Cloning, disruption and protein secretory phenotype of the GAS1 homologue of *Pichia pastoris*

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β-1,3-glucanosyltransglycosylase; cell wall; heterologous protein secretion; *Pichia pastoris*; gene disruption; yeast.

Abstract

The aim of the study was the identification, cloning and disruption of the GAS1 homologue of *Pichia pastoris*. Gas1p is a glycoprotein anchored to the outer layer of the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor. Gas1p is a β-1,3-glucanosyltransglycosylase (EC 2.4.1.-). This cross-linking enzyme highly affects the structure and permeability of the yeast cell wall. The gene coding for the GAS1 homologue of *P. pastoris* was cloned by PCR, and its functionality was proven in a *Saccharomyces cerevisiae* GAS1 null mutant. Based on the nucleotide sequence information of the *P. pastoris* GAS1 homologue, a disruption cassette was constructed for the knockout of the GAS1 in *P. pastoris*. The morphology of ΔGAS1 *P. pastoris* was identical to that of *S. cerevisiae* GAS1 mutants. Finally, the impact of GAS1 disruption on secretion of three recombinant model proteins in *P. pastoris*, human trypsinogen, human serum albumin and Rhizopus oryzae lipase, was evaluated. While the disruption had no effect on the secretion of trypsinogen and albumin, the amount of lipase released from the cells was doubled.

Introduction

The methylotrophic yeast, *Pichia pastoris* is widely used today for the production of heterologous proteins (Cereghino & Cregg, 2000). Compared with other yeast expression systems, the advantage of *P. pastoris* is, among others, the potential to synthesize and secrete high levels of protein (Porro et al., 2005).

In recent years, limitations of protein secretion have been analyzed for *Saccharomyces cerevisiae*, several fungi and *P. pastoris*. Folding and disulfide bridge formation may be limited, leading to the unfolded protein response (UPR) (Mori et al., 1992). Artificial upregulation of the UPR can lead to an improved secretion of recombinant proteins (Valkonen et al., 2003; Gasser et al., 2006), indicating that folding and disulfide bridge formation in the endoplasmatic reticulum (ER) are a major bottleneck for the secretion of certain classes of proteins. On the other hand, some proteins, even if containing several cystins, are highly secreted from *P. pastoris* without inducing the UPR, e.g. human serum albumin (HSA) (H. Hohenblum, and D. Mattanovich, unpublished).

Once a protein has been released out of the ER–Golgi system, it has to penetrate the cell wall. While the cell wall is apparently not a limiting barrier for many heterologous proteins, there are some indications of significant retention in the cell wall of *S. cerevisiae* (Rossini et al., 1993; Venturini et al., 1997). In one case, it was shown that deletion of the cell wall cross-linking enzyme glycoprophospholipid-anchored surface protein (Gas1p), leads to an almost seven-fold increase in the level of human insulin-like growth factor 1 (hIGF1) in *S. cerevisiae* (Vai et al., 2000). Gas1p is a glycoprotein anchored to the outer layer of the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor (Popolo & Vai, 1999). The enzymatic function of Gas1p is a β-1,3-glucanosyltransglycosylase activity, a cross-linking enzyme that catalyzes a transglycosylation with β-1,3-glucan as a substrate (Mouyna et al., 2000a). Disruption of GAS1 results in several morphological defects: yeast cells lose their typical ellipsoidal shape, become larger than wild-type cells, also being defective in bud maturation and in cell separation. The cell wall of GAS1 null mutants is highly resistant to zymolyase (β-1,3-glucanase), more sensitive to weakening by cell wall-perturbing agents such as...
Calcofluor White (Lesage et al., 2005) and the cells are less protected against osmotic destabilizing agents such as sodium dodecyl sulfate (SDS) (Vai et al., 1996).

