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The *TSR1* Gene of *Yarrowia lipolytica* Is Involved in the Signal Recognition Particle-dependent Translocation Pathway of Secretory Proteins*

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We have isolated suppressors (*tsr1* to *tsr5*) of the thermosensitive growth of the *scr2.II-13* mutation, which affects the stability of the signal recognition particle. The growth of these mutants is largely affected in the *SCR2* context at 34 °C. We have studied the synthesis and secretion of an alkaline extracellular protease (AEP) in both wild-type and *tsr1-1(SCR2$^+$)* thermosensitive mutant strains. Pulse-chase labeling and immunoprecipitation of this protein showed that the level of AEP precursors in the *tsr1-1(SCR2$^+$)* strain is 70% less than in the wild-type strain under conditions where the global protein synthesis is practically unaffected. This defect was observed as early as 10 min after the shift to nonpermissive temperature. In neither strain was there any effect on the kinetics of secretion, and no cytoplasmic accumulation was detected. We have cloned the *TSR1* gene by complementing the thermosensitive phenotype of a *tsr1-1(SCR2$^+$)* mutant. Analysis of the *TSR1* DNA sequence revealed an open reading frame of 1383 base pairs, encoding a serine-rich protein of 461 amino acids with an amino-terminal signal peptide, and a membrane-spanning domain of 20 amino acids that could act as a stop transfer signal to ensure membrane localization of Tsr1p. Two homologues of the *TSR1* gene were identified in *Saccharomyces cerevisiae* (*YHC8*) and *Hansenula polymorpha* (*YLU2*). Disruption of the *TSR1* gene revealed that it is an essential single-copy gene. The *TSR1* gene encodes a single mRNA of 1.5 kilobase pairs. The study of the synthesis and secretion of AEP in the complemented *tsr1-1(SCR2$^+$,TSR1$^+$)* strain revealed that the *TSR1* gene ensures complete recovery of the synthesis defect and thus could encode an important component of the endoplasmic reticulum membrane involved in the early steps of the signal recognition particle-dependent translocation pathway.

The signal recognition particle (SRP)[1] is required for the targeting of growing secretory and membrane protein chains toward the endoplasmic reticulum membrane. Current models assume that the SRP binds to the signal sequence on a nascent polypeptide upon its emergence from the translation machinery (Walter and Lingappa, 1986). This interaction causes a tight binding of the SRP to the ribosome and simultaneously an elongation delay in the translation of the nascent polypeptide (Siegel and Walter, 1985; Wolin and Walter, 1989), which appears to be necessary in order to maintain the protein in a translocation-competent state (Rapoport, 1992). The SRP then interacts with a docking protein (or SRP receptor) anchored in the membrane of the endoplasmic reticulum (ER). This triggers the release of translation arrest and subsequently ensures translocation across the ER membrane (Gilmore *et al.*, 1982; Meyer *et al.*, 1982; Walter and Lingappa, 1986).

The SRP, in higher eukaryotic cells, is a small ribonucleoprotein complex composed of one molecule of the 300-nucleotide 7 SL RNA and six polypeptides that are bound to the SRP RNA: Srp19p, Srp54p, Srp9p, Srp14p, Srp68p, and Srp72p (Walter and Blobel, 1982; Walter and Lingappa, 1986). Recently, investigations have been carried out to isolate and characterize components of the SRP from a wide variety of organisms, including mammals, yeasts, plants, bacteria, and archaebacteria (Ribes *et al.*, 1988, 1990; Poritz *et al.*, 1988a, 1988b, 1990). The isolation of mutations affecting components of the SRP have made the identification of its *in vivo* function possible (Hann and Walter, 1991; Hann *et al.*, 1992; He *et al.*, 1992; Yaver *et al.*, 1992).

In *Yarrowia lipolytica*, a dimorphic heterothallic yeast, two functional genes (*SCR1* and *SCR2*) encode the SRP RNA. Disruption of just one gene has no obvious effect on growth or secretion, but disruption of both genes is lethal (He *et al.*, 1988, 1990).

A thermosensitive mutation (*scr2.II-13*) and other lethal and viable mutations have been induced in the *SCR2* gene by site-directed mutagenesis. All mutations are substitutions at Gly-128 and Ala-130 of stem-loop 1 of 7 SL RNA, which forms the presumed binding site of Srp19p (He *et al.*, 1992). Defects include a decrease in the synthesis of secretory proteins, preferential cleavage of the 7 SL RNA, and a gene dosage-dependent lethality. The *scr2.II-13* mutation is lethal as a monocopy, but results in a thermosensitive phenotype when present on a replicative plasmid.

To gain a better understanding of the role of the different components of SRP and to identify additional partners that may interact with it, we have initiated a selection and genetic characterization of extragenic suppressors of the thermosensitive mutation *scr2.II-13*. In this paper, we describe a new component of the translocation pathway, the *TSR1* gene, that codes for a membrane-spanning, serine-rich protein located in the ER membrane.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Z69781.

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[1] The abbreviations used are: SRP, signal recognition particle; ER, endoplasmic reticulum; AEP, alkaline extracellular protease; kb, kilobase pairs(s).

