Mol Biotechnol (2009) 41:165–172
DOI 10.1007/s12033-008-9123-y

RESEARCH

The NADP-dependent Glutamate Dehydrogenase Gene from the Astaxanthin Producer *Xanthophyllomyces dendrorhous*: Use of Its Promoter for Controlled Gene Expression

Marta Rodríguez-Sáiz · Ramiro P. Godio · Vanessa Álvarez · Juan Luis de la Fuente · Juan F. Martín · José Luis Barredo

Published online: 18 November 2008

Abstract The *gdhA* gene encoding the NADP-dependent glutamate dehydrogenase (GDH) activity from *Xanthophyllomyces dendrorhous* has been cloned and characterized, and its promoter used for controlled gene expression in this red-pigmented heterobasidiomycetous yeast. We determined the nucleotide sequence of a 4701 bp DNA genomic fragment, showing an open reading frame of 1871 bp interrupted by five introns with fungal consensus splice-site junctions. The predicted protein (455 amino acids; 49 kDa) revealed high identity to GDHs, especially to those from the fungi *Cryptococcus neoformans* (70%), *Sclerotinia sclerotiorum* (66%), and several species of *Aspergillus* (66–67%). Gene phylogenies support the grouping of *X. dendrorhous* GDH close to those from the majority of the filamentous fungi. The promoter region of the *gdhA* gene (P*gdhA*) contains a TATA-like box and two large pyrimidine stretches. The use of P*gdhA* for gene expression was validated by electrotransformation of *X. dendrorhous* using an in-frame fusion with the hygromycin resistance gene (hyg^R) as a reporter. *X. dendrorhous* transformants were able to grow in YEME complex medium and in Czapek minimal medium supplemented with 50 μg/ml hygromycin, but gene expression in Czapek medium was repressed when using ammonium acetate as a nitrogen source. P*gdhA* is a valuable tool for controlled gene expression in Basidiomycetes.

M. Rodríguez-Sáiz · V. Álvarez · J. L. de la Fuente · J. L. Barredo (✉)
R&D Biology, Antibióticos S.A, Avenida de Antibióticos 59-61, 24009 León, Spain
e-mail: JBarredo@antibioticos.it

R. P. Godio · J. F. Martín
Instituto de Biotecnología de León (INBIOTEC), Parque Científico de León Av. Real 1, 24006 León, Spain

Keywords Glutamate dehydrogenase · Astaxanthin · Hygromycin · *Xanthophyllomyces dendrorhous* · Introns

Introduction

Xanthophyllomyces dendrorhous, the teleomorphic state of *Phaffia rhodozyma*, is a red-pigmented heterobasidiomycetous yeast that accumulates astaxanthin as a main carotenoid [1]. Because the astaxanthin production level of *X. dendrorhous* needs to be increased to become commercially competitive versus the chemically synthesized astaxanthin [2], several strategies have been approached including isolation of astaxanthin hyperproducing strains by classical random mutation, optimization of culture media and fermentation conditions, and the use of recombinant-DNA technology to enhance gene expression [3, 4]. Genetic strategies to enhance astaxanthin production and pathway engineering involve gene expression under the control of strong promoters such as those from the actin or glyceraldehyde-3-phosphate dehydrogenase (*gpd*) encoding genes. Some of the genes involved in primary metabolism present high expression level, suggesting that their promoters contain specific enhancers or regulatory sequences that contribute to the high transcription level.

Glutamate dehydrogenase (GDH), a key enzyme of the primary metabolism, links carbohydrate (energy) and nitrogen metabolism [5]. NADP(H)-dependent GDHs (EC 1.4.1.4) are essentially involved in ammonia assimilation and glutamate biosynthesis, catalyzing the reductive amination of 2-oxoglutarate to form glutamate. The genes encoding NADP-dependent GDHs (*gdh*) from the fungi *Neurospora crassa* [6], *Saccharomyces cerevisiae* [7], *Aspergillus nidulans* [8], *Schwanniomyces occidentalis* [9],

Agaricus bisporus [10], *Aspergillus awamori* [11], *Penicillium chrysogenum* [12], and *Hebeloma cylindrosporum* [13] have been described, and some *gdh* promoters have been used for gene expression [11, 12]. However, the use of a *gdh* promoter from Basidiomycetes for gene expression has not yet been described.

We report in this article the cloning and characterization of the *gdhA* gene of *X. dendrorhous* and the use of its promoter (P*gdhA*) for the expression of the hygromycin resistance gene (hyg^R) from *Escherichia coli* in *X. dendrorhous*.

Materials and Methods

Microorganisms, Phages, and Plasmids

The astaxanthin producing strain *X. dendrorhous* VKPM 2410 [14] was used as a source of DNA. *E. coli* SURE (Stratagene) and *E. coli* LE392 [15] were the hosts for λGEM12 (Promega) phage derivatives. Phage λGEM12 was used to construct a genomic library of *X. dendrorhous*. *E. coli* DH5α [15] was the recipient for high-frequency plasmid transformation. pGEM-3Zf(+) (Promega), pBluescript II KS (+) (Stratagene), and pBC KS (+) (Stratagene) were used for subcloning and sequencing. General procedures for plasmid DNA purification, cloning, and transformation of *E. coli* were those previously described [15].

Construction and Screening of a *X. dendrorhous* Genomic Library

X. dendrorhous was grown [14, 16, 17] and total DNA was purified according to described methods [18]. Fragments of genomic DNA (17–22 kb) were selected from *Sau*3AI partially digested DNA, ligated to λGEM12, and packaged using the "Packagene" kit (Promega) to yield approximately 2.6×10^5 pfus. For screening, the library was amplified in *E. coli* LE392, plated to obtain around 5×10^4 pfu, and hybridized according to standard methods [15] using an 1.3-kb *Hind*III fragment from the *gdhA* gene of *P. chrysogenum* [12] as a probe. Recombinant phages were amplified in liquid medium to purify their DNA according to standard procedures [15].

