**Introduction**

*Yarrowia lipolytica* is a yeast species widely used in industrial applications such as citric acid production, peach flavor production, and single cell protein production. Moreover, *Y. lipolytica* was shown to secrete a set of proteins (alkaline or acid proteases, RNase, lipases) into the medium in amounts interesting for industrial applications. To improve these properties and to understand possible problems for heterologous protein secretion, the protein secretion pathway has been studied for several years. We will give an overview of results and recent progress obtained in this field. In the first part, we will describe the reporter proteins used in these studies; this will give also information on the main secreted proteins in *Y. lipolytica*. In a second part, studies on the early steps of protein secretion will be reported. In conclusion, synthesis of these data with other results obtained in *Y. lipolytica* and in other organisms will be presented.

**Secreted proteins**

Among the secreted proteins, work was focused on the alkaline extracellular protease (AEP) processing and secretion pathway. For secretion studies, two other proteins are available: the acid protease (AXP) and Kar2p, that is a component of the endoplasmic lumen (ER), and therefore allows to restrict the field of experiments to the early steps of the secretion pathway between cytoplasm and ER.

The alkaline extracellular protease is the major secreted protease in *Y. lipolytica*. Expression levels up to 1–2 g/l were reported [38, 56]. Its expression and secretion are controlled by a complex regulation: (i) it needs a neutral or alkaline extracellular pH, (ii) it depends on the nature of the carbon, nitrogen and sulfur sources; and (iii) it is induced by the presence of exogenous polypeptides [12, 26, 37]. *XPR2*, its structural gene, has been cloned by several laboratories [13, 35, 53] and its sequence is indicative of a complex maturation pathway (Fig. 1A). Starting from the N-terminal extremity are found successively a 15 aminoacid long signal peptide, a stretch of 9 X-Ala, X-Pro dipeptides susceptible to cleavage by a dipeptidyl aminopeptidase, a 124 aminoacid long proregion displaying a glycosylation site and eventually the mature moiety. Along the proregion, there are two Lys Arg dipeptides, potential substrates for a KEX2 like endoprotease. It appeared that only the second dipeptide was physiologically effective and that after X-Ala X-Pro removal, the main maturation step is cleavage at position K<sup>156</sup> R<sup>157</sup> to release the mature AEP [15, 16]. This complex maturation pathway gives a very efficient tool for the analysis of the secretion pathway. In pulse chase experiments [30], a 55 kDa precursor appears first. It was shown to have undergone signal sequence cleavage and N-glycosylation. It is further processed into 32 kDa intracellular mature form with a striking precursor/product relationship with the 55 kDa precursor. The proregion and the mature part are both secreted outside in the...
medium [30]. Due to the high level of AEP expression, pulse duration could be as short as 40 seconds and the different maturation products could be timely resolved during the chase (Fig. 1B). The transit time at 30°C is about 3 min. It is impossible to show evidence for a cytoplasmic AEP intermediate. However, the P17M AEP mutant, which has undergone a substitution of the second amino acid after the signal sequence cleavage site in the proregion, was shown to accumulate a cytoplasmic precursor form; this indicates, first, that an AEP form can be maintained in the cytoplasm without being quickly degraded, second that an AEP form is susceptible to be translocated by a post-translational translocation pathway [59]. Maturation of the precursor is thought to take place in the late Golgi apparatus where are located the KEX2-like endoprotease Xpr6p and the dipeptidyl aminopeptidase which cleaves the X-Ala X-Pro stretch. This removal seems dispensible for maturation [31]. The proregion is required for AEP transit. Mutants deleted from part or all of the proregion do not secrete AEP and polypeptides accumulate in a membrane-bound compartment indicating that they are blocked after their translocation [16]. Expression of this proregion from an ectopic locus could rescue these deleted AEPs in trans allowing secretion of blocked molecules. Interaction of the proregion with the mature part of AEP could confer to the complex a conformation compatible with secretion [17]. This proregion was used in the construction of chimaeric genes for foreign proteins heterologous production. This allows to keep the environment of the initiator ATG codon, to use the genuine XPR2 signal peptide and to take advantage of the supposed good adaptation of the proregion to translocation. However, fusion between the proregion and rice α-amylase led to unprecise cleavage in position Pro150 Ala151 and Val135 Leu136 instead of Lys156 Arg157 [40] indicating that the specificity of the KEX2-like protease depends on the environment of the cleavage site.

