Mannitol-1-phosphate dehydrogenase from *Cryptococcus neoformans* is a zinc-containing long-chain alcohol/polyol dehydrogenase

Kalavati Suvarna,¹ Ann Bartiss² and Brian Wong^{1,2}

Author for correspondence: Brian Wong. Tel: +1 203 937 3446. Fax: +1 203 937 3476.
e-mail: brian.wong@yale.edu

¹ Department of Internal Medicine, Yale University School of Medicine, New Haven, CT, USA

² Infectious Diseases Section, VA Connecticut Healthcare System, West Haven, CT, USA

***Cryptococcus neoformans*, the causative agent of cryptococcosis, produces large amounts of mannitol in culture and in infected mammalian hosts. Although there is considerable indirect evidence that mannitol synthesis may be required for wild-type stress tolerance and virulence in *C. neoformans*, this hypothesis has not been tested directly. It has been proposed that mannitol-1-phosphate dehydrogenase (MPD) is required for fungal mannitol synthesis, but no MPD-deficient fungal mutants or cDNAs or genes encoding fungal MPDs have been described. Therefore, *C. neoformans* was purified from a 148 kDa homotetramer of 36 kDa subunits that catalysed the reaction mannitol 1-phosphate + NAD $\leftrightarrow$ fructose 6-phosphate + NADH. Partial peptide sequences were used to isolate the corresponding cDNA and gene, and the deduced MPD protein was found to be homologous to the zinc-containing long-chain alcohol/polyol dehydrogenases. Lysates of *Saccharomyces cerevisiae* transformed with the cDNA of interest (but not vector-transformed controls) contained MPD catalytic activity. Lastly, Northern analyses demonstrated MPD mRNA in glucose- and mannitol-grown *C. neoformans* cells. Thus, MPD has been purified and characterized from *C. neoformans*, and the corresponding cDNA and gene (*MPD1*) cloned and sequenced. Availability of *C. neoformans* *MPD1* should permit direct testing of the hypotheses that (i) MPD is required for mannitol biosynthesis and (ii) the ability to synthesize mannitol is essential for wild-type stress tolerance and virulence.**

Keywords: mannitol-1-phosphate dehydrogenase, *Cryptococcus neoformans*, polyol/alcohol dehydrogenases

INTRODUCTION

The pathogenic basidiomycetous yeast *Cryptococcus neoformans* produces large amounts of the six-carbon polyol mannitol both in culture and in infected mammalian hosts (Wong *et al.*, 1990). The functional and pathogenic significance of the abilities of *C. neoformans* and other fungi to synthesize mannitol is not known. However, it is known that the three-carbon polyol glycerol functions in *Saccharomyces cerevisiae* and other fungi as an intracellular osmolyte and stress protectant (Albertyn *et al.*, 1994; Wang *et al.*, 1994). Moreover,

earlier studies from this laboratory suggested that the ability of *C. neoformans* to synthesize and accumulate mannitol may be required for wild-type environmental stress tolerance and virulence. Specifically, we showed that a *C. neoformans* mutant that underproduced and underaccumulated mannitol was (i) hypersusceptible to heat and hyperosmolar stresses; (ii) profoundly hypovirulent in mice (Chaturvedi *et al.*, 1996a); and (iii) hypersusceptible to oxidative killing by human neutrophils and by oxidants generated by the iron–H₂O₂–halide system (Chaturvedi *et al.*, 1996b). Furthermore, heterologous expression of bacterial *mtlD* [which encodes mannitol-1-phosphate dehydrogenase (MPD)] conferred on transgenic tobacco plants (Tarczynski *et al.*, 1993) and on glycerol-underproducing *S. cerevisiae* *gpd1* mutants (Chaturvedi *et al.*, 1997) the abilities to synthesize mannitol and to tolerate hyperosmolar stresses.

Abbreviations: M1Pase, mannitol-1-phosphatase; MPD, mannitol-1-phosphate dehydrogenase.

The GenBank accession numbers for the nucleotide sequences for the *C. neoformans* mannitol-1-phosphate dehydrogenase cDNA and gene are AF175685 and AF186474, respectively.

The findings summarized above suggested that mannitol may function in *C. neoformans* and other fungi as an intracellular osmolyte and stress protectant and that it may be required for wild-type virulence. However, this hypothesis has not yet been tested directly because the pathway by which *C. neoformans* synthesizes mannitol is not yet known. In 1980, Hult *et al.* proposed that several other fungi synthesize and catabolize mannitol via a unidirectional mannitol cycle. In the biosynthetic arm of this pathway, fructose 6-phosphate is reduced to mannitol 1-phosphate by NAD-dependent MPD, after which mannitol-1-phosphatase (M1Pase) dephosphorylates mannitol 1-phosphate to yield mannitol. MPD and M1Pase have been demonstrated in several fungi (Jennings, 1984) and the biochemical properties of some fungal MPDs and M1Pases have been described (Jennings, 1984; Wang & Le Tourneau, 1972; Kiser & Niehaus, 1981). However, neither fungal mutants lacking either MPD or M1Pase nor cDNAs or genes encoding fungal MPDs or M1Pases have been described. Therefore, the goals of the present study were (i) to purify and characterize MPD from *C. neoformans* and (ii) to clone and analyse the corresponding cDNA and gene.

METHODS

Strains and media. *C. neoformans* strains H99 (wild-type, serotype A) and MLP (a UV-generated mutant of strain H99 that underproduced and underaccumulated mannitol) (Wong *et al.*, 1990; Chaturvedi *et al.*, 1996a) were cultured in YPD (1% yeast extract, 2% peptone, 2% glucose) or synthetic complete medium (0.67%, w/v, yeast nitrogen base with amino acids) containing either 2% glucose or 2% mannitol. *Saccharomyces cerevisiae* YPH252 (*Mata ura3-52 lys2-801 ade2-101 trp1-1Δ his3-Δ200*) and YPH252 *gpd1Δ::leu2* (Eriksson *et al.*, 1995) were cultured in YPD, and uracil prototrophs were cultured in synthetic complete (SC) medium containing 2% (w/v) glucose but no uracil (SC glu – ura) or in SC medium containing 2% (w/v) galactose but no uracil (SC gal – ura).

Libraries. A library of *C. neoformans* H99 cDNAs in the *GAL1*-regulated yeast expression plasmid pYES2.0 (Invitrogen) was constructed as follows. *C. neoformans* H99 was grown to mid- to late-exponential phase in YPD, and the cells were harvested by centrifugation and disrupted by passage through a French pressure cell (SLM Instruments) at 40000 p.s.i. (276000 kPa). Total RNA in the cell lysate was isolated using the Bio101 Fast RNA kit. The mRNA was isolated from the total RNA using the Fasttrack 2.0 kit (Invitrogen), and cDNAs were synthesized using oligo(dT)/*NotI* primers and AMV reverse transcriptase from the Copy kit (Invitrogen) according to the manufacturer's instructions. *EcoRI* adapters were added to the size-selected cDNAs (≥ 1 kb), and they were cloned unidirectionally into pYES2.0. The final cDNA library consisted of 3.9×10^6 independent clones with a mean insert size of 1.75 kb, and it was amplified twice in *Escherichia coli* prior to use. A library of *C. neoformans* H99 genomic DNA fragments in LambdaZapII (Stratagene) was provided by John Perfect (Duke University, Durham, NC, USA).

