4-Dihydromethyltrisorate dehydrogenase from *Mucor mucedo*, an enzyme of the sexual hormone pathway: purification, and cloning of the corresponding gene

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We have purified the NADP-dependent 4-dihydromethyltrisorate dehydrogenase from the zygomycete *Mucor mucedo*. The enzyme is involved in the biosynthesis of trisporic acid, the sexual hormone of zygomycetes, which induces the first steps of zygothecium development. Protein was obtained from the (–) mating type of *M. mucedo* after induction with trisporic acid, and purified by gel filtration and affinity chromatography steps. On SDS-PAGE a band with an apparent molecular mass of 33 kDa was ascribed to the enzyme. After transferring onto PVDF membranes the protein was digested with endoprotease Lys-C, and several peptides were sequenced. Oligonucleotides derived from protein sequence data were used for PCR amplification of genomic *M. mucedo* DNA. The PCR fragment was used as probe for isolation of the corresponding cDNA and complete genomic DNA clones. Comparison of protein and DNA sequence data showed that the cloned fragment corresponded to the purified protein. Search for similarity with protein sequences of the Swiss-Prot database revealed a relationship to enzymes belonging to the aldo/keto reductase superfamily. Southern-blot analysis of genomic DNA with the labelled cloned fragment detected a single-copy gene in both mating types of *M. mucedo*. PCR with genomic DNA from other zygomycetes gave rise to several fragments. Hybridization analysis with the cloned *M. mucedo* fragment showed that a fragment of similar length cross-hybridized in *Blakeslea trispora* (Choanephoraceae) as well as in *Parasitella parasitica* and *Absidia glauca* (Mucoraceae). The promoter region of the gene contains DNA elements with similarity to a cAMP-regulated gene of *Dictyostelium discoideum*.

Keywords: mating type, trisporic acid, oxidoreductase, *Mucor mucedo*, aldo/keto reductase superfamily

INTRODUCTION

Early studies of sexual reactions between different species of zygomycetes often revealed the development of structures which resembled stages of sexual differentiation, but which could not develop to fertile zygospores (Burgeff, 1924; Blakeslee & Cartledge, 1927). These experiments

were used to classify (+) and (–) mating types of zygomycetous strains. Later, the sexual pheromone pathway of *Mucor mucedo* and *Blakeslea trispora* was described in detail (Nieuwenhuis & van den Ende, 1975; Bu'Lock *et al.*, 1976; see Gooday, 1994, for a review). The two mating types produce different precursors which can be converted exclusively by the complementary mating type to the active form, the sexual hormone trisporic acid (TA). The (+)-type-specific precursor 4-dihydromethyl-TA is converted to methyl-TA by a dehydrogenase of the (–) mating type and subsequently to TA by a (–)-type-specific esterase. TA stimulates its own production and

Abbreviations: RT, reverse transcriptase; TA, trisporic acid.

The EMBL accession number for the sequence reported in this paper is Z73640.

also the activity of the dehydrogenase (Werkman, 1976). NADP and to some degree also FAD are accepted as cofactors. Enzyme activity could be found in cell extracts of both mating types of *M. mucedo*. However, no conversion of 4-dihydromethyl-TA to TA could be found *in vivo* with the (+) mating type (Werkman, 1976).

We are interested in the mycoparasitic interaction between *Parasitella parasitica* and its host *Absidia glauca*, both belonging to the family Mucoraceae. Some physiological observations in this system led to the hypothesis that links exist between the sexual development and the parasitic interaction. One argument for this assumption is the fact that the parasite discriminates between the mating types of the host. Analogous to the mating-type reaction, only (+)-type *P. parasitica* strains infect (-)-type *A. glauca* strains, and only (-)-type *P. parasitica* strains infect (+)-type *A. glauca* strains (Burgeff, 1924). Some similarities between sexual and parasitic development could be found. In the sexual reaction cytoplasmic fusion occurs between special regions of hyphal tips, the progametangia, of the two mating types. The interaction between parasite and host also involves cytoplasmic fusion between the partners. Recently, we demonstrated an active parasexual gene transfer system from the parasite to the host (Kellner *et al.*, 1993). *P. parasitica* infects a wide variety of other fungal species, all belonging to the order Mucorales. The sexual communication system works between different species of this order. It was demonstrated that TA isolated from *B. trispora* induces zygophores in *M. mucedo* (Werkman & van den Ende, 1973). We also showed by thin-layer chromatography that complementary combinations of *P. parasitica* and *A. glauca* were able to synthesize TA cooperatively. One idea is that the sexual pheromone system in this parasitic interaction also plays a role in host recognition. Thus, we are interested in isolating genes involved in sexual differentiation. In another approach we characterized cDNA clones of *P. parasitica*, the expression patterns of which are regulated by trisporic acid. Some clones could be identified, most of them belonging to multigene families (Vetter *et al.*, 1994).

In this paper we describe the identification and isolation of the gene for 4-dihydromethyl-TA dehydrogenase of *M. mucedo*.

