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## Regulated expression of green fluorescent protein in *Debaryomyces hansenii*

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**Abstract** The broad range of environmental conditions under which *Debaryomyces hansenii* can grow, and its production of lipolytic and proteolytic enzymes, have promoted its widespread use. The present work represents a preliminary characterization of *D. hansenii* for heterologous expression and secretion of green fluorescent protein (GFP). Six heterologous expression vectors were used to address protein production efficiency under regulated expression conditions. Protein expression in *D. hansenii* seems to be similar to that in *Saccharomyces cerevisiae*, with transcription being controlled by almost all of the *S. cerevisiae* and *D. hansenii* inducible promoters tested, with the exception of the alcohol dehydrogenase 2 gene promoter from *S. cerevisiae*. Extracellular protein levels in *D. hansenii* were lower than in *S. cerevisiae* when *Saccharomyces* signal peptides were used.

**Keywords** *Debaryomyces hansenii* · GPD1 · Glycerol-3-*P*-dehydrogenase

### Introduction

*Debaryomyces hansenii* is a ubiquitous unicellular haploid yeast that is commonly found in freshwater and

seawater [10, 23, 30], as a parasitic opportunistic organism in fish and humans [29, 36, 49, 58], and in vegetable, animal, and high sugar food products [53]. *D. hansenii* survives in high salt environments [1, 2, 30], digests aromatic hydrocarbons such as naphthalene, biphenyl, and benzo(a)pyrene [13], and survives in the presence of heavy metals such as cobalt, copper, zinc, and iron [20].

This yeast is also an important co-starter for the cheesemaking industry [19] due to its proteolytic and lipolytic activity, its compatibility and stimulating action with lactic acid starter cultures, inhibition of growth of spoilage bacteria [9, 16, 18, 34, 38, 45] and tolerance to changes in salt conditions. *D. hansenii* is one of the most common yeast species found in different types of cheese. *D. hansenii* has also become an important component in the wine-making industry due to its beta-glucosidase activity and its capacity of releasing flavor compounds from glycosidically bound nonvolatile precursors in wine [6, 44, 50].

*Debaryomyces hansenii*, in addition, is one representative of the 14 non-*Saccharomyces* yeast species chosen for the Génolevures project (genomic exploration of the hemiascomycetous yeasts) [17, 31]. Génolevures is a large-scale comparative genomic project between *Saccharomyces cerevisiae* and 14 other yeasts species of the various branches of the *Hemiascomycetous* class chosen to explore eukaryotic genome evolution and to address basic questions such as rate of divergence, distribution of genes among functional families, species-specific and class-specific genes, and mechanisms of chromosome shuffling, among others.

All these features, coupled with its ease of culturing (almost as easy as *S. cerevisiae*), make *D. hansenii* a potentially attractive model for biotechnological applications [43]. The present work represents a preliminary characterization of *D. hansenii* as a potential organism for heterologous expression and secretion using plasmid-based shuttle vectors.

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## Materials and methods

### Bacterial and yeast strains

*Escherichia coli* strain XL1 Blue (Stratagene, La Jolla, Calif.) was used for plasmid propagation and amplification. *S. cerevisiae* strain 288C [ $\alpha$ MAT, *ura3-52*] was obtained from the American Type Culture Collection (ATCC 90971). *S. cerevisiae* strain YH 252-1 [ $\alpha$ MAT, *ura3-52*, *leu2-3*, *ade1-X*, *pbs2*, *-112 suc2-9*], was obtained from L. Adler (University of Gotenborg, Sweden). *D. hansenii* strain NRRL Y 7426 was obtained from Dr. C. Kurtzman (National Center for Agriculture Utilization Research, US Department of Agriculture, Peoria Ill.). *D. hansenii* strain 7426-1(*ura3<sup>-</sup>*) was isolated and characterized by Ricaurte and Govind [43]. The above yeast strains were used to test all the heterologous expression vector constructs.

### Transformation

*Escherichia coli* strain XL1 blue was transformed by either electroporation or calcium chloride methods as described in [7]. *S. cerevisiae* and *D. hansenii* were transformed by electroporation [7].

### Chemicals and enzymes

Yeast nitrogen base, D-glucose, tryptone, peptone, ampicillin, chloroform, phenol, and sodium chloride were purchased from Sigma (St. Louis, Mo.). Bacto-agar was purchased from Difco (Detroit, Mich.). Yeast extract, chloramphenicol, agarose, and Tris-base were purchased from Fisher Scientific (Pittsburgh, Pa.). *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Sac*I, *Sal*I, *Sma*I, and *Xma*I restriction enzymes, *Taq* DNA polymerase, T4 DNA ligase and their buffers were purchased from Roche Applied Science (Indianapolis, Ind.). Oligonucleotide synthesis was performed in a Beckman Oligo 1000 DNA Synthesizer (GMI, Albertville, Minn.) as per the manufacturer's instructions.

### Culture media

*Escherichia coli* cell cultures were grown at 37°C with shaking in LB medium (1% tryptone, 0.5%

yeast-extract, 1% NaCl) with the appropriate selection supplement, until the desired optical density was reached. *S. cerevisiae* and *D. hansenii* yeast cells were grown at 30°C to log phase in YPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% bacto-agar) or in a synthetic yeast nitrogen base (YNB) medium supplemented with the necessary growth requirements.

