APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2004, p. 961–966 Vol. 70, No. 2
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.2.961–966.2004


# Viral Preprotoxin Signal Sequence Allows Efficient Secretion of Green Fluorescent Protein by *Candida glabrata*, *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*

Antje Eiden-Plach, Tatjana Zagorc,† Tanja Heintel,‡ Yvonne Carius, Frank Breinig, and Manfred J. Schmitt*

*Angewandte Molekularbiologie, Universität des Saarlandes, D-66041 Saarbrücken, Germany*

Received 9 September 2003/Accepted 30 October 2003

**Besides its importance as model organism in eukaryotic cell biology, yeast species have also developed into an attractive host for the expression, processing, and secretion of recombinant proteins. Here we investigated foreign protein secretion in four distantly related yeasts (*Candida glabrata*, *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*) by using green fluorescent protein (GFP) as a reporter and a viral secretion signal sequence derived from the K28 preprotoxin (pptox), the precursor of the yeast K28 virus toxin. In vivo expression of GFP fused to the N-terminal pptox leader sequence and/or expression of the entire pptox gene was driven either from constitutive (*PGK1* and *TPI1*) or from inducible and/or repressible (*GAL1*, *AOX1*, and *NMT1*) yeast promoters. In each case, GFP entered the secretory pathway of the corresponding host cell; confocal fluorescence microscopy as well as sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western analysis of cell-free culture supernatants confirmed that GFP was efficiently secreted into the culture medium. In addition to the results seen with GFP, the full-length viral pptox was correctly processed in all four yeast genera, leading to the secretion of a biologically active virus toxin. Taken together, our data indicate that the viral K28 pptox signal sequence has the potential for being used as a unique tool in recombinant protein production to ensure efficient protein secretion in yeast.**

Over the years, heterologous protein expression in yeast species such as *Candida glabrata*, *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* has become an important tool in the production of therapeutic and commercially relevant proteins. The advantage of a yeast-based expression system is due to the fact that as a eukaryotic microorganism, yeast combines ease of genetic manipulation and cell growth with a well-known capacity to perform complex posttranslational protein modifications (4, 22, 29). Because yeast per se secretes only low levels of endogenous proteins, a secreted recombinant protein usually contributes significantly to the total amount of proteins in the culture medium. Thus, directing a foreign protein for secretion is an important initial step in its subsequent purification.

While the vast majority of heterologous proteins has been expressed within the cytosol of the corresponding host, only a few proteins have been successfully secreted into the extracellular medium. In most eukaryotic proteins, the critical initial step in protein secretion is their co- and/or posttranslational translocation into the lumen of the endoplasmic reticulum (ER) followed by subsequent sorting into the Golgi network. Foreign protein import into the ER is usually achieved by fusing the protein of interest in frame to a homologous secretion signal sequence derived from a naturally secreted protein of the corresponding host, thus conferring secretion competence to the desired protein fusion (13, 23).

In yeast, the most widely used secretion signals are those derived from yeast invertase (Suc2p), acid phosphatase (Pho5p), inulinase (Inu1p), α-galactosidase (Mel1p), or pheromone α-factor or from the plasmid-driven killer toxin of *Kluyveromyces lactis* (6, 12, 16, 23). In addition to the results seen with leader sequences derived from naturally secreted host cell proteins, Heintel et al. recently showed that a viral secretion signal from the K28 killer virus is equally functional in baker's yeast and fission yeast, indicating that a preprotoxin (pptox)-based signal sequence might be a novel and unique tool for manipulating heterologous protein secretion in biotechnologically relevant yeast species (12). We now demonstrate—to our knowledge for the first time—that a viral pptox leader sequence is indeed capable of ensuring efficient green fluorescent protein (GFP) secretion in four distantly related yeast species (*C. glabrata*, *P. pastoris*, *S. cerevisiae*, and *S. pombe*), indicating that this signal sequence might be of general importance for foreign protein secretion in yeast.

