

Characterization of the *Yarrowia lipolytica* *YISRP72* gene, a component of the yeast signal recognition particle

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Summary. The *Yarrowia lipolytica* *SRP72* gene product (*YISRP72*), a homolog of the 72-kDa subunit of the mammalian SRP, encodes a putative protein of 602 amino acids. Northern blot analysis revealed a unique *YISRP72*-specific transcript of 1.8 kb. The deduced amino acid sequence showed higher identities with the Srp72 proteins of euascomycetes than with hemiascomycetes. Chromosomal hybridization experiments showed that the *YISRP72* gene is located in chromosome V of the standard E150 strain of *Y. lipolytica*. Fluorescent microscopy revealed that the *YISRP72-GFP* fusion protein was expressed in the cytoplasm and nucleus. The *YISRP72* gene was interrupted by the pop-out method; however, deletion of the gene proved to be lethal. This is in contrast to the results described for the *Saccharomyces cerevisiae* *SRP72* gene, which is not essential for cell growth, and supports our previous finding with another component of the yeast recognition particle, *YISEC65*. The present work suggests that SRP-dependent targeting is the main secretory pathway in *Y. lipolytica*, as has been described for higher eukaryotes. [Int Microbiol 2007; 10(4):283-289]

Key words: *Yarrowia lipolytica* · signal recognition particle · protein secretion · gene *SRP72*

Introduction

The signal recognition particle (SRP) is a stable cytosolic ribonucleoprotein particle present in organisms from all domains of life [22,23,38]. In eukaryotes, it is responsible for targeting proteins to the endoplasmic reticulum (ER). SRP binds to the ribosome and to a hydrophobic signal peptide at the N-terminus of a nascent polypeptide chain, thereby delaying or pausing translation (“elongation arrest”) [36]. The ribosome-nascent chain (RNC)-SRP complex is then directed to the membrane of the ER, in eukaryotes, and to the

plasma membrane, in prokaryotes, by the interaction of SRP with the SRP receptor (SR). Finally, the signal peptide is released from SRP, and the nascent chain is inserted into the translocation pore before SRP dissociates from SR. The nascent chain is transferred to the translocation site where crossing takes place simultaneous with translation [22,37]. Mammalian SRP consists of 7SL RNA and six protein subunits: Srp54p, Srp19p, Srp68p, Srp72p, Srp14p, and Srp9p. In the particle, the polypeptides are attached to 7SL RNA, either as monomers (Srp19p and Srp54p) or as heterodimers (Srp9/14p and Srp68/72p).

The SRP of *Saccharomyces cerevisiae* has been extensively studied. *S. cerevisiae* SRP (16S) is significantly larger than mammalian SRP; it contains a larger SRP RNA of 519 nucleotides [11,16], and an additional 21-kDa subunit (Srp21) for which there is no homolog in mammalian SRP [5]. Srp54p is involved in signal sequence recognition. Srp9/14p is required for elongation arrest and promotes translocation. Srp68/72p interacts with the SRP receptor; mutants in these proteins lose the capacity to promote translocation [5,32]. Surprisingly, all

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SRP components of *S. cerevisiae* are important but none are essential for growth [5,20,33].

The *Yarrowia lipolytica* and *Schizosaccharomyces pombe* SRP RNA genes were the first SRP components discovered in two genetically tractable yeasts [28]. *Y. lipolytica* SRP is similar in size (10S) to mammalian SRP and the SRP RNAs can potentially fold into similar structures. In *Y. lipolytica*, several genes involved in the SRP-dependent pathway of secretory proteins have been isolated: *SLS1* is involved in the pre-protein translocation process [6,15]. The protein product of *TSR1* functions in the early steps of the SRP-dependent translocation pathway and deletion of the gene is lethal [3]. *SCR1* and *SCR2* encode SRP RNA; the disruption of these genes is also lethal [17,18]. Deletion of *SRP54* is not lethal either in *S. cerevisiae* or in *Y. lipolytica*. [21]. We have observed that, in *Y. lipolytica*, the *YISEC65* gene homolog of *SRP19* is essential for cell viability [31]. Taking into account that *Y. lipolytica* is phylogenetically distant from *S. cerevisiae* and *S. pombe* as these two species are from each other [26], the study of *Y. lipolytica* should be valuable for understanding the role of SRP in protein translocation.

