Strong Hybrid Promoters and Integrative Expression/Secretion Vectors for Quasi-Constitutive Expression of Heterologous Proteins in the Yeast *Yarrowia lipolytica*

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Abstract

The industrial yeast *Yarrowia lipolytica* secretes high amounts of an alkaline extracellular protease encoded by the XPR2 gene. The industrial use of the XPR2 promoter was however hindered by its complex regulation. We designed hybrid promoters, based on tandem copies of the XPR2 promoter UAS1 region. In contrast to native XPR2 promoter, these hybrid promoters were not repressed by the preferred carbon and nitrogen sources, nor by acidic conditions, and they did not require the presence of peptones in the culture medium. They exhibited a strong quasi-constitutive activity, similar when carried on either integrative or replicative plasmids. We used these hybrid promoters to direct the production of bovine prochymosin, using XPR2 secretion signals. The production of active chymosin was several fold higher than with previously available *Y. lipolytica* promoters (up to 160 mg/l). Integrative vectors based on the hybrid promoters, allowing the easy insertion of a heterologous gene and its expression or expression/secretion in *Y. lipolytica*, were designed. We also designed new *Y. lipolytica* recipient strains with good secreting abilities, able to grow on sucrose, and devoid of extracellular proteases. These new tools will add to the interest of *Y. lipolytica* as a host for heterologous protein production.

Introduction

Heterologous gene expression is of considerable importance for the industrial production of proteins with pharmaceutical or commercial interest. Yeasts offer a number of advantages as expression systems for complex (*i.e.* mammalian) proteins: they combine the ease of manipulation and growth of unicellular organisms to an eukaryotic subcellular organization enabling post-translational processing and modifications. Considering the limitations of *Saccharomyces cerevisiae* as a host for heterologous gene production, alternative yeasts have been extensively studied: *Schizosaccharomyces pombe*, and several “non-conventional yeasts” like *Hansenula polymorpha*, *Kluveromyces lactis*, *Pichia pastoris*, *Schwanniomyces occidentalis*, and *Yarrowia lipolytica* (reviewed in Buckholz and Gleeson, 1991, and Dominguez et al., 1998). A recent comparative study found *Yarrowia lipolytica* to be one of the most attractive host organisms (Müller et al., 1998). This dimorphic yeast has high secreting capacities (Barth and Gaillardin, 1996), and can grow on a limited number of carbon sources (including glucose and glycerol, paraffines such as n-alkanes and alkenes - Klug and Markovetz, 1967; Bassel and Mortimer, 1973), on lipids and proteins (Ogrydziak et al., 1977), but not on sucrose. It was used in industrial applications such as the production of single cell protein, peach flavor or citric acid (process classified as GRAS - generally recognized as safe - by the American Food and Drug Administration). The XPR2 gene from *Y. lipolytica* encodes an inducible alkaline extracellular protease (AEP) which is the major protein secreted by this yeast (Ogrydziak et al., 1977; Davidow et al., 1987). The XPR2 promoter (pXPR2) has been used to direct heterologous protein production in *Y. lipolytica* (Franke et al., 1988; Nicaud et al., 1989a and 1991; Tharaud et al., 1992; Hamsa and Chattoo, 1994; Park et al., 1997; Müller et al., 1998) but its complex regulation hinders its industrial use. Actually, derepression of the pXPR2 occurs at pH above 5.5 on media lacking preferred carbon and nitrogen sources, and full induction requires high levels of peptones in the culture medium (Ogrydziak et al., 1977). A deletion analysis of the pXPR2 evidenced two major upstream activator sequences (UASs) essential for promoter activity (Blanchin-Roland et al., 1994). In order to dissect functionally these regions, outside of their original pXPR2 context, we inserted DNA fragments from UAS1 and UAS2 in front of a minimal LEU2 promoter (minimal pLEU2) directing the expression of the lacZ gene (Madzak et al., 1999). The activity of these hybrid promoters was assessed under different physiological conditions, following integration into *Y. lipolytica* genome. In all tested media, the UAS1B fragment, carrying the whole distal UAS, was able to promote an enhancement of activity which was orientation-independent and increased with the number of UAS copies (Madzak et al., 1999). Hybrid promoters containing this fragment were thus poorly affected by environmental conditions (either carbon/nitrogen sources or pH), and appeared to drive a quasi-constitutive expression. We describe here the construction of strong hybrid promoters based on iteration of UAS1B, and the analysis of their properties. We also studied the influence of the minimal promoter part, and of the vector type.
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(integrative or replicative). In order to improve heterologous protein production in *Y. lipolytica*, we designed new strains (combining good secreting abilities, growth on sucrose and absence of extracellular proteases) and a series of cloning vectors based on the hybrid promoters.