The identification of Gas1p homologues in yeast species, fungi and human fungal pathogens has led to the definition of a new family of glycosyl hydrolases (family GH-72) (http://afmb.cnrs-mrs.fr/CAZY/fam/GH72.html) on the basis of sequence similarity. Moreover, PHR1 of Candida albicans or GEL1 or GEL2 of Aspergillus fumigatus corrects the mutant phenotype of a GAS1 null mutant, indicating that these homologues are not only structurally but also functionally similar (Vai et al., 1996; Mouyna et al., 2000a, 2005).

As the factors limiting protein folding and secretion are interdependent, it is not trivial to optimize such a system dependent on several variables. A practical solution is to identify the major bottleneck of a production system, which, in general, is both host strain and product dependent. This bottleneck may overrule the influence of other variables and thus limit the productivity of a system irrespective of optimization of other variables.

As for this work, the given production strains were already optimized in respect of promoter, gene copy number, secretion signal as well as fermentation media and process development (Hohenblum et al., 2003, 2004; Resina et al., 2004, 2005), the major target was the modulation of the cell wall composition to improve the secretion into the culture supernatant. With the intention to broaden the basis by the evaluation of potential effects of cell wall engineering, three model proteins were tested: human trypsinogen (hTryp), human serum albumin (HSA) and Rhizopus oryzae lipase (ROL).

**Materials and methods**

**Strains and media**

*Saccharomyces cerevisiae* W303-1B (MATα ade2-1 can1-100 ura3-1 leu2-3, 112 trp1-1, his3-11,15); *S. cerevisiae* WB2d (gas1::LEU2) generated from strain W303-1B by a one-step gene disruption procedure (Vai et al., 1996); *P. pastoris* X-33 (wild type), GS115 (his4); GS115+pPICZzA_B_rh trypsinogen1 (his4; expresses secreted hTryp; Hohenblum et al., 2003), GS115+pPICZzA_HSA (expresses secreted HSA), X-33+pPICZFLDzROL (expresses secreted ROL under the transcriptional control of the PFLD1 promoter; Resina et al., 2004). Escherichia coli TOP10F’ (Invitrogen), E. coli Novablu (Novagen).

Standard media were used if not otherwise specified. Special media were as follows:

- YPGR-SDS: 2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) galactose, 1% (w/v) raffinose and 0.0075% (w/v) SDS. YNBGR-Congo Red: 0.67% (w/v) YNB (Difco), 2% (w/v) galactose, 1% (w/v) raffinose and 0.005% (w/v) Congo Red. For screening of hTryp and HSA-expressing clones, preculture medium and main culture medium according to Hohenblum et al. (2004) were used. For screening of ROL expression levels, the following media were used instead: BMS (buffered minimal sorbitol) containing 1% (w/v) sorbitol, 1.34% (w/v) YNB without amino acids and ammonium sulfate, 0.4% (w/v) methylamine hydrochloride, 4 × 10⁻³ (w/v) biotin and 100 mM potassium phosphate pH 6.0.

**Vectors**

pSTBlue-1: standard cloning vector (Novagen). pYX212: an *S. cerevisiae* multicopy expression vector containing the *S. cerevisiae* triose phosphate isomerase (TPI) promoter, the 2μm origin and the URA3 auxotrophic selection marker (R&D Systems). pFA6a_kanMX4 carries the kanamycin resistance gene, which confers resistance to the drug gentamicin (G418) in yeast, flanked by the PTEF promoter and the TTEF terminator of Ashbya gossypii (Wach et al., 1994).

**DNA manipulation**

If not stated otherwise, standard procedures were used for DNA manipulation. PCR was carried out with KOD XL Polymerase supplied by Novagen, according to the Novagen user protocol.

Transformation of *S. cerevisiae* by the LiAc/ss carrier DNA/PEG method was performed following the protocol of Gietz & Woods (2002) (see also www.umanitoba.ca/faculties/medicine/biochem/gietz/method.html).

Transformation of *P. pastoris* by electroporation was performed following the standard protocol provided by the Pichia manuals from Invitrogen.

