that were recovered and selected on LB SPEC 100 plates (Teknova). Plasmids were digested with BsrGI, and the XI section 1-2-3-4 insert was verified at 250 bp. The resulting pENTR//D-TOPO XI section 1-2-3-4 plasmid was then used as a template in the next PCR gene assembly step with short forward primer (A) and the XI section 4 long reverse oligo (E) (Fig. 1, Step 1, right column). The resulting PCR product was purified, TOPO cloned into pENTR//D-TOPO vector, and transformed into TOP10 cells that were recovered and selected on LB SPEC 100 plates (Teknova). Plasmids were digested with BsrGI, and the XI section 1-2-3-4 insert was verified at 250 bp. The resulting pENTR//D-TOPO XI section 1-2-3-4 plasmid was then used as a template in the next PCR gene assembly step with short forward primer (A) and the XI section 4 long reverse oligo (E) (Fig. 1, Step 1, right column). The resulting PCR product was purified, TOPO cloned into pENTR//D-TOPO vector, and transformed into TOP10 cells that were recovered and selected on LB SPEC 100 plates (Teknova).
pCR8/GW/TOPO vectors in order to isolate only those vectors containing inserts with the newly added section via SPEC or kan selection, but not select the previous template (Fig. 1, Step 1, right column).

The final plasmid, pCR8/GW/TOPO XI sections 1–17, was used in a polishing PCR with the short forward primer (A) and the short reverse primer (EE) to generate the full XI wild-type ORF (Kuyper et al., 2003) from clone #59 (designated Lyt-1 C3 variant), as well as all possible 20 amino acids at each of the 25 amino acid positions in the Lyt-1 peptide (GenBank Accession No. NP_671009) was moved from pDONR221 (Zuo et al., 2007) via LR clonase into pSUMOduo/LEU to generate the plasmid pSUMOduo/LEU/TAL. This plasmid was transformed into the two-plasmid INVSc1-XI-Lyt-1 strain to produce the strain pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 AASM-pSUMOduo/LEU/TAL INVSc1 (or INVSc1-XI-Lyt-1-TAL).

2.5. Polyacrylamide gel electrophoresis and Western blot procedures

Transformed yeast strains carrying the pSUMOduo plasmids were inoculated into 1.0 mL of medium and grown two 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 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 a Hard-Shell 96-well plate (Bio-Rad) and 30 µL of 2× tris–glycine SDS loading buffer (Invitrogen) with beta-mercaptoethanol (BME) were added to each well. To the remaining 220 µL of sample in the 1.5 mL tube were added 100 µL of Super Flow Ni beads (Qiagen), and the sample 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 were added. These samples and the samples in the Hard-Shell plates were heated to 95 °C for 10 min, loaded onto 15-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 PVDF cut sheets (Invitrogen). The transfer took place in the Novex transfer apparatus with 1× transfer buffer (Invitrogen) for 10 h at a constant 200 mA. Westerns were chromogenic and performed with the Western Breeze kit (Invitrogen) according to the manufacturer’s directions using a Qiagen Penta-His antibody resuspended in 1 mL of 1× phosphate buffered saline (PBS) with magnesium and calcium (secondary antibody was anti-mouse antibody). Western blot images were captured using the Alpha Innotech 3400. The Alpha Innotech 3400 was used to analyze Western blot images of the cell lysates for the amount of His-tagged protein expressed. The AlphaEaseFC software for spot densitometry measurement for Windows 2000 was used to determine band density compared to known molecular weight markers from the Qiagen 6×His-tagged set treated in the same fashion as the samples on the gels.
2.6. Coomassie gel analysis of ethanol wash of cell surface and ethanol extraction of cell lysate

The yeast strains were grown four days at 30 °C in 25 mL CM xylose selective medium in a shake flask. Cells from 2 mL of culture were harvested by centrifugation and the cell pellets were treated with ethanol to a final ethanol: cell pellet ratio of 9:1. The ethanol washed were recovered and air dried. Twenty micro liters of 2× tris–glycine loading buffer were added. The washed cells were treated with 50 μL of Y-PER solution, vortexed vigorously, and added to 50 μL of 2× tris–glycine SDS/BME loading buffer. The ethanol wash samples and the cell lysate samples were heated to 95 °C for 5 min, allowed to cool, and loaded onto 1.5-mm 16% tris–glycine 15-well SDS–PAGE gels (Invitrogen), and run at 110 V for 60 min. The gels were stained with Simply Blue stain (Invitrogen) for 1 h and destained with water for 10 h. The resulting gels were imaged and analyzed using the Alpha Innotech 3400 (as for Western blot gels).

2.7. Transmission electron microscopy

Yeast cells from 10 μL of culture (grown two days in glucose liquid medium) were collected on a 200 mesh, nickel, Formvar coated grid. The grid was placed on the specimen drop for 5 min, washed gently with water, and drained. The grid was negatively stained by placing it, membrane side down, onto a drop of 2% aqueous uranyl acetate for 2 min. The sample was scanned and photographed using a JEM 100 C Transmission Electron Microscope (JEOL).

2.8. Scanning electron microscopy

Yeast cells from 1.0 mL of culture (grown six days in xylose liquid medium) were suspended in 1.0 mL saline (0.85% NaCl) and centrifuged to remove residual medium. Following a modified procedure of Bang and Pazirandeh (1999), 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, respectively, in solutions containing 50%, 70%, 80% and 100% ethanol successively for 15 min for each treatment. Fifty microliters of the cell suspension in 100% ethanol 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).