### Results

#### Design of Strong Hybrid Promoters: β-Galactosidase Expression

We previously showed that a hybrid promoter, carrying the UAS1B fragment from pXPR2 (from nucleotide -805 to -701) upstream from minimal pLEU2, was poorly sensitive to regulations affecting the native promoter (Madzak et al., 1999). This fragment was therefore a good candidate for the design of optimized promoters. New hybrid promoters, based on minimal pLEU2 and carrying one to four tandem copies of UAS1B fragment, were constructed (Table 1; pINA993 in Figure 1) and tested for lacZ expression. They are hereafter abbreviated hpN(d or i) where “hp” stands for hybrid promoter, “N” is the number of UAS1B copies, and “d” (for direct) or “i” (for inverted) indicates their orientation. All plasmids were targeted to the pBR docking platform of *Y. lipolytica* JM23SB strain (Table 2) by NotI digestion. The β-galactosidase activity of the integrants was measured, on early stationary phase cultures, in five different media: (1) inducing peptone-containing YPDm (pH 6.8) medium, in which native pXPR2 activity was maximum,

### Table 1. Description of Plasmids Used

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Transformation</th>
<th>Relevant features</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIN303' integrative</td>
<td>URA3 in pBR322</td>
<td>(Xuan et al., 1990)</td>
<td></td>
</tr>
<tr>
<td>pIN303 replicative</td>
<td>Clal-Xbal fragment of XPR2 (pXPR2 and prepro) in Bluescript</td>
<td>(Fabre et al., 1991)</td>
<td></td>
</tr>
<tr>
<td>pIN752 replicative</td>
<td>pBR322/Bluescript-based plasmid carrying LEU2 and ARS68</td>
<td>(Fournier et al., 1993)</td>
<td></td>
</tr>
<tr>
<td>p3L4561YL integrative</td>
<td>Po1d, translational fusion pPHO2:lacZ</td>
<td>(Tritton et al., 1992)</td>
<td></td>
</tr>
<tr>
<td>pNU49 integrative</td>
<td>URA3, and BamHI-Sst fragment from XAP2, deleted of 665 bp NcoI fragment, in Bluescript</td>
<td>(this work)</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. *Yarrowia lipolytica* Strains Used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Phenotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM12</td>
<td>MatB, leu2-35, lys5-12, ura3-18</td>
<td>Leu+, Lys+, Ura-</td>
<td>(Nicaud et al., 1989b)</td>
</tr>
<tr>
<td>JM23SB</td>
<td>MatB, leu2-35, lys5-12, ura3-18:URA3, xpr2::LYS5</td>
<td>Leu-, AEP, pBR docking platform</td>
<td>(Blanchin-Roland et al., 1994)</td>
</tr>
<tr>
<td>Pot1</td>
<td>MatA, leu2-270, ura3-302, xpr2-322</td>
<td>Leu-, Ura-, AEP, Suc+</td>
<td>(Le Dall et al., 1994)</td>
</tr>
<tr>
<td>Pot1e</td>
<td>MatA, leu2-270, ura3-302::URA3, xpr2-322</td>
<td>Leu-, AEP, Suc+, pBR docking platform</td>
<td>(this work)</td>
</tr>
<tr>
<td>Pot1f</td>
<td>MatA, leu2-270, ura3-302::URA3, xpr2-322, aph-2</td>
<td>Leu-, Ura-, AEP, Suc+, pBR docking platform</td>
<td>(this work)</td>
</tr>
<tr>
<td>Pot1g</td>
<td>MatA, leu2-270, ura3-302::URA3, xpr2-322, aph-2</td>
<td>Leu-, AEP, AAXP, Suc+, pBR docking platform</td>
<td>(this work)</td>
</tr>
</tbody>
</table>
Figure 1. Maps of some of the plasmids carrying hp4d. The unique NotI restriction site was used to direct the integration of the plasmids into Y. lipolytica genome, at the pBR docking platform of JM23SB, Po1e or Po1g strains. "Xt" stands for XPR2 terminator, "Xpp" for XPR2 prepro region (secretion signals), and "pRen" for prorennin A cDNA.

Table 3. β-Galactosidase Activity Driven by Different Promoters

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Plasmid</th>
<th>Inducing (pH 6.8) YPDm medium</th>
<th>Non-inducing YEg medium</th>
<th>Repressing MMAm medium</th>
<th>Acidic (pH 4) YPDm medium</th>
<th>Rich YPD medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Part A/ a single integrated copy of a plasmid with a hybrid promoter based on minimal pLEU2:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>minimal pLEU2</td>
<td>pINA781</td>
<td>10.8</td>
<td>20.2</td>
<td>20.0</td>
<td>10.0</td>
<td>12.1</td>
</tr>
<tr>
<td>hp3d</td>
<td>pINA991</td>
<td>204.0</td>
<td>191.5</td>
<td>157.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>hp4d</td>
<td>pINA993</td>
<td>319.3</td>
<td>294.0</td>
<td>256.4</td>
<td>338.6</td>
<td>418.4</td>
</tr>
<tr>
<td>native pXPR2</td>
<td>pINA404</td>
<td>303.2</td>
<td>70.2</td>
<td>1.6</td>
<td>3.6</td>
<td>65.1</td>
</tr>
<tr>
<td>Part B/ a plasmid, carrying hp4i, integrated as two tandem copies into Y. lipolytica genome:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hp4i (x 2)</td>
<td>pINA994 (x 2)</td>
<td>631.5</td>
<td>616.4</td>
<td>326.8</td>
<td>ND</td>
<td>853.4</td>
</tr>
<tr>
<td>Part C/ a single integrated copy of a plasmid with a hybrid promoter based on minimal pXPR2:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>minimal pXPR2</td>
<td>pINA1204</td>
<td>0.25</td>
<td>ND</td>
<td>0.023</td>
<td>0.35</td>
<td>0.16</td>
</tr>
<tr>
<td>+1 dir. UAS1B</td>
<td>pINA1207</td>
<td>9.2</td>
<td>ND</td>
<td>0.22</td>
<td>32.4</td>
<td>8.9</td>
</tr>
<tr>
<td>+4 dir. UAS1B</td>
<td>pINA1205</td>
<td>1059.4</td>
<td>ND</td>
<td>134.2</td>
<td>732.7</td>
<td>681.9</td>
</tr>
<tr>
<td>+4 inv. UAS1B</td>
<td>pINA1206</td>
<td>1050.4</td>
<td>ND</td>
<td>165.8</td>
<td>689.2</td>
<td>695.8</td>
</tr>
</tbody>
</table>