**Preparation of plasmid DNA from yeast**

Total DNA was isolated from yeast cells according to Hoffman & Winston (1987) (www.fhcrc.org/science/labs/gotts/ch/yeast/qgprep.html). Subsequently, the prepared total DNA was purified with the GFX PCR DNA Purification Kit supplied by Amersham Biosciences. To obtain the desired plasmid DNA, *E. coli* was transformed with an aliquot of the GFX eluate.

**Small-scale cultivation of *P. pastoris***

Selected clones of GS115+pPICZzA_B_rh trypsinogen1 and GS115+pPICZzA_HSA were grown in 5 mL of preculture
medium overnight at 28 °C and used to inoculate 5 mL main culture medium at an OD<sub>600 nm</sub> = 0.1. Expression of hTrp and HSA was induced by adding 1% (v/v) methanol. This step was repeated every 12 h. After 48 h shaking at 28 °C, the OD<sub>600 nm</sub> was measured and the supernatants were harvested for analysis. Expression of ROL was induced in BMS medium by 0.4% (w/v) methyleamine hydrochloride.

### Fed-batch cultivations of *P. pastoris*

High cell density fed-batch cultivations of *P. pastoris* strains expressing ROL were carried out using a mineral medium and the operational conditions described in Resina et al. (2005). The fed-batch phase was carried out under carbon-excess conditions (i.e. at a specific growth rate near μ<sub>max</sub>), where the carbon source, sorbitol, was maintained at about 8 g L<sup>-1</sup> by manually programming the substrate feeding rate according to the consumption rate estimated from the offline-measured residual sorbitol concentration in the cultivation broth. During this phase, methyleamine was used as a sole nitrogen source and inducer of the FFLD1.

### Quantification of products

Trypsinogen quantification was performed as described before (Hohenblum et al., 2004) with the TAME assay. HSA secretion was determined by SDS-PAGE, followed by Coomassie Blue staining and densitometry. Lipase activity from cell extracts and cultivation broth samples was measured using the Lipase colorimetric assay (LIP kit Ref. No. 1821792) from Roche Diagnostics (Resina et al., 2004).

### Experiments and results

#### Isolation of the *P. pastoris* homologue of GAS1

The initial strategy to isolate the *P. pastoris* homologue of GAS1 was to transform the GAS1 deficient *S. cerevisiae* strain WB2d with a cDNA expression library from *P. pastoris*. To obtain transformants carrying the desired cDNA insert, transformed cells were plated on selection medium, either containing SDS or Congo Red. Although resistant colonies could be selected, none of the clones contained an insert with any homology to GAS1.

#### PCR cloning for *P. pastoris* GAS1 homologue

As the strategy for isolating the *P. pastoris* GAS1 homologue by selection of a cDNA library did not succeed, we attempted to clone the gene by PCR. To design primers for amplification of the unknown nucleotide sequence of *P. pastoris* GAS1, an alignment of already known GAS1 coding sequences was made by the MEGALIGN program provided by DNASTAR Inc. The nucleotide sequences of the GAS1 homologues of *S. cerevisiae*, *Neurospora crassa*, *Candida dubliniensis*, *Candida glabrata*, *Candida maltosa* and *Candida albicans* were aligned to find regions of high homology within the coding sequences of the different yeast species. The result of the alignment can be seen in the supplementary data. Regions showing the highest homology were chosen for primer design (Fig. 1). To amplify subsequently the full-length cDNA, primers located on the vector pSCGAL10-SN of the *P. pastoris* cDNA library have also been designed.