2.9. Light microscopy and immunofluorescent microscopy

Selected colonies from plates containing each of the yeast strains were inoculated into 1 mL of the appropriate liquid media in a 96-well deepwell plate and grown for 48 h at 30 °C on an incubator shaker. The resulting cultures were pelleted at 300g for 3 min at room temperature, and the supernatant was removed. The pelleted cells were washed three times by resuspending in 500 μL of PBS buffer, pH 7.4, per well. Cells were fixed using 1 mL per well of a non-toxic ‘Prefer’ fixative solution (Anatech Ltd.), incubated for up to 2.5 h at room temperature, washed again, and then subjected to staining. In addition, cells from each of the same yeast strains were digested using Zymolyase enzyme (Biosciences or MP-Biomedicals) prior to fluorescence staining to allow entry of the dye into the cell interior. Cells were incubated in 500 μL of PBS buffer with a ‘25 U’ concentration of active enzyme (e.g., 9 μL of 1 mg/mL (1%) zymolyase stock) for a maximum of 45 min at room temperature. After digestion, the cells were pelleted, washed twice to remove the zymolyase, and resuspended in a dye solution.

Nuclear staining of undigested and digested yeast cells was carried out using DAPI dye (1/500th dilution of 0.1 mg/mL stock solution). Location of His-tagged proteins was determined using a Penta-His Alexa Fluor 532 anti-His antibody conjugated dye (Qiagen, CA) prepared (1/100th dilution of 200 μg/mL stock solution) in a blocking buffer containing 5 mg/mL BSA to reduce non-specific staining. Approximately 100 μL of dye solution was added per well and incubated up to 1.5 h at room temperature followed by a single wash step and resuspension in 500 μL PBS buffer. Dilute suspensions of cells (approximately 200 μL; OD 600 below 0.2) were loaded into a flat-bottom 96-well plate (ThermoFisher Scientific) prior to imaging. Brightfield and fluorescence images were taken using the BioRyx 200® platform (Arroyx Inc.) with the Nikon TE2000 microscope modified to accomodate flat-bottom 96-well plates, automated stage movement (Prior Scientific), a 40X Nikon air objective (numerical aperture = 0.95), a Retiga Exi camera (Qimaging), and LabRyx Software.

For stained cells, ‘blue’ fluorescence was collected from the DAPI dye using a Nikon UV–2A fluorescence cube (excitation wavelength/bandwidth: 355/50 nm; emission wavelength: 420 nm longpass) and ‘red’ fluorescence was collected from the antibody-based Penta-His Alexa Fluor 532 conjugate dye using a Nikon Cy3 HYQ fluorescence cube (excitation wavelength/bandwidth: 545/30 nm; emission wavelength/bandwidth: 610/75 nm). Exposure times were 10 ms for brightfield and 1 s for fluorescence with minimal offset and gain. A series of sequential images were taken for each sample (brightfield, ‘blue’, and ‘red’) and composite (overlay) images were created for analysis.

2.10. Aerobic and anaerobic growth on selective media plates and in liquid media

For growth on glucose, samples were streaked on complete minimal (CM) 2.0% glucose URA selective plates for the INVSc1-XI yeast strain, on CM 2.0% glucose-URA/TRP selective plates for the INVSc1-XI-Lyt-1 yeast strains or on CM 2.0% glucose URA/TRP/LEU selective plates for the INVSc1-XI-Lyt-1-XKS (or TAL) yeast strains. For growth on xylose, similar plates were used but CM 2.0% xylose was substituted for CM 2.0% glucose. For aerobic growth, the plates were placed into an incubator at 30 °C. For anaerobic growth, the plates were first placed in an anaerobic chamber for at least 2 h and then samples were applied. The plates were wrapped and placed back into the anaerobic chamber for six days. Anaerobic conditions are
maintained using BD GasPak™ EZ Gas Generating Container Systems with Indicator (Becton–Dickinson). Pictures of all plates were taken digitally. Cultures were grown for two days, diluted 10,000-fold in sterile water, and 100 μL were spotted onto the respective plates and allowed to grow for three days. For growth in CM 2.0% glucose liquid medium (Teknova), 100 μL of the sample (absorbance at 660 nm = 0.1) were inoculated into a flask containing 50 mL of the appropriate selective CM 2.0% glucose liquid medium, either minus uracil, or minus uracil and tryptophan, or minus uracil, tryptophan, and leucine (Teknova). For growth on xylose, CM 2.0% xylose liquid medium was substituted for CM 2.0% glucose liquid medium.

3. Results

3.1. Yeast strain produced using three-plasmid yeast expression technology

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, consists of three vectors, pSUMOduo/URA, pSUMOduo/TRP, and pSUMOduo/LEU, each one having a different yeast selectable marker. Three plasmids were constructed and placed into INVSc1 yeast, a fast growing diploid strain that carries the mutations MATα his3D1 leu2 trp1-289 ura3-52 (Kuyper et al., 2003, 2004, and 2005) to generate a strain with the ability to metabolize xylose into xylulose. The yeast strain engineered with these plasmids expressed (1) a PCR assembled xylose isomerase ORF, (2) a library of mutagenized Lyt-1 ORFs, and (3) genes for one of two enzymes for metabolic correction to enable the yeast to utilize xylose more efficiently (Fig. 1).