Yeast transformants were grown initially in SD medium composed of 0.67% bacto-yeast nitrogen base without amino acids, 0.02% of selected amino acids mixture, 2% glucose, and 1 M sorbitol. SD medium (without sorbitol) was used for the assessment of basal level expression. Level of green fluorescent protein (GFP) expression by promoter activation was performed by supplementation of each required inducer after 24 h of growth (when almost all the glucose was depleted). Typically, liquid and solid SD media were as follows: non-supplemented for basal level expression and *CyC1* gene promoter activation or supplemented with either 2% ethanol for *ADH2* promoter activation, 2–16% NaCl for *GPD1* and *GdPD1* promoter activation, 1% potassium acetate for *SME1* promoter activation, or exposure to changes in temperature (from 30 to 37°C) for *HSP12* promoter activation (Table 1).

### Bacterial plasmid DNA extraction and analysis

After transformation and selection for ampicillin resistance, plasmid extraction from *E. coli* XL1 blue was performed using the a plasmid purification kit (Qiagen, Chatsworth, Calif.). Final plasmid preparations were performed by resuspension of plasmid in distilled water. Plasmid sizes and orientation of sequence inserts were analyzed by restriction enzyme digestion followed by 1.5% agarose gel electrophoresis.

### Plasmid constructions

Six yeast expression plasmids were constructed for *D. hansenii* using five inducible (regulated) heterologous promoters (from *S. cerevisiae*) and one endogenous gene promoter. A red-shifted mutant of the GFP from the jellyfish *Aequorea victoria* [14, 46, 59] was used as a reporter gene and extracellular targeting was directed by the signal sequence (leader peptide) of the  $\alpha$  galactosi-

**Table 1** Promoters used in this study and their inducers

Promoter	Description	Organism	Inducer/activator
ADH2	Alcohol dehydrogenase II	<i>Saccharomyces cerevisiae</i>	Non-fermentable carbons (ethanol, glycerol, raffinose); temperature shift 30–25°C
CYC1	Isocytochrome C I	<i>S. cerevisiae</i>	Barely constitutive; temperature shift 30–25°C
GPD1	Glycerol 3-P-dehydrogenase I	<i>S. cerevisiae</i>	Osmotic changes (presence of NaCl, glycerol)
GPD1d		<i>Debaryomyces hansenii</i>	
HSP12	Heat shock protein 12	<i>S. cerevisiae</i>	Temperature shift 25–35°C; osmotic changes (presence of NaCl, glycerol)
SME1	Protein kinase I	<i>S. cerevisiae</i>	Potassium acetate and stationary phase entrance

dase enzyme from *S. cerevisiae* [15, 48]. The inducible promoters from *S. cerevisiae* used in this study were alcohol dehydrogenase ADH2 [40, 41], cytochrome CyC1 [12, 24–26], glycerol-3-phosphate dehydrogenase GPD1 [3–5, 11, 35, 39], heat shock protein HSP12 [21, 27, 28, 32, 55, 56], and protein kinase SME 1 [60]. From *D. hansenii*, partial sequence of the glycerol-3-phosphate (GPD1) promoter was recently published [52] (GenBank accession number AX333427). Independently, our laboratory has isolated over 1,000 bp of the upstream sequence the GPD1 (GenBank accession number AY 570295); 500 bp of this extended upstream sequence was used as the endogenous GPD1d promoter. In addition, a 3' terminal sequence of 192 bp of the alpha mating pheromone factor gene from *S. cerevisiae* [22] was cloned in frame with GFP gene to ensure proper transcriptional termination.

All the expression plasmid constructions were based on plasmid pRGM (see below). The pRGM plasmid was designed to harbor the URA3 auxotrophic marker for selection in yeast, the ampicillin resistance gene for selection in bacteria, an autonomous replicating sequence from *D. hansenii* and an origin of replication for bacteria (Fig. 1). The URA3 auxotrophic marker gene from *S. cerevisiae* and the autonomously replicating sequence (ARSD) from *D. hansenii* were obtained from plasmid pAB83 [23]. The construction of pRGM was based on plasmid pUC19 (GenBank accession number M77789), which provided a series of unique restriction enzyme sites for directional cloning of all the sequences required for protein expression and export.

#### Signal sequence and GFP gene cloning

The 54 bp of the  $\alpha$ Gal signal sequence was incorporated at the 5' end of the GFP gene by PCR amplification. The signal sequence-GFPm3.1 gene amplification was performed using pGFPm3.1 plasmid DNA as template (pGFPm3.1 is commercially available from Clontech, Palo Alto, Calif.). Primers were designed to incorporate the signal sequence at the 5' end of the GFPm3.1 gene as well as *Sma*I and *Sac*I restriction enzyme sites at the 5' and 3' ends, respectively, to allow directional cloning. A *Bam*HI restriction enzyme site was also incorporated flanking the GFP gene. Primers sequences were 5' CCCGGGATGTTTGCTTTCTACTTTCTCACCGCATGCATCAGTTTGAAGGGCGTTTTTGGGGGATCATGCGTAAAGGAGAAGAAC 3' and 5' GAGCTCGGATCCATTTGTATAGTTCATCCAT 3' as sense and antisense primers, respectively. Amplification was performed using a 94°C, 5 min hot start, followed by 30 cycles of 94°C, 30 s denaturing, 38°C, 45 s annealing and 74°C, 45 s extension. After digestion of the PCR product ( $\alpha$ Gal-GFP cassette) and plasmid pRGM with *Xma*I and *Sac*I enzymes, the  $\alpha$ Gal-GFP cassette was cloned into pRGM plasmid by ligation overnight at 15°C with T4 DNA ligase. The resulting plasmid, pRGM $\alpha$ Gfp (Fig. 1), was used to transform *E. coli* XL1