Chemicals. All chemicals, sugar phosphates, sugar alcohols, nucleotide cofactors, and protease inhibitors were from Sigma.

Enzyme assays. MPD activity was measured by monitoring NADH appearance at 340 nm when the enzyme was added to

0.5 mM mannitol 1-phosphate and 5 mM NAD in 10 mM HEPES (pH 9.0). Fructose-6-phosphate reductase activity was measured by monitoring NADH disappearance after the enzyme was added to 3 mM fructose 6-phosphate and 5 mM NADH in 10 mM HEPES (pH 7.0).

S. cerevisiae strains YPH252 and YPH252 *gpd1Δ::leu2* were transformed with plasmids using the lithium acetate method (Sherman *et al.*, 1986) and the transformants were tested for MPD activity as described above. The *S. cerevisiae* transformants were also tested for the ability to produce mannitol from glucose by culturing in SC gal – ura and by analysing log- and stationary-phase cells by gas chromatography as previously described (Wong *et al.*, 1989). Lastly, the transformants were tested for their abilities to tolerate osmotic stress by culturing in YPD supplemented with 0.2–2.5 M NaCl.

Purification of MPD. *C. neoformans* H99 was grown to late-exponential phase in 12 l YPD and the cells were harvested by centrifugation. After harvesting, 240 ml packed cells were suspended to form a paste in 150 ml 50 mM Tris/HCl buffer (pH 7.5) containing 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 1 μ g leupeptin ml⁻¹ and 1 μ g pepstatin ml⁻¹ and the paste was passed twice through a French pressure cell at 40000 p.s.i. (276000 kPa). Cell debris was removed by centrifugation at 16000 g for 30 min, and the supernatant, after centrifugation at 100000 g for 1 h, was used for the further purification. Protamine sulphate solution (2%, w/v; 0.1 vol.) was added, and the suspension was centrifuged at 16000 g for 10 min. The protamine sulphate supernatant was loaded onto a Q12 ion-exchange column (15 $\times$ 68 mm; Bio-Rad) that had been equilibrated with 50 mM Tris/HCl buffer (pH 7.5) using a Bio-Rad Biologic medium-pressure liquid chromatograph. The column was washed with 25 ml 50 mM Tris/HCl buffer (pH 7.5) and bound proteins were eluted with a 0–200 mM NaCl gradient (75 ml). Fractions containing MPD activity were pooled, and the buffer was changed to 10 mM HEPES (pH 8.0). The sample was applied to a Matrex blue A gel column (15 $\times$ 100 mm, Millipore), the column was washed with 40 ml 10 mM HEPES (pH 8.0) and the MPD was eluted with 5 ml 0.1 mM NADH. This fraction was concentrated and the buffer was changed to 50 mM Tris/HCl (pH 7.5), 100 mM NaCl. The sample was subjected to gel filtration on a FPLC Superdex 200 HR 10/30 size-exclusion column (Amersham), and the active fractions were collected and concentrated by ultrafiltration; the final sample was stored at 4 °C after addition of 10% (v/v) glycerol and 1 mM DTT. Protein concentrations were determined by the Bradford method, using bovine serum albumin as the standard (Bradford, 1976).

Properties of *C. neoformans* MPD. SDS-PAGE was performed by the method of Laemmli (1970) and two-dimensional electrophoresis by the method of O'Farrell (1975). Gels were stained for proteins with Coomassie blue and non-denaturing gels were stained for MPD activity in 10 mM HEPES (pH 9.0), 2 mM NAD, 0.75 mg nitro blue tetrazolium ml⁻¹, 0.125 mg phenazine methosulphate ml⁻¹, and 0.38 mM mannitol 1-phosphate at 37 °C for 5 min.

The native molecular size of MPD was estimated by size-exclusion chromatography with a FPLC Superdex 200 HR 10/30 column that had been equilibrated with 50 mM Tris (pH 7.5), 100 mM NaCl. Sweet potato β -amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), bovine erythrocyte carbonic anhydrase (29 kDa) and horse heart cytochrome *c* (12.4 kDa) were used as molecular mass standards.

The substrate specificities of MPD were assayed by monitoring A_{340} after the enzyme was added to 5 mM of the polyol, sugar phosphate or polyol phosphate of interest and either 0.5 mM NAD in 10 mM HEPES (pH 9.0) or 0.5 mM NADH in 10 mM HEPES (pH 7.0). Cofactor specificity was assessed by substituting NADP for NAD and NADPH for NADH.

Kinetic constants were determined with 0.2 U purified MPD. The concentrations of mannitol 1-phosphate and NAD were varied from 0.005 to 0.07 mM and 0.01 to 0.2 mM, respectively. The concentration for ethanol varied from 0.5 mM to 3.0 mM. Kinetic constants for the reverse reaction were obtained using fructose 6-phosphate concentrations from 0.02 to 0.5 mM and NADH concentrations ranging from 0.01 to 0.1 mM. K_m and V_{max} values were calculated from Lineweaver–Burk plots.

The effects of pH were determined in 10 mM MES buffer at pH 6.0 and 6.5, 10 mM HEPES buffer at pH 7.0, 7.5, 8.0, 8.5 and 9.0, and 10 mM CAPS buffer at pH 9.5 and 10.0. The effects of temperature were determined by incubating the reaction mixture at various temperatures ranging from 25 °C to 65 °C and measuring NADH appearance at 340 nm.

Peptide sequence determination. N-terminal sequencing of purified *C. neoformans* MPD and of tryptic peptides that had been purified by reverse-phase HPLC was performed by the Macromolecular Resources Laboratory at the University of Kentucky.

Isolation and analysis of the MPD cDNA and gene. Standard methods (Sambrook *et al.*, 1989) were used to synthesize oligonucleotide primers based on partial peptide sequence data, to amplify DNA by PCR and to prepare [α - 32 P]dATP-labelled probes. These probes were used to screen the *C. neoformans* cDNA library by colony hybridization and the *C. neoformans* genomic DNA library by plaque hybridization. Positive clones were purified, mapped, subcloned and sequenced using standard methods. Multiple sequence alignments were performed by CLUSTAL W (Thompson *et al.*, 1994).

Southern and Northern hybridizations. *C. neoformans* H99 genomic DNA was isolated by the method of Sherman *et al.* (1986). Genomic DNA (5 µg per lane) was digested with the restriction enzymes *Bam*HI, *Hind*III, *Cla*I, *Sma*I, *Sal*I, *Pst*I, *Bgl*II and *Xba*I, separated by agarose gel electrophoresis, transferred to a nylon membrane and hybridized with a 1083 bp 32 P-labelled MPD cDNA using standard methods (Sambrook *et al.*, 1989).