## MATERIALS AND METHODS

**Strains and growth conditions.** All yeast strains used in this study are summarized in Table 1. *Escherichia coli* DH5α cells [F⁻ *recA1 endA1 gyrA96 thi hsdR17 supE44 relA* Δ(*argF-lacZYA*) U169 ϕ80(*dlacZΔM15*)λ⁻] grown in Luria-Bertani medium were used as a host for the amplification and propagation of all constructed plasmids. Yeast cultures were grown at 30°C either on complex yeast extract-peptone-dextrose medium or on synthetic complete minimal medium containing 0.67% yeast nitrogen base (YNB) without amino acids supplemented with ammonium sulfate and the appropriate amino acid-base requirements of each strain (12, 17). As indicated and previously described (9), the culture medium for methanol-induced pptox and/or GFP expression in *P. pastoris* was

* Corresponding author. Mailing address: Angewandte Molekularbiologie (FR 8.3), Universität des Saarlandes, Gebäude 2, Postfach 15 11 50, D-66041 Saarbrücken, Germany. Phone: (49) 681-3024730. Fax: (49) 681-3024710. E-mail: mjs@microbiol.uni-sb.de.

† Present address: Chamber of Commerce and Industry of Slovenia, SI-1504 Ljubljana, Slovenia.

‡ Present address: Medizinische Mikrobiologie und Hygiene (Abteilung Virologie), Universität des Saarlandes, D-66421 Homburg/Saar, Germany.

TABLE 1. Yeast strains and plasmids used in this study

| Yeast strain or plasmid | Genotype or characteristic(s) | Reference(s) or source |
|---|---|---|
| Yeast strains | | |
| *S. cerevisiae* 192.2d | *MAT*α *ura3 leu2* | 27 |
| *S. cerevisiae* SEY6210 | *MAT*α *ura3-52 leu2-3 112 his3-*Δ*200 trp1-*Δ*901 lys2-801 suc2-*Δ*9* [pYX242] | 21 |
| *P. pastoris* GS115 | *his4* | Invitrogen |
| *C. glabrata* BG14 | *ura3*Δ*(−85 +932)*::Tn*903neo* | 8 |
| *S. pombe* PW260 | h$^-$ *leu1-32 ura4-D18 ade6-M210* | 12 |
| Plasmids | | |
| pSL-K28 | 2μm vector for pptox expression in *S. cerevisiae* from the *TP11* promoter | This study |
| pPGK-M28-I | 2μm vector for pptox expression in *S. cerevisiae* from the *PGK1* promoter | 28 |
| pFB-pptoxGFP | *S. cerevisiae* 2μm vector for pptox-driven GFP secretion from the *GAL1* promoter | This study |
| pGRB2.2-K28 | *C. glabrata* ARS/CEN plasmid for pptox expression from the *PGK1* promoter | This study |
| pPIC3.5-K28 | *P. pastoris* integrative plasmid for pptox expression from the *AOX1* promoter | This study |
| pUG35 | *S. cerevisiae* ARS/CEN vector expressing yEGFP from the *MET25* promoter | 7, 18 |
| pTZ-pptox | Integrative *S. pombe* vector containing the pptox secretion signal under control of the *nmt1* + promoter | This study |
| pTZ-pptoxGFP | Integrative vector for pptox-driven GFP secretion in *S. pombe* from the *nmt1* + promoter | This study |

either minimal dextrose medium [0.34% YNB, 1% $(NH_4)_2SO_4$, 0.00004% biotin, 2% dextrose], buffered minimal glycerol medium [100 mM potassium phosphate (pH 6.0), 0.34% YNB, 1% $(NH_4)_2SO_4$, 0.00004% biotin, 1% glycerol], minimal methanol medium [0.34% YNB, 1% $(NH_4)_2SO_4$, 0.00004% biotin, 0.5% methanol], or minimal glycerol medium [0.34% YNB, $(NH_4)_2SO_4$, 0.00004% biotin, 10% glycerol]. All other media have been previously described (12, 21).