Nucleic Acid Hybridization and Sequencing

Southern hybridizations were made with digoxigenin-labeled probes (Non-radioactive labeling and immunological detection kit, Roche Molecular Biochemicals) according to the recommendations of the manufacturer. Clones to be sequenced were constructed with the "Erase a base" system (Promega) and sequenced with an ABI PRISM 377 DNA sequencer (Applied Biosystems). DNA sequences were analyzed with the Dnastar and Winstar packages (DNASTAR Inc.), and compared to known sequences by using the BLASTP and BLASTX programs [19]. The codon-preference algorithm (Geneplot, Winstar) was used to detect the presence of open reading frames (ORFs). Multiple protein alignments were made with the Clustal V algorithm and pair wise alignments with the Lipman–Pearson method (MegAlign, Winstar). The nucleotide sequence corresponding to the gene encoding GDH has been deposited in the GenBank with the accession No. EU791456.

hyg^R Gene Expression Using P*gdhA*

Plasmid pGDHAHg was constructed to express the hyg^R gene from *E. coli* under the control of P*gdhA* from *X. dendrorhous*. P*gdhA* was amplified by PCR with primers XDF (5′ CGGTACCCGGGGATCCGGGGTGAGTACCT GAACTAACCAG 3′; *Bam*HI site underlined) and XDR (5′ GAGTTCAGGCTTTTTCATGGTTGATGGAAGATG TGGAGGA 3′). Optimized amplification reactions (50 µl) contained about 20 ng of *X. dendrorhous* genomic DNA as a template, 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 20 pmoles of each primer, 1.25 U of Platinum® Pfx DNA polymerase (Invitrogen), and dNTPs 200 mM each. A Biometra TGradient Thermocycler was used for amplification with a program of 29 cycles [95°C, 40 s (5 min for the first cycle); 60°C, 40 s; 68°C, 30 s]. The hyg^R gene was amplified by PCR with primers HPHF (5′ CATCTTCCATCAACCATGAAAAAGCCTGAACTC ACCGCGA 3′) and HPHR (5′ GAATACTCAAGCTTG CATGCGGGGGATCTGGATTTTAGTACTGG 3′).

After sequencing to verify the lack of mutations, P*gdhA* and hyg^R were subcloned into *Bam*HI/*Sph*I digested pGEM-3Zf(+) using the In-Fusion™ 2.0 PCR Cloning Kit (Clontech) to give pGDHAHg (P*gdhA*-hyg^R-T*35S*). The DNA sequence showed the correct in-frame fusion P*gdhA*-hyg^R-T*35S*. The transcriptional terminator signal (T*35S*) was from the 35S cauliflower mosaic virus transcriptional terminator. Plasmid pGDHAHg confers hygromycin resistance in *X. dendrorhous* and ampicillin resistance in *E. coli*.

Transformation of *X. dendrorhous* by Electroporation

A method for electrotransformation of *X. dendrorhous* was developed based on described protocols [20]. Electrocompetent cells were mixed with 10 µg of linearized DNA, and the mixture was transferred to a pre-cooled electroporation cuvette (0.2 cm). A pulse (0.8 kV, 1000 Ω, 25 µF) was applied with a Gene Pulser (Bio-Rad), and

1.5 ml of YEME medium was added immediately. Then, the mixture was transferred to a sterile 10-ml tube. After incubation for 3.5 h at 21°C, 200 µl aliquots were spread onto YEME (Difco) plates supplemented with 15–30 µg/ml hygromycin (Sigma). The plates were incubated at 20°C for about 10 days. Hygromycin resistance levels were checked in YEME complex medium and Czapek minimal supplemented with either ammonium acetate (10 and 30 mM) or glutamate (15 mM) as nitrogen sources.

Analysis of Carotenoids

X. dendrorhous flask fermentations for astaxanthin production were done as previously described [17]. Carotenoids were extracted with methanol/dichloromethane (50/50) and re-dissolved in acetone. High performance liquid chromatography (HPLC) analyses were performed at 474 nm with a Nucleosil 100 NH2 column (resin particle diameter, 5 µm; 250 × 4.6 mm) and hexane/ethyl acetate (50:50 ratio) with a flow rate of 1 ml/min as the mobile phase. The astaxanthin HPLC standard was obtained from Sigma.

Results and Discussions

Cloning and Characterization of the *gdhA* Gene from *X. dendrorhous*

To identify the *gdhA* gene, a genomic library of *X. dendrorhous* was screened using an 1.3-kb *Hind*III DNA fragment from the *gdhA* gene of *P. chrysogenum* [12] as a probe. Three positive clones were identified and characterized by restriction mapping and Southern analysis. A 4.7-kb *Eco*RI fragment common to all the three clones was subcloned in pBluescript II KS (+) to yield pALPR6 and pALPR7 (both orientations). A DNA region of 4701 bp including the *gdhA* gene was sequenced in both strands and analyzed with the codon-preference algorithm (Geneplot), showing the presence of an ORF of 1871 bp interrupted by five introns at positions 1004–1169 (166 bp), 1203–1280 (78 bp), 1329–1404 (76 bp), 1489–1577 (89 bp), and 1652–1748 (97 bp) (Fig. 1). The presence of many introns per gene is a common phenomenon in most of *X. dendrorhous* known

