4-Dihydrotrisporin-Dehydrogenase, an Enzyme of the Sex Hormone Pathway of *Mucor mucedo*: Purification, Cloning of the Corresponding Gene, and Developmental Expression†*

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Received 11 July 2008/Accepted 7 October 2008

The NADP-dependent 4-dihydrotrisporin-dehydrogenase is a (−) mating-type-specific enzyme in the pathway from β-carotene to trisporic acid. This substance and its isomers and derivatives represent the general system of sexual communication in zygomycetes. The (−) mating type of *Mucor mucedo* was stimulated by trisporic acid and the enzyme was purified by ion exchange and affinity chromatography. Several peptides of the 26-kDa protein, digested with trypsin, were sequenced by mass spectrometry. Oligonucleotides based on protein sequence data were used for PCR amplification of genomic DNA. The primary PCR fragment was sequenced and the complete gene, TSP2, was isolated. A labeled TSP2 hybridization probe detects a single-copy gene in the genome of *M. mucedo*. Northern blot analysis with RNAs from different growth stages reveals that the expression of the gene depends on the developmental stage of the mycelium in both mating types of *M. mucedo*. At the enzyme level, activity is found exclusively in the (−) mating type. However, renaturation of proteins in sodium dodecyl sulfate-containing gels revealed the TSP2 gene product in both mating types. Analyzing the protein sequence places the enzyme in the short chain dehydrogenase superfamily. Thus, it has an evolutionary origin distinct from that of the previously isolated 4-dihydromethyltrisporate dehydrogenase, which belongs to the aldo/keto reductase superfamily. Apart from the TSP2 genes in the three sequenced zygomycetous genomes (*Phycomyces blakesleeanus*, *Rhizopus oryzae*, and *Mucor circinelloides*), the closest relative is the *Myxococcus xanthus* CsgA gene product, which is also a short chain dehydrogenase, involved in C signaling and fruiting body formation.

The existence of a conserved system for sexual communication in zygomycetes had been assumed very early based on observations of the ability of mucoralean fungi belonging to different species to differentiate into early sexual morphological stages in cocultures on petri dishes, although without leading to zygospores (4, 7). These observations allowed consistent assignment of zygomyces across species, genus, and family borders to (+) or (−) mating types.

In contrast to the modified peptide pheromones in asco- and basidiomycetes, the sexual reaction of zygomycetes is mediated by trisporic acid and its numerous derivatives and isomers. Trisporoids are involved in partner recognition, sexual morphogenesis, and feedback regulation of trisporoid synthesis. Especially in *Mucor mucedo*, they also induce the first discernible sexual differentiation structures, the zygophores. Physiological aspects of the trisporic acid system for sexual communication have recently been reviewed (27, 38).

Although the synthesis of trisporic acid from β-carotene (2) is still hypothetical in some parts of the pathway, the best and most consistent model assumes a shared and cooperative synthesis by both complementary mating types, accomplished by exchanging mating-type-specific precursors between complementary sexual partners (5, 6, 23, 35, 36). Although this general scheme applies to all mucoralean zygomycetes, and trisporoids are even recognized by Mortierellales (29), the spectrum of trisporoid derivatives and isomers differs in detail (38). Also, the amounts of trisporic acid and its precursors differ considerably between species. Whereas *Phycomyces blakesleeanus* and *M. mucedo* produce only small amounts of trisporoids even in mated cultures, and no detectable levels if the mating types are cultivated separately, the natural overproducer *Blakeslea trispora* secretes approximately 20 mg/liter trisporic acid in mated cultures, and even in individual cultures the trisporoid amounts are sufficient to induce zygophores in *M. mucedo*. There are only marginal observations for other mucoralean fungi, although the basic principle of trisporic acid synthesis, shared between complementary mating types, seems to be fulfilled.

Individual mating types of *M. mucedo* degrade carotene to 4-dihydrotrisporin, which is subsequently converted to specific precursor molecules. These cannot be processed further but are instead released into the medium and passed to the mating partner, which subsequently accomplishes conversion to trisporic acid.

Little is known about the reactions from β-carotene to the last precursor common to both mating types, 4-dihydrotrisporin (5). Recently, two reports were published on carotene cleavage in zygomycetes. One essentially hypothetical publication focused on possible cleavage products of β-carotene and...
two alternative possible pathways (12), and the second one identified the gene for the until-then hypothetical oxygenase in the trisporoid synthesis pathway. In M. mucedo, the respective reactions are catalyzed by two independent enzymes, 4-dihydrotrisporin dehydrogenase and 4-dihydromethyltrisporate dehydrogenase.