METHODS

Strains and growth conditions. The following strains were used: *M. mucedo* CBS 109.16(-) and CBS 144.24(+); *B. trispora* CBS 131.59(-), CBS 130.59(+) and ATCC 14060(-); *A. glauca* CBS 101.48(+) and CBS 100.48(-); *P. parasitica* CBS 208.28(-), CBS 207.28(+), ATCC 11077(-) and CBS 412.66(+); and *P. blakesleeana* strains of (-) and (+) mating type. The *M. mucedo* strains and *B. trispora* CBS 131.59 and CBS 130.59 were a gift from H. van den Ende (University of Amsterdam). The *P. blakesleeana* strains were from the Pilzkultursammlung Weimar, Jena. The other strains were from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, or the American Type Culture Collection. For enzyme preparations *M. mucedo* strain CBS 109.16(-) was grown for 3–4 d in liquid culture of complete medium (Wöstemeyer, 1985) with 10 g maltose l⁻¹ as carbon source. At 12 h before harvesting, mycelia were induced with TA at a final

concentration of 10 µg ml⁻¹. Zygophore induction by TA and 4-dihydromethyl-TA was tested according to van den Ende & Stegwee (1971). For this purpose *M. mucedo* was grown for approximately 4 d on Petri dishes containing the following medium: 2% (w/v) maltose, 0.1% (w/v) yeast extract, 100 mM KNO₃, 37 mM KH₂PO₄, 10 mM MgSO₄·7H₂O, 1.5% agar (Oxoid). After induction with appropriate substances for 12 h, the zygophores were counted under the stereomicroscope.

Purification and sequencing of 4-dihydromethyl-TA dehydrogenase. Enzyme was isolated from mycelia of (-)-type *M. mucedo*. Mycelia were washed once with H₂O in a Büchner funnel, pulverized under liquid nitrogen and resuspended in 50 mM Tris/HCl pH 8 on ice. After centrifugation at 41 000 g and 4 °C for 50 min the supernatant was concentrated by ultrafiltration on membranes with a cut-off size for proteins of 30 kDa (YM30, Amicon). Protein amounts were determined (Stoschek, 1990) and the retentate containing the enzyme activity was purified by gel filtration on a Sephadex G 200 column (volume 200 ml, flow rate 0.02–0.05 ml min⁻¹). The column was equilibrated with 50 mM Tris/HCl pH 8, 1 mM EDTA. Fractions containing enzyme activity were pooled and concentrated by ultrafiltration on Centrprep-30 (Amicon). The retentate was separated by affinity chromatography on a Blue Sepharose CL-6B (Pharmacia) column (volume 10 ml, flow rate 0.2–0.5 ml min⁻¹) equilibrated with 50 mM Tris/HCl pH 8, 1 mM EDTA. After washing the column with the same buffer, proteins were eluted with 10 mM NADP in 50 mM Tris/HCl pH 8, 1 mM EDTA. Proteins were concentrated and desalted by washing in Centricon-10 (Amicon) ultrafiltration tubes to remove NADP ions. Fractions containing enzyme activity were separated by SDS-PAGE according to Laemmli (1970). After electrophoresis, proteins were transferred onto PVDF membranes (Immobilon, Millipore). After staining with 0.5% Ponceau S (Sigma) in 1% (v/v) acetic acid, the appropriate protein band was cut out, fragmented with endoprotease Lys-C, separated by HPLC on a Vydac C18-column, and sequenced as described by Braun *et al.* (1994).

Enzyme assay and isolation of TA and 4-dihydromethyl-TA.

TA was isolated as described by van den Ende (1968) from mixed cultures of both mating types of *B. trispora*. TA was extracted from acidified culture medium with chloroform containing 5% (v/v) 2-propanol. 4-Dihydromethyl-TA was obtained by methylation and reduction of isolated TA suspensions (Werkman, 1976). Bioactivity of TA and 4-dihydromethyl-TA was measured by zygophore induction and the compounds were analysed by thin-layer chromatography on silica gel 60 F₂₅₄ (Merck) according to Werkman & van den Ende (1973). Dehydrogenase activity was measured as previously described (Werkman, 1976). Appropriate protein amounts were incubated for 1 h at 35 °C in the dark in a total volume of 0.5 ml with 200 µg 4-dihydromethyl-TA, 100 mM Tris/HCl pH 7.8, 0.24 mM nitro blue tetrazolium (Sigma), 0.13 mM phenazine methosulfate, 0.78 mM NADP, 0.2% gelatin. The reaction was stopped by acidifying the solution to pH 1 with HCl. The difference in absorbance was measured at 540 nm. The standard curve was established by reducing nitro blue tetrazolium non-enzymically to formazan with NADPH according to Nachlas *et al.* (1960). One unit of 4-dihydromethyl-TA dehydrogenase activity is defined as the amount of enzyme forming 1 µg formazan under the conditions described above.

PCR conditions, sequencing and hybridizing conditions. PCR conditions were similar to those described previously (Burmester & Czempinski, 1994). The PCR reaction contained, in a volume of 20 µl: 25 ng genomic DNA, 10 pmol of each primer, 0.2 mM dNTPs, 3 mM MgCl₂, 10 mM Tris/HCl

pH 8.3, 50 mM KCl, 0.01% gelatin and 2 U *Taq* polymerase (Boehringer). Fragments were amplified in a programmable water bath (Grant AG005 Autogene IT, CLF analytische Laborgeräte) for 30 cycles with the following temperature profile: 0.3 min at 94 °C, 0.3 min at 45 °C, 1 min at 72 °C. The following primers were chosen: P1, 5'-CGAATTCIC G/T G/A TCIA A/G IGCCCA-3'; and P2, 5'-CCAGCTGGAA T/C ACITT C/T CA-3' (I: dIMP).