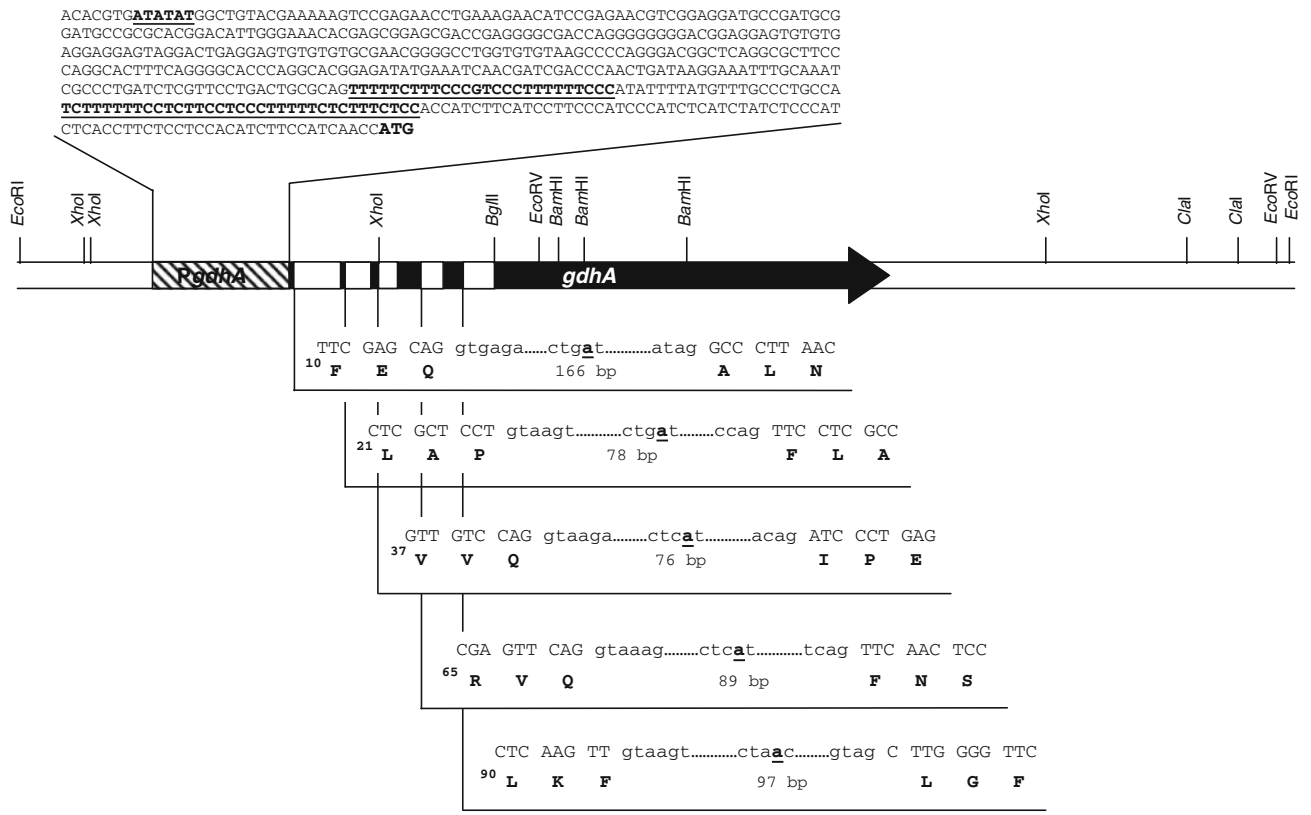


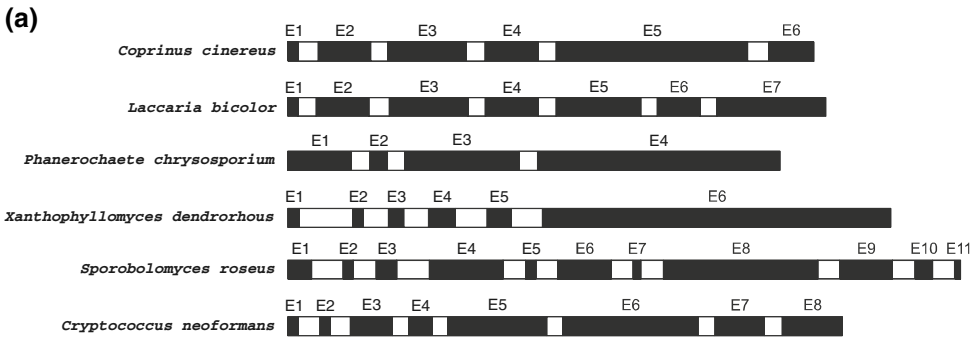
Fig. 1 Restriction map of the genomic DNA region of *X. dendrorhous* including the *gdhA* gene. The *gdhA* gene is represented by an *arrow*, P*gdhA* is *dashed*, and *white boxes* inside the *arrow* represent five introns. Potential regulatory sequences identified in the 5′ noncoding region such as pyrimidine-rich stretches and TATA-like box are *bold* and *underlined*. Location and partial sequence of exons (upper case) and introns (lower case) in the deduced protein are indicated. Fungal consensus intron processing sites for intron/exon boundaries at 5′ (g/GTAHGTY) and 3′ (MYAG/g) and splice signal for lariat formation (WRCTRAC) are conserved. H is A or C or T, D is G or A or T, Y is C or T, W is A or T, R is A or G, M is C or A, and K is G or T, according to the IUB codes. The conserved adenosine at the lariat branch site is *bold* and *underlined*

genes; remarkably the *crtS* gene, encoding a cytochrome-P450 hydroxylase involved in the astaxanthin biosynthetic pathway, has 17 introns [14, 21]. The *gdhA* exons code for 12, 11, 16, 28, 25, and 363 amino acids, respectively. Although there is still little information on consensus splicing sequences in Basidiomycetes, fungal consensus sequences described for intron/exon boundaries and splice signal for lariat formation [22] were detected (Fig. 1). The consensus of the lariat sequences maintains an adenosine (bold and underlined in Fig. 1) at the branch site, which has been shown to be crucial for the chemical steps of splicing in other genes [23]. The consensus lariat sequence for the *gdhA* gene of *X. dendrorhous* (CTVAY) is similar to that found in higher plants (CTRAY) (V is A or G or C, Y is C or T, R is A or G, according to the IUB codes).

Intron comparisons of *X. dendrorhous gdhA* gene (Basidiomycota, Tremellomycetes) were done with *gdh* genes from the Basidiomycota *Coprinus cinereus* (Agaromycetes), *Laccaria bicolor* (Agaromycetes), *Phanerochaete chrysosporium* (Agaromycetes), *Sporobolomyces roseus* (Microbotryomycetes), and *Cryptococcus neoformans* (Heterobasidiomycetes) (Fig. 2a). Curiously, the number (from 3 to 10) and location of the introns in these *gdh* genes do not match, even when compared between the Basidiomycota *X. dendrorhous* and *C. neoformans*, that appeared very closely related according to the phylogenetic tree of Fig. 3.