Total RNA was isolated by the method of Sherman *et al.* (1986) from *C. neoformans* strains H99 and MLP that had been cultured in SC-glucose or SC-mannitol at 30 °C and from *C. neoformans* H99 that had been cultured in YPD at 25 °C, 30 °C or 37 °C and in YPD containing 0.2–1.5 M NaCl at

30 °C. RNA (10 µg per lane) was fractionated by electrophoresis on 1% agarose gels, transferred to nylon membranes, and the membranes were hybridized with a 600 bp 32 P-labelled partial MPD cDNA, followed by autoradiography. Gel loading was normalized by probing duplicate blots for actin mRNA; the actin probe was amplified from *C. neoformans* genomic DNA by PCR using primers 5'-TCGCCG-CCTTGGTCATTG-3' and 5'-CGTATTCGCTCTTCGC-GA-3' (Cox *et al.*, 1995). Band densities were measured with the EagleEye photodocumentation system and EagleSight software (Stratagene).

RESULTS

Purification and physical properties of *C. neoformans* MPD

Crude extracts of YPD-grown *C. neoformans* contained 0.098 U (µmol NAD utilized min⁻¹) MPD activity (mg protein)⁻¹. MPD was purified 1190-fold, to a specific activity of 116 U (mg protein)⁻¹ and with an overall recovery of 2.7% (Table 1). The final enzyme preparation was homogeneous by SDS-PAGE (Fig. 1) and by two-dimensional gel electrophoresis (data not shown). When purified MPD was subjected to PAGE under non-denaturing conditions and stained for activity, a single band was detected. When this band was eluted from the activity gel and subjected to denaturing SDS-PAGE, it

Fig. 1. Purification of *C. neoformans* MPD. Aliquots obtained at each step during purification were separated by 10% SDS-PAGE and stained with Coomassie blue. Lanes: 1, crude extract (20 µg); 2, 100 000 g supernate (20 µg); 3, protamine sulphate supernate (20 µg); 4, Q12 column eluate (20 µg); 5, Matrex blue A column eluate (5 µg); 6, Superdex 200 size-exclusion column eluate (5 µg); 7, molecular size marker; 8, material that was eluted from a band showing MPD activity in a native activity gel and then resolubilized in SDS.

Table 1. Purification of *C. neoformans* mannitol-1-phosphate dehydrogenase

Sample	Total U	Total protein (mg)	Specific activity (U mg ⁻¹)*	Purification (fold)	Recovery (%)
Crude extract	340	3480	0.098	1	100
Supernate at 100 000 g	332	3330	0.10	1.02	98
Protamine sulphate supernate	328	2050	0.16	1.63	96
Q12 column eluate	93	21	4.4	44.6	27
Matrex blue A gel eluate	70	1.2	61	624	21
Size-exclusion eluate	9.3	0.08	116	1190	2.7

* Expressed as µmol NAD utilized (mg protein)⁻¹ min⁻¹.

1	gacggaccctttccgcccgtagtgtaataaccagatccccagactaagaaaagagcaat	1
101	aattagagattatatgatgacgatgtcgtccaggcgaatctcaaccttcaccacaaccc	
201	ataagaccctttccacacgtctcttctgccccttcacttcaccatccatccatccaca	
	actatactctgtaccatccatccacaacccaagcaaac	ATG
		M
300	TCT ACT CTC TCT AAC TTC ACC ATT CCC AAG ACC CAG ACT GCC GCT GTT GTC AAG ACA ACC GGC GCT GAG CTC GCG	1
	S T L S N F T I P K T Q T A A V V K T T G A E L A	26
375	ATT GAA AAG GAA CAC CCT GTC AAG CAG GCT TCC GAG CTT GGG CCC GGT CAA TGC TTG GTC AAA ATC TCC CAC ACT	
	I E K E H P V K Q A S E L G P G Q C L V K I S H T	51
450	GGT GTC TGC CAT ACC GAC TTG CAC GCC AAG TCT GGC GAC TGG CCT GTT CCC CCA TCT CAT CCC CTT ATC GGA GGT	
	G V C H T D L H A K S G D W P V P P S H P L I G G	76
525	CAT GAA G gtgagtctaatttgggcactgcgtctcttctcgtccggccattgcttcaaaaataaaaacgtgcgaggggccccgctgacgcaatgggaa	
	H E G	Intron A
622	ttttcccgcttttcacag GT GTC GGT ATT ATC GTC GCT ATT GGT GAC CAC ACT GCT GCT TCT CCC GTC AAG CTT GGT GAC	
	V G I I V A I G D H T A A S P V K L G D	99
701	CGA GTC GGT ATC AAG TGG CTC GCC GAC AGC TGT CTC AGC TGC GAA TAC TGT CGA CGC GGC TAT GAG ATG A gtaagt	
	R V G I K W L A D S C L S C E Y C R R G Y E M N	123
777	cgatatactatttcaagacagcaagcaattatcgattgacccaccattgtag AC TGT CCC AAC GTC AAG CTC TCT GGC TAT ACC ACC	
		Intron B
865	GAC GGT ACT TTC TCC GAA TAC GTC GTT TCC TTC GTC AAC CAT GTG ACT CCT ATT CCT GAA TCC CTC GAC TCT GCC	
	D G T F S E Y V V S F V N H V T P I P E S L D S A	159
940	GGC GCC GCC TCC ATC CTG TGT GCT GGT GTC ACC ACC TAC AAG GCT CTC AAG GTC TCC AAC ACC AAG GTC GGT GAC	
	G A A S I L C A G V T T Y K A L K V S N T K V G D	184
1015	TGG GTC GCT CTT CCC GGC GCT GGC GGT GGT CTC GGT CAT CTT GCT GTC CAA TAT GCG AAG GCC ATG GGC CTC AAG	
	W V A L P G A G G G L G H L A V Q Y A K A M G L K	209
1090	GTC ATC GCT ATC GAT AGT GGC GCT GCC AAG GAG AAG CTC GTT AAG TCT CTC GGT GCT GAC GCT TGG GTC GAC TTT	
	V I A I D S G A A K E K L V K S L G A D A W V D F	234
1165	AAG ACT TCC AAG GAT ATT GTG GCC GAC ATC AAA GCC GCT ACT GGA GGG GAC GGT CCT GCC GCC GCT GTG GTC ACG	
	K T S K D I V A D I K A A T G G D G P A A A V V T	259
1240	GCT GCC AAC AAG ACG GGT TAC GCT CAG GCT ATC GAG TAC TTG AAG CCT AGC GGC ACC CTT GTC GCT GTC GGT ATG	
	A A N K T G Y A Q A I E Y L K P S G T L V A V G M	284
1315	CCT GAT GCT GAG ATG GGC GCA AAC GTT TTC TGG ACA GTG TTC AAG AGC ATC AGG ATC CAG GGT AGC TAT GTC GGT	
	P D A E M G A N V F W T V F K S I R I Q G S Y V G	309
1390	AAC AGA CAG GAT GCG ATT GAG GCG CTT GAT ATT GCC GCG TCC GGC CAA GTC AAG GTC GTT TTT GAA GAG AAG GGT	
	N R Q D A I E A L D I A A S G Q V K V V F E E K G	334
1465	CTT GAC TCT TTA AAG GG gtaagttgtgcttccttcttgccttgcgaaatcaacactgacatgggtggtatttgaag C GTC TTC GAG	
	L D S L K G	Intron C
1551	GAT CTT GAG GCT GGT AAG ATT GCC GGC CGT GTT GTC CTC AAG GTT TCC GAC GAG TAA aagtcctgcgcgtgtctgtctcta	
	D L E A G K I A G R V V L K V S D E *	361
1631	ggagtctcatagcgtttcttttggaggttgaggggtatttttcaggttgtaggggaatccgtttttgtcatttttaaaagtacaactgtagcagtggtgtgt	
1730	gtgtgtgtacagaagagaagagaagaaagaccagaatgcataagctttccatttaccatgtgtgaaacctttgtatagcccagtagagacgaa	