To facilitate the cloning procedure, the primers contained restriction sites for *Eco*RI or *Pvu*II, respectively (shown underlined). PCR fragments were restricted with both enzymes and ligated into the *Eco*RI and *Sma*I restriction sites of the polylinker region of pBluescript KS⁺, pBluescript SK⁻ (Stratagene) or pTZ18R (Pharmacia Biotech). Ligation products were transformed by standard procedures into *Escherichia coli* XL1 Blue (Bullock *et al.*, 1987). Stringent hybridization conditions for Southern blots (Southern, 1975) were as described previously (Burmester & Czempinski, 1994). A partial gene library was constructed of *M. mucedo* (–)-type genomic DNA. Genomic DNAs of *M. mucedo* strain CBS 109.16 were prepared according to the method described previously (Burmester & Wöstemeyer, 1986). DNAs were restricted with *Sac*I and *Bam*HI, separated on 0.8% agarose gels and fragments 4–5 kb in size were eluted from the gels. Fragments were ligated into the *Bam*HI and *Sac*I site of the polylinker region of pTZ18R and transformed into *E. coli* XL1 Blue. Approximately 10⁴ transformants were picked on Petri dishes and used for colony filter hybridization with the radioactively labelled PCR fragment as probe. A genomic clone was isolated, containing a 4.5 kb *Sac*I/*Bam*HI fragment with the whole gene. RNA was prepared from (–)-type *M. mucedo* liquid cultures which were induced with TA 4 h before treatment. Liquid cultures of mycelium were harvested by filtration and homogenized in a mortar under liquid nitrogen. After evaporation of liquid nitrogen, the resulting powder was suspended in 8 M guanidine/HCl, 20 mM MES/KOH pH 7.0, 20 mM EDTA, 1% (v/v) β -mercaptoethanol. After centrifugation at 5000 g for 5 min the supernatant was extracted with 1 vol. phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). The aqueous phase was mixed with 0.05 vol. 1 M acetic acid and 0.7 vol. 99% ethanol and precipitated overnight at –20 °C. After centrifugation at 5000 g for 10 min at 4 °C the RNA pellet was washed twice with 3 M sodium acetate pH 5.2 and twice with 70% ethanol, and dried under vacuum. The RNA pellet was redissolved in H₂O and used for RT-PCR following the instructions of the manufacturers (3' RACE system, Gibco BRL). The following primers were used: P3, 5'-CGACTGCACTGGAACACGTTTCACAGC-3'; an oligo(dT)-adapter primer (AP); and the universal amplification primer (UAP) of the 3' RACE system kit. To facilitate cloning, primer P3 contains a *Pst*I restriction site (shown underlined) and primer UAP a *Sac*I site. The RT-PCR products were eluted from agarose gel, restricted with *Sac*I and *Pst*I and ligated into the *Sac*I and *Pst*I sites of the polylinker region of pBluescript SK⁺. The ligation products were transformed into *E. coli* XL1 Blue. A cDNA clone was obtained and DNA sequences of the genomic PCR and the cDNA clones were determined by standard procedures. Also the 2.1 kb *Bam*HI/*Pst*I fragment, a part of the genomic clone containing the whole 4-dihydromethyl-TA dehydrogenase gene, was sequenced on both strands.

RESULTS

Isolation and characterization of the 4-dihydromethyl-TA dehydrogenase

In order to optimize protein yield, cultures of (–)-type *M. mucedo* were pretreated with TA, which increased the

Fig. 1. Separation of purified 4-dihydromethyl-TA dehydrogenase by SDS-PAGE. Lanes 1 and 2, samples of several 4-dihydromethyl-TA dehydrogenase preparations; the arrow marks the band cut out for protein sequencing. Proteins were visualized with Ponceau S staining solution.

enzyme activity up to 15-fold. This is in accordance with previous results (Werkman, 1976). Induction of enzyme activity was also found with β -ionone, which stimulates the production of β -carotene (Eslava *et al.*, 1974). After affinity chromatography the proteins were separated by SDS-PAGE. Three bands, with molecular masses of 33, 36 and 57 kDa, could be detected (Fig. 1). Some preparations of activity peaks after affinity chromatography did not contain the 57 kDa protein. The 33 kDa protein was eluted and renatured according to Hager & Burgess (1980), and enzyme activity was measured. The renatured fraction retained 87% of the original activity. Renaturation experiments with the 36 kDa protein were not successful. Therefore, we ascribed the 4-dihydromethyl-TA dehydrogenase activity to the 33 kDa band.