Analysis of Regulatory Elements in the 5′ and 3′ Flanking Sequences of the *gdhA* Gene

Potential regulatory elements were found in the promoter region of the *gdhA* gene. Two large (26- and 35-bp)



(b)

	Xde	Sce	Ani	Aor	Acl	Pch	Eni	Ssc	Pan	Gze	Cne	Hcy	Lbi	Abi	Cci	Pha	Uma	Tbo	Sro
Xde	100%	61% 75%	66% 77%	67% 77%	66% 76%	65% 78%	66% 78%	66% 78%	63% 75%	65% 79%	70% 83%	65% 76%	64% 76%	62% 76%	63% 76%	63% 75%	64% 77%	64% 78%	65% 76%
Sce		100%	66% 66%	66% 78%	64% 76%	63% 78%	65% 77%	65% 76%	61% 73%	62% 75%	60% 74%	57% 71%	56% 71%	57% 71%	57% 72%	58% 70%	55% 70%	64% 77%	56% 71%
Ani			100%	90% 94%	88% 93%	84% 91%	84% 90%	75% 84%	70% 78%	73% 83%	64% 76%	62% 75%	62% 75%	61% 75%	61% 74%	62% 75%	62% 76%	67% 77%	61% 75%
Aor				100%	89% 95%	87% 94%	86% 93%	74% 85%	69% 79%	73% 83%	65% 77%	61% 76%	60% 76%	60% 75%	61% 75%	62% 76%	62% 76%	68% 78%	61% 76%
Acl					100%	84% 91%	83% 91%	74% 84%	69% 78%	74% 83%	64% 76%	62% 75%	61% 74%	61% 74%	60% 74%	61% 75%	61% 76%	66% 77%	60% 74%
Pch						100%	83% 92%	74% 84%	68% 79%	70% 83%	65% 76%	60% 76%	59% 75%	58% 74%	60% 75%	62% 76%	62% 76%	67% 78%	61% 75%
Eni							100%	73% 84%	66% 77%	71% 83%	65% 77%	62% 77%	61% 76%	59% 75%	61% 76%	61% 75%	63% 78%	66% 77%	60% 74%
Ssc								100%	74% 83%	76% 89%	64% 77%	61% 77%	63% 76%	61% 76%	62% 75%	62% 75%	64% 77%	67% 78%	62% 78%
Pan									100%	70% 81%	63% 75%	59% 72%	59% 71%	60% 71%	60% 72%	59% 70%	58% 71%	64% 75%	60% 73%
Gze										100%	64% 77%	62% 78%	62% 77%	60% 76%	61% 75%	61% 75%	60% 75%	63% 76%	59% 75%
Cne											100%	65% 77%	62% 76%	62% 76%	62% 76%	62% 75%	63% 76%	61% 74%	63% 78%
Hcy												100%	85% 91%	83% 90%	82% 92%	74% 85%	62% 74%	59% 72%	62% 75%
Lbi													100%	81% 90%	82% 92%	74% 84%	62% 74%	60% 74%	60% 74%
Abi														100%	79% 91%	72% 83%	61% 73%	59% 72%	59% 73%
Cci															100%	75% 85%	60% 74%	58% 73%	61% 75%
Pha																100%	61% 74%	60% 72%	59% 72%
Uma																	100%	58% 72%	60% 74%
Tbo																		100%	59% 73%
Sro																			100%

Fig. 2 **a** Intron/exon structure of the *gdh* genes of several species of Basidiomycota. *C. cinereus* (Agaromycetes), *L. bicolor* (Agaromycetes), *P. chrysosporium* (Agaromycetes), *X. dendrorhous* (Tremellomycetes), *S. roseus* (Microbotryomycetes), and *C. neoformans* (Heterobasidiomycetes). Exons (E1, E2, etc.) are in *black* and introns in *white*. **b** Identity among fungal GDH protein sequences. First result shows conserved amino acid residues, and second result corresponds to conservative substitutions. Xde, *X. dendrorhous*; Sce, *S. cerevisiae*; Ani, *A. niger*; Aor, *A. oryzae*; Acl, *A. clavatus*; Pch, *P. chrysogenum*; Eni, *Emericella nidulans*; Ssc, *S. sclerotiorum*; Pan, *Podospora anserina*; Gze, *Gibberella zeae*; Cne, *C. neoformans*; Hcy, *H. cylindrosporum*; Lbi, *L. bicolor*; Abi, *A. bisporus*; Cci, *Coprinopsis cinerea*; Pha, *P. chrysosporium*; Uma, *Ustilago maydis*; Tbo, *Tuber borchii*; Sro, *S. roseus*

pyrimidine-rich regions were detected between positions 813–838 and 861–895 (Fig. 1), suggesting possible transcription start sites. Usually, the region immediately upstream of the transcription start point is pyrimidine rich in the coding strand, particularly in highly expressed genes [24]. A TATA-like box (ATATAT) (position 488) was located upstream (474 bp) from the translation initiation site ATG (position 968) (Fig. 1). The majority of promoters in higher eukaryotes contain this box 25–30 bp upstream from the transcription initiation site [24].

The polyadenylation-like sequence TTATATT, potentially involved in the polyadenylation of the 3′ terminus, was found at position 2998.

Analysis of the Deduced Amino Acid Sequence Encoded by the *gdhA* Gene

The 455 amino acid-deduced protein has a predicted molecular mass of M_r 49 kDa and an isoelectric point of 6.2. Comparison to SwissProt & PIR databases gave high identity scores with fungal NADP-dependent GDHs, especially with those from *C. neoformans* (70%), *Aspergillus oryzae* (67%), *A. nidulans* (66%), *Aspergillus niger* (66%), *Aspergillus clavatus* (66%), *Sclerotinia sclerotiorum* (66%), and *P. chrysogenum* (65%) (Fig. 2b).

A phylogenetic tree was constructed with NADP-dependent GDH sequences from fungal representatives of the different taxonomical classes and some examples of higher eukaryotes (Fig. 3). GDH phylogenies perfectly agree with taxonomical classifications, discriminating among Fungi, Metazoa, and Viridiplantae.