Several mutants defective in AEP activity mapping in the XPR6 locus were shown to be deficient in pro-AEP processing and secrete precursor AEP into the extracellular medium [15]. They are impaired in vitro in a Kex2p-like activity. The XPR6 gene cloned by functional complementation encodes a 976 aminoacid long polypeptide, displaying a N-terminal signal peptide and a transmembrane domain near the C-terminal end. This enzyme is thought to be localized into the late Golgi compartment. The XPR6 strain appeared to be viable, but it grew poorly on rich media at neutral pH displaying an altered cell morphology with very few mycelial forms. Moreover, it was defective in mating type B function. Therefore, XPR6p could be required for maturation of some polypeptides involved in dimorphic transition and in the mat B pathway.

The AXP acid protease is secreted into media at acidic pH (pH range from 2.0 to 6.0) [33, 60]. The structural gene has been cloned and it was shown to encode a 397 residue long polypeptide including a 17 aminoacid long signal peptide, a 27 aminoacid long proregion and the 353 aminoacid long mature part [60]. The cleavage site between pro and mature
regions is located between Phe and Ala. Therefore, there is no Lys-Arg processing site and the maturation pathway is different from that of AEP. AXp expression was shown to be regulated at the transcriptional level by external pH [60]. AXp can be used as a reporter polypeptide in protein secretion studies in pulse chase experiments but intracellular events analysis is less easy than with AEP.

The KAR2 gene encodes the major hsp70 chaperone of the ER lumen in Saccharomyces cerevisiae [48]. It was extensively used as a marker of the ER compartment and as a reporter protein in translocation experiments [22]. It was shown to depend on both the co- and post-translational translocation pathways [34]. The KAR2 gene from Y. lipolytica was cloned by homology [27]. It encodes a 672 aminoacid long polypeptide which is not glycosylated. As expected it displays a 26 aminoacid long signal sequence at its N-terminus and a HDEL ER retention sequence at its C-terminal end. It was shown to be suitable for translocation experiments and it was used to show existence of post-translational translocation. Kar2p in S. cerevisiae is a major component of both the translocation machinery and the quality control pathway in the ER lumen. Therefore, interaction with Kar2p was used in Y. lipolytica as an evidence for the involvement of new components in these processes.

Early steps in protein secretion

The 7SL RNA, a small cytoplasmic RNA involved in the early steps of protein secretion was used as a starting point to dissect these steps. In this chapter, we will review successively the data concerning the 7SL RNA, then the properties of its companions Srp54p and Sec65p, finally the gene products Sls1p, Tsr1p and Tsr3p which were shown to interact genetically with the 7SL RNA, and a thermosensitive modified allele of the 7SL RNA gene from Y. lipolytica was cloned by homology [23, 42]. This RNA has the properties of the mammalian 7SL RNA which is a component of the signal recognition particle (SRP). SRP is a central component of the cotranslational translocation [1, 58]. SRP recognizes signal peptides as soon as they emerge from ribosomes and then induces a stop or at least a slow-down of translation elongation giving time to the SRP-ribosome complex to diffuse to the ER membrane where a SRP receptor complex is located. Upon interaction with ER membrane components, the nascent polypeptide is transferred to the translocon, SRP is released and translation resumes simultaneously to the threading of the polypeptide through the translocation pore. The 7SL RNA plays the role of a scaffold for the binding of six polypeptides; SRP9 and SRP14 are involved in translation inhibition; SRP 68 and 72 are thought to interact with ER membrane; SRP19 (Sec65p in S. cerevisiae and Y. lipolytica) plays a role in SRP biogenesis; SRP54 was shown to interact with signal peptide and to regulate the SRP cycle through a GTPase cycle [47, 52]. The members of the 7SL RNA family display a high conservation of their secondary structure as well as the position of the stems as for the conservation of the bases in the internal loops [3]. In S. cerevisiae, the situation is slightly different as the RNA is much larger (519 nucleotides) and adopts a different secondary structure [18]. In Y. lipolytica, the 7SL RNA is encoded by two genes SCR1 and SCR2 which share 94% homology. These genes control an essential cell function as their double disruption is lethal [24]. In order to study their physiological function, SCR1 [59] and SCR2 [25] were modified by directed mutagenesis and thermosensitive mutations were selected in strains where both these 7SL RNA genes were disrupted. For both genes, a thermosensitive mutation was identified in the loop involved in Sec65p binding (A129G-A131T for SCR1 and G128T-A130G for SCR2). Both mutations conferred the same phenotype pattern: a thermosensitive growth phenotype at 33°C when expressed on a plasmid vector; a lethal phenotype when integrated as a single copy in the LEU2 locus; and a thermosensitive translocation defect. To study this protein translocation defect, AEP pulse chases were performed after one hour or two hours shift at 33°C: the transit time was not modified but the amount of AEP precursor decreased as compared to the reference strain. All synthesized AEP had undergone ER posttranslational modifications, i.e., signal peptide cleavage and N-glycosylation. Therefore, all transport events after the translocation step appeared unmodified but there was a specific deficit in AEP synthesis. A prolonged translational arrest induced by a defective targeting to ER membrane could account for this effect if the SRP pool was sufficiently large so that no ribosome synthesizing AEP could escape to SRP interaction. Another hypothesis could be put forward: the AEP precursor would be quickly degraded targeting to ER membrane could account for this effect if the SRP pool was sufficiently large so that no ribosome synthesizing AEP could escape to SRP interaction. 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homology to SRP19 homologs from *S. cerevisiae*, human, rice and *Drosophila* (39%, 34%, 35% and 25% identity respectively). Moreover, it was possible to complement the thermosensitive growth defect of the *S. cerevisiae sec65-1* mutant by *SEC65* from *Y. lipolytica* carried on YRp7 plasmid. Co-immunoprecipitation of Sec61p and 7SL RNA was obtained with antibodies raised against Sec65p indicating that they are part of the same SRP. Disruption of *ylSEC65* as well as point mutations appeared to be lethal indicating that this gene is essential as *SCR1* and *SCR2*. A thermosensitive mutation induced in *SEC65* conferred a thermosensitive AEP secretion defect which appeared as soon as 30 min after temperature shift. Altogether, these results indicate that a bona fide SRP19 homologue was identified in *Y. lipolytica*.