The assembly of the xylose isomerase ORF (Fig. 1, Step 1) was performed using a robot-adapted strategy that involved production of increasingly longer templates with long oligonucleotide additions to the end of the previous section of the sequence, alternating between cloning into pENTR/D-TOPO with the KAN selectable marker and pCR8/GW/TOPO with the SPEC selectable marker to ensure the previous template was not selected. For each of the TOPO cloning steps a fragment was always produced and at least one clone was always obtained. The pENTR/D-TOPO plasmid with the complete xylose isomerase ORF was cloned into the yeast high-copy expression vector pSUMOduo/URA and transformed into the INVSc1 yeast strain to produce the INVSc1-XI yeast strain (Fig. 1, Step 1).

A library of mutagenized wolf spider lycotoxin-1 ORFs that was produced by amino acid scanning mutagenesis from lycotoxin-1 clone #59 (also called C3) on the automated robotic platform, as described previously (Hughes et al., 2007), was placed in the second SUMO vector to express toxin variants optimized for lethality to a test insect pest. The resulting library was transformed using the automated protocols into the INVSc1 yeast already containing the SUMO expression plasmid for xylose isomerase to produce the INVSc1-XI-Lyt-1 yeast strain (Fig. 1, Step 2).

To improve xylose utilization by the yeast strain, an additional open reading frame, either for xylulokinase or for transaldolase, was placed in the third SUMO vector, pSUMOduo/LEU, using the automated protocols, and the resulting plasmid was used to produce the INVSc1-XI-Lyt-1-XKS (or TAL) yeast strains (Fig. 1, Step 3). Expression of these enzymes is suggested as a means of enabling yeast to metabolize xylose more rapidly through the pentose phosphate pathway (Jin et al., 2003; Kuyper et al., 2004, 2005; Van Maris et al., 2007). The SUMO plasmids are particularly well suited for integration with the automated protocols on the robotic platform and complement the PCR assembly and TOPO directional in-frame cloning strategy (Hughes et al., 2005).

3.2. Results of XI expression in INVSc1-XI yeast

A transmission electron micrograph of the INVSc1-XI yeast strain compared to that of the INVSc1 yeast strain (Fig. 2A) grown aerobically on CM glucose URA selective plates shows that the XI-yeast is expressing large amounts of additional material in comparison to the strain without the XI plasmid. As a result, the shape of the cell is changed from balloon-shaped to almost cube-shaped with a dent in the center. The growth of the INVSc1-XI yeast strain on CM glucose URA selective plates and in CM glucose URA selective liquid medium was compared to growth of the strain on CM xylose URA selective plates and CM xylose URA selective liquid medium (Fig. 2B). Yeast expressing XI grew well aerobically both on glucose plates and in glucose liquid medium. It grew aerobically to a very limited extent on xylose plates after two days, but it did not grow in xylose liquid medium. The Western gels (Fig. 2C) for both the cell lysates (left) and for the Ni bead-purified cell lysates (right) show a strong attB1HisXI band at 53.7 and a 16.6 kDa band corresponding to the 6×HisSUMO fragment in the INVSc1-XI yeast strain. These bands are absent in the lanes corresponding to the INVSc1 yeast strain. The light microscope images of the INVSc1-XI yeast strain also show changes in the shape of the cells compared to the INVSc1 strain (Fig. 3) in agreement with the TEM images (Fig. 2A). The strong staining (red) seen in the immunofluorescence images of the undigested and digested cells (Fig. 3) in the INVSc1-XI yeast strain confirm the expression of His-tagged proteins, HisXI and HisSUMO; no red staining is seen in the digested cells of the INVSc1 yeast strain.

3.3. Expression and analysis of lycotoxin-1 variants

The doubling times of INVSc1-XI-Lyt-1 variants C3, A6, B9, C6 yeast strains grown aerobically in CM glucose URA/TRP selective liquid medium are presented in Table 2. Growth on glucose liquid medium for the strains expressing the lycotoxin-1 variants is similar to the INVSc1-XI strain, with doubling times the same as that for the XI yeast strain without Lyt-1 (Table 2). No growth is seen for the INVSc1-XI-Lyt-1 strains in CM xylose URA/TRP selective liquid medium (data not shown). The INVSc1-XI-Lyt-1 yeast strains were also examined under a light microscope and by using immunofluorescent
microscopy (Fig. 3). The light microscope images show more extensive shape changes than those seen for the INVSc1-XI strain. The immunofluorescence images demonstrate increased staining of expressed His-tagged proteins, HisXI, HisLyt-1, and HisSUMO from both vectors, in the undigested INVSc1-XI-Lyt-1 yeast cells (more for A6, B9, and C6 than C3) compared to the undigested INVSc1-XI yeast strain (Fig. 3). When the permeability of the cells is increased with zymolyase to allow immunostaining of His-tagged expressed proteins in internal locations of the cells, the INVSc1-XI-Lyt-1 strains, particularly A6, B9, and C6 (Fig. 3), show intense spots within the cell interior.

3.4. Analysis of strains expressing metabolic correction enzymes

The doubling times of INVSc1-XI-Lyt-1 (variants C3, A6, B9, or C6)-XKS (or TAL) yeast strains grown aerobically in CM glucose or CM xylose URA/TRP/LEU selective liquid medium and of the INVSc1-XI strain grown aerobically in CM glucose URA selective liquid medium are presented in Table 2. Aerobic growth in glucose for the strains expressing the lycotoxin-1 variants and the metabolic correction enzymes is unchanged, with doubling times very close to that for the INVSc1-XI yeast strain.