First a fragment of c. 300 bp was amplified using primers gas500fw (5′-AAC ACT GAT GCT TCT GCT TTG AAG-3′) and gas800bw (5′-TTA CAA CCG TAT TCA GAG AAG AA-3′) and subcloned into vector pSTBlue-1. The nucleotide sequence of this fragment was used to design new primers with full identity to *P. pastoris* GAS1. Using these primers, the 3′ half of the gene was amplified with gas500fw (5′-TGC CTC GGC CGA GTA CTA TTG G-3′) and vector2bw (5′-TTC GGT TAG AGC GGA TGT GG-3′), and the 5′ half was amplified with primers vector2fw (5′-AGG TCC TTT GTA GCA TAA ATT ACT ATA-3′) and gas650bw (5′-ACT CTG CGG TTC TGT CCT TAT-3′). Fragments of the correct size were subcloned and sequenced. Based on the sequences obtained, the full cDNA sequence of *P. pastoris* GAS1 could be determined (Genebank accession DQ444263).

To clone the full-length genomic copy of GAS1, primers at the start and stop codons of the ORF were used: GAS1ATG (5′-AAT ATG TTT AAA TCT CTG TGC-3′) and GAS1STOP (5′-AAC ACT GAT GCT TCT GCT TTG AAG-3′). Using these primers, the 5′ half was amplified with primers gas800fw (5′-AAG AAG AAA-3′) and gas500bw. (b) Alignment region with high homology around numbers in brackets). (a) Alignment region with high homology around 800 bp downstream from the start codon, chosen for designing the primers gas500fw and gas500bw. (b) Alignment region with high homology around 800 bp downstream from the start codon, chosen for designing the primers gas800fw and gas800bw.

![](https://academic.oup.com/femsle/article-abstract/264/1/40/548767/fig1)
(5’-GTG TGC TTT TAA TTA ATG TGA ACC-3’). The genomic GAS1 fragment had the same length as the cDNA fragment, indicating that there are no introns present in the GAS1 homologue of \( P. \) pastoris. The genomic fragment was subcloned into pSTBlue-1 and sequenced with a result identical to the assembled sequences described above.

**The \( P. \) pastoris homologue complements GAS1 deletion in \( S. \) cerevisiae**

To verify the functionality of the GAS1 homologue of \( P. \) pastoris in \( S. \) cerevisiae, the \( P. \) pastoris GAS1 insert was cloned into the \( S. \) cerevisiae 2 \( \mu \)m plasmid pYX212 and transformed into the GAS1-deficient \( S. \) cerevisiae strain WB2d by the LiAc/ss carrier DNA/PEG method. Expression of the \( P. \) pastoris GAS1 homologue in WB2d converted the morphological phenotype (round cells with daughter cells attached to the mother cell) back to wild type (data not shown). Additionally, transformants were spotted in serial 1:10 dilutions on agar plates with selection media YPGR-SDS and YNBGR-Congo Red, respectively. While the untransformed WB2d GAS1-deficient strain did not show any growth on selection medium, colonies were observed from the W303-1B strain and the WB2d transformants carrying the pYX212-\( Pp \)GAS1 vector (Fig. 2). Both the morphological and growth phenotype were restored, thus providing evidence that the GAS1 homologue of \( P. \) pastoris is functional in \( S. \) cerevisiae.

**GAS1 disruption in \( P. \) pastoris**

The kanMX4 marker cassette conferring resistance to G418 (Wach et al., 1994) was inserted into the BoxI restriction site of the \( P. \) pastoris GAS1 homologue, interrupting the gene leaving homologous ends of c. 800 bp each flanking the selection marker for homologous recombination. The \( P. \) pastoris strains X-33, X-33+\( P \)pICZFZn\( D \)zROL, GS115, GS115+\( P \)pICZFZn\_rh trypsinogen1, GS115+\( P \)pICZFZnA_\_HSA, were transformed by electroporation with the linearized disruption cassette and plated on YPDS-agar containing Geneticin (G418; 250 \( \mu \)g mL\(^{-1}\)). Colonies appeared after 3 days at 30°C. The inactivation of GAS1 was verified by colony PCR and microscopy, showing the same morphology of two buds on mother cells as \( \Delta \)GAS1 \( S. \) cerevisiae (Fig. 3).