This work reports the cloning, sequencing, and disruption of the *SRP72* homolog from *Y. lipolytica*, as well as the localization of the *YISRP72* gene in the cytoplasm and nucleus of the cell.

Materials and methods

Strains and media. The *Y. lipolytica* strain E-150, 135658, was used as wild-type strain [2]. The LMX3 strain was selected after integration of plasmid pLMX3, which contains the *URA3* gene and a deletion of the *YISRP72* gene in the strain E-150. The plasmid was linearized by digestion with *ClaI*. Strain LRP1 was selected after transformation of strain E-150 with plasmid pLRP, which contains the *LEU2* and *YISRP72* genes from *Y. lipolytica*. Strain LPV was selected after transformation of strain E-150 with plasmid pLPV, containing a *SRP72-GFP* gene fusion and the *LEU2* gene. Yeast strains were grown in YEPD (1% yeast extract, 1% glucose, 1% bacto-peptone) and YNB (0.7% yeast nitrogen base without amino acids, 1% glucose, amino acids and uracil as required). Ura⁻ strains were selected by the addition of 1.25 mg 5-fluoro-orotic acid (5-FOA)/ml to solid media.

The *Escherichia coli* strain used for transformation and amplification of recombinant DNA was DH5 α [14]. Bacterial cultures were grown in LB or 2 \times YT medium [29]; when required, the media were supplemented with ampicillin (50 mg/ml).

Yeast transformation. *Y. lipolytica* was transformed using the lithium acetate protocol developed by Xuan et al. [39]. In some cases, a faster low-efficiency protocol was employed.

DNA and RNA manipulations. Total DNA from *Y. lipolytica* was prepared as described for *S. cerevisiae* [19]. Restriction enzyme digestions and DNA ligations were done according to the recommendations of the manufacturers. Transformation of *E. coli*, DNA ligations, Southern blotting and other DNA manipulations were accomplished using standard techniques [29]. The LMX3 strain was transformed with plasmid pLRP1. Plasmid incorporation was verified by Southern blot in the three strains: wild-type

(E-150), LMX3, and LRP1. DNA was digested with *NcoI* and *PvuII* and hybridized with probe. The corresponding fragments were consistent with the theoretically calculated values. DNA fragments used as probes were labeled by random priming with digoxigenin-dUTP (Boehringer, Mannheim, Germany) and employed according to the instructions of the manufacturer. Total RNA was prepared with the RNeasy Mini Kit (QIAGEN). Prehybridization and hybridization were carried out according to standard procedures [29].

Amplification by PCR. All fragments were amplified by PCR (Thermocycler Perkin-Elmer) using *Thermus aquaticus* Taq DNA polymerase (Boehringer).

Construction of a yeast genomic DNA library and screening. Total DNA from strain E-150 was digested with *BamHI*. The resulting 5-kb DNA fragment was isolated and cloned into the poly-linker site of pGEM7Zf(-) vector, as described by the manufacturer (pGEM-T vector systems, Promega, Madison, WI, USA) to generate plasmid pLMX. *E. coli* was transformed with the resultant plasmid carrying the *YISRP72* gene. Colony hybridization was done using standard techniques [29].

Nucleotide sequence analysis. Sequencing was carried out using AmpliTaq polymerase with a BigDye Terminator v1.1 cycle sequencing kit on an automated DNA sequencer (ABI PRISM 377). The amino acid sequence was deduced from the nucleotide sequence and compared with protein sequences deposited in the DNA Data Bank of EMBL-EBI network server, using the FASTA program [25]. Amino acid sequences were aligned using CLUSTAL W [34].