TABLE I
Strains and plasmids used in this study

Strains/plasmids	Description	Source of Ref.
E. coli		
TG1	K12, Δ(lac-pro), supE, thi, hsdD5, F′ traD36, proA$^+$B$^+$, lacIq, lacZΔM15	Gibson (1984)
DH5α	supE44, ΔlacU169(ϕ80lacZDM15), hsdR17, recA1 endA1, gyrA96, thi-1, relA1	Hanahan (1983)
Y. lipolytica		
Wild-type	MatB, *his1-1, leu2-35, ura 3-303, SCR1*::*ADE1, ΔSCR2 (LEU2,SCR2*$^+$*)*	He *et al.* (1992)
E150	MatB, *his1-1, leu2-270, ura3-303*	Nicaud *et al.* (1989)
E150CClaI	MatB, *his1-1, leu2-270, ura3-303 (LEU2,pCClaI)*	This work
INAG 36461	MatB, *his1-1, leu2-35, ura3-303, SCR1*::*ADE1, ΔSCR2 (LEU2,scr2.11-13)*	He *et al.* (1992)
Plasmids		
pINA237	pBR322 carrying *Y. lipolytica ARS18* and *LEU2* genes	This laboratory
pINA443	pBR322 carrying *Y. lipolytica ARS68* and *URA3* genes	This laboratory
pINA956	Derivative of plasmid pINA443 carrying *scr2.II-13* ts[a] allele of *SCR2* gene	This laboratory
pINA829	Derivative of plasmid pINA237 carrying *scr2.II-13* ts allele of *SCR2* gene	This laboratory
pINA441	Derivative of plasmid pINA237 carrying *SCR2* gene	This laboratory
pINA943	Derivative of plasmid pINA443 carrying *SCR2* gene	This laboratory
pINA214	pBR322 carrying *Y. lipolytica LEU2* gene	This laboratory
pC10	Derivative of pINA240 carrying 5.9-kb fragment including *TSR1* gene	This work
pCClaI	Derivative of pC10 after deletion of noncomplementing *Cla*1 fragment	This work
pHSa	pCClaI derivative carrying *tsr1*::*URA3*	This work

[a] ts, thermosensitive.

EXPERIMENTAL PROCEDURES

Strains and Media—The strains and plasmids used are listed in Table I. YEA medium contained 0.5% yeast extract, 1% glucose, and 2% agar. YPD medium contained 1% yeast extract, 1% Bacto-peptone, and 1% glucose. Selective minimal medium contained 1% glucose, 0.17% yeast nitrogen base without ammonium sulfate (Difco), and 0.1% proline as nitrogen source and was supplemented with appropriate nutrients. Minimal 5-fluoroorotic acid medium is the same as the selective minimal medium, but contained 10 mg/liter uracil and 1.25 g/liter 5-fluoroorotic acid. For pulse-chase labeling, GYX medium contained 0.5% yeast extract and 2% glycerol. GG medium contained 2% glycerol, 0.5% yeast nitrogen base (Difco), 0.02% polyethylene glycol, 0.2% proteose peptone, and appropriate nutrients in 50 mM phosphate buffer, pH 6.8. GC medium was the same as GG medium except that 0.2% casein replaced the proteose peptone.

Transformation of Y. lipolytica and DNA Techniques—The protocol of Davidow *et al.* (1985) as modified by Xuan *et al.* (1988) was used for the transformation of *Y. lipolytica*. Plasmid DNA was linearized before transformation to target integration at a chosen genomic locus. Faithful integration of a single copy at the expected locus was routinely checked by Southern hybridization on chromosomal DNA, digested by the appropriate enzyme, resolved by gel electrophoresis, transferred to Hybond-N$^+$ nylon (Amersham Corp.), and hybridized to the appropriate probe. All enzyme reactions and DNA preparations were performed as described by Maniatis *et al.* (1982).

Pulse-Chase Labeling and Immunoprecipitation of AEP—The synthesis and secretion of AEP were monitored by pulse-chase and immunoprecipitation experiments as described by He *et al.* (1992) with the following modifications. Immunoprecipitates were separated on 10% SDS-polyacrylamide gel at a low voltage. The gel was fixed with a mixture of methanol/acetic acid/water (25:65:10) and treated with Intensify (DuPont NEN) according to the manufacturer's directions. The gels were dried overnight. AEP precursors and secreted mature AEP were detected by autoradiography.

RESULTS

Revertant Isolation—To isolate *scr2.II-13* suppressors, we used INAG 36461 as a parent strain, which is disrupted for the two genes *SCR1* and *SCR2* and carries the thermosensitive mutation *scr2.II-13* associated with the *LEU2* marker (pINA829) on an autonomously replicating plasmid. Cells were mutagenized for 20 s by UV irradiation (10% viability). Five-hundred thermoresistant colonies were isolated by direct plating at 33 °C. To eliminate plasmid-linked suppressor mutations, the revertants were transformed with a *URA3* plasmid carrying the *scr2.II-13* allele (pINA956) and cured of the *LEU2* plasmid, and Leu$^-$Ura$^+$ transformants were then tested for stable growth at 33 °C. This permitted the selection of 80 chromosomal revertants. Forty of them were then tested for bypass of the SRP pathway and for the presence of an associated phenotype (Fig. 1).