The β-galactosidase activity of Y. lipolytica JM23SB strain carrying the integrated plasmids is expressed in units/ml x mn x u DO600. At least three independent integrants were analyzed for each plasmid, and the activity was measured in duplicate, on early stationary phase cultures. ND = not determined.
(ii) peptone-free YEg medium, (iii) rich YPD medium, (iv) minimal MMAm medium, containing preferred carbon and nitrogen sources, and (v) acidic YPDm (pH 4) medium. Native pXPR2 activity was very low in these two later media. The activity of hybrid promoters increased with the number of UAS1B copies (Madzak et al., 1999, and Table 3, Part A). The constructs with four UAS1B copies (hp4d and hp4i) exhibited a very high activity in the different media, of the same order of magnitude than that of the native pXPR2 under inducing conditions (Table 3, Part A).

The evolution of the β-galactosidase expression driven by hp4i during the culture growth was analyzed in YPDm (pH 6.8), MMAm and YPD media, and compared to that driven by native pXPR2 (Figure 2). The results obtained with hp4d were similar (data not shown). In striking contrast with those observed for native pXPR2, the activities driven by hp4i were of the same order of magnitude in the three media. However, they did not parallel biomass formation, but followed a more complex curve. Thus, although equally active in all media tested, these hybrid promoters cannot be considered as constitutive stricto sensu.

**Tandem Genomic Integration of High-Expression Plasmids**

Following the integrative transformation of *Y. lipolytica* JM23SB strain with pINA994 plasmid (hp4d), two integrants were found to carry two complete copies of the plasmid, inserted in tandem (checked by Southern blotting - data not shown). The β-galactosidase activity of these integrants was found to be roughly twice that of an integrant with a single copy of pINA994, in the different media (Table 3, Part B), indicating the possibility to enhance expression by the way of multiple integration.

**Influence of the TATA Box Region on Hybrid Promoter Properties**

In order to test if the minimal pLEU2 part (TATA box and ATG region) present in the hybrid promoters had some influence on their properties, we replaced it by either the minimal promoter from *Y. lipolytica* PHO2 gene, or that from the XPR2 gene itself, in plasmids pINA781, pINA795 (hp1d), pINA993 (hp4d) and pINA994 (hp4i).

We constructed hybrid promoters based on a pPHO2 fragment encompassing the TATA box and ATG region (Tréton et al., 1992), and tested them in YPD medium. The β-galactosidase activity driven by minimal pPHO2 (pINA1056 plasmid integrated into JM23SB strain) was very low: 0.12 units. It was slightly enhanced by the addition of one direct copy of UAS1B (pINA1059, in JM23SB: 0.27 units). The addition of four direct or inverted copies of UAS1B (respectively pINA1057 and 1058, in JM23SB) enhanced the activity to 8.7 and 7.3 units, respectively. Although these activities were higher than that of the native pPHO2 (p3L4/61YL plasmid integrated into JM12 strain, isogenic to JM23SB: 1.65 units), the minimal pPHO2 does not appear convenient for the design of optimized hybrid promoters.

The β-galactosidase activity of JM23SB strains carrying integrated plasmids with hybrid promoters based on minimal pXPR2 are shown in Table 3, Part C. In all tested media, the minimal pXPR2 activity was very low, but the addition of UAS1B fragments strongly enhanced the expression. However, the activity of the hybrid promoters varied greatly with the medium, and was the lowest in MMAm. In this repressing medium, the minimal pXPR2 also exhibited an activity tenfold lower than in the inducing one. Although only 12 bp upstream from the XPR2 TATA box, and 58 bp between this box and the ATG, were conserved in the minimal pXPR2 (without homology to known regulatory sequences), this fragment seems to have retained some sensitivity to repression by carbon and/or nitrogen sources. Hybrid promoters based on minimal pXPR2 thus remained sensitive to some regulations and exhibited levels of expression markedly dependent on the medium composition (6-8 fold differences in activity).