Fig. 2. Nucleotide and deduced amino acid sequences of the *C. neoformans* MPD1 gene. The three peptide sequences obtained from purified MPD protein are underlined. Intron sequences are shown as lower case letters labelled Intron A, Intron B and Intron C. The CAAT and TATA boxes are underlined.

co-migrated with purified MPD (Fig. 1). The subunit molecular mass of MPD as assessed by SDS-PAGE was approximately 36 kDa, and the native molecular size as determined by gel filtration was approximately 148 kDa.

Catalytic properties

C. neoformans MPD reduced NAD in the presence of 5 mM mannitol 1-phosphate, sorbitol 6-phosphate and ethanol (38.8% and 56%, respectively, compared to mannitol 1-phosphate), but not in the presence of mannitol or sorbitol. Ethanol activity was measured when it was observed that the cDNA had 47% identity to various alcohol dehydrogenases from yeast. MPD

oxidized NADH in the presence of 5 mM fructose 6-phosphate, glucose 6-phosphate and acetaldehyde (6.2% and 54%, respectively, compared to fructose 6-phosphate), but not in the presence of xylulose 5-phosphate, ribulose 5-phosphate, fructose 1,6-phosphate and ribose 5-phosphate. Lastly, MPD was absolutely specific for NAD and NADH; there was no reduction of NADP or oxidation of NADPH with any substrate tested.

The kinetic constants for the NAD-dependent oxidation of mannitol 1-phosphate and for NADH-dependent reduction of fructose 6-phosphate were determined with 0.2 U enzyme. The following constants were derived from the Lineweaver-Burk plot: $V_{\max}$ 0.40 $\mu\text{mol mg}^{-1}$

MPD_CRYNE	1	MSTL-----SNFTLPKQTAAVVKKTGAELAIER	29
ADH3_EMENI	1	M-----SVPEVQWAVVEKAGTPEVY-K	22
ADH1_CANAL	1	M-----SEQIPKTKAVVFDNTNGGLVY-K	24
ADH1_ASFFL	1	M-----SIPEMQWAVVAKQKGLPLVY-K	22
ADH1_EMENI	1	M-----CIPTMQWAVVAKKVGLPLVY-K	22
ADH4_KLULA	1	MRLRAQTALANKASVRSFLRLNSFAIPETQKGVIFYENGRLLEY-K	49
MPD_CRYNE	30	EPVVKQASELGGQCLVVKISHTGVCHTDLHAKSGDWPLVPPSHPLTGGHEG	79
ADH3_EMENI	23	QVPVPKFP--GPDEILVKMRYSVGVCHTDLHAMKGDWPLPSKMPPLGGHEG	69
ADH1_CANAL	25	DYPVPKFP--EPNELLVHKYSVGVCHTDLHAKKGDWPLAKKLPLVGGHEG	71
ADH1_ASFFL	23	QVPVPKFP--GPDEILVKMRYSVGVCHTDLHAKKGDWPLVPMPLVGGHEG	69
ADH1_EMENI	23	QVPVPKFP--GPDEILVKIRYSVGVCHTDLHAMKGDWPLVPMPLVGGHEG	69
ADH4_KLULA	50	DLPVPKFP--KANELLNVKYSVGVCHTDLHAMKGDWPLVKKLPLVGGHEG	96
MPD_CRYNE	80	VGIIVAGLDHTAASPVKLGDRVGIKWLDSCLSCRYCRRGYEMNCPNVKL	129
ADH3_EMENI	70	AGVVVAKGELVKDEDEKIGDRAGIKWLNGSCSCCEMCMQADEPLCPHASL	119
ADH1_CANAL	72	AGVVVANGENVKGG--KKIGDFAGIKWLNGSCSCCEFCQQGAEPNCGEADL	119
ADH1_ASFFL	70	AGVVVAKGLDYTE--FEIGDHAGIKWLNGSCSCCEFCQKQADEPLCPNASL	117
ADH1_EMENI	70	AGVVVAKGELVHE--FEIGDQAGIKWLNGSCSCCEFCQKQADEPLCARAQL	117
ADH4_KLULA	97	AGIVVAKGSENVKN--FEIGDYAGIKWLNGSCSCCEFCQKQADEPLQADL	144
MPD_CRYNE	130	SGYTDGTFSFVVSVFNHVTPTPESLDSAGAASTLCAGVTYKRLKVS	179
ADH3_EMENI	120	SGYTDGTFOQYTIKGAALASKIPDNVPLDAAAPILCAGITVYKGLKESG	169
ADH1_CANAL	120	SGYTDGTGSEFYATADAVQAARKIPAGTDLANVAPILCAGITVYKGLKTD	169
ADH1_ASFFL	118	SGYTDGTGTFQYAIKGAHASKIPKNVPLDAAAPVLCAGITVYKGLKESG	167
ADH1_EMENI	118	SGYTDGTGTFQYALGKASHASKIPAGVPDAAAPVLCAGITVYKGLKEAG	167
ADH4_KLULA	145	SGYTDGTGTFQYRTADAVQAARKIPKGTDLAEIAPILCAGITVYKGLKTD	194
MPD_CRYNE	180	TKVSGDWALPAGAGGGLGLAVQYAKAMGLKVTIDSSGAARLKLKSLGAD	229
ADH3_EMENI	170	AREGGQVAIVGAGGGLGLSLAQYAKAMGLRTIAIDSSDEKKAMCEQLGAE	219
ADH1_CANAL	170	LAAQGVVAIVGAGGGLGLSLAVQYAKAMGLRVVAIDGGDEKGEFVKSLGAE	219
ADH1_ASFFL	168	VRFPGQVAIVGAGGGLGLSLALQYAKAMGLRVVAIDGGDEKQAMCEQLGAE	217
ADH1_EMENI	168	VRFPGQVAIVGAGGGLGLSLAQYAKAMGLRVVAIDGGDEKRAMCESLGT	217
ADH4_KLULA	195	LKPGQVVAIVGAGGGLGLSLAVQYAKAMGLRVVGLDGGDGKELFKQCSGE	244
MPD_CRYNE	230	AVVDFTSKDIIVADLKAATSGD-GPAAVVTANKTGYAATIEYLKPSFT	278
ADH3_EMENI	220	VFIIDFSKSDIVADVKAATFGGLGAHAVILLAVAEKPFQQAITEYVRSHGS	269
ADH1_CANAL	220	AVVDFTSKDIIVADVKAATFGGLGAHAVILLAVAEKPFQQAITEYVRSHGS	267
ADH1_ASFFL	218	AVVDFTSKDIIVADVKAATFGGLGAHAVILLAVAEKPFQQAITEYVRSHGS	266
ADH1_EMENI	218	TVYDFTSKSDIVADVKAATFGGLGAHAVILLAVAEKPFQQAITEYVRSHGS	266
ADH4_KLULA	245	VFIIDFSKSDIVADVKAATFGGLGAHAVILLAVAEKPFQQAITEYVRSHGS	292
MPD_CRYNE	279	LVAVGMPDLEAMGAMVFWVEKSKITLGSYVGNRQDAIEALDIAASGLQVR	327
ADH3_EMENI	270	VVALIGLPANAFKAPVFTTVVRMINIKGSYVGNRQDGVVEALDFFARGLIK	319
ADH1_CANAL	268	VVLVGLPAHAKVTAAPVFDVVKSTIEIKGSYVGNRQDPTAEALDFFARGLIK	317
ADH1_ASFFL	267	VVALIGLPAGAFVLRAPVFNVTVRMINIKGSYVGNRQDGVVEALDFFARGLIK	316
ADH1_EMENI	267	VVALIGLPEDAYLKAFLVNTVVRMINITIKGSYVGNRQDGVVEALDFFARGLIK	316
ADH4_KLULA	293	VVLVGLPADAVVKSSEVFSHVVKSTLTKGSYVGNRQDPTREALTDFTRGLVVK	342
MPD_CRYNE	328	VVFEEKGLDLSLKGVEFDELAGKIAGRVLKVSDE	361
ADH3_EMENI	320	AFKKAPLQDLPQIFELMEQKTIAGRVLLEIP-E	352
ADH1_CANAL	318	CEFLKIVGLSLDLPVEFKLMEQKTIAGRVLDT-S-K	350
ADH1_ASFFL	317	AFKFTAPLQDLPKIEELMEQKTIAGRVLLEIP-E	349
ADH1_EMENI	317	AFKFTAPLQDLPKIEELMEQKTIAGRVLLEMP-E	349
ADH4_KLULA	343	SEFLKIIIGLSLPEAYALMEQKTIAGRVLVDTY-K	375