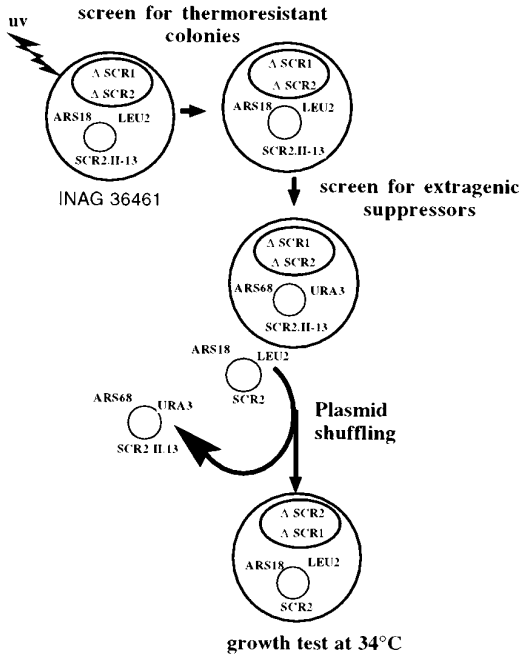
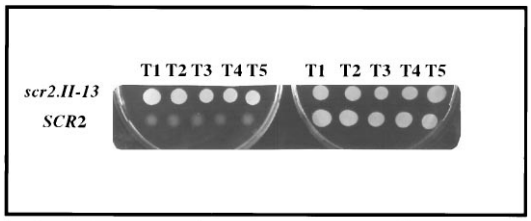


FIG. 1. **Schematic representation of the genetic procedure used to select extragenic suppressors of the *scr2.II-13* mutation.**

The revertant strains carrying pINA956 were tested for growth on 5-fluoroorotic acid medium to promote plasmid curing. No revertant was able to grow, suggesting that the SRP-dependent pathway was still essential in these strains. To determine whether the revertant strains retained a distinct phenotype in the presence of a wild-type 7 S RNA, we replaced pINA956 with a *LEU2,SCR2*$^+$ plasmid (pINA441). Five mutants (*tsr1* to *tsr5*) were then unable to grow at 34 °C (Fig. 2). The doubling time of the *tsr1-1(SCR2*$^+$*)* strain, which was further analyzed, increased from ≅180 to ≅720 min when the temperature was raised from 23 to 34 °C, whereas the wild-type strain had a doubling time of ≅200 min at 34 °C. At 23 °C, the doubling time of the thermosensitive *tsr1-1(SCR2*$^+$*)* strain was indistinguishable from that of the wild-type strain (data not shown).

Alkaline Extracellular Protease Synthesis Is Affected in the tsr1 Strain, whereas Secretion Is Unaffected—To assess the effect of the *tsr1* mutation on the translocation efficiency of



	25°C	32°C	34°C
TSR1$^+$(*SCR2*$^+$)	+	+	+
TSR1$^+$(*scr2.II-13*)	+	+/-	-
tsr1-1(*scr2.II-13*)	+	+	+
tsr1-1(*SCR2*$^+$)	+	+/-	-

FIG. 2. **Temperature conditional growth defects.** The *tsr1* to *tsr5* strains (denoted *T1* to *T5* in the *scr2.II-13* and *SCR2* genetic backgrounds) were patched on YPD medium and incubated for 3 days at the indicated temperatures. The *upper panel* displays growth on YPD plates at 34 and 25 °C. Strains carrying the *scr2.II-13* allele on the replicative plasmid pINA829 were grown on the *upper row*. Strains carrying the wild-type *SCR2* allele on the replicative plasmid pINA943 were grown on the *lower row*. The *lower panel* displays the growth pattern of the strains used to define the *tsr* phenotype.

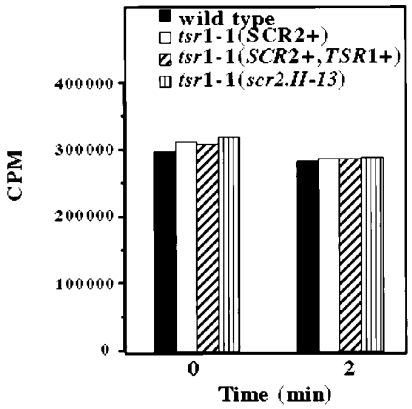


FIG. 3. **Total incorporation of the protein labels.** Shown are the total counts incorporated in the cells grown at 23 °C, transferred to 33 °C for 10 min, and labeled for 45 s with [^{3}H]leucine. 100-μl samples of labeled cell suspension were precipitated with 5 ml of 5% trichloroacetic acid, boiled for 30 min, then passed through filters washed twice in 5% trichloroacetic acid and once in 95% ethanol. Total incorporation of the protein labels was measured 0 and 2 min after the chase by scintillation counting.

secretory proteins, we used AEP as a reporter molecule in pulse-chase and immunoprecipitation experiments. AEP is the major protein secreted under induction conditions. A 55-kDa precursor is the earliest and largest form detected in wild-type cells; it lacks the signal sequence and is cotranslationally translocated and glycosylated (Matoba *et al.*, 1988). We compared the wild-type strain and *tsr1* carrying either *SCR2* (thermosensitive strain) or *scr2.II-13* (thermoresistant strain) on an autonomously replicating plasmid. AEP synthesis and secretion were monitored in cultures either at the permissive temperature of 23 °C or after a shift to the nonpermissive temperature for the *tsr1-1(SCR2$^+$)* mutant (33 °C).