In the (+) mating type the common precursor molecule 4-dihydrotrisporin is oxidized to 4-dihydromethyltrisporate acid by an unknown mechanism, whereas the (−) mating type forms trisporin by a dehydrogenase reaction. Both substances are released and reach the complementary mating partner by diffusion. The (+) mating type converts trisporin by uncharacterized oxidase reactions via trisporol to trisporic acid, while the (−) mating type uses an additional dehydrogenase from 4-dihydromethyltrisporate to methyltrisporate (10, 36). Finally, trisporic acid is formed by action of a methylsterase (36).

The conversion of 4-dihydrotrisporin to trisporin and the formation of methyltrisporate from dihydromethyltrisporate are reactions specific for the (−) mating type. Both reactions involve oxidation of the hydroxyl group at the C-4 position of the ionone ring and both are catalyzed by dehydrogenases using NADP as cofactor (Fig. 1). Regarding the identical chemical reactions and the similar substrates, both reactions could be catalyzed by the same enzyme. Using mating-deficient mutants of P. blakesleeanus, Sutter et al. (34) provided genetic arguments for two distinct enzymes. Mutants of the (−) mating type that produce glycopeptides themselves but do not induce sexual structures in the (+) mating type were isolated. These mutants were shown to be defective in pheromone biosynthesis. Complementation tests by heterokaryon analysis after grafting showed that the conversions of dihydrotrisporin to trisporin and of dihydromethyltrisporate to methyltrisporate are correlated with different mutations and, thus, presumably with different genes. In this communication we show the existence of two separate enzymes encoded by two independent genes in M. mucedo. The 4-dihydrotrisporin dehydrogenase was purified, the corresponding gene was cloned, and its expression was studied at the transcriptional level and at the level of enzyme activity.

**MATERIALS AND METHODS**

**Microbial strains and growth conditions.** Mucor mucedo FSU 620 (−) and FSU 621 (+) were used for molecular experiments, and Blakeslea trispora FSU 331 (+) and FSU 332 (−) were used for isolating trisporic acid. All strains are available from the Fungal Reference Centre, Chair of General Microbiology and Microbe Genetics, University of Jena. Strains were maintained on solid supplemental medium (37). For isolating 4-dihydrotrisporin dehydrogenase, 10^3 germinal spores of M. mucedo (−) were grown on petri dishes on a medium that induces sexual differentiation (29), covered with sterile cellophane sheets, for 60 h at 18°C in the dark. Eight to 12 h before harvest of the mycelia, the cultures were sexually stimulated by spraying with approximately 100 µg trisporic acid in 20% ethanol which were isolated from mated cultures of Blakeslea trispora.

For time course experiments, approximately 10^3 spores of M. mucedo (+) and (−) in equal ratios were grown on induction medium using the cellophane technique. Mycelia were harvested at different growth stages. To investigate the influence of trisporoids at the transcriptional level, some plates were stimulated with trisporoids for 12 h.

For DNA isolation, M. mucedo (−) was cultured in liquid supplemental medium (37) for 3 days on a rotary shaker at 120 rpm and 20°C. Plasmids were propagated in Escherichia coli strains XL1-Blue (Stratagene) and DH5α (14).

**Isolation of 4-dihydrotrisporin dehydrogenase and peptide sequencing.** The mycelium was harvested and frozen in liquid nitrogen. The frozen mycelium was ground to a fine powder in a mortar. The powder was suspended in 0.05 M Tris, pH 9.0, for the isolation of the 4-dihydrotrisporin dehydrogenase or in 0.05 M Tris-Cl, pH 8.0, for time course experiments and was subsequently centrifuged at 16,000 × g for 20 min at 4°C. The supernatant was stored at −20°C until further use. The protein concentration of the crude protein extract was determined using the bicinchoninic acid reaction (32).

The crude protein was mixed with a DEAE-cellulose ion exchanger and was stirred with a glass rod for 30 min on ice. The mixture was poured in a column (3 by 15 cm; Bio-Rad) and washed with three volumes of 0.05 M Tris-Cl, pH 9.0, with increasing concentrations of NaCl. Protein concentrations of the fractions were estimated spectrophotometrically at 280 nm. Fractions with enzyme activity were pooled and concentrated approximately 10 times in a stirred ultrafiltration cell (type 8050; Amicon/Millipore). The concentration was separated by affinity chromatography on Blue Sepharose CL-6B (Pharmacia). The protein solution was incubated with the affinity matrix in a 1:1 ratio for 30 min on ice. Subsequently, the Blue Sepharose was washed with 0.05 M Tris-Cl, pH 9.0, and 0.1 M NaCl in the same buffer. The dehydrogenase was eluted with 10 mM NADP for 30 min. The supernatant was dialyzed for 3 h each against three changes of 1 liter of 0.05 M Tris-Cl, pH 9.0. This preparation was separated on sodium dodecyl sulfate (SDS)-containing polyacrylamide gels (20), and the 4-dihydrotrisporin dehydrogenase band was cut from the gel. In-gel digestion of the enzyme with trypsin was performed according to the Bruker protocol (Bruker Daltonics), as adapted by Shevchenko et al. (30). De novo sequencing of the peptides was carried out using a microOTOF-Q mass spectrometer (Bruker Daltonics). For analyses, Bruker BioTools 3.0 (RapiDENovo) was used.