**Vector construction, transformation, and DNA sequencing.** The open reading frames of K28 pptox and yeast-enhanced GFP were amplified by PCR using *Taq* DNA polymerase and vectors pPGK-M28-I (25) and pUG35 (7, 18) as templates (Table 1). To construct pFB-pptoxGFP, the corresponding pptox/GFP fusion was PCR amplified and subsequently cloned into the galactose-inducible yeast expression vector pYES2.1/V5-His-TOPO according to the instructions of the manufacturer (Invitrogen, Groningen, The Netherlands). Oligonucleotide primers for PCR amplification introduced 5′- and 3′-flanking restriction endonuclease cleavage sites that were required for the oriented cloning of the PCR product into the corresponding yeast vector (Table 2). DNA sequence analysis of all constructs (performed by fluorescent cycle sequencing on an automated DNA sequencer [LI-COR 4200; MWG Biotech]) confirmed the predicted sequences at the fusion junction. Transformation of *C. glabrata* was carried out using a slightly modified lithium acetate (LiOAc) method (11). Briefly, cells were grown to early log phase ($10^7$ cells/ml), collected by centrifugation, washed twice with water, and resuspended in a solution containing 0.1 M LiOAc, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.5). After an incubation for 1 h, transforming DNA (1 μg) was added to the cells together with 100 μg of denatured salmon sperm DNA, 0.5 ml of 0.1 M LiOAc, 40% polyethylene glycol (PEG-3350), 1 mM EDTA, and 10 mM Tris-HCl (pH 7.5). The mixture was incubated at 30°C for 30 min, and cells were heat shocked for 15 min at 45°C. Transformants were selected on synthetic YNB medium supplemented with the appropriate amino acid-base composition of each strain. *P. pastoris* strain GS115 (Invitrogen, Table 1) was transformed by electroporation at 1.5 kV, 200 Ω, and 25 μF with a Gene Pulser II system (Bio-Rad, Munich, Germany). Plasmids used for *P. pastoris* transformation were linearized with *Sal*I to favor integration into the chromosomal *HIS4* locus. After growth on minimal dextrose plates at 30°C for 3 days, transformants were selected by plating onto minimal medium lacking histidine according to the protocol of the manufacturer (Invitrogen).

**Toxin activity assay.** The killer phenotype, i.e., in vivo toxicity of yeast transformants, was determined in a standard agar diffusion assay on methylene blue agar plates (pH 4.7) as previously described (10). Briefly, $10^7$ cells (in a total volume of 10 μl) of pptox-expressing strains of *S. cerevisiae*, *S. pombe*, *C. glabrata*, and *P. pastoris* were spotted onto methylene blue agar plates that had been seeded with an overlay of the sensitive tester strain *S. cerevisiae* 192.2d. For the determination of toxin activity in *P. pastoris*, transformants were cultivated under inducing conditions in the presence of methanol for up to 96 h and aliquots (100 μl) of a concentrated and cell-free culture supernatant were pipetted into wells (10 mm in diameter) that had been cut into the methylene blue agar (pH 4.7). After the plates were incubated for 4 days at 20°C, a clear zone of growth inhibition surrounding the well indicated K28 killer phenotype expression and toxin secretion.

**Western blot analysis.** To estimate the amount of secreted proteins after transformation with the various pptox-based expression vectors, the appropriate yeast transformants were grown in synthetic minimal medium at 30°C for 2 days until cell densities of $5 \times 10^8$ cells ml$^{-1}$ were reached (10, 21). Extracellular (secreted) proteins were concentrated from the cell-free culture supernatant by ethanol precipitation, and protein samples were electrophoretically separated in Tris-Tricine gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Schagger and von Jagow (24). After electrotransfer onto polyvinylidene difluoride membranes, blots were incubated with either a monoclonal anti-GFP antibody (Roche) (diluted 1/1,000) or a polyclonal antibody against the toxin's β-subunit (21) followed by treatment with an alkaline phosphatase-coupled secondary anti-rabbit or anti-mouse immunoglobulin G antibody (Sigma) (diluted 1/3,000) and were finally developed in a nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) stock solution (18.75 mg of nitroblue tetrazolium chloride/ml and 9.4 mg of 5-bromo-4-chloro-3-indolylphosphate [toluidine salt]/ml in 67% [vol/vol] dimethyl sulfoxide).