Under permissive conditions, no difference was observed in AEP maturation and secretion when the wild-type strain and the two *tsr1* strains were compared (data not shown). Under nonpermissive conditions, samples were analyzed 10 or 60 min after the shift. A clear secretory defect was observed in *tsr1-1(SCR2$^+$)* both 10 and 60 min after the shift; the results obtained at 10 min are presented below. No significant effect was detected at 33 °C on global protein synthesis (Fig. 3). In the *tsr1-1(SCR2$^+$)* context, the amount of labeled 55-kDa precursor and mature AEP secreted at all time points was reduced relative to the levels detected in wild-type and *tsr1-1(scr2.II-13)* cells, without the kinetics of AEP secretion and maturation being detectably modified (Fig. 4). Identical results were obtained in three independent experiments, which confirmed that *tsr1-1(SCR2$^+$)* affected the synthesis efficiency of the AEP precursor. The 55-kDa precursors detected in *tsr1-1(SCR2$^+$)* cells at chase times of 0, 1, and 3 min were treated with endoglycosidase H (data not shown), and no non-glycosylated AEP precursors were observed, indicating that in the *tsr1-1(SCR2$^+$)* strain, the 55-kDa precursors were translocated into the ER lumen.

Cloning of the TSR1 Gene—Suppressors of the temperature-sensitive phenotype of *tsr1-1(SCR2$^+$)* were cloned from the replicative *LEU2*-based *Y. lipolytica* genomic library described by Barth and Gaillardin (1996). Forty-thousand Leu$^+$ transformants were selected at 25 °C, and the resulting transformants were tested for growth at 34 °C by replica plating. Three colonies grew at 34 °C, and plasmids (pC10, pC12, and pC13) isolated from all three colonies restored temperature resistance when introduced into *tsr1-1(SCR2$^+$)* cells. Restriction enzyme mapping indicated that the three inserts were different (data not shown).

To test whether a single copy of the inserts could suppress the *tsr1-1(SCR2$^+$)* defect, we deleted the *ARS* sequence from the plasmids and linearized them to target integration into *LEU2* or to their own genomic locus. Only the pC10 derivative was able to restore temperature resistance when present in a single ectopic copy (data not shown).

Several overlapping fragments of the 5.9-kb genomic insert of pC10 were cloned into the pINA240 replicative vector, and the suppression ability of the subclones was analyzed (Fig. 5*B*). A 2.4-kb *Pst*I-*Nhe*I fragment, containing an open reading frame (hereafter referred to as *TSR1*), was sufficient to confer temperature resistance on the *tsr1-1(SCR2$^+$)* strain.

The TSR1 Gene Restores Synthesis of AEP—To determine whether the *TSR1* gene could complement the defect in AEP synthesis in the *tsr1-1(SCR2$^+$)* mutant, pulse-chase and immunoprecipitation experiments were conducted in a *tsr1-1* strain carrying both *SCR2* and the *TSR1* gene on separate replicative plasmids under nonpermissive conditions. The results given in Fig. 4 show that no difference in the level of AEP precursor or secreted mature AEP was observed between this and the wild-type strain. This experiment revealed that complementation of the *tsr1-1(SCR2$^+$)* thermosensitive growth defect paralleled restoration of AEP synthesis.

Sequence of the TSR1 Gene and of Its Putative Product—The nucleotide sequence of the *TSR1* gene is given in Fig. 5*A*. DNA sequence analysis revealed an open reading frame of 1386 base pairs. This was confirmed by a Northern blot experiment that revealed a single transcript of ~1.5 kb (data not shown). The ATG codon has a standard nucleotide environment for translation initiation. A typical TATA consensus sequence was present around position −315 relative to the ATG start codon. The *TSR1* gene is predicted to encode a protein of 461 amino acids residues with a molecular mass of ~50 kDa (Fig. 5*A*). This protein shows an N-terminal signal peptide and a predicted signal cleavage site between amino acids 19 and 20. Computer analysis predicts the presence of a transmembrane domain of 20 amino acids. This could act as a stop transfer signal dividing the polypeptide in a C-terminal cytoplasmic and an N-terminal

