JCB
Online Supplemental Material

Umebayashi et al. http://www.jcb.org/cgi/doi/10.1083/jcb.200303088

Plasmids and antibodies

The 2.9-kb PstI-EcoRI fragment of *TAT2* was prepared from YCp*TAT2* (Schmidt et al., 1994; Nakamura et al., 2000), and was subcloned into YEplac195 (Gietz and Sugino, 1988) to generate pKU68 (YEp*TAT2*).

Three tandem copies of the HA epitope were placed just before the stop codon of *TAT2* as follows: the 2.9-kb PstI-EcoRI fragment of *TAT2* was subcloned into pUC118 to generate pKU1. Using pKU1 as a template, the XbaI site (underlined in the sequences below) was introduced by PCR just before the stop codon of *TAT2*. The upstream or downstream region of the stop codon was amplified using the primer combination 5′-GGCCACGTGCATTGTCTGG-3′ and 5′-GTATTA TCTAGAACACCAGAAATGGAACTG-3′, or 5′-TGTTCTAGATAATACCGAAGAAACAAG-3′ and 5′-GTTATGCATTAAATGATCT-3′, respectively. After annealing these two PCR products, the entire 0.57-kb fragment was amplified, cut with PmaCI and EcoT22I, and then sequenced for confirmation. This fragment was used to replace the corresponding region of pKU1 to generate pKU42. The NheI-NheI cassette containing three copies of the HA epitope was prepared from pYT11 (Takita et al., 1995), and inserted into the XbaI site of pKU42. The 3.0-kb PstI-EcoRI fragment of *TAT2–3HA* from pKU42 was subcloned into YCplac33 to generate pKU46 (YCp*TAT2–3HA*).

GFP was fused to the COOH terminus of Tat2p as follows: the ORF of GFP lacking the stop codon was amplified by PCR using pEGFP-1 (BD Biosciences) as a template. Primers used were 5′-AAACTAGTATGGTGAGCAAGGGC-3′ and 5′-AAACTAGTCTTGTACAGCTCGTC-3′, both of which contained the SpeI sites (underlined). The 0.7-kb SpeI-SpeI fragment of GFP was sequenced, and was inserted into the XbaI site of pKU42. *TAT2-GFP* was prepared as the 3.6-kb PstI-EcoRI fragment and subcloned into YCplac33 to generate pKU76 (YCp*TAT2-GFP*).

Plasmids for disruption of *TAT2* are based on pKU1. The 1.5-kb EcoT14I-EcoT14I fragment of *TAT2* was replaced by *ADE2* or *hisG-URA3-hisG*. *ADE2* was prepared as the 2.2-kb BglII-BglII fragment from pASZ11 (Stotz and Linder, 1990). *HisG-URA3-hisG* was prepared as the 3.8-kb BamHI-BglII fragment from pNKY51 (Alani et al., 1987). Either pKU41 (*tat2Δ::ADE2*) or pKU82 (*tat2Δ::hisG-URA3-hisG*) was digested with EcoRI and SphI, and then used for transformation. Yeast transformation was performed by the lithium thiocyanate method (Keszenman-Pereyra and Hieda, 1988). Correct integrants (*tat2Δ::hisG-URA3-hisG*) were grown on 5-fluoroorotic acid (5-FOA) medium to select *ura3* auxotrophs (*tat2Δ::hisG*).

For integration of *TAT2–3HA* into the genome, the 1.2-kb StuI-EcoRI fragment of *TAT2–3HA*, which corresponds to the COOH-terminal and 3′ noncoding regions, was prepared from pKU46. This fragment was inserted between the SmaI and EcoRI sites of YIplac211 (Gietz and Sugino,1988). The resultant *URA3*-marked plasmid pKU51 was cut with PmaCI and used for transformation. The integrants were grown on 5-FOA medium to replace the *TAT2* locus with *TAT2–3HA*. The integration and the replacement was confirmed by PCR and immunoblotting to detect Tat2–3HAp.