Fig. 3. Multiple alignment of alcohol dehydrogenases from several fungi with the deduced peptide sequence of *C. neoformans* MPD. The sequences shown are *C. neoformans* (MPD-CRYNE), *A. nidulans* (ADH3.EMENI) (McKnight *et al.*, 1985), *Candida albicans* (ADH1-CANAL) (Shen *et al.*, 1991), *Aspergillus flavus* (ADH1-ASFFL) (Woloshuk & Payne, 1994), *A. nidulans* (ADH1.EMENI) (Gwynne *et al.*, 1987), *Kluyveromyces lactis* (ADH4.KLULA) (Saliola *et al.*, 1991). Residues that are identical are boxed. The conserved region for cofactor binding is shown with a black bar. Conserved residues for the active site of zinc ligand are shown with an asterisk. Gaps (indicated by hyphens) were used to optimize the alignments.

min⁻¹ and K_m 0.055 mM for mannitol 1-phosphate; V_{max} 0.17 μ mol mg⁻¹ min⁻¹ and K_m 0.11 mM for NAD; V_{max} 0.31 μ mol mg⁻¹ min⁻¹ and K_m 0.30 mM for fructose 6-phosphate; V_{max} 0.21 μ mol mg⁻¹ min⁻¹ and K_m 0.099 mM for NADH. The K_m and V_{max} for ethanol were 0.94 mM and 0.04 mmol mg⁻¹ min⁻¹, respectively.

NAD-dependent oxidation of mannitol 1-phosphate was optimal at pH 9.0–9.5 and NADH-dependent reduction of fructose 6-phosphate was optimal at pH 7.0–7.5. Optimal MPD activity was at 37 °C and MPD was stable for 1 week at 4 °C. Addition of CaCl₂ (1 mM), NaCl (200 mM), EDTA (5 mM), MgSO₄ (5 mM), NiSO₄ (5 μ M) and ZnSO₄ (2 μ M) had little effect on MPD activity (<10% difference compared to standard buffer), but addition of MnSO₄ (5 mM) increased activity by 220%. However, neither the salts listed above nor EDTA changed the NAD-dependent oxidation of ethanol.

Peptide sequences

Efforts to sequence the N terminus of purified MPD were unsuccessful, which suggested that the N terminus was blocked. Therefore, a tryptic digest of purified MPD was separated by reverse-phase HPLC and three well-resolved tryptic peptides were sequenced. The partial

sequences obtained from these three peptides were (i) LSGYTTDGTFSFY, (ii) (K or R)GVFEDLEAG and (iii) (K or R)SLGADAWVDF (Fig. 2).

Cloning and analysis of the MPD cDNA

The following oligonucleotide primers were based on the partial MPD peptide sequences: KS1: 5'-TT(A/G)TCT GGT TAC ACC AC(A/G/C/T) GA(T/C) GG-3'; KS2: 5'-GAA (A/G/C/T)GT ACC (G/A)TC (A/G/C/T)GT GGT GTA AC-3'; KS3: 5'-GGT GTC TTC GAA GAC TTG G-3'; KS4: 5'-GGT TCA GAA GCT TCT GTG G-3'; and KS5: 5'-CCA AGT CTT CGA AGA CAC C-3'. When the *C. neoformans* cDNA library was amplified with *Taq* DNA polymerase and primers KS1 (derived from peptide 1) and KS5 (derived from peptide 2), a single 600 bp PCR product was obtained. When this PCR product was cloned and sequenced, its deduced peptide product contained the third peptide sequence (KSLGADAWVDF) that had previously been determined from *C. neoformans* MPD. Since this implied that the 600 bp PCR product was derived from the MPD cDNA, it was labelled and used to identify the full-length *C. neoformans* MPD cDNA. A plasmid identified by colony hybridization (pYcnmpd) contained 1380 bp of insert DNA, which consisted of (i) 45 bp of 5' untranslated DNA, (ii) a 1083 bp ORF

Fig. 4. Expression of the *C. neoformans* MPD cDNA in *S. cerevisiae* YPH252. Cell extracts (30 µg protein) of *S. cerevisiae* YPH252 transformed with pYES2.0 (lane 1) or with plasmid pYCnmpd (lane 2) were subjected to PAGE under non-denaturing conditions and stained for MPD activity. Only the pYCnmpd transformant contained MPD activity.

encoding a deduced peptide of 361 aa and M_r 37513, and (iii) a 3' polyA tail.