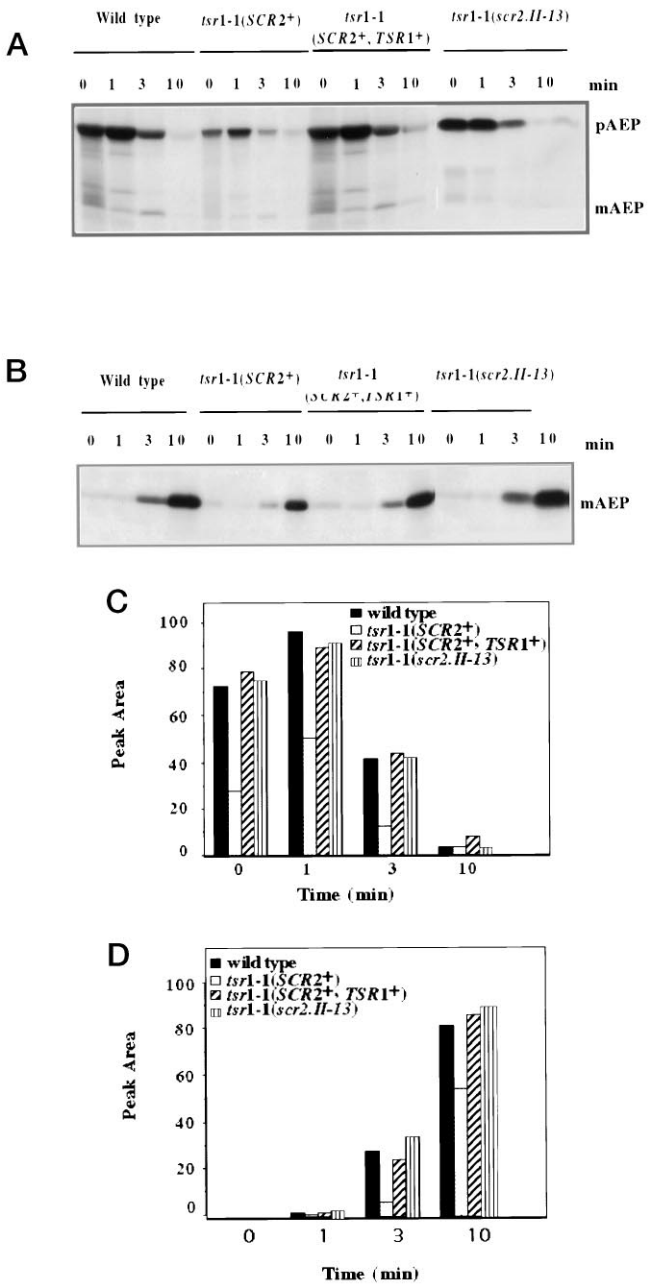


FIG. 4. **Pulse-chase labeling and immunoprecipitation of AEP from *tsr1-1(SCR2$^+$,TSR1$^+$)*, *tsr1-1(SCR2$^+$)*, *tsr1-1(scr2.II-13)*, and wild-type strains.** Cells were shifted from 23 to 33 °C. 10 min after this, the cells were labeled for 45 s with [^{3}H]leucine and chased with a large excess of unlabeled leucine for 0, 1, 3, and 10 min as indicated. Total proteins from extracellular samples precipitated with trichloroacetic acid (*A*) and intracellular AEP immunoprecipitated with polyclonal anti-AEP antiserum and protein A-Sepharose (*B*) were analyzed by 10% SDS-polyacrylamide gel electrophoresis and fluorography. The positions of the 55-kDa precursor (*pAEP*) and mature AEP (*mAEP*) are indicated. A densitometer was used to scan the gels. The relative amounts of AEP precursors (*C*) and secreted mature AEP (*D*) were compared.

luminal portion containing cysteine- and serine/threonine-rich domains.

Homology searches conducted for the nucleotide sequence and the deduced protein sequence revealed a significant similarity to two yeast proteins: Yhc8p encoded by *YHL028w* from chromosome VIII of *Saccharomyces cerevisiae*, which was identified by systematic sequencing, and Ylu2p from *Hansenula polymorpha*, which was partially sequenced as a neighbor of the *LEU2* gene (Agaphonov *et al.*, 1994) (Fig. 6*B*). These sequences share 43 and 33% identity, respectively, with Tsr1p according to BESTFIT from the Genetics Computer Group package using the default settings. As with Tsr1p, the N-terminal region of Yhc8p and Ylu2p displays a signal peptide with a predicted cleavage site at positions 26 and 14, respectively. From this sequence homology, five different domains could be defined as shown in Fig. 6.

A cysteine-rich conserved domain was located just after the signal peptide (Fig. 6, *A* and *B*). The length of this domain is relatively well conserved among the three homologues (88 amino acids for Yhc8p, 90 for Tsr1p, and 93 for Ylu2p). The homology to Tsr1p was relatively high (55 and 32% identity, respectively, to Yhc8p and Ylu2p using BESTFIT). This domain appeared to be related to a family of scorpion neurotoxins (Fig. 7) (Fontecilla-Camps *et al.*, 1981). The homology was mainly due to conservation of the cysteines and, to a lesser extent, to their immediate environment. This family of neurotoxins is composed of a large range of polypeptides having rather divergent sequences but sharing a common core of internal disulfide bonds. As a consequence, according to the three-dimensional structures (Bontems *et al.*, 1991), cysteines 43 and 61 and cysteines 46 and 63 should be linked by a disulfide bridge (Fig. 7). It can be hypothesized that the N-terminal cysteine 21 is bonded to one of the C-terminal cysteines (position 76, 80, or 88) connecting the two extremities of this domain, as is the case with variant-3 of *Centruroides sculturatus* (Almassy *et al.*, 1983). This domain may define a compact module of the protein that could interact with membrane channels as do scorpion neurotoxins (Bontems *et al.*, 1991).

The following domains are serine/threonine-rich (Fig. 6*A*): 92% of 100 amino acids in Tsr1p, 75% of 194 amino acids in Yhc8p, and 75% of 104 amino acids in Ylu2p. The domain of the *S. cerevisiae* homologue is twice as large as the others. Many proteins display such serine/threonine-rich domains, but these three homologues present this domain at the same position between the transmembrane segment and the cysteine-rich domain. Before the transmembrane segment, an intermediary domain with a lower proportion of serine/threonine residues is not significantly conserved among the three proteins. Here also, the corresponding *S. cerevisiae* region appears twice as larger as the others. A transmembrane domain of 20 amino acids was predicted in the three proteins by different programs (Kyte and Doolittle (1982), TMAP on the EMBL server, and HCA). This domain suggests a putative membrane localization.

The last putatively cytoplasmic region is the same length (~170 amino acids) in *Y. lipolytica* and *S. cerevisiae* (the *YLU2* sequence is not complete) (Fig. 6*A*). The last 30 amino acids of this domain are highly conserved in Tsr1p and Yhc8p (Fig. 6*C*). This motif may be accessible for interaction with the SRP and/or ribosomes. The upstream region of the three genes displayed no significant homology.