Determination of the protein sequence and cloning of the gene for 4-dihydromethyl-TA dehydrogenase

The proteins from the final electrophoresis purification step were stained with Ponceau solution, cut out and subjected to Edman degradation. Because the N-terminus was blocked, successful sequencing required digestion with the endoprotease Lys-C. Several peptide sequences were determined (Table 1). Protein sequence data were used to design the degenerate primers P1 and P2 (see Methods). These primers were used for PCR, and products could be found at an annealing temperature of 45 °C. A major PCR product with a length of 0.7 kb was isolated from an agarose gel and cloned in pTZ18R and pBluescript KS⁺/– in order to determine the DNA sequence. The PCR clone detected a 4.5 kb *Bam*HI/*Sac*I fragment in Southern-blot analysis with (–)-type *M.*

Table 1. Sequences of polypeptides of the 4-dihydromethyl-TA dehydrogenase obtained by digestion with endoprotease Lys-C

The asterisks mark uncertain positions. Numbers indicate codon positions of the amino acid sequence of the ORF.

No.	Peptide sequence	Codon position
1	MGYRLFDGAEDYG * IETQSIKL A S TAG P F V	40–52
2	REDLFIVTK	72–80
3	LWNTFHSK	81–88
4	TYTSLLEHASVK	233–244
5	NQVLLRWALDREFAVIK *	255–272

mucedo genomic DNA. A partial gene library was screened, containing restriction fragments with the expected length, and the corresponding genomic clone was isolated. For isolation of a cDNA clone, RNA was isolated from TA-induced (–)-type *M. mucedo* cultures. A cDNA clone was obtained by reverse transcription of the RNA combined with PCR (RT-PCR).

Sequence analysis of the cloned gene for 4-dihydromethyl-TA dehydrogenase

DNA sequence data revealed that all the sequenced polypeptides could be localized in the gene (Table 1). Deviations between DNA and protein sequence were found in peptide 1 in codon position 49. This is probably due to contamination of this fragment with other peptides. A second deviation was found in peptide 5 at codon position 271, where the proline predicted by the DNA sequence was determined as isoleucine in the peptide. In both codon positions no sequence deviations were found between cDNA and genomic DNA. This excludes an RNA-editing mechanism for these peptide sequence changes. No further sequence deviations were found between cDNA clone, genomic sequence and protein sequence. The poly(A) tail was added to nucleotide 1458 of the genomic sequence (Fig. 2). A putative polyadenylation site was found at position 1430.

A short (74 bp) intron disrupts the open reading frame, starting at position 687 of the nucleotide sequence (Fig. 2). The cDNA clone contains no part of the intron sequence, indicating that the intron is spliced correctly. The intron characteristics are very similar to those of other introns of zygomycetes, such as those of the gene for elongation factor 1 α of *Mucor racemosus* (Sundstrom *et al.*, 1987) and *A. glauca* (Burmester, 1995) and the gene for orotidine-5'-phosphate decarboxylase of *P. blakesleeana* (Díaz-Mínguez *et al.*, 1990), *Mucor circinelloides* (Benito *et al.*, 1992) and *Rhizopus niveus* (Horiuchi *et al.*, 1995). Only introns of small size have been found in zygomycetes, and