**Design of Replicative High-Expression Plasmids**

Autonomously replicating sequences (ARS) from *Y. lipolytica* ensure both centromeric and replicative functions (Fournier et al., 1993; Vernis et al., 1997). ARS-carrying plasmids therefore exhibit relative mitotic stability and low copy number. In order to test the hybrid promoters, based on minimal pLEU2, on autonomously replicating plasmids, the *Y. lipolytica* ARS68 was inserted into pINA781, pINA993 (hp4d) and pINA994 (hp4i). The resulting plasmids, respectively pINA1053, 1054 and 1055, were
electroporated into *Y. lipolytica* JM23SB strain. Their transformation efficiencies were in the range of \(7.5 \times 10^4\) to \(2 \times 10^6\) per \(\mu g\), similar to that of a control replicative plasmid (pINA752: \(8.5 \times 10^4\) per \(\mu g\)). The proportion of \(\text{Leu}^+\) cells in the culture, after 4 generations under non-selective conditions (rich YPD medium), was measured on two transformants for each plasmid. They were in the range of \(63.5\) to \(70\)\%, comparable to that obtained with pINA752 (\(68\)\%). This shows that the *ARS* was fully functional in these plasmids, and that the expression of \(\beta\)-galactosidase did not confer any detectable selective disadvantage to the transformants.

No plasmid rearrangement was detected upon back-transformation of the plasmids into *E. coli* (data not shown). The region of the hybrid promoters was analyzed more precisely by a PCR reaction involving two flanking oligonucleotides. In each case, a unique band of the expected size was obtained (data not shown), showing that this region was not rearranged during passage in yeast and that the four UAS1B copies were conserved in the rescued plasmids pINA1054 and 1055.

The \(\beta\)-galactosidase activity of strains carrying the *ARS*-containing plasmids was measured after growth in MMAm selective medium. In parallel, an aliquot of the same cultures was taken during the late exponential growth phase, and used to determine the percentage of cells carrying plasmids under selective conditions. We also checked the mean copy number of the replicative plasmid, on the same transformants, by determining the ratio of plasmidic to genomic *LEU2* fragments (Table 4). The mean \(\beta\)-galactosidase activity of pINA1053 (minimal pLEU2) was 2.5 fold higher than previously observed for pINA781. In contrast, the mean activities of pINA1054 (hp4d) and pINA1055 (hp4i) were of the same order of magnitude than those previously observed for pINA993 and 994. The mean copy number was around 0.8 plasmid per cell, for the three replicative plasmids. As the percentage of cells carrying the replicative plasmid in selective medium is similar (Table 4), this indicates that only one copy of pINA1053, 1054 and 1055 plasmids was present per JM23SB plasmid-containing cell. The higher activity driven by minimal pLEU2, when carried on a replicative plasmid, cannot therefore be attributed to the copy number. It could be due to the absence of a negative effect exerted by some genomic sequence, in the integrative context. The low copy number, expected from the centromeric contribution of *ARS* in *Y. lipolytica* (Fournier *et al.*, 1993), did not allow to increase the expression directed by the hybrid promoters on replicative vectors. Nevertheless, the fact that hp4(d or i) were able to drive similar expression levels when carried on replicative or integrative plasmids shows that their properties were intrinsic, and not dependent on the integration site, at the pBR platform.

### Design of *Y. lipolytica* Strains Optimized for Heterologous Protein Production

In order to develop an efficient system for heterologous production in *Y. lipolytica*, we designed new yeast strains combining an easy-to-use vector integration mechanism with a high secretion efficiency, and minimizing the risks of heterologous protein degradation. The *Y. lipolytica* Po1d strain is a derivative of the wild-type W29 strain (ATCC 20460), which was deleted for the *XPR2* gene, and carries the *S. cerevisiae* *SUC2* gene under the control of *XPR2* promoter and secretion signals. The strains carrying this fusion were shown to display invertase activity which permits growth on sucrose as a sole carbon source (Nicaud *et al.*, 1989a). Growth kinetics of Suc\(^+\) transformants, on pure sucrose or on sugar beet molasses, showed that sucrose was a carbon source as efficient as glucose, under conditions favourable for invertase gene expression (Wojtatowicz *et al.*, 1997). We constructed a derivative of Po1d strain deleted for the *AXP* gene: the Po1f strain, unable to produce any extracellular protease (neither neutral-alkaline AEP nor acidic AXP). In order to allow the easy integration of pBR-based expression/secretion vectors, we constructed Po1e and Po1g recipient strains, by integration of a pBR322 docking platform at the *URA3* locus of respectively Po1d or Po1f strains.

### Expression/Secretion of Bovine Prochymosin, Using Strong Hybrid Promoters

We tested *Y. lipolytica* for the expression and secretion in the culture medium of bovine chymosin. We compared, on the one hand, our hybrid promoters hp4(d or i) to other promoters available in *Y. lipolytica*, and, on the other hand, the newly designed recipient strains to the JM23SB laboratory strain.