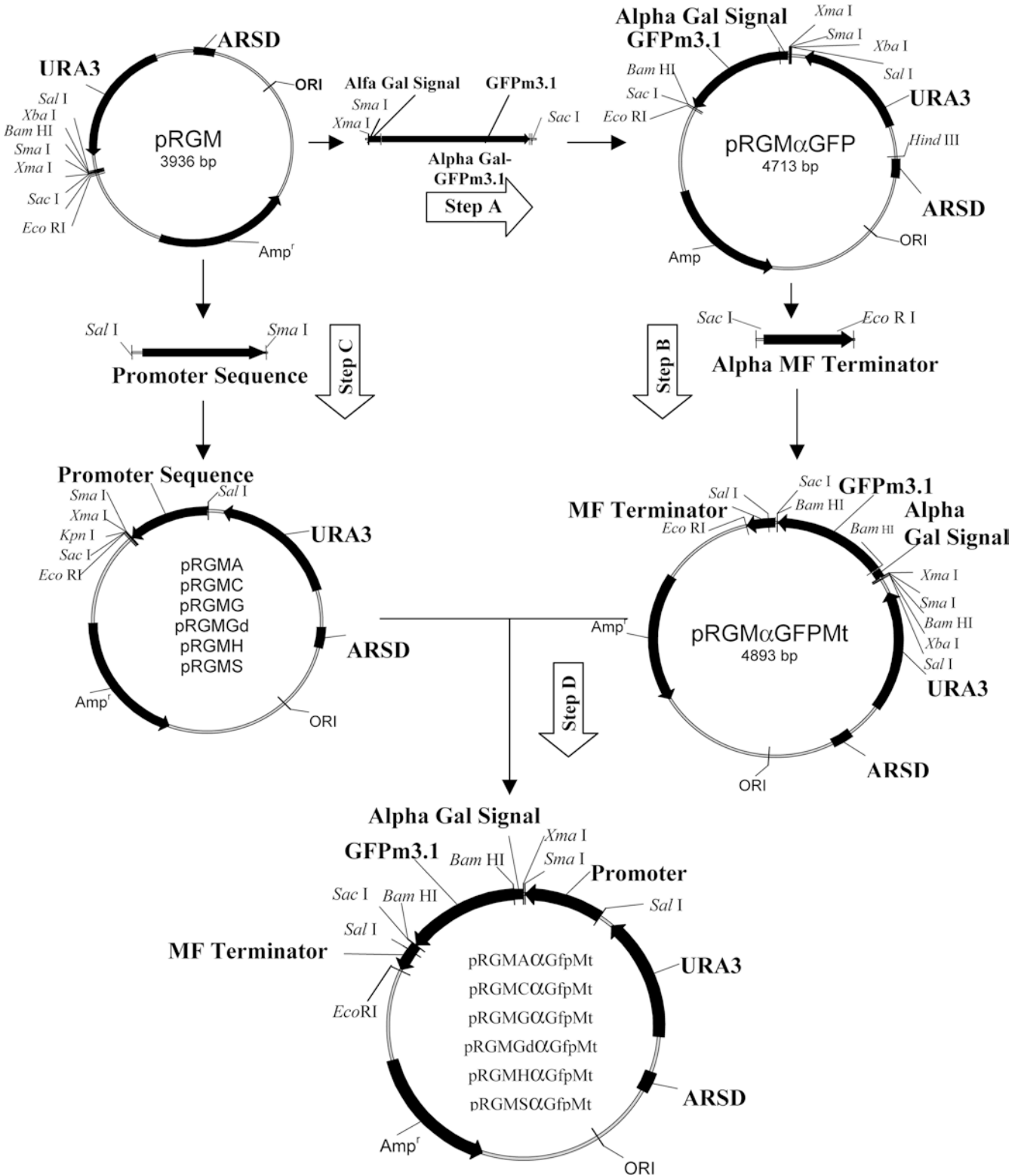
Blue, and colonies were screened for ampicillin resistance. Plasmid size and  $\alpha$ Gal-GFP cassette insert orientation were analyzed by plasmid miniprep and enzyme digestion followed by 1.5% agarose gel electrophoresis (results not shown).