GGATCCGCGGAAAAATAGTAAAGAAGTTGGGTGCGTTAGTGCTTACAACCTTAACAAGAA	60
TATCTCTAACTTACACACACACACATATATAACGGACAGTTTTATATCACAAGTA	120
AGGTAAAGGCATTCAAATGTGGGTAGAAAATCAAACCTTTTGATGTGCTGATTATATA	180
TTAGGTGAATTTTTTTTTTTCATTAGTGCCACAGTTTGAAGCGGCATTAAACCCC	240
AGGAAAAAAGGCGCTATATAACAAGAAGTTTGGGTCTGTTCTTACTTTTTTT	300
TTTTCTCTCTTGATTATATTACACACACATACCTCAAATTAATAATGCCACTGATTA	360
M S T D Y	5
CCTTACCTTGAACAGAACCGGCGACAAGATGCCCATTCGCGGTTTCGGCTGTTGAAGAT	420
L T L N R T G D K M P I R G F G C W K I	25
TGACACCAAGGATTGTGAGGAGACCGTCTACCAAGCTATCAAGACTGGTTACCGTCTTT	480
D T K D C E E T V Y Q A I K T G Y R L F	45
CGATGGTCCCTGTGATTATGGTAACGAAGTCGAGGTGGTGGTATTAACAAGGCCAT	540
D G A C D Y G N E V E V G R G I N K A I	65
CAACGAAGTTTGGTCAAGAGAGAAGATCTTTTCATCGTCACCAAGCTCTGGAACACCTT	600
N E G L V K R E D L F I V T K L W N T F	85
CCACGACGAAGACGTCGGTGGCTTGTTCGATAGACAATTAAGGATACCGGTCTTGA	660
H S K K H V R A L F D R Q L K D T G L E	105
ATACTTGTGATCTTATCTCATTCATTGTAAGTACATTTTAAGACGCGTCATTGTTTGT	720
Y F D L Y L I H	113
GGGGGGTTATTAGTATACTAATCTTACATCTTAACTAGTCCCGGTTCCTTGCAATAT	780
F P V P L Q Y	120
GTTGACCCGTGCTACCGTCTACCCCTCCCGGTGGTATGTGCGGTGATGCCAAGTCTCTCAA	840
V D P A T V Y P P G W Y V G D A K S L Q	140
TTGAGCAATCTCCCATCCACGAATGTGGGCTGAGTTAGAAAAGATTGTCGATGCTGGT	900
F E Q S P I H E C W A E L E K I V D A G	160
TTGGCTCGTAACATTGGTGTGCTAAGTCTCAAGTATCTTGGACTTGTCTACC	960
L A R N I G V A N F N C Q A I L D L L T	180
TATGCCAGATTAAGCCTGCCGTTTACAAATCGAAGTCCATCTTACTTGCCTCAAGAG	1020
Y A R I K P A V L Q I E L H P Y L P Q E	200
CGTTTGGTCAAGTGGTCAAGGAACAAGGATTCAAATCTGCTTACTCTCTTCTCGGT	1080
R L V K W V K E Q G I Q I T A Y S S F G	220
CCTACCTCTTATGTTGACTTGACTGAATCTGGTAAGACTTATACCTCTTGTCTGCAACAC	1140
P T S Y V D L T E S G K I Y T S L L E H	240
GCCAGTGTCAAGTCTGTTGCCGATAAGCACAAGCTTCCACTGGTCAAGTCTTGTCCGT	1200
A S V K S V A D K H N V S T G Q V L L R	260
TGGGCTTGGACCGTGAATTGCGTGTATATCCCAAGAGTGAACGCTGGTAGAATGAAG	1260
W A L D R E F A V I P K S V N A G R M K	280
GCTAAGTGGAACTTGGATATCAAGTCTGATGCTGAGGATAACAAGACTTTGGATCTT	1320
A N L E I L D I K L D A E D N K T L D S	300
CTCAAGACCAACCAAGATTCAACGATCCCATGACCTACGGTTTCGGTTTGCCTCTTTTC	1380
L K T N Q R F N D P M T Y G F G L P L F	320
GATTAAGCAATACACACATACACACACACACACACCCCTTGTAATTAATAAATACCG	1440
D -	321
CAATTTTTTTTTTTCATCAGTCACTTTTTTTTTTATTAACATAATGTTGTTCAATACCGCT	1500
*	
TCTAGCATGGCATGCCGTCATGATACCTTGAATCGTCTACGTTTCGCATCGATCCAA	1560
AGCATAGTTCTGCATAGGTTTTGGGTAAAGATACGACGCAATTCACCTTATCGTTTCT	1620
GAAAGCAAGGTGAATGGAAACATTGGTAGTTTCACGCGTACCCCTTTCAATATTATG	1680
ACCTCTGCAATGACACGGACTTGACTGACCGATGTTGGGTATCCCTCGGATAAACAATA	1740
CCTGATCTCAAAATCAATATGACACATGCACTGGCTTTCGAAAGTGACTTCATCC	1800
ATCGACACAAGGTAGGTGAGGAAGTCTTCATGAAGAGAGCAGTATTGTCATACGCCAAT	1860
TCATAGGCACGACGCATCAAGTAACCAACCAAGATATTGTTGTCAGGTTACGATCTGA	1920
GGCTGCATAAAAAATTAATTTCAATCTTTGATCAGCATCCAACTAGGTAGAGGC	1980
AATGGCTGCATTTTCATCAGACGATTGAGAGTTTATAAAGCGCTCATCACCTGTACCGTTG	2040
GTGACTGAGAACTACTGCA	2060

Fig. 2. Nucleotide sequence of the 4-dihydromethyl-TA dehydrogenase. Underlined parts of the nucleotide sequence correspond to the cDNA clone. Sequenced parts of the protein are also underlined. The asterisk marks the position of the poly(A) tail in the cDNA sequence. The CACA boxes are shown in bold.

the 5' and 3' motifs match with consensus motifs known for other eukaryotes. The intron presented here disrupts codon 34, for phenylalanine, of the protein sequence.

Table 2. Percentage identity in pairwise comparison of the protein sequence for the dihydromethyl-TA dehydrogenase with other members of the aldo/keto reductase superfamily

Top half, percentage identity; bottom half, number of identities/number of residue pairs compared (without gaps). Proteins were as follows: 1, rat 3 α -hydroxysteroid dehydrogenase (Pawlowski *et al.*, 1991); 2, human aldose reductase (Chung & LaMendola, 1989); 3, *Pichia* xylitol reductase (Amore *et al.*, 1991); 4, *Mucor* 4-dihydromethyl-TA dehydrogenase; 5, *Hordeum* aldose reductase (Bartels *et al.*, 1991); 6, *Saccharomyces* GCY protein (Oechsner *et al.*, 1988); 7, *Leishmania* putative reductase (Samaras & Spithill, 1989); 8, *Pseudomonas* morphine dehydrogenase (Willey *et al.*, 1993).