Disruption of the *YISRP72* gene. *Y. lipolytica* *YISRP72* was disrupted with the pop-out method [4] using an integrative plasmid (pLMX3) that contained a deleted copy of the *YISRP72* gene and the *URA3* gene as a selection marker. Genomic integration of the plasmid was carried out by homologous recombination. Yeast colonies were selected for resistance to 5-FOA.

Subcellular location of the *YISRP72-GFP* fusion. To determine the subcellular location of the *SRP72* gene product, a hybrid construct consisting of the *SRP72* promoter and coding regions fused in-frame with the green fluorescent protein (*GFP*) reporter gene was introduced into a *Y. lipolytica* integrative plasmid (pINA240) containing the *LEU2* gene as a marker. The resulting plasmid was designated pLPV. The sequence was confirmed by DNA sequencing (ABI PRISM 377). Strain E-150 was transformed with pLPV; two independent transformants were then grown and prepared for immunofluorescence microscopy. Nucleic acids were stained by DAPI (Sigma Chemical Co.). Cells were visualized under a Leica DMXRA microscope, and fluorescence was detected using the recommended filters.

Nucleotide sequence accession number. The sequences reported in this work for *YISRP72* and the NIP7 homolog of *S. cerevisiae* are available from EMBL/GenBank/DBJ under accession nos. AJ567758 and AJ567759.

Results and Discussion

Isolation and sequencing of the *YISRP72* gene.

The sequence of the *YISRP72* gene was identified from a partial sequence obtained from *Y. lipolytica* sequencing results. That sequence revealed identity with other *SRP72* genes homologs, suggesting that the PCR product was specific for the *SRP72* gene. Of about 800 transformants screened from a