An INVSc1 strain expressing XI and XKS used as a control shows aerobic growth in CM xylose URA/LEU selective liquid medium with a doubling time approximately three times that of aerobic growth in glucose liquid medium (Table 2). Strains expressing Lyt-1 variants C6 or C3 in addition to XI and XKS also show aerobic growth in xylose liquid medium but with doubling times two to three times longer, respectively, than that for the INVSc1-XI-XKS strain (Table 2). However, strains expressing Lyt-1 variants A6 or B9 in addition to XI and XKS show essentially no growth in xylose liquid medium (Table 2). No growth was seen in xylose liquid medium for the control strain expressing only XI and TAL (data not shown), for strains expressing XI and the Lyt-1 variants (data not shown), or for strains expressing XI, the Lyt-1 variants, and TAL (Table 2).

Western blot analysis of the INVSc1-XI-Lyt-1 and INVSc1-XI-Lyt-1-XKS (or TAL) strains using penta-His antibody is shown in Fig. 4. All 12 strains are expressing a lycotoxin-1 variant and xylose isomerase and eight strains are also expressing XKS or TAL as indicated above the lanes. Lysates prepared after growth on glucose plates and run directly on a 16% polyacrylamide gel show three major bands for all 12 strains, at 53.7 kDa for the attB1HisXI recombinant protein, at 16.6 kDa for the HisSUMO tag removed from HisXI, HisLyt-1, and XKS or TAL by yeast SUMO protease 1, and at 10.7 kDa for the attB1HisEntKLyt-1 toxin band (Fig. 4A). Lysates prepared after growth on glucose plates, subjected to Ni bead purification of the His-tagged proteins, and run on a 16% polyacrylamide gel, show the same three bands and an additional band at 21.4 kDa, attributed to a dimer of the attB1HisEntKLyt-1 toxin band (Fig. 4A). The concentration of His-tagged protein in each band in Fig. 2C for XI and Fig. 4A for XI and Lyt-1 was calculated using AlphaEaseFC software for Windows 2000 to determine band density compared to known molecular weight markers from the Qiagen 6×His-tagged set treated in the same fashion as the XI and Lyt-1 samples. The results are shown in Table 3. Protein concentrations for the individual XI and Lyt-1 bands for the two-plasmid strain are on the
Fig. 3. Sequential light microscope (brightfield), immunofluorescence (red), and DAPI-stained (blue) images for undigested and permeabilized (with zymolyase) cells from each strain after two-days growth in glucose liquid selective medium, and composite (overlay) images. Red staining indicates location of expressed His-tagged proteins using a Penta-His Alexa Fluor 532 anti-His antibody-conjugated dye. Blue staining with DAPI indicates the location of cell nuclei.
order of 12–14 μg/mL. For the three-plasmid strain, expression of Lyt-1 is slightly greater than that of XI, with the Lyt-1 concentrations ranging from 14 to 19 μg/mL and the XI concentrations from 9 to 12 μg/mL. However, the sum of the concentrations determined for the XI and Lyt-1 bands is similar in all strains expressing them, about 25–29 μg/mL. The concentrations of XKS and TAL cannot be determined directly since they are not His-tagged, but the increased concentration of the His-tagged SUMO band (14–16 μg/mL) in the triply transformed yeast strains over that in the doubly transformed yeast strains (8–9 μg/mL) gives a measure of the expression of XKS or TAL.

Data from growth analysis of the strains that were transformed with TAL or XKS genes in addition to the XI and Lyt-1 ORFs (variant C6, B9, A6, or C3) are displayed in Fig. 5. All the triply transformed strains show extensive growth aerobically and anaerobically on CM glucose URA/TRP selective plates. These strains also show growth aerobically and anaerobically on CM xylose URA/TRP/LEU selective plates. These strains also show growth aerobically and anaerobically on CM glucose or CM xylose URA/TRP/LEU selective liquid medium (control is INVSc1-XI-XKS) and of INVSc1-XI-Lyt-1 (variants A6, B9, C3, or C6)-XKS (or TAL) grown aerobically in CM glucose or CM xylose URA/TRP/LEU selective liquid medium (control is INVSc1-XI-XKS).