**Overexpression of trypsinogen and albumin in \( \Delta \)GAS1 strains**

Twenty clones of GS115+\( P \)pICZFZn\_rh trypsinogen1 \( \Delta \)GAS1 and four clones of the parental strain GS115+\( P \)pICZFZn\_rh trypsinogen1 were precultivated on preculture medium, and induced with methanol (see Small-scale cultivation of \( P. \) pastoris).

The amount of recombinant human trypsinogen in the supernatant of the shake flask cultures was determined with the TAME assay. Statistical analysis by a \( t \)-test revealed that there was no significant difference between the specific productivity of trypsinogen of the two compared populations at a confidence level of \( p = 0.05 \). These results provide evidence that the GAS1 disruption in hTryp secreting \( P. \) pastoris did not have a significant effect on secretion of hTryp. Similarly, no increase of HSA secretion was detected in GAS1-deleted clones as compared with the respective parental strains.

**ROL extracellular expression in a \( P. \) pastoris \( \Delta \)GAS1 strain**

Four clones of X-33+\( P \)pICZFZFLDzROL \( \Delta \)GAS1 and four clones of the parental strain X-33+\( P \)pICZFZFLDzROL were precultivated in shake flasks using BMS medium, and methylamine as an inducer substrate. The amount of secreted recombinant ROL in culture supernatants was tested after 24 h. The \( \Delta \)GAS1 transformants showed an average increase of 20% in specific activity levels (0.12 ± 0.031 vs. 0.10 ± 0.006 AU/OD). The X-33+\( P \)pICZFZFLDzROL \( \Delta \)GAS1 with the highest specific activity

![Fig. 2. Spotting of Saccharomyces cerevisiae cell suspensions on selection media inhibiting the growth of GAS1-deficient strains. Two millilitres of three dilution steps (1:10) of a colony suspension were spotted on agar plates of (a) YPGR-SDS medium, and (b) YNBGR-Congo Red medium. The strains used were: (1) \( S. \) cerevisiae W303-1B; (2) \( S. \) cerevisiae WB2d, GAS1 deficient. (3–5) Three clones of \( S. \) cerevisiae WB2d transformed with an expression plasmid (pYX212) carrying the \( Pichia \) pastoris GAS1 homologue.](https://academic.oup.com/femsle/article-abstract/264/1/40/548767)

![Fig. 3. Morphological phenotype of Pichia pastoris wild-type and \( \Delta \)GAS1 strains. (a) Strain GS115. (b) Strain GS115 \( \Delta \)GAS1. The \( \Delta \)GAS1 cells show the typical phenotype by two buds attached to the mother cell (Mickey Mouse-like appearance) indicated with arrows, while the wild-type cells show normal budding morphology.](https://academic.oup.com/femsle/article-abstract/264/1/40/548767)
was selected for further expression studies in fed-batch cultivations.

Previous studies (Resina et al., 2005) have shown that ROL production and productivity levels in high cell density fed-batch cultivations are best when the induction phase (when cells are growing on sorbitol and methylamine as a carbon and nitrogen source, respectively) is carried out under carbon-excess conditions, i.e. near $\mu_{\text{max}}$ (about 0.025 h$^{-1}$). Therefore, we performed a fed-batch cultivation with the new strain using the same conditions. The results are summarized in Fig. 4. The $P. pastoris$ X-33 $\Delta$GAS1 strain performed significantly different when compared with the corresponding parental strain grown under the same conditions (Resina et al., 2005): first, the maximum specific growth rate achieved by the $\Delta$GAS1 strain during the induction phase was significantly lower compared with the parental strain (0.01 vs. 0.02 h$^{-1}$). Second, the final extracellular lipase activity levels were about 20% higher after 90 h of cultivation with the $\Delta$GAS1 strain, but intracellular activity levels per cellular protein remained consistently lower (c. 0.25 vs. 0.5 AU µg$^{-1}$) after 60 h of cultivation. Third, the specific production rates ($q_p$) remained almost constant during virtually the entire induction phase of the $\Delta$GAS1 strain cultivation, in contrast to the situation in the cultivations performed with the parental strain, where a sharp decrease in $q_p$ values was observed shortly after the start of the induction phase.