Neither this cDNA sequence nor its deduced protein product was homologous to the nucleotide or deduced peptide sequences of mannitol-1-phosphate dehydrogenases from several bacteria (Brown & Bowles, 1977; Fischer *et al.*, 1991; Novotny *et al.*, 1984) or the protozoon *Eimeria tenella* (Schmatz, 1997). However, a BLAST search of the GenBank and SWISS-PROT databases (Altschul *et al.*, 1997) revealed that the deduced peptide was 43–47% identical to long-chain alcohol/polyol dehydrogenases from several fungi and that it was most similar to alcohol dehydrogenase III from *Aspergillus nidulans*. The deduced peptide also contained conserved residues for cofactor binding (Wierenga & Hol, 1983) and conserved residues for two active sites containing zinc (Jornvall *et al.*, 1987) (Fig. 3).

Expression of MPD in *S. cerevisiae*

To verify that pYCnmpd indeed encoded MPD, extracts of pYCnmpd- or pYES2-transformed *S. cerevisiae* YPH252 cells were cultured in SC-galactose and the cell lysates were tested for MPD catalytic activity. Because of the high background levels of non-specific NAD reductase activity, we were unable to demonstrate MPD activity in the pYCnmpd transformants using the

Fig. 5. Northern blot analysis. (a) Total RNA (10 µg) isolated from *C. neoformans* grown in YPD medium alone and YPD medium with 0.2–1.5 M NaCl, (b) total RNA (10 µg) isolated from *C. neoformans* grown in YPD medium at 25 °C, 30 °C and 37 °C, (c) total RNA (10 µg) isolated from *C. neoformans* H99 and *C. neoformans* MLP grown in SC-glucose and SC-mannitol. The amount of RNA loading was normalized with actin probe.

standard fluid-phase enzyme assay. However, non-denaturing polyacrylamide gels prepared from lysates of pYCnmpd transformants contained MPD catalytic activity, whereas gels prepared from pYES2-transformed controls did not (Fig. 4).

Since these results implied that pYCnmpd encoded MPD, we next examined the phenotypic consequences of transforming *S. cerevisiae* with pYCnmpd. We found that pYCnmpd did not confer on galactose-grown *S. cerevisiae* YPH252 *gpd1Δ::leu2* transformants the ability to synthesize mannitol from glucose, nor did pYCnmpd reverse the inability of these transformants to grow in YPD supplemented with 0.2–2.5 M NaCl.

Northern blot analysis

Northern blotting experiments demonstrated the 1.6 kb MPD mRNA in *C. neoformans* cells grown in SC-glucose, SC-mannitol, or YPD and they showed that MPD mRNA levels were only 1.2-fold higher in cells grown in mannitol than in those grown in glucose. When MPD mRNA levels were examined in *C. neoformans* cells grown at various salt concentrations, MPD mRNA levels increased as NaCl concentrations were increased from 0 to 0.4 M NaCl and they decreased as NaCl concentrations were increased from 0.6 to 1.5 M (Fig. 5a). In addition, MPD mRNA levels were threefold higher in cells grown at 30 °C or 37 °C than in cells grown at 25 °C (Fig. 5b). Lastly, the amounts of MPD mRNA were similar in glucose- and mannitol-grown cultures of wild-type *C. neoformans* H99 and the mannitol-underproducing mutant of *C. neoformans* MLP (Fig. 5c).

Cloning and analysis of *MPD1*

When the *C. neoformans* genomic DNA library was probed with the 600 bp partial MPD cDNA, a positive lambda clone was identified and purified. Restriction mapping and Southern blotting localized the gene of interest to a 6.0 kb *EcoRI*–*BglII* fragment, which was ligated into pBluescriptII SK+ (Stratagene). The sequence of this gene (*MPD1*) was identical to the cDNA sequence except for the presence of three introns (Fig. 2). Two of these introns (A and B) had splice sites that conformed with the consensus 5' and 3' sequences (GTNNGY and YAG) in other *C. neoformans* introns (Parker & Patterson, 1987). In contrast, the 3' splice site of the third intron (C) was AAG instead of YAG. Analysis of the 5' flanking DNA revealed the presence of potential CAAT and TATA boxes at positions –99 bp and –241 bp, respectively (Fig. 2). A BLAST search of the partial *C. neoformans* JEC21 (serotype D) genomic database revealed a homologue of *MPD1*. Lastly, the MPD probe hybridized to single bands in restriction digests of *C. neoformans* genomic DNA, which suggested that *MPD1* is a single-copy gene (data not shown).

DISCUSSION

This report describes the purification and properties of *C. neoformans* MPD and the isolation and analysis of the corresponding cDNA and gene. We found that *C. neoformans* cell extracts contained a 148 kDa homotetrameric enzyme (MPD) that catalysed the NAD-dependent oxidation of mannitol 1-phosphate to fructose 6-phosphate. Although enzymes that catalyse the same reaction have been described in several bacteria, fungi and the protozoon *E. tenella*, all of these previously known enzymes are very different from *C. neoformans* MPD. For example, the bacterial MPDs are monomers of ~40–45 kDa (Brown & Bowles, 1977; Fischer *et al.*, 1991; Novotny *et al.*, 1984), *Aspergillus niger* MPD is a homodimer of 22 kDa subunits (Kiser & Niehaus, 1981) and *E. tenella* MPD is a 67 kDa protein that was purified as a complex with a 35 kDa dimeric inhibitory protein 14-3-3 (Schmatz, 1997).

Although the most active substrates for *C. neoformans* MPD were mannitol 1-phosphate and fructose 6-phosphate, this enzyme also catalysed the interconversion of sorbitol 6-phosphate with glucose 6-phosphate, as do the MPDs of *Pyrenochaeta terrestris* and *Sclerotinia sclerotiorum* (Jennings, 1984; Wang & Le Tourneau, 1972). *C. neoformans* MPD resembled *A. niger* MPD in that it was exclusively NAD-dependent (Kiser & Niehaus, 1981). In contrast, *E. tenella* MPD can utilize NAD or NADP as cofactors (Schmatz, 1997). The K_m and V_{max} values of *C. neoformans* MPD for mannitol 1-phosphate, fructose 6-phosphate, NAD and NADH were similar to those of *A. niger* MPD (Kiser & Niehaus, 1981). Unlike the *Aspergillus parasiticus* enzyme, zinc had no effect on the thermal stability of *C. neoformans* MPD (Foreman & Niehaus, 1985). Like most dehydrogenases, the optimal activity for the oxidation of mannitol 1-phosphate was at pH 9.0–9.5, whereas op-

timum reduction of fructose 6-phosphate was at pH 7.0–7.5. *C. neoformans* MPD was highly active at 37 °C, whereas the optimal temperature for *E. tenella* MPD was 41 °C (Schmatz, 1997).