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Plasmid(s) within strain INVSc1</th>
<th>Medium used</th>
<th>Hours</th>
</tr>
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<tbody>
<tr>
<td>Yeast strain doubling time in 2.0% glucose</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>INVSc1-XI</td>
<td>pSUMOduo/URA/HisXI</td>
<td>CM 2% glucose-URA</td>
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<tr>
<td>INVSc1-XI-XKS</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/LEU/XKS</td>
<td>CM 2% glucose-URA-LEU</td>
<td>1.84</td>
</tr>
<tr>
<td>INVSc1-XI-Lyt-1 A6</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 A6</td>
<td>CM 2% glucose-URA-TRP</td>
<td>1.86</td>
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<td>INVSc1-XI-Lyt-1 B9</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 B3</td>
<td>CM 2% glucose-URA-TRP</td>
<td>1.92</td>
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<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 C3</td>
<td>CM 2% glucose-URA-TRP</td>
<td>1.91</td>
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<tr>
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<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 C6</td>
<td>CM 2% glucose-URA-TRP</td>
<td>1.90</td>
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<td>INVSc1-XI-Lyt-1 A6-XKS</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 A6-pSUMOduo/LEU/XKS</td>
<td>CM 2% glucose-URA-TRP-LEU</td>
<td>2.01</td>
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<td>CM 2% glucose-URA-TRP-LEU</td>
<td>1.98</td>
</tr>
<tr>
<td>INVSc1-XI-Lyt-1 C3-XKS</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 C3-pSUMOduo/LEU/XKS</td>
<td>CM 2% glucose-URA-TRP-LEU</td>
<td>1.98</td>
</tr>
<tr>
<td>INVSc1-XI-Lyt-1 C6-XKS</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 C6-pSUMOduo/LEU/XKS</td>
<td>CM 2% glucose-URA-TRP-LEU</td>
<td>1.96</td>
</tr>
<tr>
<td>INVSc1-XI-Lyt-1 A6-TAL</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 A6-pSUMOduo/TRP/TAL</td>
<td>CM 2% glucose-URA-TRP-LEU</td>
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<tr>
<td>INVSc1-XI-Lyt-1 B9-TAL</td>
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<td>CM 2% glucose-URA-TRP-LEU</td>
<td>1.96</td>
</tr>
<tr>
<td>INVSc1-XI-Lyt-1 C6-TAL</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 C6-pSUMOduo/TRP/TAL</td>
<td>CM 2% glucose-URA-TRP-LEU</td>
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<tr>
<td>INVSc1-XI-Lyt-1 C6</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 C6</td>
<td>CM 2% glucose-URA-TRP</td>
<td>1.90</td>
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<td>1.92</td>
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<td>CM 2% glucose-URA-TRP</td>
<td>1.86</td>
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<tr>
<td>INVSc1-XI-Lyt-1 B9</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 B3</td>
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<td>1.86</td>
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<tr>
<td>INVSc1-XI-Lyt-1 A6-XKS</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 A6-pSUMOduo/LEU/XKS</td>
<td>CM 2% glucose-URA-TRP-LEU</td>
<td>&gt;20</td>
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<td>INVSc1-XI-Lyt-1 B9-XKS</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 B3-pSUMOduo/LEU/XKS</td>
<td>CM 2% glucose-URA-TRP-LEU</td>
<td>&gt;20</td>
</tr>
<tr>
<td>INVSc1-XI-Lyt-1 C3-XKS</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 C3-pSUMOduo/LEU/XKS</td>
<td>CM 2% glucose-URA-TRP-LEU</td>
<td>&gt;20</td>
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<tr>
<td>INVSc1-XI-Lyt-1 C6-XKS</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 C6-pSUMOduo/LEU/XKS</td>
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<td>CM 2% glucose-URA-TRP-LEU</td>
<td>13.20</td>
</tr>
<tr>
<td>INVSc1-XI-Lyt-1 B9-TAL</td>
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<td>CM 2% glucose-URA-TRP-LEU</td>
<td>0</td>
</tr>
<tr>
<td>INVSc1-XI-Lyt-1 C3-TAL</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 C3-pSUMOduo/TRP/TAL</td>
<td>CM 2% glucose-URA-TRP-LEU</td>
<td>0</td>
</tr>
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<td>INVSc1-XI-Lyt-1 C6-TAL</td>
<td>pSUMOduo/URA/HisXI-pSUMOduo/TRP/HisLyt-1 C6-pSUMOduo/TRP/TAL</td>
<td>CM 2% glucose-URA-TRP-LEU</td>
<td>0</td>
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</tbody>
</table>

Among the strains expressing XI, XKS, and the Lyt-1 variants, only the strains with variants C3 and C6 were able to grow in xylose liquid medium. Strains expressing XI, XKS, and Lyt-1 variants A6 and B9 did not grow in xylose liquid medium. Fig. 7A and B shows the amino acid changes in the wild-type sequence from lysine to proline at position 3.5. Lycotoxin-1 mutations
and from leucine to tryptophan at position 25 in both C3 and C6 variants (dark green squares) and from leucine to serine at position 9 in C6 (red square). The helical-wheel projection of the amphipathic peptide (Fig. 7B) illustrates the Lyt-1 mutations relative to the hydrophobic and hydrophilic portions of the three-dimensional structure. There are no mutations in the positively charged lysine residues at positions 7, 11, 15, and 19 in the hydrophilic section which interacts with the membrane lipid headgroup negative charges. The lysine to proline mutation at position 24 increases the hydrophobic nature of the hydrophobic side of the helix and ensures two hydrophobic residues at the C-terminal, which have been found to be important for activity (Mayo et al., 2000). The mutation at position 25 replaces a leucine residue with tryptophan. Tryptophan residues in conjunction with lysines have also been shown to contribute to favorable interaction with negatively charged cell membranes (Mayo et al., 2000).

The scanning electron micrographs (Fig. 6) suggest that in the case of the triply transformed strain expressing XI, XKS, and C3, considerable expressed protein appears on the outside of the cells, and that in the case of the triply transformed strain expressing XI, XKS, and C6, most of the material appears to be on the inside of the cells with a network of tube-like structures visible on the surface. A proposed model for the effect of the C3 and C6 peptides on the yeast cell membrane is depicted in Fig. 7C.

A Coomassie gel analysis of the material on the surface of the cells compared to the material in the cell lysate after cells were grown for four days in CM xylose URA/TRP/LEU selective liquid medium is shown in Fig. 7D. Ethanol washings from the outside of the cells expressing C6 and C3 are shown in Lanes 2 and 3, respectively, using a 5 μL sample and in Lanes 7 and 8, respectively, using a 20 μL sample. Cells expressing C3, which have matted material on the outside of the cells in the SEM images, show the presence of Lyt-1 in the ethanol washings from the outside of the cells (Lanes 3 and 8; Fig. 7D), but essentially no Lyt-1 is seen in the ethanol washings from the outside of the cells expressing C6 (Lanes 2 and 7; Fig. 7D). Lysates of the cells expressing C6 and C3 are shown in Lanes 4 and 5, respectively, using a 5 μL sample and in Lanes 9 and 10, respectively, using a 20 μL sample. Cell lysates of both C6 and C3 show approximately the same total amount of Lyt-1 throughout the cells (Lanes 9 and 10; Fig. 7D).