A comparative analysis of the fed-batch cultivations carried out with the parental and $\Delta$GAS1 strains in terms of $Y_{P/X}$, productivity and specific productivity (Table 1) revealed that the overall efficiency of the system increased significantly in the $\Delta$GAS1 strain: twofold in terms of $Y_{P/X}$ and specific productivity, and 1.3-fold in terms of productivity. The performance of the $\Delta$GAS1 strain was compared with that obtained with two strains expressing ROL under the classical PAOX promoter (Cos et al., 2005) grown under the same conditions (Table 1). The best $Y_{P/X}$, productivity and specific productivity levels were achieved with the $\Delta$GAS1 strain, strongly suggesting that the cell wall poses a critical bottleneck on ROL secretion.
intra/interdomain disulfide bonds (Carotti (C74, C103, C216, C234 and C265) that might be involved in recognition and orientation/activation of the nucleophilic glu-

2004), residues corresponding to R90, G264 and Y231 of GAS1, P. pastoris GAS1 displays the typical modular organization of the other proteins of the coding sequences of the GAS1 family GH72: an N-terminal secretory signal sequence, a polypeptide with all the features of the other proteins of the same family to identify regions of high homology. With this approach, the entire P. pastoris GAS1 homologue could be amplified and sequenced.

The nucleotide sequence of GAS1 encodes a 539 aa polypeptide with all the features of the other proteins of the family: an N-terminal secretory signal sequence, several putative N-glycosylation sites, a COOH-terminal region rich in serine residues that are potential sites for O-glycosylation and a hydrophobic carboxyl terminus characteristic of GPI-anchored proteins. In addition, a multiple amino acid sequence alignment revealed that P. pastoris Gas1p displays the typical modular organization of the other members of the family. In the catalytic domain, all the strictly conserved residues are present (Papaleo et al., 2006), in particular, in the A/S-G-N-E-V/I and S-E-Y/F-G-C patterns, the two glutamic residues (E161 and E262, S. cerevisiae Gas1p numbering) that are essential for the enzymatic activity (Mouyna et al., 2000b; Carotti et al., 2004), residues corresponding to R90, G264 and Y231 of S. cerevisiae Gas1p that might be important for substrate recognition and orientation/activation of the nucleophilic glutamic catalytic residue (Papaleo et al., 2006) and five cysteines (C74, C103, C216, C234 and C265) that might be involved in intra/interdomain disulfide bonds (Carotti et al., 2004).

The cloned gene was expressed in the GAS1-deficient S. cerevisiae strain WB2d. Reversion of the phenotype back to wild type proved that this homologue is not only structurally but also functionally similar to the S. cerevisiae gene.

Disruption of GAS1 in P. pastoris revealed the influence of the gene on the cell wall morphology. The GAS1 null mutants of P. pastoris showed the same phenotype as reported for S. cerevisiae (Popolo et al., 1993), where budded cells frequently appear with two buds (Mickey Mouse-like appearance). The similarity of the GAS1 null mutant phenotypes and the functionality of the P. pastoris GAS1 homologue in S. cerevisiae indicate the similarity of the cell wall structure of P. pastoris compared with the cell wall structure of S. cerevisiae.

To improve the expression level of a heterologous secreted protein systematically, the synthetic pathway of the protein from transcription over translation to secretion, has to be observed (Sreekrishna et al., 1997). As outlined in the Introduction, the production strains used in this work were already optimized regarding the promoter, gene copy number, secretion signal as well as fermentation media and process development. Considering the secretory pathway of a secreted protein, it was speculated whether the protein becomes retarded in the cell wall after release from the plasma membrane.