No.	1	2	3	4	5	6	7	8
1		45	29	30	27	27	25	22
2	133/ 295		37	35	32	36	35	31
3	87/ 299	111/ 299		47	31	24	29	28
4	90/ 299	105/ 299	142/ 299		29	24	25	28
5	81/ 295	93/ 291	93/ 299	86/ 299		30	27	26
6	80/ 295	105/ 291	73/ 299	71/ 299	86/ 283		31	28
7	75/ 295	101/ 291	86/ 299	76/ 299	75/ 276	87/ 283		38
8	64/ 295	89/ 291	83/ 299	84/ 299	73/ 276	79/ 283	102/ 269	

A sequence motif of CACA repeats flanked the open reading frame. It could be detected at nucleotide positions 323–335, in the vicinity of the putative translational start codon region, and at positions 1391–1417, immediately after the putative translational stop codon. The last region is also part of the cDNA clone. Whether the 5' box is also transcribed cannot be answered, because the cDNA clone does not contain the complete 5' end. A third CACA box is at positions 72–87 of the promoter region. Additionally, the promoter contains pyrimidine-rich stretches, similar to those found in the promoter of the elongation factor 1 α from *A. glauca* (Burmester, 1995). A putative TATA box starts at position 317.

Protein sequence comparison with data from the Swiss-Prot database shows similarity to proteins belonging to the aldo/keto-reductase superfamily. Highest similarity is found to the NADPH-dependent xylose reductase of the yeast *Pichia stipitis* (Amore *et al.*, 1991). The proteins share an identity of 47% at the amino acid level (Table 2). Fig. 3 shows alignments of some protein examples, all belonging to the aldo/keto-reductase superfamily. There was no significant similarity to dehydrogenases belonging to other superfamilies, such as the short-chain dehydrogenases, or the zinc-containing alcohol dehydrogenases.

The percentages of identical amino acid positions of some

1) Rattus	HDH	MDSISLRVA	9
2) Homo	ALR	ASRLL	5
3) Pichia	XYR	MPSIK	5
4) Mucor	TDH	MSTDYLT	7
5) Hordeum	ALR	MASAKATMGQGEQDH	16
6) Saccharomyces	GCY	MPATLHDSKILS	13
7) Leishmania	PUR	MAGVDKAMVT	10
8) Pseudomonas	MDH	MAGKSP	6

1) LN-DGNFIPVLGFGTTVPEKVADEVIKATKIAIDNGFRHFSAYLYEVE	58
2) LN-NGAKMPLGLGTW---KSPPGQVTEAVKVAIDVGYRHIDCAHVYQNE	51
3) LN-SGYDMPAVGFGCW---KVDVDTCEQIYRAIKTYGLFDGAEDYANE	51
4) LNRTGDKMPIRGFGCW---KIDTKDCEETVYQAIKTYGLFDGACDYNE	54
5) L-KSGHAMPVGLGTWRAGSDTAHSVRTAI---TEAGYRHVDTAAYGVGE	62
6) LN-TGAGIPQIGLGTW---QSKENDAYKAVLTALKDGYRHIDTAAYRNE	59
7) LSN-GVKMPQFGLGVW---QSPAGEVTENAVKWCAGYRHIDTAAYKNE	57
8) LNN-GVKMPALGLGVF---AASAE-TASATASAISSGYRLIDTARSYNNE	52

1) EEVGQATRSKIEDGTVKREDIFVYSKLWSTFHRPELVRTCLETKLSTQL	108
2) NEVGVAIQEKLREQVVKREELFVSKLWCTYHEKGLVKGACQKTLSDLLK	101
3) KLVGAGVKKAIIDEGIVKREDLFTSKLWNNYHHPDNVEKALNRTLSDLQ	101
4) VEVGRGINKAINEGLVKREDLFIIVTKLWNTFHSKKHVRALFDRQLKDTGL	104
5) KEVGKGLKAAMEAG-IDRKDLFVTSKIWCTNLAPERRVPALENTLKDQL	111
6) DQVGQAATK---DSGVPREEIFVTTKLWCTQHHEPEV---ALDQSLKRLGL	103
7) ESVGAGLR---ASGVPRDVFITTKLWNTTEQGYSTLAAFEESRQKLG	103
8) AQVGEGIR---NS-VDRAEFMVITTKLNCQDGYERALRAFDLSRLGL	97

1) DYVDLYIHFPMALQ---PGDIFFPD---EHGKLLFETVDICDTWEA	150
2) DYDLLYLIHWPTGFK---PGKEFFPLD---ESGNVVPSTNILDWAA	143
3) DYVDLYLIHFVPTFK---FVPLEKYPGFGCGDNFDYDVPILETWKA	149
4) EYFDLYLIHFVPLQ---YVDPATVYPPGWYVGDAKSLQFEQSPIHECWA	152
5) DYIDLHYIHWPFRLK---DGAHMPPE---AGEVLEFDMEGV---WKE	149
6) DYVDLYLMHWPARLDPAYIKNEDILSVPTKKDGSRAVDITNWNFIKTWEL	153
7) DYIDLHYIHWPR-----GKDLILSKGKKYLDWSRA	133
8) DYVDLYLHWPR-----TKDWNATIQSWKA	121

1) MEKCKDAGLAKSIGVSNFNCRLERILNKPGLKYKPVNCQVECHLYNQS	200
2) MEELVDEGLVKAIGISNFHNLQVEMILNKPGLKYKPAVNCQIECHPYLTQE	193
3) LEKLKAGKIRISIGVSNFPGALLDLRLGATIK---PSVLQVEHHPYLQOP	197
4) LEKIVDAGLARNIGVANFNQATLDLLTYARIK---PAVLQIELHPYLPQE	200
5) MENLVKDGVLKDIGVCNYTIVTKLNRILRSKIP---PAVCQEMHPGKND	197
6) MQELPKTGKTKAVGVSNFISNNLKDLLASQGNKLPAAHQVEIHPLLPDQ	203
7) FEQLYKEKVRIGVSNFHIHLEDVLAQCMTVT---PMVNGVELHPLNQA	181
8) AEKILGDGRARIGVCNFLEDQDELTAASDVV---PAVNQIELHPYFAQK	169