Three signature sequences have been developed for family I hexameric GDH in the Prosite database [25]: (1) ^{83}PSVNL^{87}, (2) ^{91}KFLGFEQ^{97}, and (3) ^{191}RPEATGY^{197}. One of the conserved regions in the sequence includes the putative NADP-binding motif ^{226}**GSG**NV**A**QYAALKV-IEL**G**^{242}. The primary structure of NADP-dependent enzymes was identified by amino acid fingerprinting [26] and by homology comparison [27]. A lysine residue potentially involved in the binding of 2-oxoglutarate and in the catalytic activity of the enzyme was identified at position 112.

Gene Expression Under the Control of the Nitrogen Regulated P*gdhA*: Promoter Validation Studies

The functionality of P*gdhA* was tested in *X. dendrorhous* using the *hyg*^{R} gene from *E. coli* as a reporter [28]. Plasmid pGDHAHg (Fig. 4) was constructed by in-frame fusion of P*gdhA* to *hyg*^{R} at the ATG translation initiation codon. pGDHAHg contains the P*gdhA*-*hyg*^{R}-T*35S* cassette and the ampicillin resistance gene for selection in *E. coli*.

Hygromycin (30 μg/ml) resistant transformants were selected and tested by Southern hybridization with an 800 bp *Eco*RI/*Xmn*I probe from the *hyg*^{R} gene (Fig. 5a), and with a 550-bp probe amplified from P*gdhA* (Fig. 5b). All of them showed a 4.7-kb *Eco*RI hybridization band corresponding to the endogenous *gdhA* gene, and a 2.7-kb *Eco*RI hybridization band corresponding to the P*gdhA*-*hyg*^{R}-T*35S* cassette. Transformation resulted in one or more integration events of pGDHAHg into the *X. dendrorhous* genome. The intensities of the 2.7 and 4.7 kb

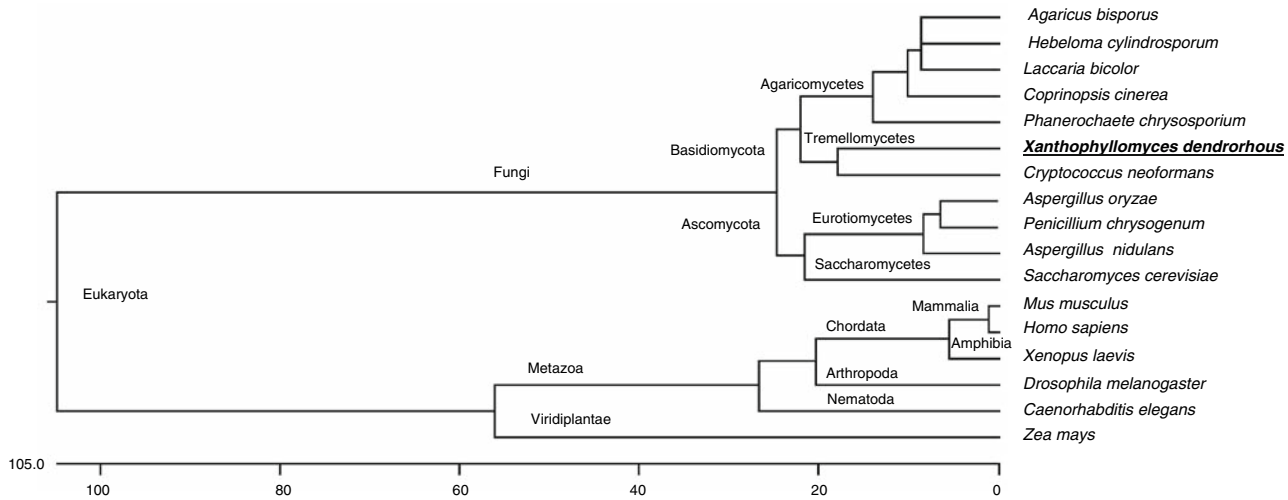


Fig. 3 Phylogenetic tree of NADP-dependent GDHs. The protein sequence of *X. dendrorhous* (Basidiomycota, Tremellomycetes) was compared with sequences from fungi Basidiomycota (Agaricomycetes, Tremellomycetes) and Ascomycota (Eurotiomycetes, Saccharomycetes), Metazoa (Chordata, Arthropoda, Nematoda), and Viridiplantae (Streptophyta). The alignment was performed with the program Megalign by using the Clustal method with the PAM250 residue weight table. Multiple alignment parameters were: gap penalty 10 and gap length penalty 10. Pairwise alignment parameters were: ktuple 1, gap penalty 3, window 5, and diagonals 5. The length of each pair of branches represents the distance between sequence pairs. The units at the bottom of the tree indicate the number of substitution events

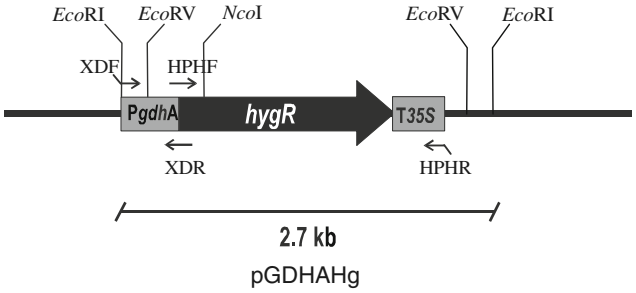


Fig. 4 Restriction map of the plasmid pGDHAHg used for hygromycin resistance (hyg^R) gene expression under the control of the *gdhA* gene promoter (P*gdhA*) from *X. dendrorhous*. T*35S* is the 35S cauliflower mosaic virus transcriptional terminator. XDF and XDR primers were used to amplify P*gdhA*, and HPHF and HPHR primers to amplify hyg^R

bands in the transformants showed in Fig. 5b agree with the presence of two copies of pGDHAHg. Depending on the locus and number of integrations, hybridization bands of different size and intensity were detected in other transformants (data not shown), but only the hybridization signal corresponding to the *gdhA* gene (4.7 kb *Eco*RI) appeared in the parental untransformed strain (Fig. 5b).

