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CLONING AND SEQUENCE ANALYSIS OF *MUCOR CIRCINELLOIDES* GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE GENE

KLÁRA ÁCS[1], ZS. KASZA[1], GYÖNGYI LUKÁCS[1], H. SCHWAB[2] AND CS. VÁGVÖLGYI[1]

[1]Department of Microbiology, Faculty of Sciences, University of Szeged, P.O. Box 533, H-6701 Szeged, Hungary, [2]Institut für Biotechnologie, Technische Universität Graz, Petersgasse 12, A-8010 Graz, Austria

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A genomic library of *Mucor circinelloides* ATCC 1216b has been constructed in Lambda Fix II vector. The library has an average insert site of 10 kb and covers the genome 12 times. The *M. circinelloides* gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gpd*) was isolated from this library by hybridization of the recombinant phage clones with a *gpd*-specific gene probe generated by PCR reaction. The complete nucleotide sequence encodes a putative polypeptide chain of 339 amino acids interrupted by 3 introns. The predicted amino acid sequence of this gene shows a high degree of sequence similarity to the GPD proteins from other filamentous fungi. The promoter region, containing a consensus TATA and CAAT box and a 298 nucleotid long termination region were also determined.

Keywords: *Mucor circinelloides*, cloning of glyceraldehyde-3-phosphate dehydrogenase

Introduction

Mucor circinelloides is a ubiquitous filamentous fungus belonging to the Zygomycetes. Its special biochemical, morphological and physiological features established a longstanding interest of both applied and theoretical research. The fact that the species of the genus *Mucor* are able to produce and secrete a large amount of extracellular enzymes [1], their peculiar feature to show dimorphic transition under

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given conditions [2] or their unique sexual processes [3] all attract substantial attention. Recently, further biotechnological interest has been generated because of the potential of *M. circinelloides* to synthetize carotenoids [4] and an enzyme having amydalase activity degrading cyanogenic glycosides (the HCN released from these glycosides may cause acute and/or chronic poisoning in humans) [5].

The use of classical genetic approach in these fungi is problematic because of the absence of an easily utilizable sexual recombination system. Until now, the single transformation system developed in *M. circinelloides* is based on complementation of leucine auxotrophy [6–7]. Due to an ARS sequence in the flanking region of the cloned *leu*A gene high transformation rates could be obtained: most of the transformants contain autonomously replicating plasmids [80–10]. In spite of the efficiency this transformation system has a serious drawback: a stable mutant deficient in the synthesis of α-isopropylmalate isomerase has to be isolated from each strain want to be transformed. Therefore, it seems desirable to establish a transformation system based on a strong native promoter allowing efficient expression of heterologous reporter genes as selection markers.

Glyceraldehyde-3-phosphate dehydrogenase is an important enzyme in both glycolysis and gluconeogenesis. It catalyzes the oxidation and phosphorylation of the glyceraldehyde-3-phosphate to 1,3-bis-phosphoglycerate. As a main housekeeping enzyme its amino acid sequence shows a strong structural conservation by different prokaryotic and eukaryotic organisms, which allow for an evolutionary analysis [11]. Furthermore, though, as an exception, the *gpd* of *Trichoderma harzianum* is known to be repressed during conidiation and mycoparasitism [12] *gpd* is usually considered as a highly and constitutively expressed, e.g. in yeast 2–5% of the poly(A)$^+$ RNA may constitute GPD mRNA [13]. Based on this feature, its regulatory flanking sequences have been used to construct efficient transformation systems in numerous fungi, e.g. *Podospora anserina* and *Claviceps purpurea* [14–15]. In contrast to other transformation systems which rely on nutritional auxotrophic markers for the selection of transformants, the combination of a *gpd* promoter sequence and a dominant selectable marker allows the transformation of wild-type strains.