4. Discussion

4.1. Three-plasmid yeast expression strategy

The small ubiquitin-like modifier (SUMO) modulates protein structure and function by covalently binding to the lysine side chains of the target proteins. Attachment of SUMO (also called Smt3) to the N-terminus of proteins
Table 3
Concentration of His-tagged protein in each band in Fig. 2C for XI and Fig. 4A for XI and Lyt-1 using AlphaEaseFC software for Windows 2000 to determine band density compared to known molecular weight markers from the Qiagen 6×His-tagged set treated in the same fashion as the XI and Lyt-1 samples

<table>
<thead>
<tr>
<th>Strain</th>
<th>Band on gel</th>
<th>Amount total of each His-tagged band</th>
<th>Sum of His XI plus His EnTK Lyt-1</th>
<th>Sum of all His-tagged</th>
</tr>
</thead>
<tbody>
<tr>
<td>INVSc1-XI</td>
<td>HIS XI; HIS SUMO</td>
<td>24477; 4771</td>
<td>N/A</td>
<td>29248</td>
</tr>
<tr>
<td>INVSc1-XI-Lyt-1 C3</td>
<td>HIS XI; HIS SUMO; HIS ENTK LYT-1</td>
<td>12404; 7845; 13101</td>
<td>25505</td>
<td>33350</td>
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<tr>
<td>INVSc1-XI-Lyt-1 A6</td>
<td>HIS XI; HIS SUMO; HIS ENTK LYT-1</td>
<td>13401; 7982; 13943</td>
<td>27344</td>
<td>35326</td>
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<tr>
<td>INVSc1-XI-Lyt-1 B9</td>
<td>HIS XI; HIS SUMO; HIS ENTK LYT-1</td>
<td>12229; 8083; 12780</td>
<td>25009</td>
<td>33092</td>
</tr>
<tr>
<td>INVSc1-XI-Lyt-1 C6</td>
<td>HIS XI; HIS SUMO; HIS ENTK LYT-1</td>
<td>13052; 8747; 11669</td>
<td>24721</td>
<td>33468</td>
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<tr>
<td>INVSc1-XI-Lyt-1 C3-TAL</td>
<td>HIS XI; HIS SUMO; HIS ENTK LYT-1</td>
<td>10478; 16357; 16026</td>
<td>26504</td>
<td>42861</td>
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<tr>
<td>INVSc1-XI-Lyt-1 A6-TAL</td>
<td>HIS XI; HIS SUMO; HIS ENTK LYT-1</td>
<td>9106; 15095; 14533</td>
<td>23639</td>
<td>38734</td>
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<tr>
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<td>11752; 13556; 14757</td>
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<td>40064</td>
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<tr>
<td>INVSc1-XI-Lyt-1 A6</td>
<td>HIS XI; HIS SUMO; HIS ENTK LYT-1</td>
<td>10097; 14925; 15877</td>
<td>25974</td>
<td>40900</td>
</tr>
<tr>
<td>INVSc1-XI-Lyt-1 B9</td>
<td>HIS XI; HIS SUMO; HIS ENTK LYT-1</td>
<td>11124; 13619; 18969</td>
<td>30093</td>
<td>43712</td>
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<tr>
<td>INVSc1-XI-Lyt-1 C3</td>
<td>HIS XI; HIS SUMO; HIS ENTK LYT-1</td>
<td>11894; 12446; 15075</td>
<td>26969</td>
<td>39415</td>
</tr>
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<td>INVSc1-XI-Lyt-1 A6-XKS</td>
<td>HIS XI; HIS SUMO; HIS ENTK LYT-1</td>
<td>10097; 14925; 15877</td>
<td>25974</td>
<td>40900</td>
</tr>
<tr>
<td>INVSc1-XI-Lyt-1 B9-XKS</td>
<td>HIS XI; HIS SUMO; HIS ENTK LYT-1</td>
<td>11124; 13619; 18969</td>
<td>30093</td>
<td>43712</td>
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<tr>
<td>INVSc1-XI-Lyt-1 C3-XKS</td>
<td>HIS XI; HIS SUMO; HIS ENTK LYT-1</td>
<td>11894; 12446; 15075</td>
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<td>39415</td>
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<td>HIS XI; HIS SUMO; HIS ENTK LYT-1</td>
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<td>40900</td>
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<td>INVSc1-XI-Lyt-1 C3</td>
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<td>11894; 12446; 15075</td>
<td>26969</td>
<td>39415</td>
</tr>
</tbody>
</table>

Known reference at 15 kDa: His-tagged control marker 250

has been found to enhance their expression (Malakhov et al., 2004). The structure of SUMO in aqueous solution consists of two α-helices and one β-sheet with 1 parallel and 3 antiparallel β-strands. Helix α1 (Leu45–Gln56) is strongly amphipathic with hydrophobic residues pointing inward and hydrophilic residues directed toward the solvent (Sheng and Liao, 2002). Yeast cells contain a protease (Ulp1 or SUMO protease 1) that can cleave a variety of SUMO fusions (Li and Hochstrasser, 2003). The enhanced expression and solubility of proteins or peptides fused to SUMO combined with the broad specificity and highly efficient cleavage properties of SUMO protease make this a useful system for obtaining high levels of expression of functional proteins (Malakhov et al., 2004).