**Recombinant pptox and GFP expression.** To induce protein expression in *P. pastoris*, transformants were grown for 16 to 18 h in buffered minimal glycerol medium to an optical density at 600 nm of 2 to 6 and subsequently diluted in minimal methanol medium to an optical density at 600 nm of 1.0. Every 24 h, methanol (100% [vol/vol]) was added to a final concentration of 1% to maintain inducing conditions for protein expression from the *AOX1* promoter. After a total incubation for 96 h, cells were removed; the secreted proteins present in the cell-free culture supernatant were detected by Western analysis as described above.

**Fluorescence microscopy.** Exponentially growing cells of GFP-expressing yeast transformants were harvested, washed twice with phosphate-buffered saline buffer (pH 7.4), and examined using either a BX51 fluorescence microscope (Olympus) or a confocal laser scanning microscope (Bio-Rad) with standard GFP settings (excitation wavelength, 488 nm; emission filter, 515 to 550 nm).

TABLE 2. PCR primers used in this study[a]

| Primer pair | Sequence (5′–3′) |
|---|---|
| PGRB2.2-K28 | GG***GAATTC***ATGGAGAGCGTTTCCTCA |
| | CC***GCGGCCGC***TTAGCGTAGCTCATCGTG |
| PGRB2.2-SP/GFP | GG***GAATTC***ATGGAGAGCGTTTCCTCA |
| | ***GAATTC***TTATTTGTACAATTCATCCATACCATGGG |
| PPIC3.5-K28 | GG***TCTAGA***ATGGAGAGCGTTTCCTCA |
| | CC***GAATTC***TTAGCGTAGCTCATCGTG |
| PPIC3.5-SP/GFP | GG***TCTAGA***ATGGAGAGCGTTTCCTCA |
| | ***GCGGCCGC***TTATTTGTACAATTCATCCAT |
| PTZ-pptoxGFP | ***GGATCC***ATGGGCAGCTCAGAAGATAAAATAACA |
| | CCCG***GGATCC***TTATTTGTACAATTCATCCATACC |
| | ATGG |

[a] Restriction endonuclease cleavage sites used for PCR cloning are shown in bold and italic characters.

## RESULTS AND DISCUSSION

**K28 pptox expression and toxin secretion in *C. glabrata*, *P. pastoris*, *S. cerevisiae*, and *S. pombe*.** In natural killer strains of *S. cerevisiae*, the virally encoded K28 pptox represents the unprocessed precursor of a secreted protein toxin (K28) that kills sensitive yeast cells in a receptor-mediated fashion by causing a cell cycle arrest at the $G_1$/S boundary followed by a rapid inhibition of DNA synthesis in the nucleus (26, 27, 28). The 38-kDa pptox consists of an N-terminal leader peptide necessary for pptox import into the ER lumen followed by the α and β subunits of the mature toxin (10.5 and 11.0 kDa, respectively) which are separated from each other by a potentially N-glycosylated γ sequence (28). During passage through the yeast secretory pathway, signal peptidase cleavage after $Gly^{36}$ removes the N-terminal leader peptide, the N-glycosylated γ sequence is excised by the action of the late Golgi endopeptidase Kex2p, and the mature and biologically active protein is finally secreted as an α/β heterodimeric toxin in which both subunits are covalently linked by a single disulfide bond (21) (Fig. 1A).

Heintel et al. recently demonstrated that fission yeast is capable of secreting a correctly processed and biologically active K28 virus toxin after in vivo expression of the K28 pptox gene under transcriptional control of the thiamine-repressible *nmt1*$^+$ promoter (12). To investigate whether such plasmid-driven killer phenotype expression is also possible in *P. pastoris* and *C. glabrata*, episomal vectors were constructed in which the entire K28 pptox gene was expressed either from the constitutive *PGK1* promoter (*C. glabrata*) or from the methanol-inducible *AOX1* promoter (*P. pastoris*). As a positive control, *S. cerevisiae* or *S. pombe* vector was also included which allows K28 pptox expression from the constitutive *TPI1* or the thiamine-repressible *nmt1*$^+$ promoter, respectively (Fig. 1B). In each case, yeast transformants were selected on an appropriate minimal medium and positive clones were subsequently analyzed for constitutive and/or regulated pptox expression. Since pptox expression in fission yeast was driven from the thiamine-repressible *nmt1*$^+$ promoter, yeast transformants were tested for toxin production after growth in liquid minimal medium (in the presence or absence of 25 μM thiamine) as previously described (12). In the case of *P. pastoris* transformants, pptox expression was induced by cultivating the cells on methanol as a carbon source (see Materials and Methods).