Three components of SRP were thus characterized. One puzzling discrepancy is the viability of *³SRP54* disruption. *³SCR1*, *³SCR2* and *³SEC65* are reported to be essential, but *³SRP54* appeared to induced a slow-growth phenotype. In all cases, the SRP structure is largely impaired. The methods to evaluate lethality can explain partly these differences: it is easier to detect slow-growing colonies when looking at tetrads than when recording replica plates (for instance, when studying to detect slow-growing colonies when looking at tetrads than evaluate lethality can explain partly these differences: it is easier to detect slow-growing colonies when looking at tetrads than when recording replica plates (for instance, when studying to detect slow-growing colonies when looking at tetrads than when recording replica plates (for instance, when studying the viability of *³SCR1* and *³SCR2* and *³SRP54* strains and confirmed that *³SCR1* *³SCR2* was lethal whereas *³SRP54* caused slow growth. Different answers of the cells to starvation in one of the SRP components could be a possible explanation: a very stringent answer would lead to a blockage of the metabolism and eventually to the death; a more relaxed answer would allow an adaptation to the stress and give time to the cells to induce alternative pathways such as post-translational translocation.

In order to study the translocon (Fig. 2), some of its components were cloned. The core of the translocon is Sec61p in *S. cerevisiae*, which is thought to constitute the translocation pore by oligomerization [21]. *SEC61* was cloned by homology [11]. This gene, that is essential for viability, encodes a 471 aminoacid long polypeptide which displays a 69% sequence identity with Sec61p from *S. cerevisiae*. *SEC61* was able to complement a sec61 null mutation in *S. cerevisiae* but the complemented strain had a retarded growth rate and accumulated some preprocarboxypeptidase Y in an untranslocated form. Sec62p is a specific component of the post-translational translocation complex. The *SEC62* gene was cloned by complementation of the *sec62-1* mutant in *S. cerevisiae* [54]. It encodes a 396 aminoacid polypeptide with two potential transmembrane domains. The identity percentage between Sec62p from *Y. lipolytica* and its homologue in *S. cerevisiae* is rather low (37.6%). Nevertheless *SEC62* from *Y. lipolytica* was shown to be able to complement a *SEC62* null mutant in *S. cerevisiae*.