Three lysine residues (10, 17, and 20) of Tat2p were replaced with arginine by using mutated PCR primers. The upstream region was amplified using the primer combination 5′-TCGTTAAATGGTACGTAGGC-3′ and 5′-GTTAGATCTTCGCTCCCTCAGCTCCTCATTTGAACGCCTGACAGA-3′. The downstream region was amplified by 5′-TCTGTCAGGCGTTCAAATGAGGAGCTGAGGGAGCGAAGATCTAAC-3′ and 5′-CTTGGATCCAAAAACCTTG-3′. Sequences where lysine codons are converted to arginine are underlined. After annealing these two PCR products, the entire 0.7-kb fragment was amplified and cut with SnaBI and BamHI. The SnaBI-BamHI fragment was used to replace the corresponding region of pKU1, and the resultant plasmid (pUC-*TAT2*$^{3K>R}$) was sequenced for confirmation. Ligation was performed among three fragments; the PstI-PvuI fragment of *TAT2*$^{3K>R}$, the PvuI-EcoRI fragment of *TAT2–3HA*, and YCplac33 cut with EcoRI and PstI. The resultant plasmid was designated as pKU158 (YCp*TAT2*$^{3K>R}$-3HA).

ERG6 was disrupted by introducing the *erg6Δ::LEU2* fragment into yeast cells. This fragment was amplified by PCR using the genome of the Δ*erg6* strain R2497 (a gift from R. Gaber, Northwestern University, Evanston, IL) as a template. The primer combination was 5′-GGGCAGTCCTCTTCTGCTTAGT-3′ and 5′-CCAGAGGAGGTCGTGAAGTTGC-3′, or 5′-AATCAACCAAACAGCTAGGGCTGG-3′ and 5′-AAGGCCTGCTAGCAATGAACGTGC-3′. The *end3Δ*::*HIS3* and the *pep12Δ::HIS3* fragments were provided by K. Iwamoto (University of Tokyo, Tokyo, Japan).

PEP4 was disrupted as follows: the 1.5-kb of EcoRI-SalI fragment of *PEP4* was isolated from pSPRA12, and subcloned into pUC18. The 0.49-kb EcoT14I-EcoT14I fragment of *PEP4* was replaced by the 1.8-kb BamHI-BamHI fragment of *HIS3*. Plasmid pKU60 (*pep4Δ::HIS3*) was digested with EcoRI and SalI, and then introduced into yeast cells.

VPS1 was amplified from the genome of YPH499. Primers used were 5′-AAGCATGCAACATATCTTCCAAGACAGACC-3′ and 5′-AAGCATGCACCATCACGCCGAAATGAAAGC-3′. The SphI sites are introduced in the primers (underlined). The PCR product was cut with SphI, and the 3.1-kb fragment was subcloned into pUC19. The internal 1.8-kb BstPI-SpeI fragment of *VPS1* was replaced by the 1.8-kb BamHI-BamHI fragment of *HIS3*. Plasmid pKU62 (*vps1Δ*::*HIS3*) was digested with SphI, and then introduced into yeast cells.

To disrupt *BUL1*, the 5.6-kb EcoRI-SacI fragment of *BUL1* was prepared from pHY06 (Yashiroda et al., 1996), and subcloned into pBlueScript® II KS+. The internal 1.8-kb BstPI-SpeI region was replaced by the 1.8-kb BamHI-BamHI fragment of *HIS3* or the 2.3-kb XhoI-SalI fragment of *LEU2* to form pKU143 or pKU121, respectively. Before transformation, pKU143 was cut with EcoRI and SalI, and pKU121 with ApaI and SacI.

YEp96 and YEp105 (Ellison and Hochstrasser, 1991), *TRP1*-marked plasmids with which ubiquitin and myc-tagged ubiquitin, respectively, are expressed by the copper-inducible *CUP1* promoter and were provided by Mark Hochstrasser (Yale University, New Haven, CT). To convert the plasmid marker to *URA3*, the 1.0-kb BamHI-ClaI fragment of P_{CUP1}-*Ub*-T_{CYC1} was prepared from YEp96 and subcloned into pRS426 (Sikorski and Hieter, 1989) to form pKU105. Similarly, pKU106 was constructed by preparing the P_{CUP1}-*myc-Ub*-T_{CYC1} fragment from YEp105.