Chymosin is expressed as azymogen precursor (prochymosin, also known as prorennin) and is converted to the active form at low pH, by autocatalytic proteolysis (removal of 42 amino acids in N-terminal). It was previously shown by Franke *et al.* (1988) that prorennin could be expressed in *Y. lipolytica* and efficiently secreted in the culture medium, using the secretion signals (prepro region) of the *XPR2* gene. In contrast to the situation in *S. cerevisiae*, no residual chymosin activity was found within the cells (Franke *et al.*, 1988 ; Nicaud *et al.*, 1991). Like the bovine enzyme, the prorennin secreted in *Y. lipolytica* was not glycosylated, although the amino acid sequence of the protein includes two putative tripeptide glycosylation

### Table 4. Copy Number and Expression from *ARS*-Carrying Plasmids Containing Hybrid Promoters, Compared with Integrative Plasmids

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Plasmid (Number of transformants analyzed)</th>
<th>Leu(^+) cells in MMAm selective medium</th>
<th>Ratio of plasmidic to genomic <em>LEU2</em></th>
<th>(\beta)-Galactosidase act. in MMAm selective medium</th>
<th>(\beta)-gal. act. of equivalent integrative plasmid in MMAm</th>
</tr>
</thead>
<tbody>
<tr>
<td>minimal p LEU2</td>
<td>pINA1053 (2)</td>
<td>77.8%</td>
<td>0.84</td>
<td>53.4</td>
<td>20.0</td>
</tr>
<tr>
<td>hp4d</td>
<td>pINA1054 (5)</td>
<td>83% (±1.4%)</td>
<td>0.82 (±0.04)</td>
<td>227.2 (±17.2)</td>
<td>256.4</td>
</tr>
<tr>
<td>hp4i</td>
<td>pINA1055 (6)</td>
<td>82.4% (±1.2%)</td>
<td>0.83 (±0.05)</td>
<td>183.0 (±6.3)</td>
<td>186.6</td>
</tr>
</tbody>
</table>

When more than two transformants were analyzed, the standard deviation is indicated between brackets after the mean value. The ratio of plasmidic to genomic *LEU2* DNA, measured by Southern blotting, corresponds to the mean copy number of the replicative plasmid per cell, under selective conditions.
signals (Davidow et al., 1986). We constructed vectors carrying a translational fusion of either minimal pLEU2 (pINA1208), hp4d (pINA1209, shown in Figure 1), or hp4i (pINA1210), with the prepro region of XPR2 gene (157 N-terminal amino acids) followed by the cDNA sequence of the prorennin A allele. The plINA1214 plasmid is similar to the above vectors but based on native pXPR2 (same fusion of prepro region to prorennin gene). The above constructs were targeted to the pBR docking platform of JM23SB, Po1e or Po1g strains, by NotI digestion. Results were compared to those obtained with native pLEU2 using pLX-34 plasmid (same translational fusion of promoter to prepro region and prorennin gene than in pINA1208 to 1210), integrated at the pBR platform of JM23SB, following Ndel digestion.

For each plasmid, the production of prochymosin was determined in an activity test, after acid activation of the culture supernatants of several transformants. The comparison of the hybrid promoters with native ones was performed in JM23SB recipient strain; the results obtained using hp4i were very similar to those of hp4d (data not shown). As shown in Figure 3, hp4d was much more efficient than either pXPR2 or pLEU2, for protein production in both rich or minimal media.

The measured rennin activity obtained using hp4d, in JM23SB strain, slightly diminished after 40 h of culture in YPD medium, but not in MMAm medium. We wondered if this was due to degradation of the produced enzyme by secreted AXP activity. Actually, JM23SB carries a wild-type AXP gene, which was shown to be expressed at pH 6, the approximate pH of the unbuffered YPD medium (Cordero-Otero and Gaillardin, 1996). This protease was also shown to retain at pH 6 a fourth of its maximum activity, observed at pH 4, whereas its activity at pH 6.8, as in MMAm medium, is negligible (Yamada and Ogrydziak, 1983). We thus compared the two isogenic recipient strains Po1e and Po1g, both XPR2-deleted, and differing by the presence or absence of the AXP gene, respectively. As can be seen in Figure 3 and Table 5, the amount of rennin activity measured for hp4d was very similar in these two strains, in both YPD and MMAm media. No decrease in rennin activity was observed with both of these strains, for which the production of active enzyme reached a plateau after 60-70 h of culture in YPD medium. The possible AXP expression in YPD medium was therefore not responsible for the decrease of rennin activity observed during culture of transformed JM23SB strain. This decrease might be due to the release of intracellular proteases in stationary phase, perhaps more important in this strain.

Growth kinetics of Po1g transformed with either pINA1209 (hp4d) or pINA1210 (hp4i) plasmids were very similar to those of the wild-type W29 strain, in YPD or MMAm media: neither the genetic modifications, nor the expression of the heterologous protein, had an effect on growth capacities (data not shown). As can be seen in Figure 3, hp4d-driven production of prorennin, in both Po1e and Po1g strains, was much higher than in JM23SB strain (20 mg/l in YPD medium, under non-optimized low cell density conditions). We also tested the capacities of hybrid promoters under industrial-type conditions (5 l reactor): in YPD medium, hp4d (in Po1g strain) produced a twofold higher amount of secreted prorennin, but this increase was lower than could be expected from the fivefold increase in cell density (Table 5). The industrial-type PPB medium, containing sucrose as the carbon source, appeared to be much more appropriate for protein production: the amount of secreted prorennin obtained using hp4d reached 160 mg/l. However, this amount remained constant after 48 h of culture, despite the continuation of cell growth (Table 5), which suggests that the process could still be improved. The results were very similar when using hp4i (data not shown).