#### Terminator sequence

Mating factor terminator sequence amplification was performed by PCR techniques in which *S. cerevisiae* DNA was used as template. Each terminator primer was designed to incorporate *Sac*I and *Eco*RI restriction enzyme sites at the 5' and 3' ends, respectively. Primer sequences were 5'-GAGCTCTAAGCCCCGACTG-ATAACAACA-3' and 5'-GAATTCATAGCTATATAAAGTATGTGTA-3' as sense and antisense primers, respectively. Amplification was performed using 94°C, 5 min hot start, followed by 30 cycles of 94°C, 30 s denaturing, 32°C, 30 s annealing and 74°C, 15 s extension. PCR product and pRGM $\alpha$ Gfp plasmid were double digested with *Sac*I and *Eco*RI restriction enzymes. After digestion, the terminator sequence amplicon was cloned into pRGM $\alpha$ Gfp between *Sac*I and *Eco*RI by ligation using T4 DNA ligase enzyme at 15°C overnight. The resulting pRGM $\alpha$ GfpMt plasmid (Fig. 1) was used to transform *E. coli* XL1 Blue and colonies were screened for ampicillin resistance. As above, plasmid size and  $\alpha$ GfpMt cassette insert orientation were analyzed by plasmid miniprep and enzyme digestion followed by agarose gel electrophoresis (results not shown).

#### Promoter sequence cloning

Each regulated gene promoter region was amplified by PCR using *S. cerevisiae* or *D. hansenii* genomic DNA as template. A 380 bp sequence of GPD1d from *D. hansenii* (GenBank accession number AY570295) was amplified using 5'-GTCGACTAGGCCTAGCAAATCACATACGCC-3' and 5'-CCCGGGTAA TCCGAATGTCTAATGATGTCATCATT-3' as sense and antisense primers, respectively, under the following conditions: hot start 95°C, 5 min; 30 cycles of 94°C, 30 s denaturing, 32°C, 45 s annealing and 74°C, 60 s extension, followed by a final extension of 74°C, 10 min. The same PCR conditions were used for the amplification of the following promoters from *S. cerevisiae*: a 512 bp sequence of ADH2 (GenBank accession number J01314) using 5'-GTCGACTTATCTAAAATTGCCTTATGATCCG-3' and 5'-CCCGGGTGTGTATTACGATATAGTTAATA-3' as sense and antisense primers respectively, a 384 bp sequence of CyC1 (GenBank accession number X03472) using 5'-GTCGACGCAAGATCAAGATGTTTCA-3' and 5'-CCCGGGTATTAATTTAGTGTGTGATT-3' as sense and antisense primers, respectively, a 320 bp sequence of GPD1 (GenBank accession number Z24454) using 5'-GTCGACCCCCCTCCTGCGCGCGG-CCTCCC-3' and 5'-CCCGGGCTTTATATTATCAA-



TATTTGTG-3' as sense and antisense primers respectively, a 486 bp sequence of HSP12 (GenBank accession number X55785) using 5'-GTCGACTAGAAGCCAA-AAGCCAGGGCGGT-3' and 5'-CCCGGGTTTTGT TTTGAGTTGTTTGTGTTGAGATTATCG-3' as sense and antisense primers, respectively, and a 560 bp sequence

of SME1 (GenBank accession number X53262) using 5'-GTCGACTTTATGTTACGGCGGCTATTTGAGT-TTTTG-3' and 5'-CCCGGGAAATGACCTATTAAG-TTAAGCTTAGTACTCTTCTT-3' as sense and antisense primers, respectively. Promoter primers were designed to incorporate *Sal*I and *Sma*I restriction enzyme sites at the

**Fig. 1** Heterologous expression vector construction. Steps: *A* Alpha Gal Signal-GFPm3.1 amplicon was cloned into *SmaI-SacI* sites of plasmid pRGM, *B* mating factor terminator sequence was cloned into *SacI-EcoRI* sites of plasmid pRGM $\alpha$ GFP, *C* promoter sequence amplicons were cloned into *SalI-SmaI* sites of plasmid pRGM, *D* alpha Gal signal-GFPm3.1-MF terminator cassette were cloned into *SmaI-EcoRI* sites of plasmids pRGM $\alpha$ GFP, pRGM $\alpha$ GFP, pRGMG, pRGMGd, pRGMH, and pRGM $\alpha$ S. *ORI* *Escherichia coli* origin of replication, *URA3* uracil auxotrophic marker, *Amp<sup>r</sup>* ampicillin resistance, *ARSD* autonomous replicating sequence from *Debaryomyces hansenii* (also functions in *Saccharomyces cerevisiae*), *GFPm3.1* green fluorescent protein gene

5' and 3' ends, respectively. Following double digestion with *SalI* and *SmaI* restriction enzymes, each PCR product (ADH2, CYC1, GPD1, GPD1d, HSP12, and SME1 gene promoters) was cloned in pRGM plasmid by ligation using T4 DNA ligase enzyme at 15°C overnight. The resulting plasmids, pRGM $\alpha$ GFP, pRGM $\alpha$ GFP, pRGMG, pRGMGd, pRGMH, and pRGM $\alpha$ S (Fig. 1) were screened and analyzed by plasmid miniprep and enzyme digestion followed by agarose gel electrophoresis (results not shown).