Plasmid 1 of the three-vector SUMO system was used to express the PCR-assembled xylose isomerase ORF. Here the full XI ORF was assembled to ensure that rapid high-level expression of properly folded XI was obtained. Misfolding has been identified as a possible issue in the functional expression of XI for efficient ethanologenic fermentation of xylose (Kuyper et al., 2003). The SUMO protease 1 in yeast is highly efficient and cleaves the SUMO tag to completion giving large amounts of functional enzyme (Malakhov et al., 2004). Plasmid 2 was used for expression of the lycotoxin-1 clone library produced by amino acid scanning mutagenesis to allow automated high-throughput plasmid production and expression of this test peptide. Alternatively, the second vector can be used to introduce other mutagenized open reading frames such as cellulases (Den Haan et al., 2007) to produce improved yeast strains for industrial use in conjunction with the XI gene to enable xylose utilization. Plasmid 3 makes possible the introduction of additional clones of interest. These could be obtained from traditionally produced cDNA libraries or from the FLEXGene collections (Zuo et al., 2007) of expression-ready fully annotated inserts for full-genome library introductions or ordered grid transformations of select FLEXGene sets. This plasmid allows expression of enzymes for corrective metabolic pathways or other desirable functions to improve tolerance, anaerobic growth, and increase ethanol output in industrial yeast strains. High levels of expression are obtained from all three plasmids. Because it has two promoters, an ADH promoter and also a T7 modified promoter for in vitro transcription/translation, this plasmid system can be used in bacterial cells and bacterial lysates as well as yeast cells. The three-plasmid system also offers the possibility of using three different libraries in three different vectors which maximizes the number of combinations possible for screening on the automated platform.

4.2. Expression of xylose isomerase

The transmission electron micrograph (TEM) of the INVSc1-XI yeast strain compared to that of the INVSc1 yeast strain (Fig. 2A) shows INVSc1-XI yeast contains large amounts of additional material not seen in the strain without the XI plasmid. Although the cells change dramatically in shape, growth does not appear to be adversely affected since the doubling time on glucose is the same for both the INVSc1 yeast strain and the INVSc1-XI yeast strain (1.8 h). The presence of expressed XI enzyme is verified by the appearance of the 53.7 kDa attB1HisXI band on the Western gel (Fig. 2C). Expression of xylose isomerase enables the XI-strain to grow aerobically on CM xylose URA selective plates to a very limited extent. This strain has potential for use in the metabolic engineering of a pentose-fermenting industrial S. cerevisiae strain for production of bioethanol from renewable lignocellulosic feedstocks (Hahn-Hägerdal et al., 2007; Kuyper et al., 2003).

4.3. Expression of lycotoxin-1 mutants

Common features of amphipathic peptides include an overall basic charge, a small size (23–39 aa residues), and the ability to form amphipathic α-helices. Whether they function by modification of the membrane lipid bilayer via the amphipathic α-helix portion or by ion channel
formation, an ordered secondary conformation such as an amphipathic α-helix and a positive charge appear to participate in their function (Corzo et al., 2002; Duguid et al., 1998; Julian and Jaynes, 1998; Kourie and Shorthouse, 2000). Both composition and sequence contribute to function (Mayo et al., 2000). Expression of the Lyt-1 variants does not appear to disrupt the yeast cells since the doubling time of the lycotoxin-containing yeast cultures on glucose (1.86–1.92 h) is similar to that for the INVSc1 and INVSc1-XI yeast strains (1.84 h), even though the Lyt-1-containing yeast strains are lethal to fall armyworms (Hughes et al., 2007, 2008). Immunofluorescence images of the INVSc1 strain show no signal, indicating absence of expression of any His-tagged species, while the INVSc1-XI strain shows extensive and intense staining of the large amount of HisXI and HisSUMO throughout the cells but most strongly in areas with the obvious dent visible in the brightfield images (Fig. 3), consistent with expression of XI. In spite of high levels of expression of XI and the presence of indentations, the cells show growth on glucose similar to INVSc1. The INVSc1-XI-Lyt-1 strains show more intense and widespread staining than the INVSc1-XI strain, also around the indentations, from expression of HisLyt-1 and more HisSUMO in addition to the HisXI and HisSUMO from plasmid 1 (Fig. 3). Both Lyt-1 and SUMO are amphipathic peptides. Localization of an amphipathic peptide is strongly dependent on the charge and hydrophobic nature of the peptide and the charge on the cell membranes (Parenteau et al., 2005; Holm et al., 2005). Immunofluorescence shows that large quantities of His-tagged material are present in the INVSc1-XI and INVSc1-XI-Lyt-1 yeast strains after two-days growth. SUMO undergoing processing is known to localize to the nucleus (Parenteau et al., 2005; Holm et al., 2005). Immunofluorescence shows that large quantities of His-tagged material are present in the INVSc1-XI and INVSc1-XI-Lyt-1 yeast strains after two-days growth. SUMO undergoing processing is known to localize to the nucleus (Parenteau et al., 2005; Holm et al., 2005).
show staining similar to the undigested cells. The Western
gels in Fig. 4 suggest that the Lyt-1 is associated with the
membrane and remains associated with membrane com-
ponents in the lysate. When the His-tagged Lyt-1 is Ni bead
purified, the Lyt-1 molecules are pulled away from the
membrane components and bound to the beads. Their
close proximity provides the opportunity for aggregation,
with dimers seemingly favored. The SEM and Coomassie
gel results suggest that more of the expressed Lyt-1 is
inside the yeast cells rather than on the surface in the C6,
B9, and A6 variant strains compared to the C3 variant
strain.