1) KMLDYCKSKDIIIVSYCTLGSSRDKTWVDQS---PVLDDPVLCAIAK	246
2) KLQYCSQSGIIVTAYSPGLSP-DRPWAKPED---PSLLEDPRIKAIK	238
3) RLIEFAQSGIAVTAYSSFGPQSFVELNQGRLANT-SPLFENETIKAIK	246
4) RLKWKVEQGIQITAYSSFGPTSYDLTES-GKTY-TSLEHASVKSVD	246
5) KIFEACKKHGIHVTAYSPGSGS-----KNLADHPVVEKVAN	234
6) ELINFCCKSGIIVVEAYSPGSTD-----APLLKEPVILEIAK	240
7) DLRAFCDAKQIKVEANSPLG-----QGKLSNPLISAIGA	216
8) PLLAKNRALGIVTEAMSPIGCHQ---RWQDGNHGRKHPITDPVITIAE	217

1) KYKQTPALVALRYQLRGVPLIRSFNAKRIKELTQVFEFLASEDMKAL	296
2) KHNKTTAQLIRFPMQRNLVVPKSVTPERIAENKVFDFELSSQDMTTL	288
3) KHGKSPAQVLLRWSSQGIATIPKSNTPVRLLENKDVSFDLDEQFADI	296
4) KHNVTGQVLLRWALDREFAVIPKSVNAGRMKANLEIDIKLDAEDNKT	298
5) KLNKTPGQVLIKWALQRTSVIPKSSKDERIKENIQVFGWEIPEEDFKVL	284
6) KNNVQPGHVVISHWVQRGVVLPKSVNPDRIKTRNIFT---LSTEDFEAT	288
7) KYNKTAQVILRWNIQKNLITIPKSVHREIEENADIFDFELGAEDVMSI	266
8) AHGRSAAQVILRWHFQNDVVAIPKSVNPERIAKNIDVDFALSDAEMAQL	267

1) DGLNRNFRYNNAKYFDDHPNHPFTDE	322
2) LSYNRNWRVALLSCTSHKDYPFHEEF	315
3) AKLDINLRFNDPMDWKIPFV	318
4) DSLKTNQRFNDPMTYGFGLPLFD	321
5) CSIKDEKRVLTGEELFVNKTHGPRYSAADVMDHEN	320
6) NNISSKEGKRVVHPNWSPEVFK	312
7) DALNTNSRYGPDDEAQF	284
8) DELDTGVRIGDPDRDVTSSFAEFV	295

Fig. 3. Alignment of protein sequences of the 4-dihydromethyl-TA dehydrogenase with other members of the aldo/keto reductase superfamily. Asterisks mark identical positions. Abbreviations: HDH, 3 α -hydroxysteroid dehydrogenase; ALR, aldose reductase; XYR, xylitol reductase; TDH, 4-dihydromethyl-TA dehydrogenase; GCY, GCY protein; PUR, putative reductase; MDH, morphine dehydrogenase.

members of the aldo/keto-reductase superfamily are shown in Table 2. Values were obtained by comparison of the same fragment length of all proteins, which corre-

Fig. 4. Southern-blot analysis of genomic DNA of *M. mucedo* with the cloned PCR fragment of the 4-dihydromethyl-TA dehydrogenase. Genomic DNAs of *M. mucedo* strains CBS 144.24(+) (lanes 1–6) and CBS 106.16(–) (lanes 7–12) were restricted with *Eco*RI, *Pst*I, *Bam*HI, *Hind*III, *Pvu*II and *Sac*I in that order. The 1 kb ladder (Gibco BRL) was used as size marker (M).

sponded to amino acids 8–306 of the 4-dihydromethyl-TA dehydrogenase.

Hybridization analysis and PCR assay with genomic DNA of other zygomycetes

Genomic DNAs of *M. mucedo* (+) and (–) strains were digested with *Eco*RI, *Pst*I, *Bam*HI, *Hind*III, *Pvu*II and *Sac*I. The corresponding blots were hybridized with a labelled probe of the cloned PCR fragment, and only one band was seen in all lanes (Fig. 4). Therefore we conclude that only a single copy of this gene is present in the genome of both mating types. No polymorphism could be detected between the two mating types, which indicates that the adjacent regions are conserved.

PCR primed by degenerated primers with genomic DNA templates of other zygomycetes, *A. glauca*, *P. parasitica* and *B. trisporea*, gave rise to a multiple band pattern on agarose gels (Fig. 5a). Hybridization of the corresponding blot with the labelled PCR fragment of *M. mucedo* showed that only one band in all lanes cross-hybridized. This band had a similar size to the PCR product from *M. mucedo* DNA (Fig. 5b). No hybridization signals were obtained for either mating type of *P. blakesleeana*.