#### Expression plasmid construction

The cassette  $\alpha$ Gal-GFP-terminator sequence was extracted from pRGM $\alpha$ GfpMt after digestion with *SmaI* and *EcoRI* restriction enzymes using 1% agarose gel electrophoresis. This cassette was cloned into pRGM $\alpha$ GFP, pRGM $\alpha$ GFP, pRGMG, pRGMGd, pRGMH, and pRGM $\alpha$ S at *SmaI* and *EcoRI* sites by ligation at 15°C overnight using T4 DNA ligase. The resulting plasmids, pRGM $\alpha$ GfpMt, pRGM $\alpha$ GfpMt, pRGMG $\alpha$ GfpMt, pRGMGd $\alpha$ GfpMt, pRGMH $\alpha$ GfpMt, and pRGM $\alpha$ S $\alpha$ GfpMt (Fig. 1), were analyzed as described above.

#### Green fluorescent protein assays

##### Fluorescence microscopy

Expression of GFP in yeast cells was examined by fluorescence microscopy using a Zeiss Axioplan 2 fluorescence microscope. Cells were grown on solid synthetic complete medium (SD) and in SD with supplements to induce GFP expression through each promoter activity. The number of cells showing GFP fluorescence (as a percentage) was expressed relative to the total number of cells in a given area. Expression in both supplemented and non-supplemented growing conditions was examined and recorded using a Canon AF-1 camera with 35 mm 100 ASA print film and 15 s exposure.

##### Fluorescence photometry

GFP product expression was quantified in extracellular and intracellular fractions of transformed cells using a Bio-Rad VersaFluor fluorometer (excitation = 495 nm,

emission = 510 nm). After mid-log growth in SD and/or SD supplemented media ( $1.42\text{--}1.7 \times 10^7$  cells/ml), cells were harvested by centrifugation at 5,000 g, 4°C for 5 min. Supernatant and cells were processed for measurement of extracellular and intracellular of GFP fluorescence, respectively.

##### Extracellular fraction

Supernatant fraction (medium) was dialyzed three times in 0.1 M  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ , pH 7.4, 1 mM EDTA buffer through a 10 kDa cut-off pressure membrane (SD medium shows high fluorescence at 510 nm), and concentrated to 1/50 its original volume. Protein concentration in the supernatants was determined using a standard Bio-Rad protein assay kit. Fluorescence due to GFP vs total protein concentration in the extracellular fraction was measured during cell growth in SD medium with and without supplement.

##### Intracellular fraction

Harvested yeast cells were washed twice with 0.1 M  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ , pH 7.4, 1 mM EDTA buffer. After resuspension, cells were disrupted by three rounds of passage through a French-pressure cell [3,000 psi (20.7 MPa), 4°C]. The lysates were then centrifuged at 10,000 g 4°C for 30 min, and supernatant fluorescence due to GFP was measured vs total intracellular protein concentration.

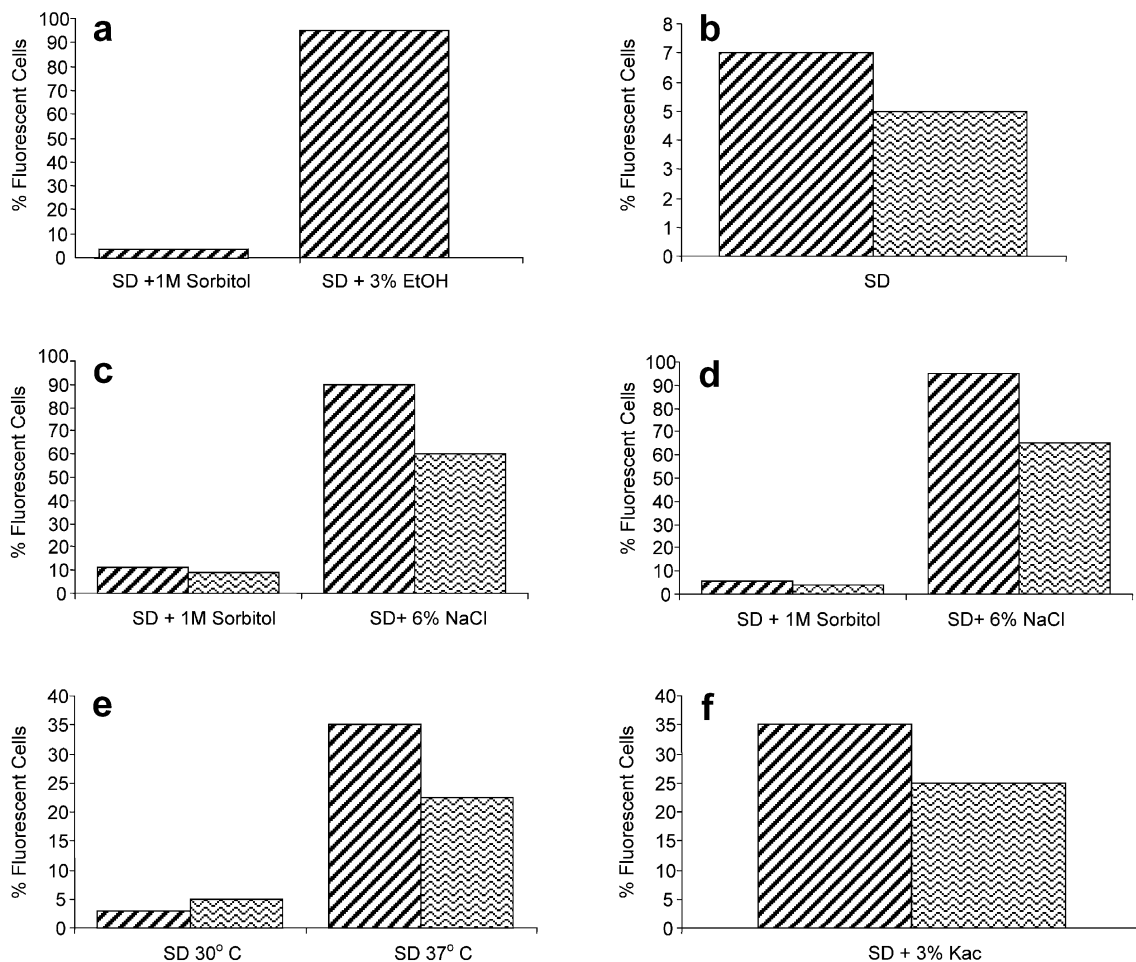
Green fluorescent protein quantification in both extracellular and intracellular fraction was reported as fluorescent units/total protein. Fold increase in GFP expression levels under activation and non-activation conditions was calculated. All in vivo experiments were conducted in duplicate.