As part of the effort to develop a new transformation system for *Mucor* based on a *gpd* promoter sequence, we cloned and sequenced the *gpd* gene of *M. circinelloides.*

Materials and methods

Strains and plasmids

Genomic DNA library and genomic DNA used as templates in PCR reactions were derived from the wild-type *M. circinelloides* strain ATCC 1216b. For genomic library construction Lambda FIX II/XhoI Partial Fill-In Vector Kit and the Gigapack III Gold Packaging Extract (Stratagene) were used. For subcloning of genomic DNA fragments pBluescript SK vector (Stratagene) was routinely used. The *Escherichia coli* strain XL1-Blue MRA (P2) (Stratagene) and DH5α (BRL) were applied for amplification of *M. circinelloides* recombinant lambda clones and subcloning experiments, respectively. Growth and selection of the *E. coli* strains were performed as described by Sambrook et al. [16].

Isolation of Mucor genomic and phage DNA

M. circinelloides was grown in YEG medium, under vigorous shaking for three days. Mycelium was harvested by filtration and powderized with a pestle and mortar under liquid nitrogen. Genomic DNA was isolated according the method of Kolar *et al*. [17].

Following the lysis of *E. coli* host culture phage DNA was isolated as described by Sambrook et al. [16].

Construction of genomic lambda library

M. circinelloides genomic DNA fragments were generated by partial digestion (*Sau*3AI) and size selection by gel electrophoresis (8–15 kbp). DNA was ligated with the lambda FIX II vector as suggested by the manufacturer after a partial fill-in and an alkalic phosphatase reaction. The GigapackIII Gold Packaging Extract was used according to the recommendations of the supplier.

Synthesis of homologous gpd probe and hybridization reactions

Two degenerated oligonucleotide primers designed for the most conserved regions of the *gpd* gene [18] were used to generate a homologous DNA probe from *M. circinelloides*. These were the followings:

5’-cgggatccaa(ag)ctiaciggiatggc-3’
(corresponds to amino acids 241–246: KLTGMA)
5’-cgggatcc(ag)taicc(cag)(ct)a(tc)tc(ag)tt(ag)tc(ag)tacca-3’
(corresponds to amino acids 331–338: WYDNEWGY)

Polymerase chain reaction (PCR) was carried out using the following parameters: one cycle (5 min 95 °C), five cycles (1 min 95 °C, 2 min 5°C, 2 min 72 °C), 25 cycles (1 min 95 °C, 2 min 55 °C, 2 min 72 °C), one cycle (10 min 72 °C).

To screen the genomic library, *gpd* probe was labeled with DIG DNA Nonradioactive Labeling and Detection Kit (Boehringer Mannheim). Hybridisation and detection of positive clones following plaque lifting were carried out as suggested by the supplier.

Computer analysis

The *gpd* sequences used in this paper were obtained from nucleotide sequence libraries (EMBL, GenBank). The derived amino acids sequences were aligned by Clustal W [19].

Results and discussion

Isolation and characterization of the glyceraldehyde-3-phosphate dehydrogenase gene of M. circinelloides

In order to isolate the *gpd* gene of *M. circinelloides* a specific homologous gene probe was synthetized by polymerase chain reaction. The two oligonucleotide primers corresponding to highly conserved regions of *gpd* gene amplified the expected 350 bp fragment from the chromosomal DNA of *M. circinelloides*. It was cloned into pBluescript SK and sequenced. Analysis of the sequence indicated that a fragment of the *gpd* gene was isolated. This fragment was used to screen the lambda genomic library by plaque filter hybridization. From the twenty positive clones isolated one was involved in further studies after three purification rounds. Phage DNA isolated from this clone was digested with several restriction enzymes (*Sal*I, *Pst*I, *Not*I, *Sac*I, *Hind*III, *Xho*I) and subjected to Southern-blot analysis using the labelled PCR fragment as a probe. A 5.2 kb *Xho*I fragment was identified and ligated into pBluescript SK vector. This cloned fragment was further digested with several enzymes and the restriction fragments were also cloned into pBluescript SK vector.