Disruption of *DOA4* was performed as follows: the upstream region of the *DOA4* ORF was amplified by PCR using the primer combination 5′-AAAGAGCTCTATTCCTTCCCCCCTGGTCC-3′ and 5′-AAGGATCCGCATTTATCTAGAAGACTGG-3′, and then cut with SacI and BamHI (each restriction site is underlined). The downstream region was amplified using 5′-AAGGATCCTTATTTCAGGGACAATACGCC-3′ and 5′-AAAGCATGCGCCCAAAATGGAGTCAAGTCC-3′, and then cut with BamHI and SphI (each restriction site is underlined). Ligation was performed among these two fragments and pUC18 to generate a 0.8-kb SacI-SphI insert in pUC18. This plasmid was cut at the BamHI site, the junction between the two fragments, and the 1.8-kb BamHI-BamHI fragment of *HIS3* was inserted. Plasmid pKU108 (*doa4Δ*::*HIS3*) was cut with SacI and SphI, and then was introduced into yeast cells.

VPS27 was amplified from the genome of YPH499. Primers used were 5′-AAGAAGATGCCTTAACCTGTGG-3′ and 5′-CAGCCACCATTGAAAAGC-3′. The PCR product was cut with BamHI and EcoRI, and the 3.2-kb fragment was subcloned into pUC18. The internal 1.4-kb BglII-ClaI fragment of *VPS27* was replaced by the 1.8-kb BamHI-BamHI fragment of *HIS3*. Plasmid pKU65 (*vps27Δ*::*HIS3*) was digested with EcoRI and SalI, and then introduced into yeast cells.

Plasmids for expression of GFP-Pep12p were constructed as follows. The *PEP12* ORF was amplified by PCR using the primer combination 5′-AAGGATCCATGTCGGAAGACGAATTTTTTGG-3′ and 5′-AAAAGCTTTTACAATTTCATAATGAG-3′. As indicated by the underlines, The BamHI or the HindIII site was introduced adjacent to the start or stop codon, respectively. The BamHI-HindIII fragment of *PEP12* was subcloned into pSKY5, at the junction between *EGFP* and the *CMK1* terminator, to form pKU84. The plasmid pSKY5 is based on pRS316 (Sikorski and Hieter, 1989), and is designed for expression of EGFP fusion proteins under the control of the *TDH3* promoter. As another plasmid marker (*TRP1*), the 3.3-kb BamHI-SalI fragment of P_{TDH3}-*GFP-PEP12*-T_{CMK1} was subcloned into pRS314 to form pKU144.

Disruption of *ERG13* was performed as follows: the upstream region of the *ERG13* ORF was amplified by PCR using the primer combination 5′-AAGAATTCTCGTGTCGAAAATGAACCGGGC-3′ and 5′-AAGGATCCACAAAAGACATGTTG-GTTTGGC-3′, and then cut with EcoRI and BamHI (each restriction site is underlined). The downstream region was amplified using 5′-AAGGATCCCTGTTTATGCCGCCTTTGCATC-3′ and 5′-AAGCATGCGCATTTATGAAGGGGGTTCAGC-3′, and then cut with BamHI and SphI (each restriction site is underlined). Ligation was performed among these two fragments and pUC18 to generate a 0.8-kb EcoRI-SphI insert in pUC18. This plasmid was cut at the BamHI site, the junction between the two fragments, and the 1.8-kb BamHI-BamHI fragment of *HIS3* was inserted. Plasmid pKU131 (*erg13Δ*::*HIS3*) was cut with EcoRI and SphI, and then introduced into yeast cells.