The TSR1 Gene Is an Essential Single-copy Gene—Southern hybridization at low stringency failed to detect any additional DNA fragments, suggesting the absence of a second *TSR1*-related sequence in the *Y. lipolytica* genome. Southern hybridization on separated *Y. lipolytica* chromosomes showed that the *TSR1* gene was located on chromosome 5 (data not shown).

A null *tsr1::URA3* allele was constructed by deleting the 1-kb *Hin*dIII-*Sal*I fragment from pCClaI (Fig. 5*B*) and replacing it with the prototrophic marker *URA3* (1.6 kb). The resulting pHSa plasmid (Fig. 5*C*) was digested with *Apa*I, and a haploid strain (E150CClaI) containing an extrachromosomal copy of the *TSR1* gene on a *LEU2*-based replicative plasmid (pCClaI) was transformed with the 2.2-kb gel-purified fragment.

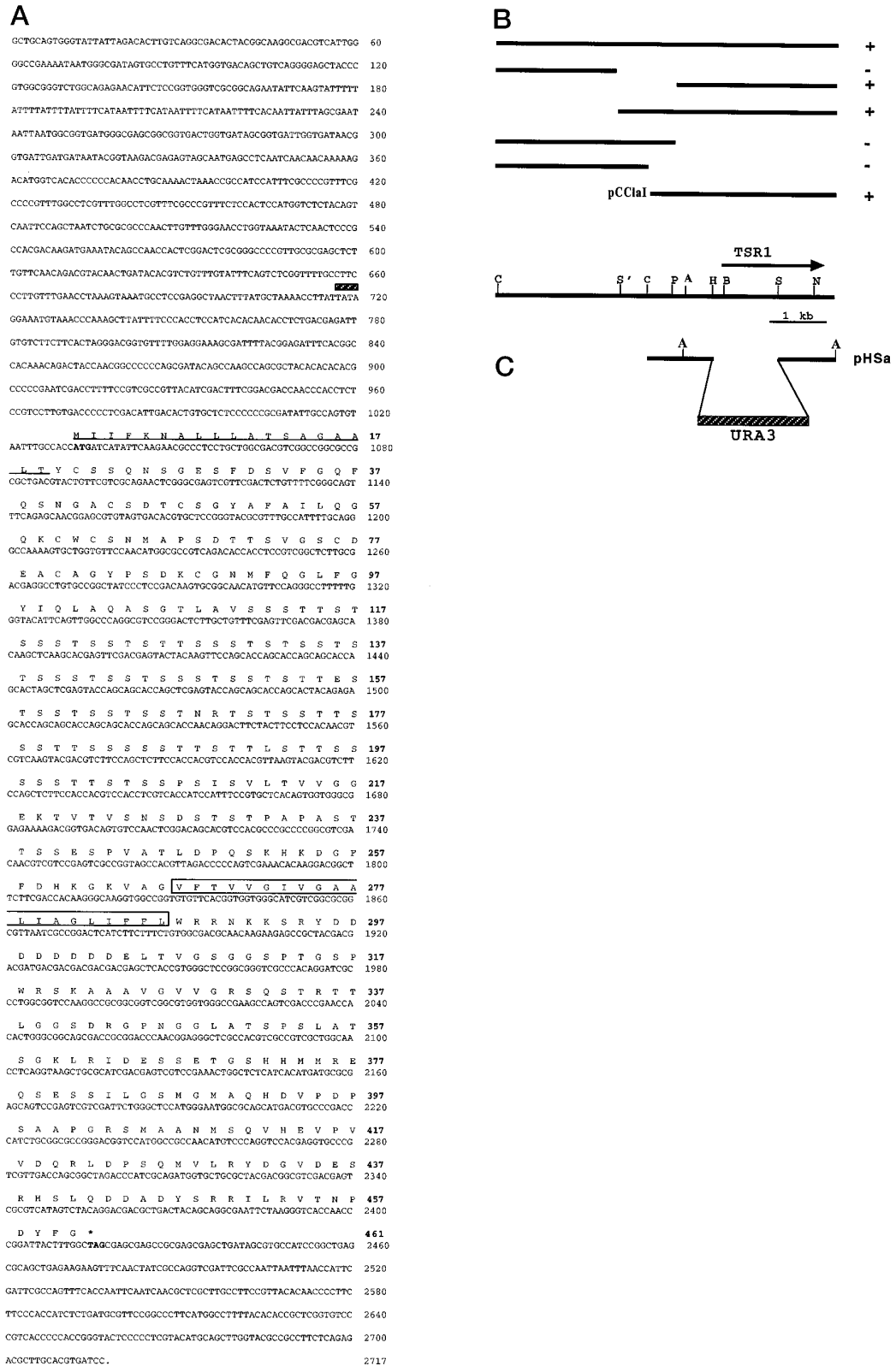


FIG. 5. **Cloning and analysis of the *TSR1* gene.** *A*, nucleotide sequence of the *TSR1* gene. The gene is shown with its upstream and downstream flanking sequences. The deduced amino acid sequence of the *TSR1* ORF is indicated in single-letter code above the nucleotide sequence and is numbered on the right in *boldface*. The potential TATA element is *highlighted* by a *striped bar* above the sequence. The putative N-terminal signal sequence is *underlined*. The membrane-spanning segment is *boxed*. *B*, complementing activity of inserts containing the *TSR1* gene. The *longest line* indicates the 5.9-kb complementing fragment from the original insert in pC10. The *arrow* shows the direction and position of the open reading frame of the *TSR1* gene. The *plus signs* denote the ability of an insert to confer 34 °C temperature resistance on the thermosensitive *tsr1* mutant. The *minus signs* denote the inability of an insert to confer 34 °C temperature resistance on the thermosensitive *tsr1* mutant. Restriction site abbreviations are as follows: *A*, *Apa*I; *B*, *Bcl*I; *C*, *Cla*I; *N*, *Nhe*I; *P*, *Pst*I; *S*, *Sal*I; and *S′*, *Sac*II. *C*, construction of chromosomal deletion at the *TSR1* locus. The pHSa plasmid used for chromosomal deletion was constructed by replacement of the 1-kb *Hin*dIII-*Sal*I fragment containing the *TSR1* open reading frame from pCClaI with a 1.6-kb *URA3*-containing fragment. A 2.2-kb fragment obtained by *Apa*I digestion was purified on a low melting agarose gel and integrated by transformation and homologous recombination at the *TSR1* chromosomal site of a haploid E150CClaI strain.

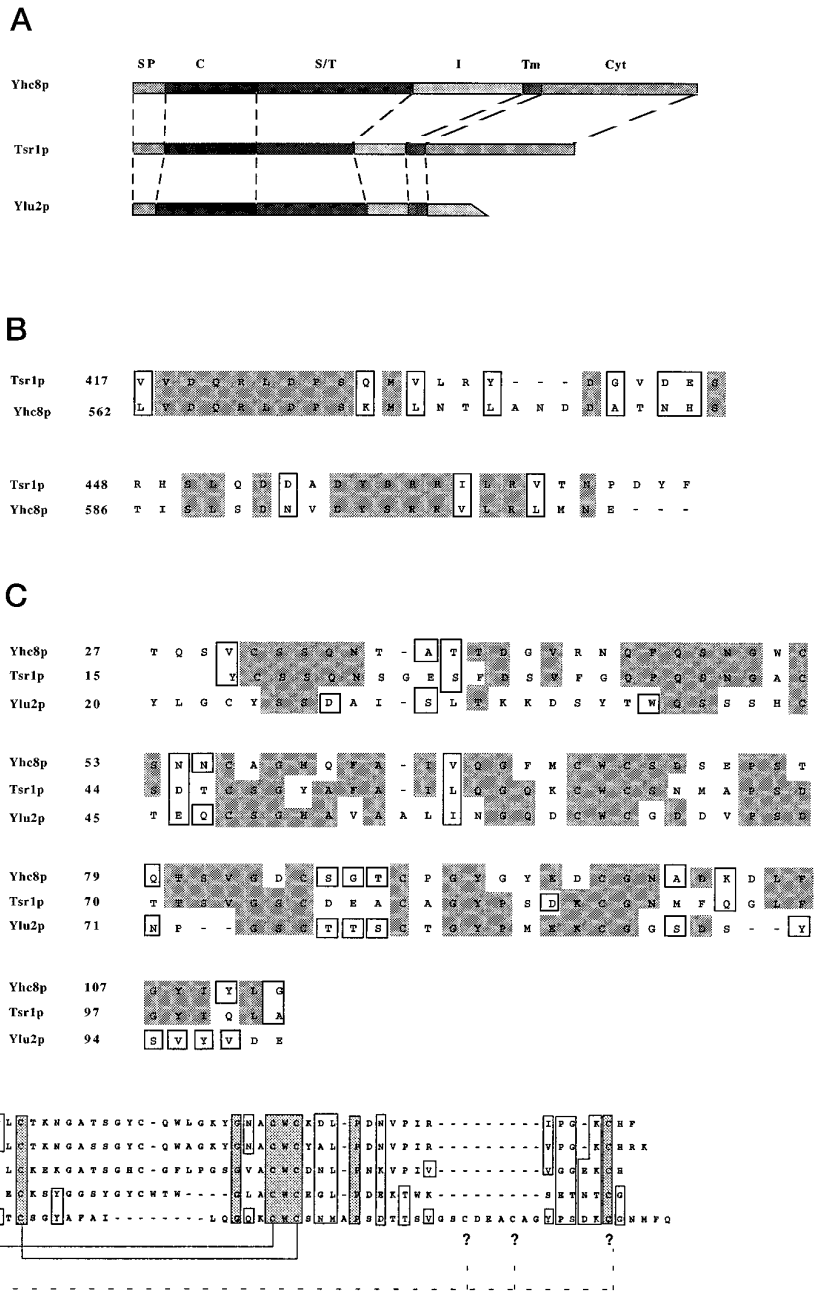


FIG. 6. **Alignment of the amino acid sequences deduced from the *TSR1*, *YHC8*, and *YLU2* genes.** *A*, diagrammatic representation of the six domains of the three proteins Tsr1p, Yhc8p, and Ylu2p. *SP*, signal peptide; *C*, cysteine-rich domain; *S/T*, serine/threonine-rich domain; *I*, intermediary domain; *Tm*, transmembrane domain; *Cyt*, C-terminal cytoplasmic domain. *B*, sequence alignment of the most conserved box of the C-terminal cytoplasmic domain between Tsr1p and Yhc8p using the BESTFIT program from the Genetics Computer Group software package with the scoring matrix of Risler *et al.* (1988). Identities are *shaded*, and similarities are *boxed*. *C*, sequence alignment of the cysteine-rich domains of the three homologous proteins using the PILEUP program from the Genetics Computer Group software package with the scoring matrix of Risler *et al.* (1988).