Stability of the transformants was proved after five consecutive cycles of growth, for 10 days each cycle, without hygromycin as a selective agent, a relevant period of time according to the extent (4–5 days) of the *X. dendrorhous* culture for astaxanthin production. All transformants analyzed so far proved to be stable in these conditions.

The hygromycin resistance level of six transformants (T13, T15, T16, T17, T20, and T21) was tested by solid and liquid cultures in minimal medium (Czapek) supplemented with either ammonium acetate (10 and 30 mM) or glutamate (15 mM) as nitrogen sources. Results (Fig. 6) showed that transformants can grow in the presence of 30 and 50 μg/ml hygromycin in Czapek-glutamate, reaching dry weights in the range of the controls without hygromycin supplementation (around 5–6 g/l). However, transformants did not grow in Czapek-ammonium acetate 30 mM supplemented with either 30 μg/ml or 50 μg/ml hygromycin (dry weights from 0.5 to 1.5 g/l). When transformants were checked in Czapek-ammonium acetate 10 mM, they were able to grow in 30 μg/ml hygromycin (dry weights about 4–5 g/l) but not in 50 μg/ml hygromycin (dry weights from 0.5 to 1.5 g/l). In brief, *X. dendrorhous* P*gdhA* expression is repressed when using ammonium acetate as a nitrogen source.

These results show that P*gdhA* is able to direct hyg^R gene expression in *X. dendrorhous*, conferring the hygromycin-resistant phenotype. Lower hygromycin resistance levels have been described in *Acremonium chrysogenum* and *P. chrysogenum* by expressing hyg^R under the control of the *A. nidulans gpdA* promoter (5–10 μg/ml) [28, 29].

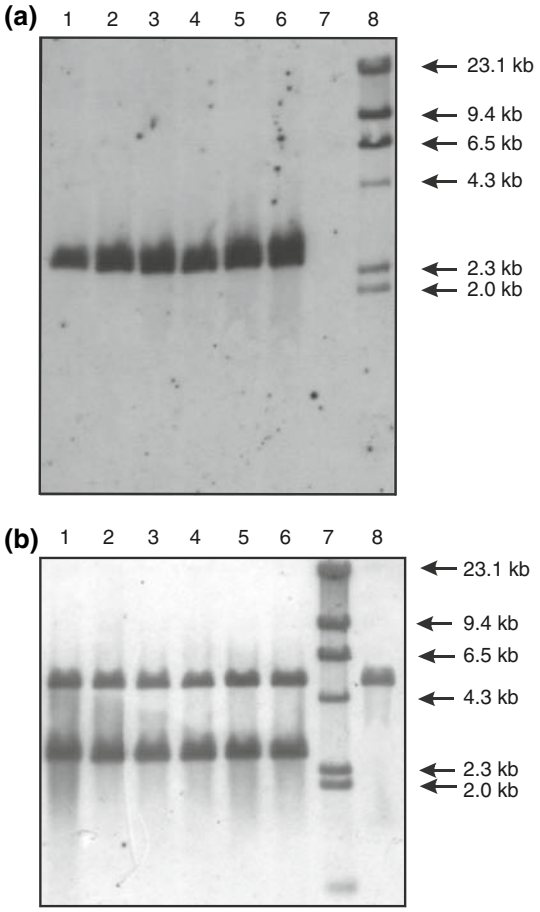


Fig. 5 Southern analyses of pGDHAHg transformants. **a** Southern hybridization of *Eco*RI digested total DNAs using an 800 bp *Eco*RI/*Xmn*I DNA fragment internal to hyg^R gene as a probe. Lanes 1–6, pGDHAHg transformants (T13, T15, T16, T17, T20, and T21, respectively). Lane 7, parental untransformed strain. Lane 8, molecular weight marker. The 2.7-kb hybridization band corresponds to the P*gdhA*-hyg^R-T*35S* cassette. **b** Southern hybridization of *Eco*RI digested total DNAs using a 550-bp probe amplified from P*gdhA*. Lanes 1–6, pGDHAHg transformants (T13, T15, T16, T17, T20, and T21, respectively). Lane 7, molecular weight marker. Lane 8, parental untransformed strain. The 4.7-kb hybridization band corresponds to the endogenous *gdhA* gene, and the 2.7-kb band to the P*gdhA*-hyg^R-T*35S* cassette

Astaxanthin Production

The parental untransformed strain *X. dendrorhous* VKPM 2410 and six hyg^R transformants (T13, T15, T16, T17, T20, and T21) were fermented under astaxanthin production conditions. After extraction and analysis of carotenoids by HPLC using an astaxanthin commercial standard (Sigma) with a retention time of 8.39 min, equivalent astaxanthin production was obtained with all seven strains (from 150 to 200 μg/g dry weight; about 0.5 mg/l) (Fig. 7).

In summary, we described a new gene from *X. dendrorhous*, a microorganism with great importance from a commercial point of view because of its potential in