Sequence analysis of M. circinelloides gpd gene

Construction of several subclones and designing a series of sequencing primers allowed to identify the complete nucleotide sequence of the *gpd* gene. Altogether, 1861 nucleotides (nt) containing 350 nt as a promoter and 298 nt in the untranslated terminal region were identified. The nucleotide sequence of the *gpd* structure gene contained

1213 nucleotides and involved 4 exons and 3 introns (Figure 1). These introns were rather small; their sizes were 77 nt, 56 nt and 61 nt for intron I, intron II and intron III, respectively. The conserved sequence motifs (nucleotide pairs GT and AG, bounding their 5' and 3' extremities, respectively) are present in all introns.

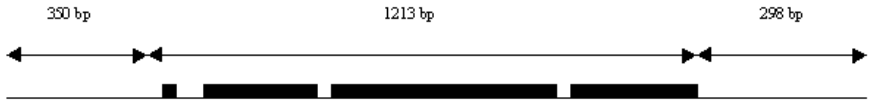

Figure 1. Organisation of the *M. circinelloides gpd* gene. Exons are indicated by black boxes and gaps between exons are introns

The 5'-flanking region contained a putative TATA box at nt –77 from the start codon (ATG). A putative CAAT box (–135 nt upstream) was also identified. A 307 nt long 3' non-coding sequence (untranslated region) is also determined. This involves (58 nt downstream from the stop codon) a putative polyadenylation site (ATAAAA), which resembles to the consensus polyadenylation site [20].

The nucleotide sequence data of the *M. circinelloides gpd* gene and its flanking regions reported in this paper have been assigned GenBank accession number AF346893.

Codon usage in M. circinelloides

Different fungi show varying patterns of codon usage with preference related, most likely, to their abundance of codon isoacceptor tRNA molecules. Furthermore, there can be a substantial bias in codon usage between different genes within the same organism. Generally, genes which are highly and constitutively expressed show more marked codon bias than genes expressed at a low level. This sitiuation is revealed in the *M. circinelloides gpd* gene where the codon bias is marked: only 42 of the 61 possible sense codons are used (Table I).

The identified *gpd* gene corresponds to a putative GPD protein with 339 amino acids. The derived amino acid sequence of *Mucor gpd* was aligned with other fungal GPD sequences using Clustal W program [17]. These alignments are manually adjusted and the result is shown in Figure 2. Though these three filamentous fungi take up highly different taxonomic positions, this comparison indicated substantial amino acid sequence similarities (Table II).

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Table I

Codon usage in the *gpd* gene of *M. circinelloides*

Amino acid	Codon	*gpd*	Amino acid	Codon	*gpd*
Phe (F)	TTT	9:2	Trp (W) Stop	TGG	3:3
	TTC	9:7		TAA	1:1
				TAG	1:0
				TGA	1:0
Leu (L)	TTG	20:6	Tyr (Y)	TAT	10:3
	TTA	20:0		TAC	10:7
	CTT	20:6			
	CTC	20:8			
	CTA	20:0			
	CTG	20:0			
Ile (I)	ATT	22:6	His (H)	CAT	3:2
	ATC	22:16		CAC	3:1
	ATA	22:0			
Met (M)	ATG	6:6	Gln (Q)	CAA	9:9
				CAG	9:0
Val (V)	GTT	40:19	Asn (N)	AAT	17:1
	GTC	40:19		AAC	17:16
	GTA	40:1			
	GTG	40:1			
Ser (S)	AGT	24:0	Lys (K)	AAA	22:0
	AGC	24:2		AAG	22:22
	TCT	24:14			
	TCC	24:8			
	TCA	24:0			
	TCG	24:0			
Pro (P)	CCT	13:8	Asp (D)	GAT	20:14
	CCC	13:5		GAC	20:6
	CCA	13:0			
	CCG	13:0			
Thr (T)	ACT	20:11	Glu (E)	GAA	16:8
	ACC	20:9		GAG	16:8
	ACA	20:1			
	ACG	20:0			
Ala (A)	GCT	37:26	Arg (R)	CGT	10:9
	GCC	37:11		CGC	10:0
	GCA	37:0		CGA	10:0
	GCG	37:0		CGG	10:0
				AGA	10:1
				AGG	10:0
Cys (C)	TGT	3:3	Gly (G)	GGT	30:29
	TGC	3:0		GGC	30:1
				GGA	30:0
				GGG	30:0