The *scr2*+ mutant was used to identify components involved in the secretion pathway (Fig. 2). To this end, suppressors of *scr2* and mutations colethal with *scr2* were selected. Among the suppressors, studies were focused on a special class, the TSR mutations which suppress the thermosensitive phenotype of *scr2* and which confer themselves a thermosensitive growth phenotype in combination with a *SCR2* allele. In pulse chase experiments, they appeared to be altered in AEP translocation in a similar manner to the *scr2* mutant. *TSR1* was cloned by complementation of the thermosensitive phenotype [8]. It encodes a 461 aminoacid long polypeptide which displays the features of a type I ER membrane protein having a N-terminal signal peptide, a long luminal region divided into a Cys-rich domain and a Ser/Thr-rich domain, a transmembrane domain and eventually a cytoplasmic C-terminal domain. This sequence analysis was correlated with experiments of subcellular fractionation and protease accessibility. Databases searches indicate that four homologues displaying the same structure can be identified in *S. cerevisiae* and one in the yeast *Hansenula polymorpha*. By co-immunoprecipitation, Tsr1p was shown to interact with Kar2p on the luminal side and with SRP components and ribosome components on the cytoplasmic side [9]. As expected for a *scr2* suppressor mutation, *tsr1-1* stabilizes the *scr2* SRP and enhances the interaction of SRP with ribosomal components such as 5S RNA used as ribosome marker. Tsr1p seems therefore to be a component of the ER membrane interacting with SRP at a step where SRP is associated to the ribosome.
view, SRP targets the nascent polypeptide-ribosome SRP complex to the ER membrane through interaction with the SRP receptor. These targeting steps are regulated through GTPase cycles of SRP54p and the SRTα subunit of the SRP receptor [4, 32, 45]. In these models, no indication is given how the vacant translocons are recruited. Potential roles for Tsr1p would be selections of empty translocons or sensing by its luminal part of availability of luminal components to the translocation machinery.

A second suppressor tsr3-1 was also investigated. It displays the same phenotype as tsr1-1, suppressing the thermosensitive growth defect of scr2+, displaying itself a thermosensitive phenotype and a translocation defect in SCR2 background. The TSR3 gene was cloned by complementation; it encodes a 260 aminoacid long polypeptide which has a single homologue as yet unstudied in S. cerevisiae. The studies of its physiological features are currently underway.

A second genetic screen was applied to the scr2+ mutant: synthetic lethal mutants were selected. These mutations conferred a strong thermosensitive phenotype in association with scr2+ and a milder phenotype when combined to SCR2. The co-lethal sls1-1 mutant displayed a thermosensitive growth phenotype and a thermosensitive translocation defect [10]. The SLS1 gene was cloned by complementation. It encodes a 426 aminoacid long polypeptide which displays the distinctive features of an ER resident luminal component. No Sls1p homologue was found but one as yet unstudied ORF exists in S. cerevisiae. Its deletion conferred a thermosensitive phenotype and a translocation defect. By pulse chase analysis, it was shown that there were two types of alteration, i.e., an inhibition of synthesis of AEP as in scr2+ strain and a delay in maturation of the AEP precursor into the intracellular mature form. These observations highlight the tight coupling of events on both sides of the ER membrane and suggest a dual role for Sls1p. On the one hand, it may interact with the translocation apparatus, which is confirmed by co-immunoprecipitation data with Sec61p; on the other hand, it may have a role in the luminal processing of the secreted polypeptides. This latter hypothesis is strengthened by evidences for interaction of Sls1p with Kar2p and by induction of Sls1p expression by unfolded polypeptides accumulation in the ER lumen, as ER chaperones. Therefore, Sls1p seems to be at the boundary between the processing of the translocating polypeptide and the folding steps in the ER lumen before packing in vesicles.

**Conclusion**

It has been demonstrated that Y. lipolytica can be used as a model organism in a number of fundamental fields of cell biology due to its specific properties and to the availability of the main tools of genetic engineering in this yeast (for a review see [6, 7]). It has been chosen to study the dimorphism as it displays a true dimorphic transition [44, 57]. It has also been shown to be a valuable tool to analyze peroxysome biogenesis [36]. In this report, it has been shown that Y. lipolytica is an efficient model to study the protein secretion pathway. The work was focused on the earlier steps (Table 1). The study of later steps was less productive: two major components were cloned by reverse genetics. Sec14p was described in S. cerevisiae as involved in the transit through the Golgi apparatus [5]; the homologous Y. lipolytica SEC14 gene was not essential neither for growth or protein secretion, but was shown to be involved in dimorphism [28, 43]. S. cerevisiae Sec4p, a member of the rab family, was shown to control the fusion step between secretory granules and the plasma membrane [20]; a homologue called RYL1 able to complement the S. cerevisiae sec4-8 mutation was studied in Y. lipolytica but failure to obtain conditional mutations prevented further study [41]. These results highlighted distant evolutionary relationships between Y. lipolytica and S. cerevisiae.