The production of prorennin driven by hp4d, in JM23SB or Po1g strains, was analyzed by Western blotting (Figure 4). Anti-rennin antibodies revealed a single band of the size expected for prochymosin, in the culture supernatant of transformed JM23SB strain. In the culture supernatant of transformed Po1g strain, a second band co-migrating with the rennin marker was also observed. It corresponds to a small amount of mature enzyme, obtained by spontaneous autocatalytic activation (probably due to pH changes during growth in unbuffered medium). The amount of secreted prorennin, determined by comparison with the rennin marker, was consistent with the quantification obtained in the activity tests (data not shown). Thus, the
protein produced exhibited a biological activity indistinguishable from that of the bovine enzyme. The glycosylation status of the prorennin produced was determined using either endoglycosidase H or concanavaline A (Figure 4). The prochymosin band showed no change in mobility following endo H treatment. After addition of concanavaline A beads and centrifugation, the protein was recovered in the supernatant, and not in the bead pellet. Both of these tests thus clearly indicate that the prorennin produced was not glycosylated. No antibody-reacting protein was detected, neither intracellularly (after cell lysis using the glass-bead method), nor in the culture supernatants of untransformed JM23SB or Po1g strains (data not shown). Thus, the high expression level driven by hybrid promoters, and the good secretion capacities of newly designed *Y. lipolytica* strains, allowed the efficient production of prochymosin, which properties were indistinguishable from those of the bovine enzyme.

**Design of Cloning Vectors for the Expression/Secretion of Heterologous Proteins**

We designed a whole set of pBR-based integrative vectors, carrying the hybrid promoters, and allowing easy insertion of a heterologous gene, and expression or expression/secretion of its product in *Y. lipolytica*. The expression vectors pINA1269 (hp4d - Figure 1) and pINA1270 (hp4i) are devoid of secretion signal: the heterologous protein can be either produced intracellularly, or secreted using its eventual own signal peptide. The *PmlI* blunt cloning site, replacing the ATG sequence of hp4(d or i) in these vectors, can be used to obtain a perfect fusion between the hybrid promoter and the heterologous gene. The expression/secretion vectors pINA1267 (hp4d - Figure 1) and pINA1268 (hp4i) carry a translational fusion of the hybrid promoter to the *XPR2* prepro region, which allows the secretion of the produced heterologous protein. The *XPR2* pro sequence was modified to create a *Sfi* cloning site permitting a perfect fusion with the heterologous gene. Depending on the protein produced, the maximum secretion efficiency can be obtained either with the cognate version of the former - Figure 1), together with another integrative expression/secretion vector based on hp4d, have already been successfully used to produce several homologous or heterologous proteins. In our laboratory, the AEP and the *Arxula adeninivorans* glucoamylase were produced in YPD medium, at levels similar to or higher than those obtained with the induced native pXPR2 (D. Swennen, personal communication). The secretion of 20 mg/l of an active anti-Ras single-chain antibody was obtained, under non-optimized conditions (L. Vernis, personal communication). We also obtained a 15 fold overexpression of the catalytic subunit of the *Y. lipolytica* caseine kinase II, and its secretion in the culture medium (T. Chardot, LCB, CBAI, personal communication). Some heterologous proteins difficult to obtain in other systems were produced successfully using hp4d: the human β-2-microglobulin (K. Uchida, Nagahama Inst. for Biol. Sciences, personal communication); the murine interleukin-6 (15 mg/l under non-optimized conditions, a level similar to that obtained in the baculovirus system, B.-C. Sang, Pharmingen, personal communication); the human α-fetoprotein (140 μg/l under non-optimized conditions, a level more than twofold higher than that obtained in *S. cerevisiae* - K. Uchida, personal communication).

**Table 5. Prorennin Production Driven by the Hybrid Promoter hp4d**

<table>
<thead>
<tr>
<th>Recipient strain</th>
<th>Medium</th>
<th>Culture conditions (number of assays)</th>
<th>Hours of culture for maximum production</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>Dry weight</th>
<th>Secreted prorennin</th>
<th>mg of prorennin per g of dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM23SB</td>
<td>YPD</td>
<td>2 ml, tube (3)</td>
<td>48 h</td>
<td>18</td>
<td>ND</td>
<td>7.5 mg/l</td>
<td>ND</td>
</tr>
<tr>
<td>Po1e</td>
<td>YPD</td>
<td>2 ml, tube (3)</td>
<td>72 to 96 h</td>
<td>18</td>
<td>ND</td>
<td>20 mg/l</td>
<td>ND</td>
</tr>
<tr>
<td>Po1g</td>
<td>YPD</td>
<td>2 ml, tube (3)</td>
<td>64 to 80 h</td>
<td>18</td>
<td>ND</td>
<td>20 mg/l</td>
<td>ND</td>
</tr>
<tr>
<td>Po1g</td>
<td>YPD</td>
<td>5 l, reactor (1)</td>
<td>74 h</td>
<td>100</td>
<td>17 g/l</td>
<td>40 mg/l</td>
<td>2.4</td>
</tr>
<tr>
<td>Po1g</td>
<td>PPB</td>
<td>5 l, reactor (2)</td>
<td>48 to 54 h</td>
<td>50 to 85</td>
<td>7.5 to 8.25 g/l</td>
<td>160 mg/l</td>
<td>21.3 to 19.4</td>
</tr>
</tbody>
</table>

Recipient strains carried one integrated copy of pINA1209 plasmid. Reproducibility of OD<sub>600</sub> measurement between independent cultures was in the range of ±10%. The results for the 2 ml cultures correspond to the same experiments than in the Figure 3.