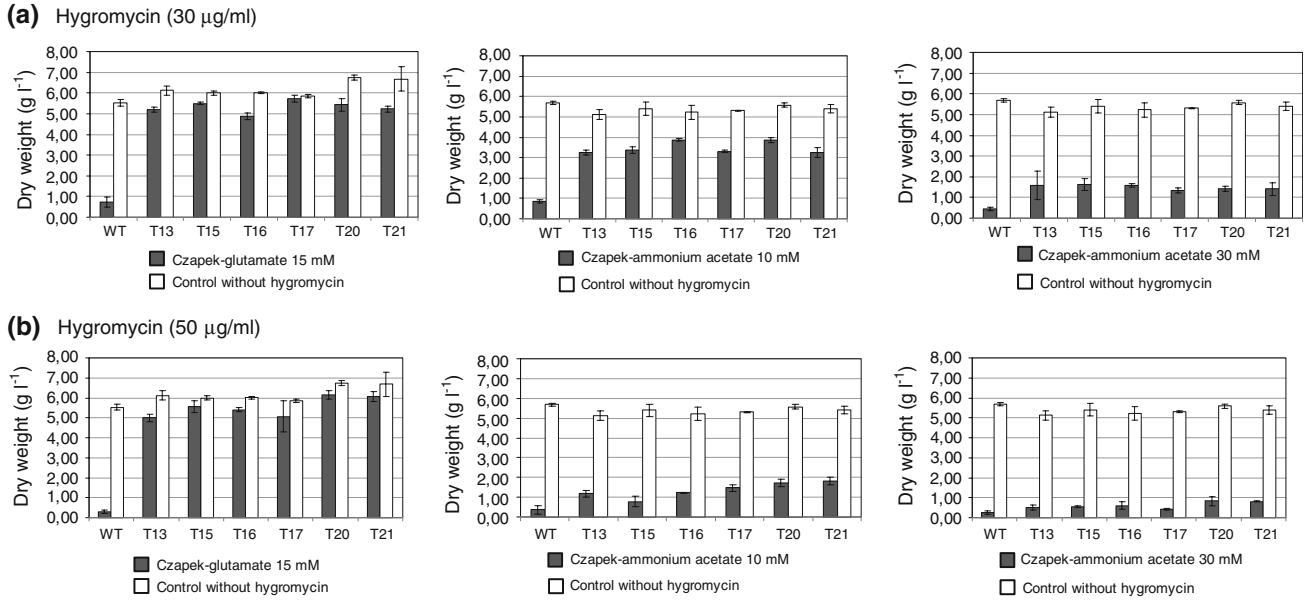


Fig. 6 Hygromycin resistance level of six transformants (T13, T15, T16, T17, T20, and T21) tested in liquid Czapek minimal medium supplemented with either ammonium acetate (10 and 30 mM) or glutamate (15 mM) as nitrogen sources. 30 µg/ml (**a**) and 50 µg/ml (**b**) hygromycin were checked, using always a control without hygromycin. Dry weight average results (g/l of dry weight) of three independent cultures ± standard deviations are shown

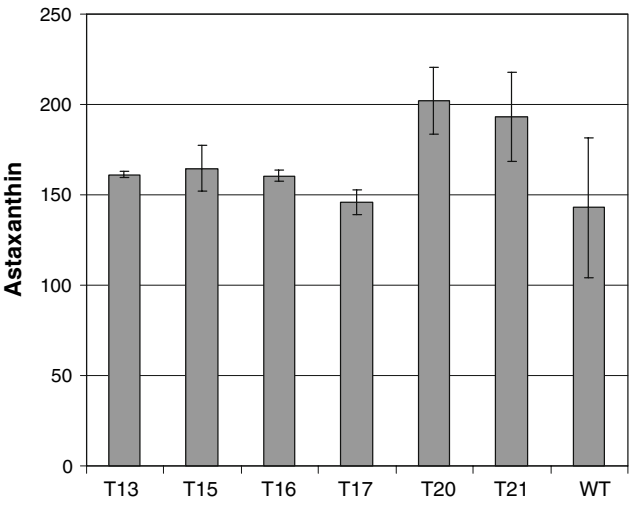


Fig. 7 Astaxanthin production by *X. dendrorhous* VKPM 2410 (WT) and pGDHAHg hygromycin-resistant transformants (T13, T15, T16, T17, T20, and T21). Astaxanthin average results (µg/g of dry weight) of three independent cultures ± standard deviations are shown

industrial use for the biotechnological production of astaxanthin. The *gdhA* promoter region can be used as a valuable tool to guide controlled gene expression for the metabolic engineering of *X. dendrorhous* and other Basidiomycetes.

Acknowledgments This study was supported in part by the PROFIT project FIT-010000-2006-20 granted to Antibióticos S.A. and INBIOTEC. The authors thank P. Merino, C. Flórez, A. Morán, M. Sandoval, and C. Cañón for their excellent technical assistance. V. Álvarez was supported by a fellowship "Acción MIT-F2" from the Spanish Ministry of Science and Technology.

References

1. Andrewes, A. G., Phaff, H. J., & Starr, M. P. (1976). Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast. *Phytochemistry, 15*, 1003–1007. doi:10.1016/S0031-9422(00)84390-3.
2. Johnson, E. A., & Schroeder, W. (1995). Astaxanthin from the yeast *Phaffia rhodozyma*. *Studies in Mycology, 38*, 81–89.
3. Visser, H., van Ooyen, A. J. J., & Verdoes, J. C. (2003). Metabolic engineering of the astaxanthin-biosynthetic pathway of *Xanthophyllomyces dendrorhous*. *FEMS Yeast Research, 4*, 221–231. doi:10.1016/S1567-1356(03)00158-2.
4. Wery, J., Verdoes, J. C., & van Ooyen, A. J. J. (1998). Efficient transformation of the astaxanthin-producing yeast *Phaffia rhodozyma*. *Biotechnology Techniques, 12*, 399–405. doi:10.1023/A:1008834600770.
5. Smith, E. L., Austen, B. M., Blumenthal, K. M., & Nyc, J. F. (1975). Glutamate dehydrogenases. In P. D. Boyer (Ed.), *The enzymes* (Vol. 11). NY: Academic Press.
6. Kinnaird, J. H., & Fincham, J. R. (1983). The complete nucleotide sequence of the *Neurospora crassa am* (NADP-specific glutamate dehydrogenase) gene. *Gene, 26*, 253–260. doi:10.1016/0378-1119(83)90195-6.
7. Nagasu, T., & Hall, B. D. (1985). Nucleotide sequence of the GDH gene coding for the NADP-specific glutamate dehydrogenase of *Saccharomyces cerevisiae*. *Gene, 37*, 247–253. doi:10.1016/0378-1119(85)90279-3.
8. Hawkins, A. R., Gurr, S. J., Montague, P., & Kinghorn, J. R. (1989). Nucleotide sequence and regulation of expression of the *Aspergillus nidulans gdhA* gene encoding NADP dependent glutamate dehydrogenase. *Molecular and General Genetics, 218*, 105–111. doi:10.1007/BF00330572.