Work on the early steps in protein secretion was more fruitful. These steps are supposed to be crucial for secretion, especially foreign protein secretion, as the polypeptides have to be selected in the cytoplasm to be addressed to the ER membrane and to be threaded through the translocation pore; on the ER luminal side, these polypeptides are folded and undergo a range of post-translational modifications such as N-glycosylation, disulfur bonds formation, and possibly oligomerization. During these steps therefore, the polypeptide should have very close interactions with the cell machinery. Understanding and control of these interactions should be very important to improve quality and yield of protein secretion. In higher eukaryotes, the translocation machinery was first investigated by biochemical techniques using in vitro systems allowing coupled translation-translocation. Experiments of crosslinkings were able to identify components interacting directly with the translocating polypeptide. The ultimate goal was to reconstitute a translocation apparatus from synthetic membranes and from purified proteins [19, 29, 39]. By this way, a minimal translocation apparatus could be defined. The use of unicellular eukaryotic microorganisms such as S. cerevisiae facilitated the genetic study of this system. In the case of mammalian cells, the identified components are related to the SRP-dependent translocation pathway as this one is prominent in these cells. In S. cerevisiae, genetic studies identified first members of the post-translational translocon such as SEC61, SEC62, SEC63 [14, 47] because in this yeast, the main pathway is post-translational. Other components were identified by reverse genetics using data from biochemistry in higher eukaryotes. Studies on S. cerevisiae benefited from the complete sequencing of this genome. This allowed for instance identification of homologues for NAC [51] or for TRAM which is a component of the translocon essential for reconstituted systems. In Y. lipolytica, where studies have begun later, some fundamental partners whose
structure and sequence are well-conserved, were cloned by reverse genetics (SCR1, SCR2, SRP54, SEC61, SEC62, KAR2). But the most original aspect of the work was to induce synthetic lethal and suppressor mutations with scr2ts. These approaches allowed identification of as yet uncharacterized partners of the translocation pathway. Homologues were found only in S. cerevisiae. It is unlikely that yeast specific genes have been identified; but only in S. cerevisiae among eukaryotes, the complete genomic sequence is available. As the screens for mutants were original, it is not surprising that as yet uncharacterized genes were identified. Moreover, in the case of Tsr1p homologues, it is possible that the multiplicity of the related genes in S. cerevisiae could correspond to a redundancy in function and that these components have as yet escaped to genetic screens. These components are also as yet undetected in biochemical studies, but in this case the reductionist approach retained in these studies led to neglect minor components or partners not strictly required for in vitro assays. The setup of such an in vitro assay in Y. lipolytica is desirable because selected phenotypes could be induced by indirect effect. It is especially the case concerning ER related events as this organelle is involved in very different metabolic pathways such as general secretion pathway, peroxysome biogenesis [55] or phospholipids biosynthesis. This could account for indirect effects such as peroxysome polypeptide import defects observed in ΔSRP54 cells [55]. Therefore, provided that biochemical assays could be developed to complete genetic studies, Yarrowia lipolytica could become a model organism for translocation studies.

In summary, the major achievements were the demonstration of the role of the proregion of AEP as an internal chaperone essential for the transit of this polypeptide, the first in vivo evidence for an inhibition of translation by SRP and the identification of new components of the translocation machinery.

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<tr>
<th>Gene identified in Yarrowia</th>
<th>Homologue in S. cerevisiae</th>
<th>Homologue in higher eukaryotes</th>
<th>Function</th>
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<tr>
<td>SCR1, SCR2</td>
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<td>7SL RNA</td>
<td>SRP component, SRP scaffold</td>
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<td>SRP54</td>
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<td>SRP component, interacts with signal peptide, GTPase</td>
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<td>SEC65</td>
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<td>SRP19</td>
<td>SRP component, role in SRP biogenesis</td>
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<td>TSR3</td>
<td>ydr489w</td>
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<td>tsr1-1 screened as scr2ts suppressor, interacts with SRP and Kar2p</td>
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<td>TSR1</td>
<td>yhc8, hrc556, UNF378, scynl283c, ylu (Hansenula polymorpha)</td>
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<td>SLS1</td>
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<td>sls1-1 screened as synthetic lethal with scr2ts, component of ER lumen, interacts with Sec61 and Kar2p</td>
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<td>KAR2</td>
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<td>Major chaperon of ER lumen, interacts with Sec63p, a component of the post-translational complex</td>
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Table 1 Summary of the genes involved in the early steps of protein translocation identified in Yarrowia lipolytica and the corresponding genes in Saccharomyces cerevisiae and higher eukaryotes. Accession numbers of the S. cerevisiae genes known only at the sequence level: yhc8 (swissprot: P38739), ylu2 (swissprot: P34735); hrc556 (swissprot: S51892); scynl283c (genbank: Z71559); unf378 (genbank: U43491); yol031c (PIR: S66714); ydr489w (PIR: S69656)
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