![Figure 4. Western blot showing the production of prorennin driven by hp4d. Two ml of supernatant from YPD cultures of JM23SB or Po1g strains transformed with pINA1209 plasmid (after 40 or 64 h of culture, respectively) were treated or not with either endoglycosidase H ("endo H") or concanavaline A beads ("conc. A": fractions associated to supernatant or to pellet), and concentrated for electrophoresis. A rennin control (20 mg/l in YPD medium) was processed in parallel. The revelation was performed using an anti-rennin antibody.](image)
Discussion

Hybrid promoters, based on iteration of UAS1B fragment from pXPR2 added to a minimal pLEU2, were able to drive a high quasi-constitutive expression: they were not repressed by the carbon and nitrogen sources, did not require the addition of peptones in the culture medium and were insensitive to extracellular pH. As a strong promoter, hp4d (or hp4i) is of interest for the production of heterologous proteins in Y. lipolytica: it allows to obtain, in a rich medium or in a defined minimal medium, high expression levels which were previously attained only with the native pXPR2 under very restrictive inducing conditions.

Surprisingly, the origin of the minimal promoter part appeared to be crucial for the properties of the hybrid promoter. When the minimal pPHO2 was used, the activities remained very low: the UAS1B copies, from pXPR2, could be unable to interact efficiently with the TATA box from pPHO2. In contrast, when the minimal pXPR2 was used, the addition of cognate UASs was able to promote a strong increase of activity in some media, but only a limited one in presence of preferred carbon and/or nitrogen sources: despite the small size of minimal pXPR2, it seems to contain unidentified elements responding to carbon/nitrogen regulation.

The use of replicative plasmids showed that the efficiency of the hybrid promoters was an intrinsic property, not dependent on the chosen integration site. They however did not allow to increase the copy number of the vector per cell and thus the expression of the reporter heterologous protein, compared to the integrative situation. Although a copy number of one or two replicative plasmids per cell was usually the norm in Y. lipolytica (Nicaud et al., 1991). The copy number that can be obtained is probably dependent on the strain used, and on unidentified elements in the structure of the replicative vector.

In order to increase the expression in our system, the use of multiple integrations appears much more interesting than that of replicative vectors. The hybrid promoters will be combined to a multiple integration method, developed in our laboratory (Le Dall et al., 1994), using repetitive genomic elements together with a defective selection marker.

The use of our hybrid promoters, combined with that of XPR2 secretion signals, allowed to obtain an efficient production of bovine prochymosin. The limited industrial-type assays performed permitted an eightfold increase of production, compared to standard laboratory conditions.

We wish however to emphasize that only a limited number of culture conditions were tested, and that the results presented here are probably underestimates of those reachable under optimized conditions. In conclusion, the hybrid promoters, together with the newly designed Y. lipolytica recipient strains, appear efficient for the production of heterologous proteins (production competitive with other systems, absence of hyperglycosylation, and normal biological activity). The new tools we have developed contribute to increase the potentialities of Y. lipolytica for heterologous production.

Experimental Procedures

Culture Media

The fully inducing medium (YPDm) contained 0.2% yeast extract, 0.1% glucose and 5% proteose peptone in 50 mM sodium phosphate buffer (pH 6.8). When the effect of acidic pH was assayed, YPDm was buffered with 200 mM sodium citrate buffer (pH 4.0). The peptone-free medium YEG contained 1% yeast extract and 0.1% glucose in 50 mM sodium phosphate buffer (pH 6.8). The peptone-free medium MMAm (Blanchin-Roland et al., 1994) contained 200 mM phosphate buffer (pH 6.8), glycerol (10 g/l) as a carbon source and ammonium sulfate (2 g/l) as a nitrogen source. The richYPD medium contained 1% yeast extract, 1% glucose and 1% bacto peptone. Minimal YNB medium (Sherman et al., 1986) was used for the selection of yeast transformants. For reactor assays, the industrial-type YPD medium was also used (20 g/l sucrose, 1.32 g/l yeast extract, 0.32 g/l NHCl, 0.32 g/l KH2PO4, 0.13 g/l MgSO4, 0.33 mg/l thiamine), the shaking was 200 rpm, the air flow 1vvm and the pH was maintained at 5.3.