We used partial peptide sequences derived from purified *C. neoformans* MPD to generate a specific hybridization probe and to clone the *C. neoformans* MPD cDNA by hybridization. Three independent lines of evidence support the conclusion that the cDNA of interest encodes *C. neoformans* MPD. First, the molecular size of the deduced product encoded by the cDNA of interest and the subunit molecular size of *C. neoformans* MPD as determined by denaturing SDS-PAGE were very similar (37.5 kDa and ~36 kDa, respectively). Second, all three partial peptide sequences derived from purified *C. neoformans* MPD (including one that was not used to generate the hybridization probe) were encoded by the cDNA of interest. Third, lysates of *S. cerevisiae* cells that had been transformed with the cDNA of interest contained MPD catalytic activity, whereas lysates of vector-transformed controls did not.

Computerized searches of the GenBank and SWISS-PROT databases showed that the *C. neoformans* deduced peptide sequence was homologous to long-chain alcohol/polyol dehydrogenases from several different micro-organisms. The alignment of *C. neoformans* MPD with other alcohol dehydrogenases from yeast revealed a conserved region around amino acids 190–200 which contains the typical GXGXXG pattern found in the coenzyme-binding domain of dehydrogenases (Wierenga & Hol, 1983). The deduced *C. neoformans* MPD sequence also contained the conserved Cys residues at positions 55 and 136 and the conserved His residue at position 78, which together form the active site for the zinc ligand. The deduced MPD sequence also contained Cys residues at positions 110, 113, 116 and 124, which are similar to the conserved Cys residues in the second zinc-binding site in yeast alcohol dehydrogenase (Jornvall *et al.*, 1987). Comparison of *C. neoformans* MPD with other alcohol dehydrogenases also revealed a tetrameric quaternary structure similar to yeast alcohol dehydrogenases, which agrees with the experimental data. Once we noted the structural similarities between *C. neoformans* MPD and other alcohol dehydrogenases, we tested *C. neoformans* MPD for alcohol dehydrogenase activity and found that it reduced ethanol at 56% the rate of mannitol 1-phosphate. The ethanol activity was not affected by the addition of salts or EDTA. The K_m for ethanol was higher than that for mannitol 1-phosphate, suggesting more efficient binding of mannitol 1-phosphate by the enzyme. Also, horse-liver alcohol dehydrogenase purchased from Sigma reduced NAD in the presence of mannitol 1-phosphate (at 23% the rate of ethanol reduction; data not shown). Based on all the similarities summarized above, we concluded that *C. neoformans* MPD is a member of the zinc-containing long-chain alcohol dehydrogenase family.

Availability of the *C. neoformans* MPD cDNA enabled us to begin to examine the functions of MPD. One

approach was to examine the phenotypic consequences of overproducing *C. neoformans* MPD in *S. cerevisiae*. Chaturvedi *et al.* (1997) showed that overproduction of bacterial MPD conferred on *S. cerevisiae* YPH252 *gpd1Δ::leu2* transformants the abilities to convert glucose to mannitol and to grow in high salt concentrations. However, we found that lysates of the *S. cerevisiae* transformants contained very low levels of MPD catalytic activity. Therefore, the observations that plasmid pYCnmpd did not confer on *S. cerevisiae* YPH252 the ability to synthesize mannitol from glucose or on *S. cerevisiae* YPH252 *gpd1Δ::leu2* the ability to grow in hypertonic media may have been due to inefficient heterologous expression rather than implying that MPD does not catalyse a key step in mannitol biosynthesis in *C. neoformans*.

We also measured MPD mRNA levels in *C. neoformans* cells that were cultured in glucose or mannitol or that were subjected to temperature and osmotic stresses. If *C. neoformans* synthesizes and catabolizes mannitol via the unidirectional mannitol cycle originally proposed by Hult *et al.* (1980) and if MPD is a key component of the biosynthetic arm of this pathway, one would predict that MPD mRNA levels would be higher in glucose-grown *C. neoformans* cells than in mannitol-grown cells. We found, however, almost as much MPD mRNA in *C. neoformans* cells cultured in SC-mannitol as in cells cultured in SC-glucose, which was consistent with the observation by Niehaus & Flynn (1994) that mannitol-grown *C. neoformans* contained both MPD and M1Pase. We also found that glucose- and mannitol-grown cells of the mannitol-underproducing mutant *C. neoformans* MLP contained as much MPD mRNA as did wild-type *C. neoformans* H99. Although this result implies that inability to express MPD mRNA was not responsible for mannitol underproduction in *C. neoformans* MLP, we have not ruled out a loss of function mutation in the gene encoding MPD in *C. neoformans* MLP. Lastly, because earlier studies suggested that mannitol may function in *C. neoformans* as an intracellular stress protectant (Chaturvedi *et al.*, 1996a), we also examined MPD mRNA levels in *C. neoformans* cells exposed to temperature and osmotic stresses and we found that MPD mRNA levels increased when *C. neoformans* was cultured in YPD supplemented with 0.2 or 0.4 M NaCl (but not higher levels) and when the incubation temperature was increased from 25 °C to 30 °C or 37 °C.

Although the results summarized above suggested that MPD may play a role in environmental stress responses, it was not possible to draw definitive conclusions about the functions of MPD in *C. neoformans*. Therefore, the logical next step will be to generate and analyse *C. neoformans* mutants lacking MPD. Since targeted gene disruption is now feasible in *C. neoformans* (Chang *et al.*, 1996; Lodge *et al.*, 1994), we used the MPD cDNA to clone the corresponding structural gene (*MPD1*) by hybridization. The coding sequence of this gene was identical to that of the MPD cDNA except for the presence of three introns, and a potential TATA box

was present 99 bp upstream and an adenosine residue (consistent with the Kozak model of translation initiation in *S. cerevisiae* (Kozak, 1986) was present 3 bp upstream of the putative ATG start codon. Since genomic Southern hybridization indicated that *MPD1* is a single-copy gene, construction of *C. neoformans mpd1* null mutants should be technically feasible and analysis of these mutants should provide definitive information about the functions of MPD in *C. neoformans*.