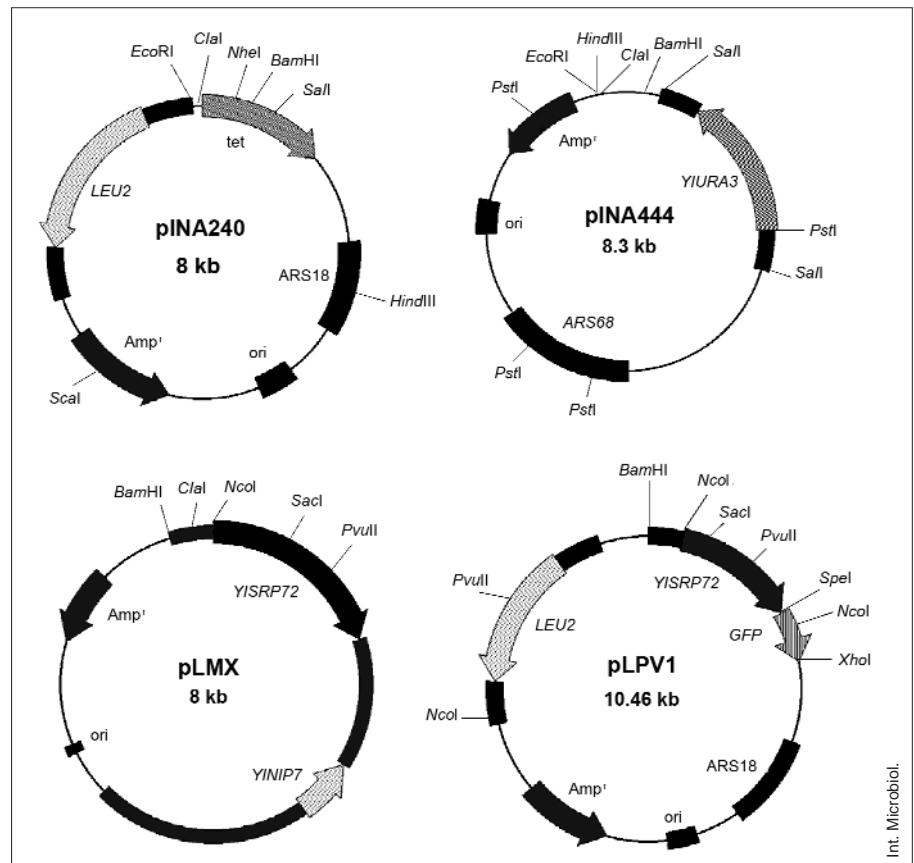


Fig. 1. Restriction maps of the plasmids constructed and used in this work: pLMX, pLMX3, pLRP1, pLPV1, pINA240, and pINA444. Plasmid pLMX containing the 5 kb insert digested with *Bam*HI and cloned in the polylinker site of pGEM7zf(-) vector (Promega, Madison, WI) was used to isolate the *YISRP72* gene. Integrative plasmid pLMX3, used to disrupt the *YISRP72* gene, contains a deleted copy of the *YISRP72* gene cloned in the polylinker site of pGEM-T vector and the *URA3* gene obtained from pINA444. Recombinant plasmid pLRP1 was constructed by inserting the 2-kb *Bam*HI-*Spe*I fragment carrying the *YISRP72* gene between the sites of *Bam*HI-*Nhe*I of pINA240. Plasmid pLPV1 was used for subcellular location of the product of *YISRP72*.

Y. lipolytica partial library, two positive transformants were obtained. Southern analysis suggested that the two clones contained an overlapping sequence. A 5-kb *Bam*HI fragment was subcloned into the pGEM7zf(-) vector (Fig.1, plasmid pLMX). This insert was sequenced on both strands, revealing two open reading frames (ORFs). The *YISRP72* ORF of 1809 bp was located between nucleotides 501 and 2309 in the first phase (+1); the second ORF, of 540 bp, corresponded to the *NIP7* gene and was situated between nucleotides 2661 and 3201 in the first phase (-1) on the opposite strand. Sequence analysis of 2010 nucleotides in the first phase (+1) revealed an ORF of 1809 bp encoding a 65.3-kDa polypeptide ($pI = 9.06$) of 602 amino acid residues. The nucleotide sequence surrounding the putative initiation codon (ATG) conformed to the consensus for translation initiation in yeast, with a conserved A and a G at positions -3 and +4, respectively [8,30]. In yeasts, multiple putative TATA elements are found [13] in the 5'-untranslated region of the *YISRP72* gene. TATA boxes have been identified at positions -210, -385, and -416 relative to the A of the initiation codon. Other features frequent in yeast promoters [9] were found in the 5' region, one hexanucleotide CACACA (position -369) and several CA and CT sequences. In *Y. lipolytica*, the

TAA...TAGT/TATGT...TTT transcription termination motif [41,42] is located at the 3' end of most genes published so far [30,31,40]. The motif was also observed at the 3' end of the *YISRP72* gene (positions +1947, +1959) as were two putative polyadenylation signals (positions +1824 and +1844). Our sequencing results are in agreement with those described for the entire genome of *Y. lipolytica* strain CLIB122, sequenced by the Genolevures consortium [10], except for 15 nucleotides in the region of the *NIP7* gene.

Comparison of the predicted amino acid sequence of the protein (YISrp72p) with sequences in the databases identified matches with *e* values of $9e-33$ with *Giberella zeae*; $4e-32$ with *Aspergillus nidulans*; $8e-32$ with *Neurospora crassa*; $2e-21$ with *Candida albicans*, and $8,2e-18$ with *S. cerevisiae* Srp72p. These values are in agreement with the peculiar taxonomic position of *Y. lipolytica*. YINip7p matched values of $4e-82$ with *Debaryomyces hansenii*; $3e-79$ with *Ashbya gossypii*, and $1e-77$ with *S. cerevisiae*. While it could be argued that there is insufficient identity to classify this protein as Srp72p, the low identity between Srp72p proteins between all fungi and the global synteny of gene conservation (*SRP72* and *NIP7*) in *A. gossypii* and *Kluyveromyces lactis* (before genome duplication) and *S. cerevisiae* and *Y. lipolytica* support