Strains and Transformation Procedures

Bacterial strains used were Escherichia coli HB101 (Boyer and Roulland-Dussoix, 1969), DH5αF′ (Stratagene), and DK1 (Raleigh et al., 1989). E. coli was transformed as described by Hanahan (1985). The Y. lipolytica strains used are described in Table 2. Integrative or replicative transformations of Y. lipolytica were carried out as previously reported (Hoffman and Winston, 1987; Xuan et al., 1988; Fournier et al., 1993). The pBR docking platform, in JM23SB strain, was provided by genomic integration of plasmid pNA300', linearized by Smal digestion, at the URA3 locus (Blanchin-Roland et al., 1994). The ura3-302 mutation, in Po1d strain and its derivatives, consists in the disruption of URA3 by the fusion pXPR2:SUC2 (from S. cerevisiae). The yeast transformants were checked by Southern blotting, as previously described (Hoffman and Winston, 1987; Xuan et al., 1988). The deletion of the 665 bp NcoI fragment into the coding region of the AXP gene, in the Po1f strain, was obtained using plasmid pnU49, by the two-step method (pop in/pop out) described in Scherer and Davis (1979). Po1 and Po1g strains were obtained by integrative transformation of respectively Po1d and Po1f strains with plasmid pNA300' at the URA3 locus.

Plasmids

Plasmids used are described in Table 1. The pBR322 sequence of plasmids pINA34, pINA404, pINA781, and all derivatives, carries a NotI polylinker at the previous Pvull site (Ribet and Gaillardin, unpublished data). Consequently, they can be targeted by NotI digestion to the pBR docking platform of JM23SB, Po1e or Po1g. For all 8-galactosidase-expressing plasmids, the translational fusion of the different promoters to the lacZ gene was performed at the tenth codon (Madzak et al., 1999).

The UAS1B fragment consists in nucleotides -805 to -701 of pXPR2, with added Spht cohesive ends (Madzak et al., 1999). The minimal pLEU2 consists in the TATA box and ATG region of pLEU2, from nucleotide -94 to +16, with added Spht and BclI upstream sites, and BamHI downstream sites (Madzak et al., 1999). The derivatives of pINA781 were obtained by inserting UAS1B copies at this Spht site; plasmids with three or four copies were derived from the corresponding plasmids with two copies by ligation of the fragments from a total Notf and partial Spht digestion.

The plasmids with translational fusion of the minimal pPHO2 (TATA box and ATG region of pPHO2, from nucleotide -116 to +51) to the lacZ gene were derived from the similar plasmids with minimal pLEU2 as follows. A PCR fragment was synthesized using p3L461YL as a template and two oligonucleotides, one in pPHO2: 5'-GCCAAACGTAATGCAGAATGTTCA CCCAGGAC (mutations in bold type create the underlined XhoI site; plasmids with three or four copies were derived from the corresponding plasmids with two copies by ligation of the fragments from a total Notf and partial Spht digestion.

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promoters of pINA781, 993 or 994 and the XPR2prepro sequence of pLX-34. The upstream oligonucleotide, 5'-AGGCCCTCTCAAGGGCACCCTGCCGTC, hybridized upstream from the SaI site in the pBR region of pINA781, 993 or 994. The fusion oligonucleotide, 5'-ATACCAACACACACATCATCATACATAG GCC TTT ACT TT ATG ATG ACT G, hybridized to pLX-34 in the region of the fusion between pLEU2 and the XPR2 prepro, and also partly hybridized to pINA781, 993 or 994 in the region of the minimal pLEU2.

The downstream oligonucleotide, 5'-GAGAAAGCGGACATCCGCTTG (noted oligo XPR), hybridized downstream from the SaI site in the XPR2 prepro region of pLX-34. This PCR fusion fragment was then digested by SaI and SaU, and recombined to (i) a SaI-Boll fragment from pLX34 (translational fusion of XPR2 prepro region to CDNA of the preprorennin A gene), and (ii) a SaI-BamHI fragment from pINA781 (XPR2 terminator, LEU2 gene and pBR region).

The mean copy number of replicative plasmids was determined using pINA303 as a template and two oligonucleotides, one in pXPR2 upstream from the Apa site, 5'-CAGATATGCTAGCAAGGCGGCAACTA, and the oligo XPR. This PCR fragment was then digested by ApaI and SaU, and recombined to (i) a SaI-NotI fragment from pINA1208 (translational fusion of XPR2 prepro region to preprorennin, XPR2 terminator, LEU2 gene and pBR region), and (ii) a NotI-Apal fragment from pINA354 (major part of pXPR2).

The modification of the TATA box, creating a PmI cloning site, in pINA1265 and 1266, was performed as gift from Dr Christine Strick (Pfizer Co., USA) and the AXP gene fragment was a gift from Dr Thomas Young (University of Birmingham, U. K.). We are indebted to Dr Chouki ben Mamoun for helpful discussion about the construction of hybrid promoters with three or four UAS1B copies, and to Dr Laurence Verme for technical advice about replicative plasmids. We are grateful to Dr Claude Gaillardin for his constant interest in this work. The hybrid promoters based on iteration of UAS1B copies were patented by INRA and the Institut National Agronomique Paris-Grignon (EP 0 747 484 A1). The testing of the hybrid promoters under industrial conditions was performed at the Plateforme pour le Processement d’Acide (INRA-Bourgogne Technologies, Dijon, France) and was partly supported by the Agence Nationale pour la Valorisation de la Recherche (ANVAR).

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References


216 Madzak et al.