## Results

### In vivo GFP expression

*Saccharomyces cerevisiae* SC288 and *D. hansenii* NRRL Y 7426-1 were able to express GFP in vivo under promoter activation conditions (Table 1) upon transformation of cells with all the expression plasmid constructs tested here (Fig. 2). The only exception was plasmid pRGM $\alpha$ GfpMt in *D. hansenii*, which did not express GFP when the reporter gene was under control of the ADH promoter (see below).

No GFP expression in either *S. cerevisiae* or *D. hansenii* was found when cells were transformed with plasmid pRGM or plasmids without promoters, such as pRGM $\alpha$ GfpMt and pRGM $\alpha$ Gfp, under activated and non-activated conditions. This finding suggests that GFP expression was controlled solely by the promoter sequences used and was not due to any underlying basal expression.



**Fig. 2a-f** Percentage of cells fluorescent due to GFP expression in *S. cerevisiae* Sc288 and *D. hansenii* Dh7426-1 under non-induced (SD) and induced (SD + supplement) conditions of activation for each promoter: **a** ADH2 promoter, **b** CyC1 promoter, **c** GPD1 promoter, **d** GPD1d promoter, **e** HSP12 promoter, **f** SME1 promoter. Diagonal hatching *S. cerevisiae* Sc288, wavy hatching *D. hansenii* Dh7426-1. SD Synthetic yeast medium (0.67% Bacto-yeast nitrogen base, 0.02% selected amino acids, 2% glucose), EtOH ethyl alcohol, NaCl sodium chloride, KAc potassium acetate

#### ADH2 promoter activation

Cells of *S. cerevisiae* and *D. hansenii* transformed with pRGMAzGfpMt plasmid were grown in SD, SD + 1 M sorbitol, and SD + 3% glycerol. When transformants were grown in solid SD medium without supplements, cells showed no evidence of GFP expression. When cells were grown in medium supplemented with 1 M sorbitol (as after transformation by electroporation), an average of 4% of *S. cerevisiae* cells showed GFP expression. During growth in medium supplemented with 3% glycerol as a carbon source, 95% of *S. cerevisiae* cells showed GFP fluorescence. No evidence of GFP expression under ADH2 activation in *D. hansenii* was found during activation (SD + 3% glycerol) or non-activation (SD or SD + 1 M sorbitol) conditions.

The activation of *S. cerevisiae* ADH2 gene when cells are exposed to non-fermentable carbon sources such as

glycerol or ethanol seems to have no counterpart in *D. hansenii*. Only a pentose fermentative process is known in *D. hansenii* [33, 37, 47, 51].

#### CyC1 promoter activation

*Saccharomyces cerevisiae* and *D. hansenii* cells transformed with pRGMCAzGfpMt plasmid showed GFP expression under normal growth in SD basal medium. An average of 7% of *S. cerevisiae* and 5% of *D. hansenii* cells showed fluorescence due to GFP expression. The low number of cells expressing GFP could be attributable to the low basal expression level of cytochrome *c* gene during cell growth.

#### GPD1 promoter activation

*Saccharomyces cerevisiae* (95%) cells transformed with plasmid pRGMGAzGfpMt showed fluorescence in the presence of 6% NaCl. An average of 60% of *D. hansenii* transformed cells showed fluorescence under the same conditions. No GFP expression was observed when either transformed yeast species (*S. cerevisiae* or *D. hansenii*) were grown in SD medium without salt

supplementation. A low percentage of cells (11% for *S. cerevisiae* and 9% for *D. hansenii*) expressed GFP when cells were grown in SD + 1 M sorbitol after electroporation.