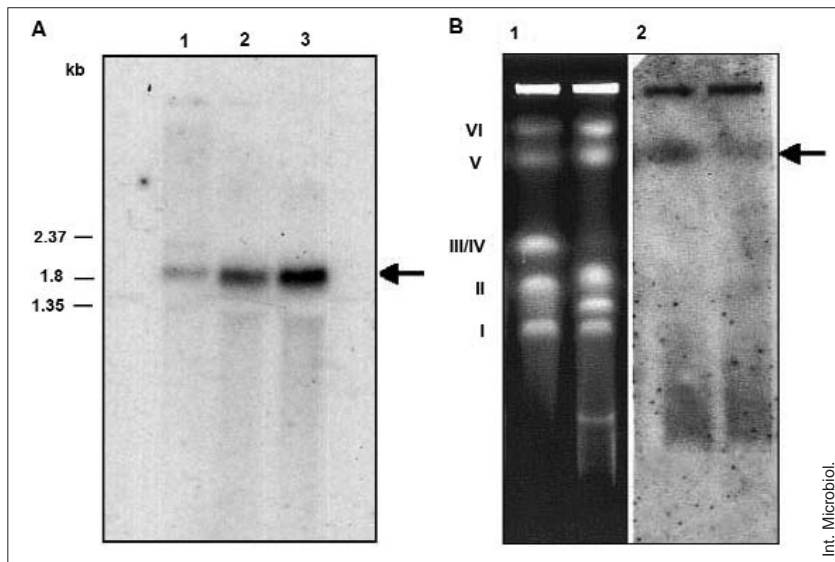


Fig. 2. (A) Northern blot hybridization of the *YISR72* gene using a probe of the gene. Lane 1, 2.5 µg; lane 2, 5 µg; and lane 3, 10 µg RNA isolated from *Y. lipolytica* strain E-150. (B) Chromosomal localization of *YISR72* in *Y. lipolytica* strain E-150: (1) transferring chromosomes of strain E-150 [7]; (2) chromosomal location of the *YISR72* gene.

our hypothesis. All SRP proteins are very rich in leucine, and both Srp68p and Srp72p contain sequences that perfectly match the nuclear export signal (NES) consensus motif $L(X)_2$ $_3L(X)_{2,3}LXL$ [12,24]. Srp72p has three sequences that match the NES consensus: at positions 264 (LRKLQDHGLGLS), 340 (LEDLIETLR), and 436 (LAYALSLLE).

Northern blotting using a fragment obtained from *YISR72* gene as a probe revealed a single RNA band of about 1.8 kb in strain E-150 cells, indicating that the *YISR72* gene is transcribed into a single specific mRNA (Fig. 2A).

Chromosomal location of the *YISR72* gene.

The number of chromosomal bands in *Y. lipolytica* is between four and six in natural isolates from different geographic origins. The numbers and sizes of the chromosomes differ between strains [24]. The karyotype of reference strain

E-150 consists of six chromosomal bands, ranging from 2.6 to 4.9 Mb [7]. Southern blotting revealed that the *YISR72* gene was located on chromosome V, again in agreement with the entire *Yarrowia* genome (Fig. 2B).

Subcellular location of YISrp72p protein.

Srp72p protein has been detected in the nucleolus as well as in the cytoplasm [27]. SRP RNA and three SRP proteins (Srp19p, SRP68p, and SRP72p) are temporarily present in the nucleolus, suggesting that it is the site of partial SRP assembly or of another unidentified activity of the SRP components. In order to localize the YISrp72 polypeptide, it was expressed in *Y. lipolytica* cells (strain E-150) as a GFP fusion protein. Fluorescent *SRP72-GFP* was detected both in the nucleolus and the cytoplasm. Nuclear staining with DAPI subsequently revealed colocalization of the two fluorescent stains (Fig. 3).