FIG. 7. **Alignment of the C-terminal domain of Trs1p with scorpion neurotoxin motifs using the PILEUP program from the Genetics Computer Group software package with the scoring matrix of Risler *et al.* (1988).** Identical amino acids are *shaded*, and similar amino acids are *boxed*. *scx1*, neurotoxin I of *Buthus occitanus* (SwissProt accession number PO1488); *scxa*, α-neurotoxin of *Leirus quinquestriatus* (SwissProt accession number P17728); *scx3*, neurotoxin II of *B. occitanus* (SwissProt accession number P13488); *scx2*, neurotoxin II of *L. quinquestriatus* (SwissProt accession number P19855).

Leu$^+$Ura$^+$ transformants were selected and tested for stability of the Leu$^+$ marker; if *TSR1* controlled an essential function, pCClaI and the Leu$^+$ phenotype should be stabilized after genomic copy disruption. Among 24 transformants tested for both Ura$^+$ and Leu$^+$ phenotypes, 3 were stable and 21 simultaneously segregated both Leu$^+$ and Ura$^+$ markers (indicating integration of the disrupting fragment into the replicative plasmid). The structure of the three stable Leu$^+$Ura$^+$ clones was established by Southern blotting of total DNA digested by *Spe*I or *Pvu*II; all appeared to carry the expected disruption of the genomic *TSR1* allele and a free pCClaI plasmid. No loss of the replicative pCClaI plasmid was observed in any of these disruptants, among >2000 clones tested after 20 generations on nonselective medium, thus showing that the *TSR1* gene is essential.

DISCUSSION

We isolated five *tsr* (thermosensitive rescued) mutants that restore normal growth to *scr2.II-13* mutants, but are thermosensitive at 34 °C in the wild-type *SCR2* context. Different hypotheses could account for the *tsr* phenotype. The suppressive effect may be due to a general slowdown in cell metabolism, alleviating the effects of the *scr2.II-13* mutation. In this case, the cell growth of the suppressed strains should be affected. That was not the case because the *tsr* mutants carrying the *scr2.II-13* mutation displayed a growth rate similar to that of the wild-type strains at both 23 and 34 °C. Another hypothesis could be that the suppressors could activate a "salvage pathway," bypassing the SRP requirement for secretory proteins. This was not the case since chasing the plasmid carrying

the *SCR2* allele was impossible in the *tsr* (Δ*SCR1,*Δ*SCR2*) background.

The synthesis and translocation of the AEP reporter molecule in the *tsr1* mutant, both in *scr2.II-13* and *SCR2* backgrounds, were studied and compared to the impairment in AEP secretion observed in the *scr2.II-13* strain. A complete recovery in the synthesis and secretion of AEP was observed in the suppressed *tsr1-1(scr2.II-13)* strain. On the contrary, the thermosensitive *tsr1-1(SCR2$^+$)* strain displayed an inhibition of the synthesis of AEP precursors under conditions where global protein synthesis was unaffected. Unlike *scr2.II-13*, where a defect in AEP synthesis was detectable only after 60 min of the shift from 23 to 33 °C, the synthesis defect in the *tsr1* mutant was seen as early as 10 min after the shift. Similar results were observed when acid extracellular protease was used as a second secretory reporter molecule (data not shown). The immediate alteration of the synthesis in the *tsr1* mutant indicates that the mutation does not primarily affect the SRP biogenesis, unlike *scr2.II-13*. It suggests a direct effect of the mutation on the stability of the component(s) of the translocation pathway.

No obvious effect on the kinetics of transit or maturation of AEP was observed in this mutant, as monitored from the appearance of mature AEP in the extracellular medium (see Fig. 4, *B* and *C*), indicating that post-ER events were not affected. There was no accumulation of cytoplasmic precursors in the *tsr1-1(SCR2$^+$)* mutant, indicating that synthesis of AEP was severely affected as in the parent *scr2.II-13* mutant. In the case of the *scr2.II-13* mutant, which is impaired in the 7 SL RNA component of the SRP, it was proposed that the SRP was competent in signal sequence recognition and translational arrest, but defective in ER targeting, thus trapping AEP-synthesizing ribosomes in translational arrest (He *et al.*, 1992; Yaver *et al.*, 1992). A similar mechanism could be envisioned for the *tsr1* mutant, which may impair targeting of wild-type SRP-ribosome complexes, but restore targeting of *scr2.II-13* mutant SRP-ribosome complexes. Since Tsr1p is evolutionarily conserved, our results suggest that Tsr1p identifies a new and critical component of the SRP cycle.

The Tsr1p amino acid sequence shows extensive homology to two putative proteins, Yhc8p from *S. cerevisiae* and Ylu2p from *H. polymorpha*, and suggests a functional conservation. A signal sequence is localized in the N-terminal region of the three proteins. Five domains could be defined by sequence homology in these proteins, and the presence of a membrane-spanning domain suggests that these three proteins are integral membrane proteins of the secretory pathway. No homology was observed to docking proteins. The C-terminal cytoplasmic domain of Tsr1p, highly conserved among the three species, may play an important role in the interaction with the SRP. Isolating suppressors of *scr2.II-13*, a mutation affecting a component of the SRP, appears to be an efficient method for the selection of mutations impaired in the early events of protein secretion and for genetic exploration of this pathway.

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