#### GPD1d promoter activation

Yeasts *S. cerevisiae* or *D. hansenii* transformed with plasmid pRGMGd $\alpha$ GfpMt showed a high percentage of fluorescent cells under activation conditions. An average of 95 and 65% in *S. cerevisiae* and *D. hansenii* cells, respectively, expressed GFP during growth in medium containing 6% NaCl. As before, the number of cells showing fluorescence dropped to zero when grown in SD without NaCl, and only low number of cells (5.5 and 4% in *S. cerevisiae* and *D. hansenii*, respectively) showed GFP expression in SD + 1 M sorbitol.

The mechanism of salt acclimation in both species is very similar and involves common regulatory elements in the MAP-kinase cascade. This cascade regulates the synthesis of several enzymes in the glycerol synthesis pathway, the activation of heat shock proteins genes, as well as other enzymes, including alcohol dehydrogenase [2, 3, 4, 8, 42, 56]. Given these common regulatory elements (cis-elements) in both species, activation of expression of the reporter gene by the heterologous promoter (GPD1d in *S. cerevisiae*, and GPD1 in *D. hansenii*) can be expected.

#### HSP12 promoter activation

Yeast *S. cerevisiae* and *D. hansenii* transformed with plasmid pRGMH $\alpha$ GfpMt showed a low percentage of fluorescent cells under normal growth conditions at 30°C (3% in *S. cerevisiae* and 5% in *D. hansenii* cells). When growing conditions were changed to 37°C, *S. cerevisiae* showed GFP expression in 35% of cells, while 22.5% of *D. hansenii* cells expressed GFP under the same conditions.

As our results indicated, the common transcription activation mechanism present in both yeast species (as described above) can account for the expression of GFP in *D. hansenii* by activation of HSP12 from *S. cerevisiae*.

#### SME1 promoter activation

Under normal growing conditions (SD at 30°C), yeasts *S. cerevisiae* and *D. hansenii* transformed with plasmid pRGMs $\alpha$ GfpMt showed no evidence of GFP expression. When cells grown in SD + 3% KAc (which activates yeast stationary phase entrance), 35% of *S. cerevisiae* and 25% of *D. hansenii* showed fluorescence due to GFP expression. As above, these results suggest that both yeasts share a similar transcription mechanism for the activation of SME1 gene during the first stage of stationary phase.

#### Control of GFP expression by GPD1 and GPD1d

Intracellular and extracellular fluorescence due to the expression of GFP was measured in *S. cerevisiae* and *D. hansenii* transformed with plasmids pRGMG $\alpha$ GfpMt and pRGMGd $\alpha$ GfpMt. The results showed an increase in fluorescence relative to total proteins in both yeasts when cells were grown in medium supplemented with 0–16% NaCl.

#### GPD1 control of GFP expression

When cells were grown in 16% NaCl, intracellular GFP expression in pRGMG $\alpha$ GfpMt-transformed *S. cerevisiae* cells showed a 17-fold increase in fluorescence when compared to the initial base level (at 0% NaCl) (see Fig. 3). Dh7426-1 showed an increase of up to 18.5-fold the base level of fluorescence under the same conditions. These similarities suggest that the GPD1 promoter from *S. cerevisiae* is recognized in a similar way by the transcription machinery of both species.

In contrast, extracellular fluorescence due to GFP expression under GPD1 promoter activation showed different results in both yeast species. In *S. cerevisiae* cells, extracellular levels of fluorescence relative to total proteins at 16% NaCl reached 25-fold its base level at 0% NaCl. *D. hansenii* cells showed only a 10-fold increase under the same conditions (Fig. 3). This result suggests that the mechanism of extracellular transport and/or membrane structure/composition in *D. hansenii* is quite different from that of *S. cerevisiae*.

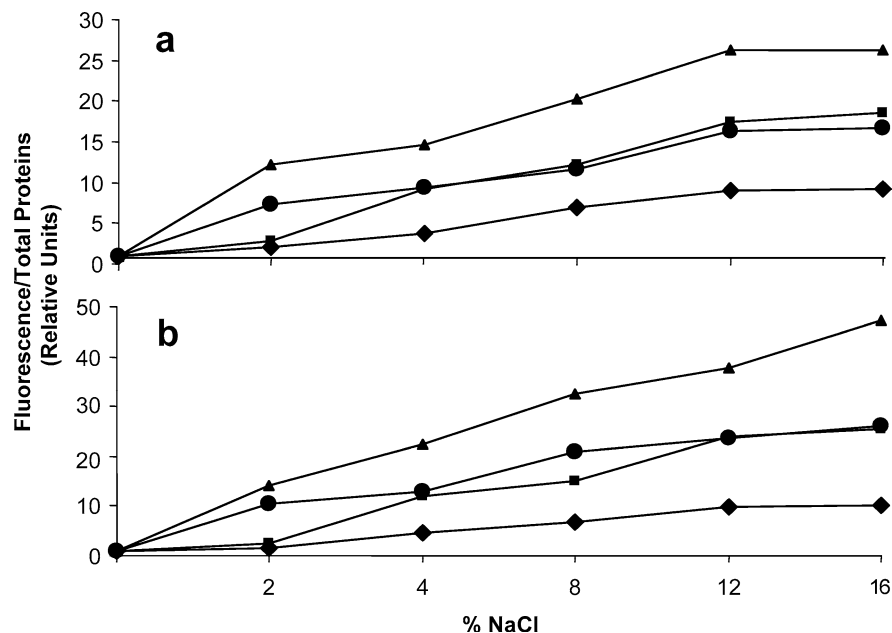
#### GPD1d control of GFP expression

Green fluorescent protein expression under the control of GPD1d promoter was quite different from its *S. cerevisiae* counterpart. Cells of *S. cerevisiae* and *D. hansenii* showed a 25-fold increase in intracellular fluorescence levels due to GFP activation when cells were grown in 16% NaCl (Fig. 3).