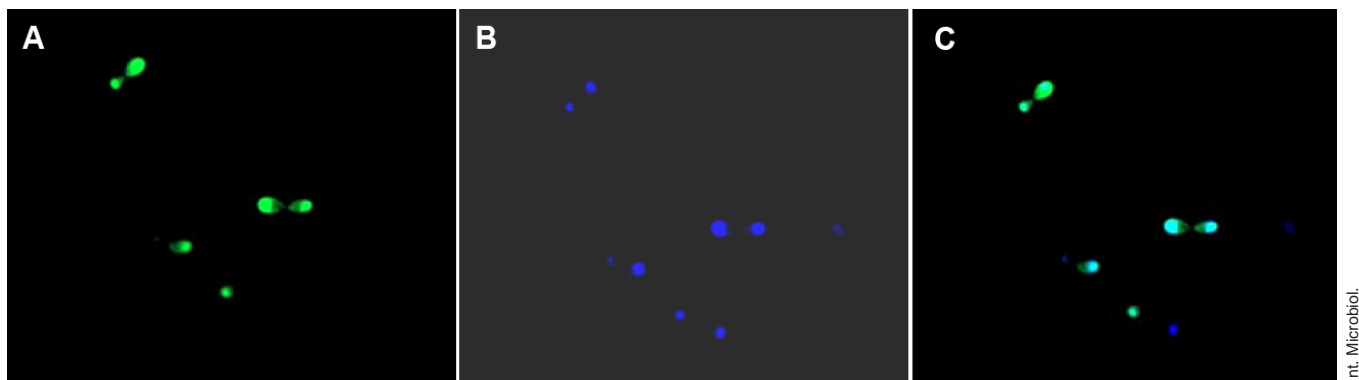


Fig. 3. Sub-cellular localization of the YISrp72p protein of *Y. lipolytica*. Strain E-150 was transformed with plasmid pLPV1, containing the *YISR72-GFP* gene fusion, and subjected to fluorescence microscopy (A); the cell nuclei were visualized with DAPI (B); merged image (C).

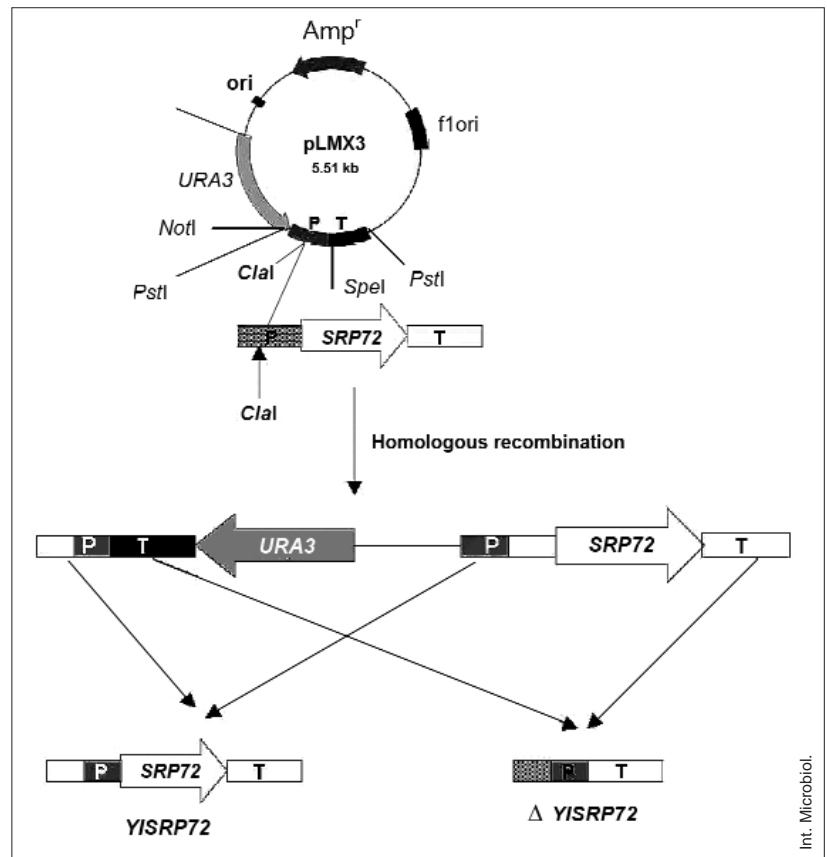


Fig. 4. Disruption of the *SRP72* gene by the pop-out method [4]. The plasmid pLMX3, used to perform the *YISR72* gene disruption, was linearized with *ClaI* and integrated into the genome of the E-150 strain of *Y. lipolytica*. Homologous recombination was favored by growing the transformed strain (named LMX3) in YPD for 48 h. *Ura⁻* strains were screened by selection in 5-FOA medium and confirmed by amplifying the *YISR72* gene by PCR.