As shown in Fig. 2, all tested yeast transformants expressed the viral pptox gene under the appropriate culture conditions and showed a clearly detectable killer phenotype with a toxin-sensitive tester strain. In addition, SDS-PAGE and Western analysis of cell-free culture supernatants further indicated that in each case the recombinantly expressed virus toxin was correctly processed to an α/β heterodimeric protein whose electrophoretic mobility after SDS-PAGE and immunoblotting was identical to that of the homologous toxin expressed and secreted by *S. cerevisiae* (Fig. 3). Thus, the N-terminal signal sequence in K28 pptox functions equally effectively in all four yeast genera, indicating that it might have the potential for being used as a novel and unique signal sequence suitable for foreign protein secretion. Furthermore, this is the first time that a biologically active virus toxin has been successfully expressed and secreted in *C. glabrata* and *P. pastoris*, confirming

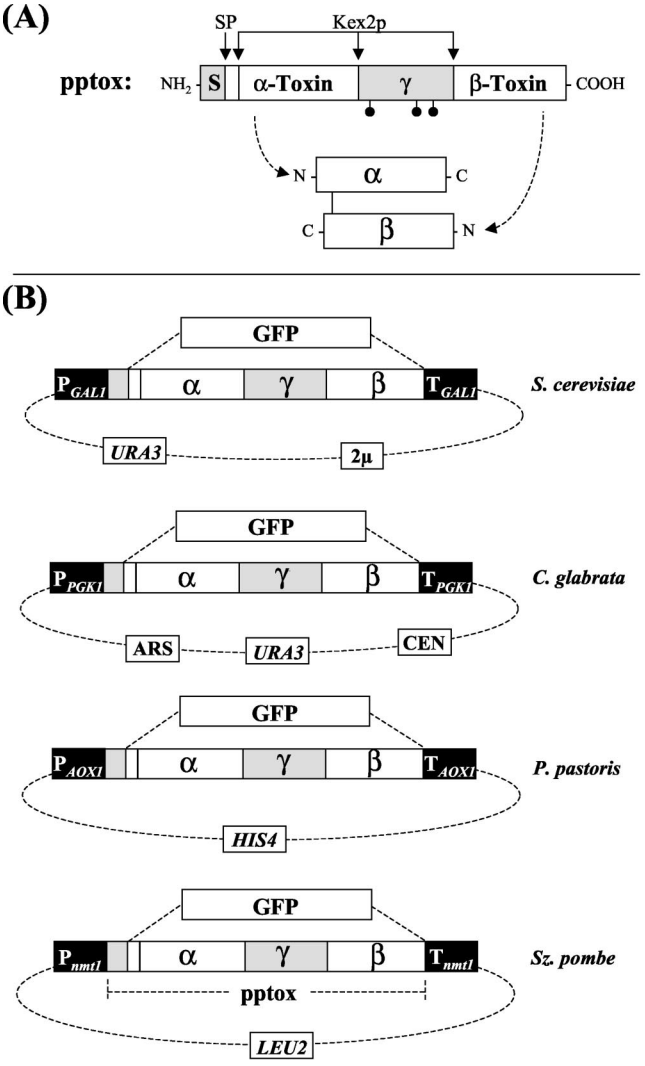


FIG. 1. (A) Kex2p-mediated pptox processing to the α/β heterodimeric K28 virus toxin in yeast. Internal cleavage sites of the ER lumenal signal peptidase (SP) and of the late Golgi endopeptidase Kex2p are indicated. The three N-glycosylation sites within the pptox γ-sequence are indicated by circles. S, secretion signal at the pptox N terminus. (B) Schematic outlines of the constructed vectors that allow constitutive or regulated expression of K28 pptox and/or GFP in the yeasts *S. cerevisiae*, *C. glabrata*, *P. pastoris*, and *S. pombe*. In each vector, the pptox and/or GFP open reading frame is under the transcriptional control of the indicated promoter and transcription termination combinations; the origins of replication (ARS) and low- or high-copy numbers of the vectors (CEN and 2μm [2μ]) as well as marker genes for the selection of yeast transformants (*HIS4*, *URA3*, and *LEU2*) are indicated.

more recent reports on the existence of Kex2p/Kex1p homologous processing machinery that is required for the in vivo processing and maturation of protein precursors (1, 2).