Extracellular fluorescence due to GFP expression in *S. cerevisiae* showed a 50-fold increase when cells were exposed to 16% NaCl. *D. hansenii* cells reached less than a 10-fold increase under the same conditions (Fig. 3).

The higher level of fluorescence due to GFP in both yeasts by GPD1d (compared with GPD1) suggests important differences in the regulation under salt activation. This difference could be the result of tight regulation in the promoter activation of *D. hansenii* during osmotic stress. Several regulatory sequences (cis-elements) found in the glycerol-3-phosphate dehydrogenase promoter of *D. hansenii* are not present in *S. cerevisiae* (R.G. Maggi and N.S. Govind, unpublished data). Some of these sequences had not been characterized and their role, as well as the trans-elements associated with them, should be studied in order to elucidate all the steps in the glycerol-3-phosphate dehydrogenase regulation.

**Fig. 3a, b** Intracellular and extracellular fluorescence due to GFP expression in media with salt concentrations of 0–16% NaCl. **a** GPD1 control by plasmid pRGMG $\alpha$ GfpMt, **b** GPD1d control by plasmid pRGMGd $\alpha$ GfpMt. *Squares* intracellular level in *D. hansenii* DH7426-1, *diamonds* extracellular level in *D. hansenii* DH7426-1, *circles* intracellular level in *S. cerevisiae* SC288, *triangles* extracellular level in *S. cerevisiae* SC288



## Discussion

The broad spectrum of environmental conditions under which it can grow, the production of extracellular and intracellular hydrolytic enzymes (such as  $\beta$ -glucosidase), its compatibility and stimulating action with lactic acid starter cultures, and the inhibition of growth of spoilage bacteria in cheese, has promoted *D. hansenii* as one of the most important non-*Saccharomyces* yeast in the wine and cheese industry worldwide [44, 50, 54, 57]. The development of *D. hansenii* as an organism for biotechnological expression of cloned gene products is then an attractive option not only for the optimization of endogenous processes but also for the heterologous production of industrial proteins.

This work demonstrates the feasibility of heterologous expression and secretion in *D. hansenii*. Expression in both *S. cerevisiae* and *D. hansenii* seems to be similar when controlled by almost all the *S. cerevisiae* and *D. hansenii* inducible promoters tested here. The only exception was the ADH2 promoter from *S. cerevisiae*, which was not able to induce expression in *D. hansenii*.

Expression regulation with changes in osmotic pressure proved stronger with the *D. hansenii* GPD1d promoter than for its counterpart in *S. cerevisiae* (GPD1). The reason for this difference could be related to the presence of several cis-elements in the *D. hansenii* promoter that are not present in *S. cerevisiae*. An analysis of these elements and their effect on transcriptional regulation due to osmotic changes is the focus of a separate paper (R.G. Maggi and N.S. Govind, in preparation).

In addition to the high level of expression achieved by the GPD1d promoter in both yeasts, a low level of protein export was found in *D. hansenii*. A 5-fold decrease in fluorescence relative to total protein levels occurred in the *D. hansenii* extracellular GFP fraction

when compared with the *S. cerevisiae* extracellular fraction under the same activation conditions. The low levels of GFP protein exported by *D. hansenii* could be related to differences in signal sequence efficiency, protein transport mechanism, or cell membrane structure. To elucidate whether these differences could be attributed to the  $\alpha$ -galactosidase signal sequence used here, we replaced it with the  $\alpha$ -mating factor signal sequence (also from *S. cerevisiae*) in the same plasmid constructs. Our results indicated that there were no major changes in the level of protein export by *D. hansenii* for either signal sequence (data not shown). The use of an endogenous signaling sequence becomes necessary if high level extracellular production of a heterologous protein is required in *D. hansenii*.

Heterologous gene expression is a complex, multi-step process, especially when the product is designed for secretion. Host physiology at high cell densities, promoter induction strength, heterologous protein stability, and the burden effect of the expressed protein on host cell metabolism can cause low yields of both intracellular and extracellular proteins. One important factor in this regard is vector (plasmid) copy number. Some integration vectors have been reported to give higher yields of secreted products than high copy number, episomal plasmids such as those described here. This is due to a bottleneck effect in transcription factor (trans-element) availability. High levels of expression of heterologous proteins can place a significant metabolic burden on the host cell by reducing its growth rate, its metabolism, or the efficiency of gene expression. Although no deleterious effects were observed in the six plasmids constructed and tested here, this effect could be particularly important in systems with constitutive promoters where growth and expression are linked. The use of tightly regulated promoters such as GPD1 and



GPD1d, therefore, becomes crucial, since the growth and the expression phases can be largely separated.

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