Role of *SRP72* in the growth of *Yarrowia lipolytica*. To determine whether *YISR72* was essential for the viability of *Y. lipolytica*, two types of gene-disruption experiments were carried out sequentially. First, a 4.6-kb *HindIII*-*BamHI* fragment carrying a deletion of the *YISR72* gene tagged with the *LEU2* marker was used to transform strain E-150. No *LEU⁺*, *spr72* transformants were detected (out of 250 analyzed), whereas in a similar approach with the *HOY1* gene, one out of four transformants was positive [35]. In the second approach, the pop-out method was used (Fig. 4). After recombination, experiments to disrupt the *YISR72*, *URA3⁺* transformants were selected. Four stable *URA3⁺* clones were confirmed by PCR (data not shown) to contain the expected integration of the construct into genomic DNA. These transformed strains were designated LMX3 to LMX6 (*leu2* and *his1*). We were unable to obtain a *spr72* mutant, even after several generations grown on YEPD and selection on 5-FOA plates supplemented with histidine and leucine. One hundred clones were analyzed by PCR (data not shown) using two oligonucleotides that belonged to the ORF *YISR72* gene and all of them were wild-type.

In order to ensure that *YISR72* gene was essential, we again attempted to disrupt the gene by transforming the recom-

binant LMX3 strain (*leu2*, *his1*), which contains the wild type *YISR72* gene, with a *LEU2* replicative plasmid (pLRP1) also carrying a *YISR72* wild-type gene (Fig. 5). *Ura⁻ Leu⁺* clones were selected after growth on YPD for 48 h and on 5-FOA plates supplemented with histidine. Several independent transformed strains were selected and named LRP1 to LRP7 (*his1*, *ura3*). Five clones of strain LRP1 (LRP1-4, LRP1-8, LRP1-11, LRP1-15, and LRP1-16) were confirmed by Southern hybridization to contain the expected chromosomal disruption of *YISR72*. Three of these *Ura⁻ Leu⁺* transformants (LRP1-11, LRP1-15, and LRP1-16) (Fig. 5) had not lost the *LEU2* replicative plasmid. This was confirmed by growing the clones (LRP1-11, LRP1-15, and LRP1-16) in YEPD for 72 and 240 h followed by selection on plates containing histidine plus uracil and YPD. The results showed that clones exhibited similar growth patterns in the two media and no slow-growing colonies were detected. Thus, our experiments confirmed that the *YISR72* gene is essential for cell growth.

Concluding remarks. A fragment containing the *SRP72* and *NIP7* genes of *Y. lipolytica* was isolated and completely sequenced. Northern analysis revealed the presence of a single transcript and confirmed expression of *YISR72*.