4.4. Expression of xylulokinase or transaldolase genes

Lysates prepared after growth on glucose of all 12 yeast
strains expressing xylose isomerase and the lycotoxin-1
variants, either with or without one of two metabolic cor-
rection enzymes, show the expected bands for attB1HisXI,
the attB1HisEntKLyt-1, and the HisSUMO tag removed
from both HisXI and HisLyt-1 by yeast SUMO protease 1
(Fig. 4A). The band for the HisSUMO tag is twice as intense
as either of the other two bands since it is released from
both the XI protein and the Lyt-1 variants. Lysates pre-
pared after growth on glucose and subjected to Ni bead
puriﬁcation of the His-tagged proteins, show bands at the
same three locations (with a decrease in intensity of the
10.7 kDa attB1HisEntKlyt-1 band) and an additional band
at 21.4 kDa, attributed to a dimer of the attB1HisEntK Lyt-1
protein for all 12 strains (Fig. 4B). Dimer formation is pos-
tulated because in the Ni bead puriﬁcation the beads con-
centrate the peptide on their surface and highly favor
aggregation. Numerous amphipathic peptides are thought
to form multimeric pores in cell membranes (Kourie and
Shorthouse, 2000). The amphipathic peptide, amyloid β-
protein, associated with senile plaques in Alzheimer’s dis-
ease, forms stable dimers (Garzon-Rodriguez et al., 1997)
as shown by ﬂuorescence resonance energy transfer and
gel ﬁltration chromatography, and aggregate formation
has been studied using SDS–PAGE (Burdick et al., 1992;
Soreghan et al., 2002). As would be expected, the monomer
band is dramatically decreased in intensity since it is being
used to form dimer. Aggregation most probably contrib-
utes to the invaginations, tube-like formations and matted
material seen in the scanning electron micrographs (Fig. 6).

4.5. Growth of triply transformed INVSc1 yeast strain

The INVSc1-XI-Lyt-1 (C3, A6, B9, or C6)-XKS (or TAL)
strains grow better aerobically, although still to a limited
extent, on xylose plates than INVSc1-XI, with the XKS strains showing slightly better growth than the TAL strains (Fig. 5). The triply transformed strains show extensive growth aerobically and anaerobically on glucose liquid medium and on glucose plates. The strains expressing XKS and C6 or XKS and C3 show measurable growth aerobically on xylose liquid medium. This would suggest that overexpression of an enzyme that contributes to xylose utilization via the pentose phosphate pathway helps to correct metabolic imbalance caused by overexpression of xylose isomerase (Jin et al., 2003; Kuyper et al., 2004, 2005; Van Maris et al., 2007). Growth is not yet at an industrial level, but this work demonstrates that the SUMO three-plasmid system can be used to express high levels of several proteins that enhance xylose utilization simultaneously. The INVSc1-XI-XKS strain showed optimum growth on xylose liquid medium with a doubling time of 5.87 h. The INVSc1-XI-XKS strain developed in the work described here will be used as the base strain for further development by addition of genes from a full-genome yeast library and from other xylose-utilizing fungi and yeast.

4.6. Effects of lycotoxin-1 mutations

The four mutants used in this work were identified as the most effective in killing a test insect pest (Hughes...
et al., 2007, 2008). They all have proline and tryptophan replacing lysine and leucine at positions 24 and 25, respectively. Tryptophan is important to activity of amphipathic peptides (Blondelle et al., 1993; Mayo et al., 2000; Salzwedel et al., 1999). The variant, C6, in the INVSc1-XI-XKS strain that showed limited growth aerobically in xylose liquid medium also had the substitution of serine for leucine at position 9. These variants retain the core set of lysine residues, K7, K11, K15, and K19, present on the hydrophobic side of the α-helix in the amphipathic peptide structure, giving the net positive charge that promotes interaction (Lamazière et al., 2007). A possible model for the effect of these peptides on membrane bilayers, including formation of tubes and amphipathic character, these peptides induce different association of amphipathic peptides with mammalian cell membranes, leading to membrane permeabilization. It also places a hydrophobic residue at the C-terminal position, which appears to be important for greatest activity (Mayo et al., 2000). The activity of amphipathic peptide toxins such as lycotoxin-1 results from their modification of insect cell membranes to form pores or channels, making them more permeable (Kourie and Shorthouse, 2000). The effect on yeast cells is seen in the dents, bulges, and irregularities on the surface of the cells in the SEM images. Association of amphipathic peptides with mammalian cell membranes have been shown to produce significant perturbations in the cell structure. Depending on their charge and amphipathic character, these peptides induce different effects on membrane bilayers, including formation of tubules and membrane aggregation and/or formation of pores (Lamazière et al., 2007). A possible model for the effect of Lyt-1 on the yeast cell membrane (Fig. 7C) shows the hydrophobic residues including the tryptophans directed toward the interior of the cell membrane and the hydrophobic residues forming the interior of a pore. Xylose uptake by the cells is dependent on hexasaccharide transporter genes. Deletion of all the hexasaccharide transporter genes results in loss of the ability to grow on xylose (Hamacher et al., 2002). It is possible that the presence of an amphipathic peptide increases transport of xylose into the cells by increasing surface area and membrane permeability to allow greater transporter activity.

In summary, the yeast strains expressing xylulokinase in addition to xylose isomerase show improved aerobic growth on xylose liquid medium compared to the INVSc1-XI yeast strain. Strains expressing lycotoxin-1 variants C6 and C3 in addition to XI and XKS also show limited aerobic growth on xylose liquid medium. These results suggest that additional enzymes in the xylose metabolic pathway or other metabolic flux improvements might be needed for metabolic correction of XI and XKS overexpression or that improving xylose transport across the cell membrane might increase the efficiency of xylose utilization. The automated three-plasmid system provides a strategy for strain optimization.

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References