The disruption of GAS1 in P. pastoris production strains for hTryp and HSA did not show an enhancement of product secretion, whereas ROL secretion could be improved twofold. The rationale behind the selection of the model proteins for this study was significant differences between the secretory behavior of these proteins. hTryp is retained in the ER at significant levels without showing enzymatic activity (Hohenblum et al., 2004), while HSA is secreted at very high levels without being retained in the cells. ROL was detected in the cells as well, with significant levels of active enzyme in cell lysates (Resina et al., 2005).

Discussion

The manipulation of the cell wall permeability can improve the release of heterologous products from yeast (Ferrer et al., 1998). Consequently, among the targets for strain improvement are the enzymes, such as GAS1, that mediate the cross-linking of cell wall components.

The first strategy to isolate the up to now unknown P. pastoris homologue of that gene, was to clone the gene by complementation of GAS1-deficient S. cerevisiae cells. As this strategy did not result in any clone related to GAS1, another cloning strategy was used, based on the alignment of the coding sequences of the GAS1 homologues of six yeast species to identify regions of high homology. With this approach, the entire P. pastoris GAS1 homologue could be amplified and sequenced.

P. pastoris GAS1, ROL under control of the AOX1 promoter, MutT phenotype; PAXO MutT, ROL under the AOX1 promoter, MutT phenotype; PFLD1, untransformed GAS1-positive strain expressing ROL under the RLD1 promoter; PFLD-AGAS1, strain deleted in GAS1, expressing ROL under the RLD1 promoter; AU max, maximal ROL activity achieved; Ymx, maximal ROL activity per biomass; mean, average specific growth rate; Productivity, ROL activity per volume and per time; Specific productivity, ROL activity per biomass and per time.

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<th>Strain</th>
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<th>Ymx (AU g⁻¹)</th>
<th>μ mean (h⁻¹)</th>
<th>Productivity (AU L⁻¹ h⁻¹)</th>
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<td>0.010</td>
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*Data taken from Cos et al., 2005
'Data taken from Resina et al., 2005

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</table>

The nucleotide sequence of GAS1 encodes a 539 aa polypeptide with all the features of the other proteins of the family: an N-terminal secretory signal sequence, several putative N-glycosylation sites, a COOH-terminal region rich in serine residues that are potential sites for O-glycosylation and a hydrophobic carboxyl terminus characteristic of GPI-anchored proteins. In addition, a multiple amino acid sequence alignment revealed that P. pastoris Gas1p displays the typical modular organization of the other members of the family. In the catalytic domain, all the strictly conserved residues are present (Papaleo et al., 2006), in particular, in the A/S-G-N-E-V/I and S-E-Y/F-G-C patterns, the two glutamic residues (E161 and E262, S. cerevisiae Gas1p numbering) that are essential for the enzymatic activity (Mouyna et al., 2000b; Carotti et al., 2004), residues corresponding to R90, G264 and Y231 of S. cerevisiae Gas1p that might be important for substrate recognition and orientation/activation of the nucleophilic glutamic catalytic residue (Papaleo et al., 2006) and five cysteines (C74, C103, C216, C234 and C265) that might be involved in intra/interdomain disulfide bonds (Carotti et al., 2004).
These observations are in line with the results of the present work, as obviously ROL is partially retained in the cell in an active form, which is released at higher levels in GAS1-deleted strains. HTrpy, on the other hand, is mainly retained due to constraints in the folding pathway, while HSA is readily released from the cell, proportional to the promoter strength and gene copy number.

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**Supplementary material**

The following supplementary material is available for this article:

Fig. S1. Alignments of GAS1 homologues from *S. cerevisiae* (NC_001145/locus_tag = “YMR307W”), *N. crassa* (XM_953585), *C. dubliniensis* (AF184908), *C. glabrata* (AJ302061), *C. albicans* (AF011386) and *C. maltosa* (AB011286) used for the design of PCR primers (Genebank accession numbers in brackets).

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2006.00427.x (This link will take you to the article abstract).

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