**The K28 pptox signal sequence allows efficient GFP secretion in various yeast genera.** For unknown reasons, causing secretion of the GFP turned out to be extremely difficult, not only in mammalian cells (19) but also in insect cells (15) as well as in yeast other than *P. pastoris* and *S. pombe* (3, 5, 14, 20). Unlike successful protein tagging with GFP for in vivo targeting to different organelles, secretion of GFP and/or GFP fu-

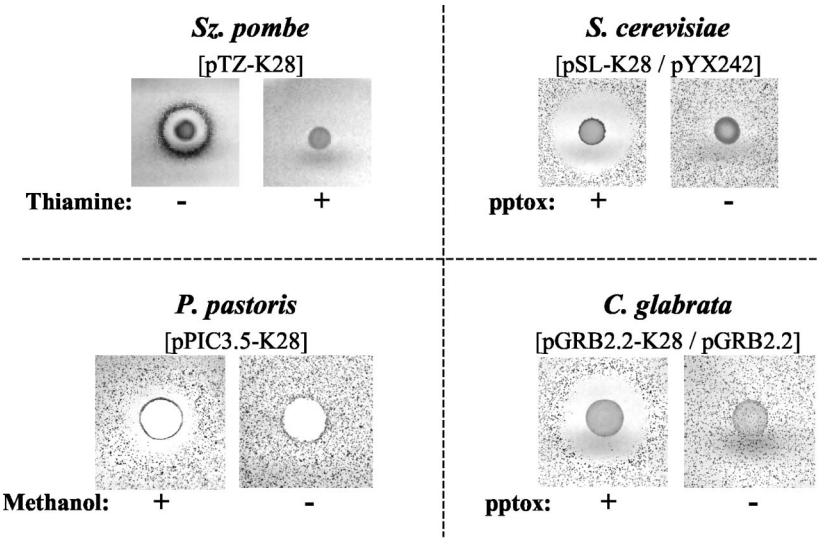


FIG. 2. Constitutive or thiamine-methanol-regulated K28 pptox expression in *S. pombe* (upper left panel), *S. cerevisiae* (upper right panel), *P. pastoris* (lower left panel), and *C. glabrata* (lower right panel). Yeast transformants harboring the indicated K28 pptox expression plasmid were grown under induced and/or repressed culture conditions (in the absence [−] or presence [+] of thiamine and/or methanol), and K28 toxin production levels were determined on methylene blue agar plates (pH 4.7) against the sensitive *S. cerevisiae* strain 192.2d. Toxin activity levels in pptox-expressing *P. pastoris* transformants were determined by pipetting a 100-μl aliquot of a cell-free culture supernatant into a 10-mm-diameter well that had been cut into the agar. After the plates were incubated for 4 days at 20°C, a cell-free zone of growth inhibition around the pprox-expressing yeast strain and/or the well indicated toxin secretion and killer activity.

sions has often been described as causing mistargeting to the vacuole (14) or ER-Golgi retention (16). Thus, until today all attempts to secrete GFP into the culture medium of *S. cerevisiae* failed, even when the well-known secretion signals derived from yeast invertase (Suc2p), acid phosphatase (Pho5p), α-galactosidase (Mel1p), inulinase (Inu1p), and/or α-mating factor were used (16) (a virally encoded pptox secretion signal has not yet been tested). To fill this gap and to show that the K28 pptox leader peptide is suitable for driving heterologous protein secretion in different yeast genera, the series of pptox-based yeast expression vectors described above was further modified in such a way that the K28 protoxin sequence (ptox) was removed and replaced by an in-frame fusion of GFP to the 36-amino-acid presequence of K28 pptox (Fig. 1B). As described above for the full-length pptox constructs, the GFP secretion vectors were transformed into each of the four yeast species tested and the intracellular localization and secretion pattern of GFP was analyzed by confocal fluorescence microscopy as well as by SDS-PAGE and Western analysis of extracellular proteins present in the cell-free culture supernatant.