9. De Zoysa, P. A., Connerton, I. F., Watson, D. C., & Johnston, J. R. (1991). Cloning, sequencing and expression of the *Schwanniomyces occidentalis* NADP-dependent glutamate dehydrogenase gene. *Current Genetics, 20*, 219–224. doi:10.1007/BF00326236.
10. Schaap, P. J., Müller, Y., Baars, J. J., Op den Camp, H. J., Sonnenberg, A. S., van Griensven, L. J., et al. (1996). Nucleotide sequence and expression of the gene encoding NADP+-dependent glutamate dehydrogenase (*gdhA*) from *Agaricus bisporus*. *Molecular and General Genetics, 250*, 339–347.
11. Cardoza, R. E., Moralejo, F. J., Gutiérrez, S., Casqueiro, J., Fierro, F., & Martín, J. F. (1998). Characterization and nitrogen-source regulation at the transcriptional level of the *gdhA* gene of *Aspergillus awamori* encoding an NADP-dependent glutamate dehydrogenase. *Current Genetics, 34*, 50–59. doi:10.1007/s002940050365.
12. Díez, B., Mellado, E., Rodríguez, M., Bernasconi, E., & Barredo, J. L. (1999). The NADP-dependent glutamate dehydrogenase gene from *Penicillium chrysogenum* and the construction of expression vectors for filamentous fungi. *Applied Microbiology and Biotechnology, 52*, 196–207. doi:10.1007/s002530051509.
13. Javelle, A., Morel, M., Rodríguez-Pastrana, B. R., Botton, B., Andre, B., Marini, A. M., et al. (2003). Molecular characterization, function and regulation of ammonium transporters (Amt) and ammonium-metabolizing enzymes (GS, NADP-GDH) in the ectomycorrhizal fungus *Hebeloma cylindrosporum. Molecular Microbiology, 47*, 411–430. doi:10.1046/j.1365-2958.2003.03303.x.
14. Álvarez, V., Rodríguez-Sáiz, M., de la Fuente, J. L., Gudiña, E. J., Godio, R. P., Martín, J. F., et al. (2006). The *crtS* gene of *Xanthophyllomyces dendrorhous* encodes a novel cytochrome-P450 hydroxylase involved in the conversion of β-carotene into astaxanthin and other xanthophylls. *Fungal Genetics and Biology, 43*, 261–272. doi:10.1016/j.fgb.2005.12.004.
15. Sambrook, K. J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning. A laboratory manual* (2nd ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
16. Verdoes, J. C., Sandmann, G., Visser, H., Diaz, M., van Mossel, M., & van Ooyen, A. J. J. (2003). Metabolic engineering of the carotenoid biosynthetic pathway in the yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). *Applied and Environmental Microbiology, 69*, 3728–3738. doi:10.1128/AEM.69.7.3728-3738.2003.
17. de la Fuente, J. L., Peiro, E., Díez, B., Marcos, A. T., Schleissner, C., Rodríguez-Sáiz, M., et al. (2004). Method of producing astaxanthin by fermenting selected strains of *Xanthophyllomyces dendrorhous*. International Patent WO 03/066875.
18. Specht, C. A., DiRusso, C. C., Novotny, C. P., & Ullrich, R. C. (1982). A method for extracting high-molecular-weight deoxyribonucleic acid from fungi. *Analytical Biochemistry, 119*, 158–163. doi:10.1016/0003-2697(82)90680-7.
19. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research, 25*, 3389–3402. doi:10.1093/nar/25.17.3389.
20. Visser, H., Sandmann, G., & Verdoes, J. C. (2005). Xanthophylls in fungi. In J. L. Barredo (ed.), *Methods in biotechnology. Microbial processes and products* (Vol. 18, pp. 257–272). Totowa, NJ: Humana.
21. Ojima, K., Breitenbach, J., Visser, H., Setoguchi, Y., Tabata, K., Hoshino, T., et al. (2006). Cloning of the astaxanthin synthase gene from *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) and its assignment as a beta-carotene 3-hydroxylase/4-ketolase. *Molecular Genetics and Genomics, 275*, 148–158. doi:10.1007/s00438-005-0072-x.
22. Balance, D. J. (1986). Sequences important for gene expression in filamentous fungi. *Yeast, 2*, 229–236. doi:10.1002/yea.320020404.
23. Bhattacharya, D., & Weber, K. (1997). The actin gene of the glaucocystophyte *Cyanophora paradoxa*: analysis of the coding region and introns, and an actin phylogeny of eukaryotes. *Current Genetics, 31*, 439–446. doi:10.1007/s002940050227.
24. Davis, M. A., & Hynes, M. J. (1991). Regulatory circuits in *Aspergillus nidulans*. In J. W. Bennet & L. L. Lasure (Eds.), *More gene manipulations in fungi* (pp. 151–189). San Diego, CA: Academic Press.
25. Benachenhou-Lahfa, N., Forterre, P., & Labedan, B. (1993). Evolution of glutamate dehydrogenase genes: Evidence for two paralogous protein families and unusual branching patterns of the archaebacteria in the universal tree of life. *Journal of Molecular Evolution, 36*, 335–346. doi:10.1007/BF00182181.
26. Wierenga, R. K., Terpstra, P., & Hol, W. G. L. (1986). Prediction of the occurrence of the ADP-binding $\beta\alpha\beta$-fold in proteins, using an amino acid sequence fingerprint. *Journal of Molecular Biology, 187*, 101–107. doi:10.1016/0022-2836(86)90409-2.
27. Scrutton, N. S., Berry, A., & Perham, R. N. (1990). Redesign of the coenzyme specific of a dehydrogenase by protein engineering. *Nature, 343*, 38–43. doi:10.1038/343038a0.
28. Punt, P. J., & van den Hondel, C. A. (1992). Transformation of filamentous fungi based on hygromycin B and phleomycin resistance markers. *Methods in Enzymology, 216*, 447–457. doi:10.1016/0076-6879(92)16041-H.
29. Velasco, J., Adrio, J., Moreno, M. A., Díez, B., Soler, G., & Barredo, J. L. (2000). Environmentally safe production of 7-aminodeacetoxycephalosporanic acid (7-ADCA) using recombinant strains of *Acremonium chrysogenum*. *Nature Biotechnology, 18*, 857–861. doi:10.1038/78467.