The anti-HA (16B12) and the anti-myc (9E10) mAbs were purchased from BAbCO. The rabbit affinity-purified pAb against the HA epitope (Y-11) was purchased from Santa Cruz Biotechnology, Inc. To eliminate nonspecific staining in immunofluorescence microscopy, these antibodies were adsorbed with fixed and Triton X-100–permeabilized yeast cells not expressing the HA epitope (Roberts et al., 1991). Anti-Pep12p mAb (2C3-G4), anti-vacuolar alkaline phosphatase mAb (1D3-A10), goat anti–mouse Alexa Fluor® 488, and goat anti–rabbit Alexa Fluor® 568 were purchased from Molecular Probes, Inc. Anti-Gas1p antibodies were provided by R. Hirata (RIKEN, Saitama, Japan). In immunofluorescence microscopy, the preadsorbed anti-HA mAb was used at 1:300, and anti–mouse Alexa® 488 at 3 μg/ml. For double staining, the anti-Pep12p mAb and the preadsorbed anti-HA pAb were used at 10 μg/ml and 1:500, respectively.

To prepare antibodies specific for Tat2p, a synthetic peptide that contains the 14 amino acids spanning from 248 to 261 residues (PDHEFIGAKYWHDP) of Tat2p was used as the antigen. Glycine and cysteine were added to the COOH terminus of this peptide. The peptide was coupled to KLH using the cross-linker *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, and linked conjugates were dialyzed against PBS. The peptide–KLH conjugate (0.2 mg) was fixed with Freund's complete adjuvant and injected into rabbits, followed by biweekly boosts with 0.2 mg of conjugate in Freund's incomplete adjuvant. The antiserum against Tat2p was affinity purified, adsorbed to Δ*tat2* cells, and used for immunofluorescence microscopy at 1:2.5 dilution.

Immunoblotting

Cells were treated with NaN_3 and KF at the final concentration of 20 mM each. Preparation of protein extracts was performed by agitation with glass beads in 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 1 mM EDTA, and 1 mM PMSF. Before SDS-PAGE, β-mercaptoethanol was added at the final concentration of 5%, and the samples were heated at 37°C for 10 min. The anti-HA mAb was used at 1:1,000.

References

Alani, E., L. Cao, and N. Kleckner. 1987. A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. *Genetics.* 116:541–545.

Ellison, M.J., and M. Hochstrasser. 1991. Epitope-tagged ubiquitin. *J. Biol. Chem.* 266:21150–21157.

Gietz, R.D., and A. Sugino. 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene.* 74:527–534.

Keszenman-Pereyra, D., and K. Hieda. 1988. A colony procedure for transformation of *Saccharomyces cerevisiae. Curr. Genet.* 13:21–23.

Nakamura, H., K. Miura, Y. Fukuda, I. Shibuya, A. Ohta, and M. Takagi. 2000. Phosphatidylserine synthesis required for the maximal tryptophan transport activity in *Saccharomyces cerevisiae. Biosci. Biotechnol. Biochem.* 64:167–172.

Roberts, C.J., C.K. Raymond, C.T. Yamashiro, and T.H. Stevens. 1991. Methods for studying the yeast vacuole. *Methods Enzymol.* 194:644–661.

Schmidt, A., M.N. Hall, and A. Koller. 1994. Two FK506 resistance-conferring genes in *Saccharomyces cerevisiae*, *TAT1* and *TAT2*, encode amino acid permeases mediating tyrosine and tryptophan uptake. *Mol. Cell. Biol.* 14:6597–6606.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae. Genetics.* 122:19–27.

Stotz, A., and P. Linder. 1990. The *ADE2* gene from *Saccharomyces cerevisiae*: sequence and new vectors. *Gene.* 95:91–98.

Takita, Y., Y. Ohya, and Y. Anraku. 1995. The *CLS2* gene encodes a protein with multiple membrane-spanning domains that is important Ca^{2+} tolerance in yeast. *Mol. Gen. Genet.* 246:269–281.

Yashiroda, H., T. Oguchi, Y. Yasuda, A. Toh-e, and Y. Kikuchi. 1996. Bul1, a new protein that binds to the Rsp5 ubiquitin ligase in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 16:3255–3263.