As shown in Fig. 4, confocal laser scanning microscopy of GFP-expressing yeast transformants resulted in a fluorescence pattern that is characteristic for a protein that is being secreted into the medium; in each case, intracellular GFP fluorescence was detectable within the ER-Golgi system as well as at the outer yeast cell surface. Because of the bigger cell size of *S. pombe* compared to that seen with the other yeast genera tested, ER-Golgi localization of GFP and its final targeting to the yeast cell surface was most prominent in fission yeast transformants (Fig. 4, bottom right panel). To further confirm that GFP is indeed secreted into the medium and not retained within the yeast cell wall, culture supernatants of each yeast transformant were subjected to SDS-PAGE and GFP secretion was determined by Western analysis and probing with a monoclonal anti-GFP antibody. As shown in Fig. 4, GFP was efficiently secreted into the medium by all four yeast species; in each case, a single GFP protein signal was detectable in the corresponding immunoblot. Efficacy of GFP secretion was most pronounced in *S. pombe* and *P. pastoris* transformants, and on the basis of a direct comparison of the amount of GFP secreted by the indicated yeasts with that corresponding to the known amount of GFP that had been used as a protein standard on the same immunoblot, it can be estimated that pptox-driven GFP secretion was in the range of 0.1 to 2 mg of GFP per liter of culture medium. This rather efficient GFP secretion result was also confirmed by the observation that in each yeast

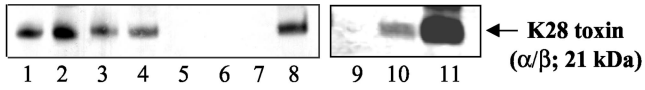


FIG. 3. Western blot analysis of K28 toxin secretion in *C. glabrata*, *P. pastoris*, *S. pombe*, and *S. cerevisiae* after in vivo expression of K28 pptox. Cell-free culture supernatants (600 μl each) of the corresponding yeast transformants grown under pptox-inducing culture conditions were concentrated by ethanol precipitation, separated by SDS-PAGE under nonreducing conditions, and probed with a polyclonal antibody against the toxin's β-subunit. Lane 1, K28 toxin (positive control); lane 2, *S. cerevisiae*(pSL-K28); lane 3, *C. glabrata*(pGRB2.2-K28); lane 4, *P. pastoris*(pPIC3.5-K28) after cultivation for 96 h under inducing conditions; lane 5, *S. cerevisiae*(pYX242) (negative control); lane 6, *C. glabrata*(pGBR2.2) (negative control); lane 7, *P. pastoris*(pPIC3.5) (negative control); lane 8, *P. pastoris*(pPIC3.5-K28) after cultivation for 120 h under inducing conditions; lane 9, *S. pombe*(pTZ) (empty vector control); lane 10, *S. pombe*(pTZ-K28) grown under repressed conditions in the presence of thiamine; lane 11, *S. pombe*(pTZ-K28) grown under induced conditions in the absence of thiamine. The position and size of the correctly processed α/β heterodimeric K28 virus toxin is indicated.