DISCUSSION

Little is known about the genetic basis of sexual differentiation in zygomycetes. This report presents the analysis of one gene of the sexual pheromone pathway and sequence comparison data show that the 4-dihydromethyl-TA dehydrogenase of *M. mucedo* belongs to the aldo/keto reductase superfamily. Most enzymes belonging to this superfamily prefer NADP/H as cofactor.

Comparison of a bacterial morphine dehydrogenase (Bruce *et al.*, 1994) with other members of the aldo/keto reductase superfamily distinguishes three subgroups in this superfamily. Subgroup I contains the mammalian aldose reductases and human aldehyde reductase; subgroup II contains other mammalian oxidoreductases with a wide variety of substrates. Members of subgroup III share lower similarities to each other; here we find bacterial enzymes, e.g. the morphine dehydrogenase of *Pseudomonas putida* (Willey *et al.*, 1993) and the 2,5-diketo-D-gluconate reductase of *Corynebacterium* sp. (Grindley *et al.*, 1988), and eukaryotic enzymes such as a putative reductase of *Leishmania major* (Samaras & Spithill, 1989) and the GCY protein of *Saccharomyces cerevisiae* (Oechsner *et al.*, 1988). Some members of the superfamily cannot be placed in the three subgroups and probably represent additional subgroups. Examples were the xylitol reductase

Fig. 5. PCR with genomic DNAs of other zygomycetes. PCR products were separated on a 1.2% agarose gel and the gel was blotted. The blot was hybridized with a radioactively labelled probe of the cloned PCR fragment of *M. mucedo*, containing parts of the 4-dihydromethyl-TA dehydrogenase gene. DNAs of the following strains were used: *M. mucedo* CBS 109.16 (lane 1) and CBS 144.24 (lane 2); *B. trisporea* CBS 131.59 (lane 3) and CBS 130.59 (lane 4); *P. parasitica* ATCC 11077 (lane 5), CBS 412.66 (lane 6), CBS 208.28 (lane 7) and CBS 207.28 (lane 8); *A. glauca* CBS 101.48 (lane 9) and CBS 100.48 (lane 10); *P. blakesleeana* (–) mating type (lane 11) and (+) mating type (lane 12). The 1 kb ladder (Gibco BRL) was used as size marker (M).

of *Pichia stipitis* (Amore *et al.*, 1991) and the aldose reductase of *Hordeum vulgare* (Bartels *et al.*, 1991). The degree of identical amino acid positions, summarized in Table 2, shows that the xylitol reductase of *P. stipitis* has the highest similarity to the 4-dihydromethyl-TA dehydrogenase of *M. mucedo*. Comparison of the three fungal sequences shows that the xylitol reductase and 4-dihydromethyl-TA dehydrogenase show a higher similarity to sequences of subgroup I and II than to the GCY protein of yeast. One might speculate that distinction between the two types of genes arose at a very early point of evolution, similarly perhaps as the homologies found between the elongation factor I and II genes, which are discussed as a result of duplication of the universal ancestor (Hasegawa *et al.*, 1992).

Analysis of the structural components of the human aldose reductase (Wilson *et al.*, 1992) gives interesting clues for the deduced protein sequence of the 4-dihydromethyl-TA dehydrogenase. In human aldose reductase the substrate-binding site is located in a large deep elliptical pocket at the COOH-terminus with a bound NADPH in an extended conformation. The hydrophobic nature of the active site favours aromatic and apolar substrates over highly polar monosaccharides. Thus compounds such as isocorticosteroids are the best substrates for aldose reductase (Wilson *et al.*, 1992). In the aldo/keto reductase superfamily substrates are often hydrophobic substances like steroids or in our case 4-dihydromethyl-TA, which is a derivative of β -carotene.

In aldose reductase two possible sites, a tyrosine at position 48 or a histidine at position 110, might act as hydrogen donors in transferring hydrogen. Both regions are highly conserved in all proteins of the superfamily. The 4-dihydromethyl-TA dehydrogenase contains both hydrogen donor/acceptor sites found by similarity at positions 51 and 113 of the amino acid sequence (Fig. 3).

The promoter region of the gene for 4-dihydromethyl-TA dehydrogenase contains sequence elements, the CACA boxes, which were also found in a cAMP-regulated stage-specific gene of *Dictyostelium discoideum* (Haberstroh & Firtel, 1990). Promoter deletion experiments showed that deletion of these boxes resulted in a loss of cAMP and developmentally induced expression (Haberstroh & Firtel, 1990). Similar promoter studies of the gene for 4-dihydromethyl-TA dehydrogenase should shed light on the role of these sequence motifs in *Mucor*.

PCR experiments with DNAs of other zygomycetes show that it is possible to identify genes homologous to the *M. mucedo* 4-dihydromethyl-TA dehydrogenase gene from *P. parasitica* and *A. glauca*. Transformation with gene replacement vectors will allow the knockout of these genes. Thus, we may be able to reveal molecular links between parasitic and sexual development.

ACKNOWLEDGEMENTS

We thank Professor H. van den Ende for his advice and help in the initial phase of the project. This work was supported by

grants from Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

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Received 22 January 1996; revised 26 April 1996; accepted 9 May 1996.