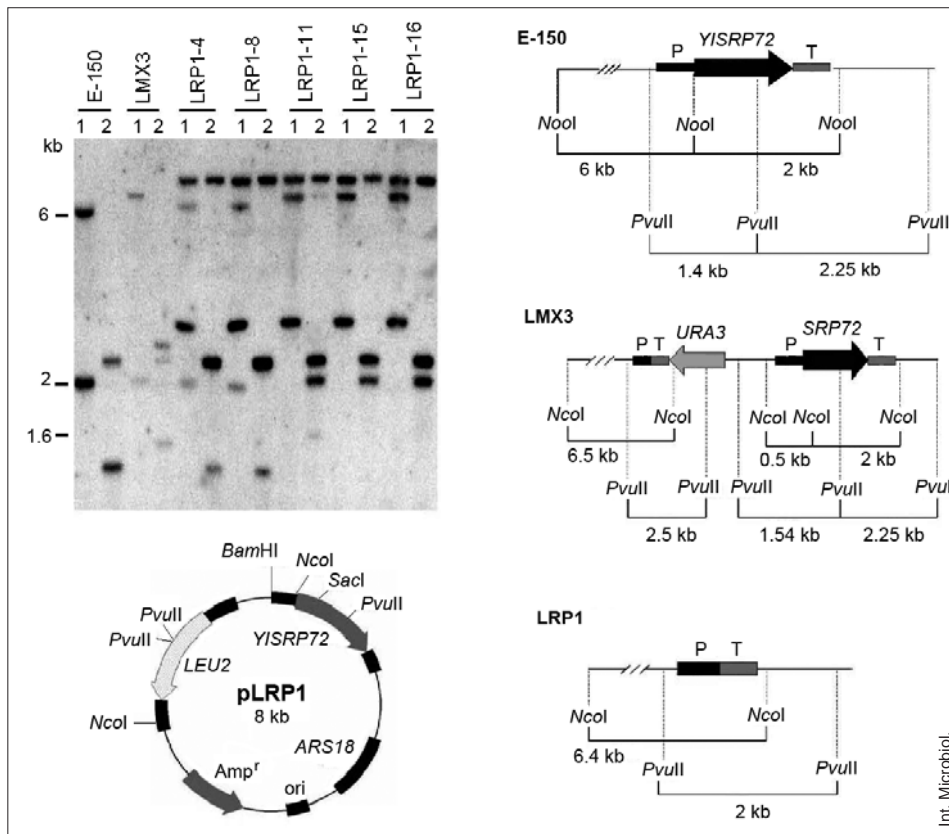


Fig. 5. Southern blot of E-150 (wild-type), LMX3 and LRP1 strains of *Y. lipolytica*. The LMX3 strain was transformed with plasmid pLRP1. The incorporation of the plasmid was verified by Southern blot in the three strains: wild-type (E-150), LMX3 and LRP1. DNA was digested with *Nco*I and *Pvu*II and hybridized with the probe. The corresponding fragments were consistent with the theoretically calculated values.

Chromosome location studies, using *YISR72* as probe, located the gene as a single signal on chromosome V of the standard E-150 strain of *Y. lipolytica*. In this yeast, *Srp72p* was located in the cytoplasm and in the nucleus of the cell. Strong nucleolar accumulation of this protein was unexpected, considering the established role of SRP in the cytoplasm. However, our data are in agreement with the published localization of the mammalian and *S. cerevisiae* SRPcore proteins *Srp19p* (the homolog of *Sec65p*), *Srp68p*, *Srp72p*, and SRP-RNA. All of these were shown to be present in both the cytoplasm and the nucleolus [12,27].

Disruption analysis led us to conclude that *YISR72* is essential for the growth of *Y. lipolytica*. The lethal effect of deleting *YISR72* contrasts with the results described for *S. cerevisiae* [5] and with those reported by Lee and Ogrzydzak [21], who showed that *YISR54* is not essential in *Y. lipolytica*. This yeast differs from *S. cerevisiae* in several features, including the development of a true yeast-hypha transition [35], the presence of a mitochondrial respiratory chain that is more similar to the mammalian one, and the predominance of the cotranslational translocation pathway from the cytoplasm to the ER [1]. Moreover, the 7S RNA component of the SRP receptor of *Yarrowia* was reported to be closely related to the human one, in contrast to the unusual 7S RNA of *S. cerevisiae* [18].

These differences suggest that, in *Yarrowia*, signal recognition is separate from elongation arrest and translocation. This is supported by the finding that disruption of signal recognition, mediated by *Srp54p*, does not result in a lethal phenotype, only slow growth, while the lack of the 7S RNA and of the SRP 9/14 and the 68/72 complexes is lethal. Accordingly, the observations described in the present study imply that *Y. lipolytica* SRP-deficient cells would be unable to utilize an alternative SRP-independent targeting pathway(s) to the ER membrane, at least for some essential proteins, thus validating the use of yeast other than *S. cerevisiae* as models to better understand general biological phenomena.

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