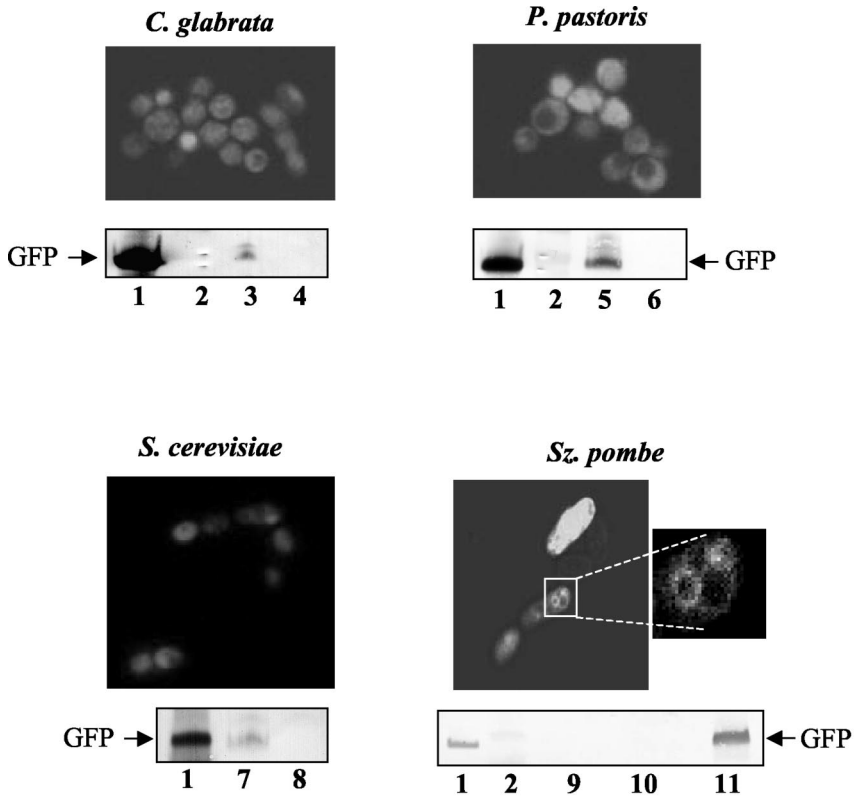


FIG. 4. pptox-driven GFP secretion in yeast. Fluorescence microscopy (upper panel in each pair of panels) and Western analysis (lower panel in each pair of panels) of the indicated yeast transformant after in vivo expression of GFP fused to the amino-terminal secretion signal of K28 pptox. The inset in the bottom right panel shows a stronger magnification of the confocal laser scanning micrograph, illustrating localization of GFP within the secretory pathway (ER and Golgi) of fission yeast. In each case, SDS-PAGE and Western analysis of a cell-free culture supernatant probed with a monoclonal anti-GFP antibody were performed as described in the legend to Fig. 3. Lanes 1, GFP marker (positive control; 5 ng); lanes 2, prestained marker protein standard; lane 3, GFP secreted by *C. glabrata*(pGRB2.2-pptoxGFP); lane 4, *C. glabrata*(pGRB2.2) (empty vector control); lane 5, GFP secreted by *P. pastoris*(pPIC3.5-pptoxGFP) grown for 96 h under inducing conditions in the presence of methanol; lane 6, *P. pastoris*(pPIC3.5-pptoxGFP) grown under noninducing conditions in the absence of methanol; lane 7, GFP secreted by *S. cerevisiae*(pFB-pptoxGFP) grown under inducing conditions on galactose; lane 8, *S. cerevisiae*(pFB-pptoxGFP) grown under repressed culture conditions on glucose; lane 9, *S. pombe*(pTZ-pptox) (empty vector control); lane 10, *S. pombe*(pTZ-pptoxGFP) grown under repressed conditions in the presence of thiamine; lane 11, *S. pombe*(pTZ-pptoxGFP) grown under induced conditions in the absence of thiamine.

transformant, GFP fluorescence was visible in a cell-free, 10-fold-concentrated culture supernatant (data not shown).

Taken together, these data demonstrate that the viral K28 pptox leader peptide is fully functional in different yeast genera and indicate the potential of this signal sequence as a unique and powerful tool in foreign protein secretion in yeast. Our future experiments will therefore focus on the pptox-driven secretion of pharmaceutically and/or biotechnically relevant proteins. Since it has recently been shown that overexpression of the unfolded-protein response pathway in yeast causes an increase in the expression of the ER chaperone Kar2p (BiP) which in turn results in a significant increase in α-amylase secretion (30), this might be a fruitful strategy to further increase pptox-driven foreign protein secretion in yeast.

## ACKNOWLEDGMENTS

We thank Brendan Cormac and Fritz Mühlschlegel for kindly providing the *C. glabrata* ARS/CEN plasmid pGBR2.2 and the *C. glabrata* strain BG14.

This work was kindly supported by a grant from Aventis Research & Technologies (Frankfurt am Main) to M.J.S. and is part of patent number DPA 10062302-6.

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