Table II

Comparison of the putative amino acid (aa) sequence of the *gpd* gene from *M. circinelloides* with other fungal *gpd* sequences

	aa homologies (%)		
	1	2	3
1. *M. circinelloides*	100	67.95	73.89
2. *A. nidulans*		100	65.97
3. *S. commune*			100

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Mucor           MVTQVGINGFGRIGRIVLRASLSNPEVQVVAINDPFIPLEYMVYMFKYDSVHGRFQGTVE 60
S.commune       MAVKVGINGFGRIGRIVLRNALQLGNIEVVAINDPFIALDYMVYMFKYDTVHGRYKGTVE 60
A.nidulans      MAPKVGINGFGRIGRIVFRNAIEAGTVDVVAVNDPFIETHYAAYMLKYDSQHGQFKGTIE 60
                *. :**************:* ::.   ::***:*****  .* .**:***: **:::**:*

Mucor           AKDGKLVVNGKEISVFSERDPAQIPWGSVEAAYVVESTGVFTSIDAASAHLQGGAKKVII 120
S.commune       VKDGKLVVDGHAITVFAEKNPADIKWGS-AADYIVESTGVFTTVEKASLHLQGGAKKVVI 119
A.nidulans      TYDEGLIVNGKKIRFHTERDPANIPGQD-GAEYIVESTGVFTTQEKASAHLKGGAKKVVI 119
                . *  *:*:*: * ..:*::**:*   .  * *:********: : ** **:******:*

Mucor           SAPSGDAPMFVCGVNLEKYTSDLKVISNASCTTNCLAPLAKVINDNFGIVEGLMTTVHAT 180
S.commune       SAPSADAPMFVVGVNLDKYDSKYQVISNASCTTNCLAPLAKVIHDKYGIAEGLMTTVHAT 179
A.nidulans      SAPSADAPMFVMGVNNETYKKDIQVLSNASCTTNCLAPLAKVINDNFGIIE-LMTTVHSY 178
                ****.****** *** :.* .. :*:******************:*::** * ******:

Mucor           TATQKTVDGPSNKDWRGGRGAGANIIPSSTGAAKAVGKVIPELNGKLTGMAFRVPTPDVS 240
S.commune       -ATQKTVDGPSHKDWRGGRSVNNNIIPSSTGAAKAVGKVIPSLNGRLTGLAFRVPTLDVS 238
A.nidulans      TATQKVVDGPSAKDWRGGRTAATNIIPSSTGAAKAVGKVIPSLNGKLTGMAMRVPTSNVS 238
                 ****.***** ******* .  ******************.***:***:*:**** :**

Mucor           VVDLTVRLEKGATYEEIKAVIKKASENELKGILGYTNDQVVSTDFVGDAQSSIFDAAAGI 300
S.commune       VVDLVVRLEKEASYDEIVATVKEASEGPLG-ILGFTDESVVSTDFTGANESSIFDSKAGI 297
A.nidulans      VVDLTVRTEKAVTYDQIDA-VKKASENELKGILGYTEDDIVSTDLNGDTRSSIFDAKAGI 297
                ****.** ** .:*::* * :*:***. *  ***:*::.:****: *  .*****: ***

Mucor           ALNDKFVKLVSWYDNEFGYSNRVIDLLAYAAKVDAAAQ 338
S.commune       AISKSFVKLIAWYDNEWGYSRRVCDLLVYAAKQDGAL- 334
A.nidulans      ALNSNFIKLVSWYDNEWGYSRRVVDLITYISKVDAQ-- 333
                *:...*:**::*****:***.** **:.* :* *.
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Figure 2. Alignment of deduced amino acid sequences coded by the *gpd* gene of *M. circinelloides* with the *gpd* genes of *A. nidulans* and *S. commune*. Perfectly and well-conserved residues are indicated by * respectively.

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