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REFERENCES

- Albertyn, J., Hohmann, S., Thevelein, J. M. & Prior, B. A. (1994). GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *S. cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. *Mol Cell Biol* **14**, 4135–4144.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.
- Brown, A. T. & Bowles, R. D. (1977). Polyol metabolism by a caries-conducive *Streptococcus*: purification and properties of a nicotinamide adenine dinucleotide-dependent mannitol-1-phosphate dehydrogenase. *Infect Immun* **16**, 163–173.
- Chang, Y. C., Penoyer, L. A. & Kwon-Chung, J. K. (1996). The second capsule gene of *Cryptococcus neoformans*, CAP64, is essential for virulence. *Infect Immun* **64**, 1977–1983.
- Chaturvedi, V., Flynn, T., Niehaus, W. G. & Wong, B. (1996a). Stress tolerance and pathogenic potential of a mannitol mutant of *Cryptococcus neoformans*. *Microbiology* **142**, 937–943.
- Chaturvedi, V. P., Wong, B. & Newman, S. L. (1996b). Oxidative killing of *Cryptococcus neoformans* by human neutrophils: evidence that fungal mannitol protects by scavenging oxidants. *J Immunol* **156**, 3836–3840.
- Chaturvedi, V., Bartiss, A. & Wong, B. (1997). Expression of bacterial *mtlD* in *Saccharomyces cerevisiae* results in mannitol synthesis and protects a glycerol-defective mutant from high-salt and oxidative stress. *J Bacteriol* **179**, 157–162.
- Cox, G. M., Rude, T. H., Dykstra, C. C. & Perfect, J. R. (1995). The actin gene from *Cryptococcus neoformans*: structure and phylogenetic analysis. *J Med Vet Mycol* **33**, 261–266.
- Eriksson, P., Andre, L., Ansell, R., Bloomberg, A. & Alder, L. (1995). Cloning and characterization of GPD2, a second gene encoding sn-glycerol-3-phosphate dehydrogenase (NAD⁺) in *Saccharomyces cerevisiae*, and its comparison with GPD1. *Mol Microbiol* **17**, 96–107.
- Fischer, R., Von Strandmann, R. P. & Hengstenberg, W. (1991). Mannitol-specific phosphoenolpyruvate-dependent phosphotransferase system of *Enterococcus faecalis*: molecular cloning and nucleotide sequences of the *enzymell^{mtl}* gene and the mannitol-1-phosphate dehydrogenase gene, expression in *Escherichia coli*, and comparison of the gene products with similar enzymes. *J Bacteriol* **173**, 3709–3715.

- Foreman, J. E. & Niehaus, W. G. (1985). Zn²⁺-induced cooperativity of mannitol-1-phosphate dehydrogenase from *Aspergillus parasiticus*. *J Biol Chem* **260**, 10019–10022.
- Gwynne, D. I., Buxton, F. P., Sibley, S., Davies, R. W., Lockington, R., Scazzocchio, A. & Sealy-Lewis, H. M. (1987). Comparison of the cis-acting control regions of two coordinately controlled genes involved in ethanol utilization in *Aspergillus nidulans*. *Gene* **51**, 205–216.
- Hult, K., Veide, A. & Gatenbeck, S. (1980). The distribution of the NADPH regenerating mannitol cycle among fungal species. *Arch Microbiol* **128**, 253–255.
- Jennings, D. H. (1984). Polyol metabolism in fungi. *Adv Microb Physiol* **25**, 149–193.
- Jornvall, H., Persson, B. & Jeffery, J. (1987). Characteristics of alcohol/polyol dehydrogenases. The zinc-containing long-chain alcohol dehydrogenases. *Eur J Biochem* **167**, 195–201.
- Kiser, R. C. & Niehaus, W. G. (1981). Purification and kinetic characterization of mannitol-1-phosphate dehydrogenase from *Aspergillus niger*. *Arch Biochem Biophys* **211**, 613–621.
- Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44**, 283–292.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lodge, J. K., Jackson-Machelski, E., Toffaletti, D. L., Perfect, J. R. & Gordon, J. I. (1994). Targeted gene replacement demonstrates that myristoyl-CoA:protein N-myristoyltransferase is essential for viability of *Cryptococcus neoformans*. *Proc Natl Acad Sci U S A* **91**, 12008–12012.
- McKnight, G. L., Kato, H., Upshall, A., Parker, M. D., Saari, G. & O'Hara, P. J. (1985). Identification and molecular analysis of a third *Aspergillus nidulans* alcohol dehydrogenase gene. *EMBO J* **4**, 2093–2099.
- Niehaus, W. G. & Flynn, T. (1994). Regulation of mannitol biosynthesis and degradation by *Cryptococcus neoformans*. *J Bacteriol* **176**, 651–655.
- Novotny, M. J., Reizer, J., Esch, F. & Saier, M. H., Jr (1984). Purification and properties of D-mannitol-1-phosphate dehydrogenase and D-glucitol-6-phosphate dehydrogenase from *Escherichia coli*. *J Bacteriol* **159**, 986–990.
- O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* **250**, 4007–4021.
- Parker, R. & Patterson, B. (1987). *Molecular Biology of RNA: New Perspectives*. Edited by M. Inouye & B. S. Dudock. New York: Academic Press.
- Saliola, M., Gonnella, R., Mazzony, C. & Falcone, C. (1991). Two genes encoding putative mitochondrial alcohol dehydrogenases are present in the yeast *Kluyveromyces lactis*. *Yeast* **7**, 391–400.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schmatz, D. M. (1997). The mannitol cycle in *Eimeria*. *Parasitology* **114S**, S81–S89.
- Shen, H. D., Choo, K. B., Lee, H. H., Hsieh, J. C., Lee, W. R. & Han, S. H. (1991). The 40-kilodalton allergen of *Candida albicans* is an alcohol dehydrogenase: molecular cloning and immunological analysis using monoclonal antibodies. *Clin Exp Allergy* **21**, 675–681.
- Sherman, F., Fink, G. R. & Hicks, J. B. (1986). *Methods in Yeast Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Tarczynski, M. C., Jensen, R. G. & Bohnert, H. J. (1993). Stress protection of transgenic tobacco by production of the osmolyte mannitol. *Science* **259**, 506–510.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Wang, H. T., Rahaim, P., Robbins, P. & Yocum, R. R. (1994). Cloning, sequence, and disruption of the *Saccharomyces diastaticus* DAR1 gene encoding a glycerol-3-phosphate dehydrogenase. *J Bacteriol* **176**, 7091–7095.
- Wang, S. Y. & Le Tourneau, D. (1972). Mannitol biosynthesis in *Sclerotinia sclerotiorum*. *Arch Mikrobiol* **81**, 91–99.
- Wierenga, R. K. & Hol, W. G. J. (1983). Predicted nucleotide-binding properties of p21 protein and its cancer-associated variant. *Nature* **302**, 842–844.
- Woloshuk, C. P. & Payne, G. A. (1994). The alcohol dehydrogenase gene *adh1* is induced in *Aspergillus flavus* grown on medium conducive to aflatoxin biosynthesis. *Appl Environ Microbiol* **60**, 670–676.
- Wong, B., Brauer, K. L., Tsai, R. R. & Jayasimhulu, K. (1989). Increased amounts of the *Aspergillus* metabolite D-mannitol in tissues and serum of animals with experimental aspergillosis. *J Infect Dis* **160**, 95–103.
- Wong, B., Perfect, J. R., Beggs, S. & Wright, K. A. (1990). Production of the hexitol D-mannitol by *Cryptococcus neoformans* in vitro and in rabbits with experimental meningitis. *Infect Immun* **58**, 1664–1670.

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