**4-Dihydrotrisporin activity assay.** Trisporic acid from mated cultures of Blakeslea trispora was isolated as described previously (29). The conversion of trisporic acid to 4-dihydromethyltrisporate was performed as described previously (36). 4-Dihydrotrisporin B (racemic mixture) was synthesized by ACR Laboratories B.V. (Apeldoorn, The Netherlands). Enzyme activity was detected in situ on nondenaturing polyacrylamide gels, as described elsewhere for 4-dihydromethyltrisporate dehydrogenase (28), and on renatured SDS gels. After electrophoresis, the gels were washed three times for 15 min in water, followed by incubation in 1% Triton X-100 for 15 and 30 min, and finally washed twice in water for 20 min. The alternative substrates, 4-dihydrotrisporin B and 4-dihydromethyltrisporate C, were added at 1.7 mM in a polyethylene bag in approximately 0.1 ml of 1.5 mM NADP, 0.75 mM nitroblue tetrazolium chloride, 0.1 mM phenazine methosulfate, 12 mM NaCl, 0.6 mM MgCl₂, and 0.08 M Tris-Cl, pH 8.0, per cm² of gel.

**Nucleic acid preparation, PCR, inverse PCR, and cloning.** Chromosomal DNAs of M. mucedo (−) for PCRs (26) and for Southern blot analysis (11) were isolated as described elsewhere. RNA was extracted using guanidinium thiocyanate and isolated by density ultracentrifugation on a cushion of cesium chloride as described previously (28).

Oligonucleotides derived from protein sequence data were used for PCR amplification of genomic M. mucedo DNA. Primer sequences are listed in Table 1. The PCR mix contained the following constituents in a volume of 25 µl: 12.5
TABLE 1. Peptide sequences obtained by MALDI-TOF mass spectrometry of 4-dihydromethyltrisporin dehydrogenase fragments

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Peptide sequence</th>
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<tr>
<td>1</td>
<td>NPSASEGLQK</td>
<td>TSP2-7f</td>
<td>5'-GGgaattcGTIGCIGGIATGGC-3'</td>
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<tr>
<td>5</td>
<td>SYDNNDNLGAVVTGSK</td>
<td>TSP2-7r</td>
<td>5'-GGgaattcGCIICICRTTTRTC-3'</td>
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<tr>
<td>6</td>
<td>ATGVLASMSOQPVSAGMAK</td>
<td>TSP2-8f</td>
<td>5'-GGgaattcGTTGCAGATCCGMC-3'</td>
</tr>
<tr>
<td>12</td>
<td>ESMGGDDAPVEEEK</td>
<td>TSP2-8r</td>
<td>Not used for cloning</td>
</tr>
</tbody>
</table>

* The enzyme was isolated from M. mucedo (−), identified on SDS gels, and digested with trypsin. Peptide sequences and the deduced degenerated primers used for amplification of part of the corresponding gene are shown. Only those peptides that proved to be unequivocally colinear with the cloned DNA sequence are shown. MALDI-TOF, matrix-assisted laser desorption ionization–time of flight analysis. Sequence portions shown in lowercase letters indicate restriction sites used for cloning.

4-Dihydrotrisporin dehydrogenase was enriched by DEAE-cellulose ion exchange adsorption. The fractions eluted with 0.1 M NaCl display a strong dehydrogenase activity on renatured SDS gels (Fig. 3A), whereas lower, 0.05 M, or higher, 0.15 M, sodium chloride concentrations elute only minor enzyme activities. Fractions with the strongest enzyme activities were used for affinity binding on Blue Sepharose CL-6B. 4-Dihydrotrisporin dehydrogenase elutes with a high yield with the cosubstrate NADP (Fig. 3B).

Protein sequencing, Southern hybridization, and cloning of the corresponding gene. Fractions enriched for 4-dihydrotrisporin dehydrogenase were separated on a denaturing polyacrylamide gel and stained with Coomassie brilliant blue. The prominent band with the molecular mass of 26 kDa was cut out and digested with trypsin, and the peptides were sequenced by a molecular mass of 33 kDa represents the 4-dihydromethyltrisporate dehydrogenase (10).

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**RESULTS**

Isolation and substrate specificity of 4-dihydrotrisporin dehydrogenase. In situ enzyme activity assays for 4-dihydrotrisporin dehydrogenase on non-denaturing gels showed that the enzyme is active in the (−) mating type of *Mucor mucedo* irrespective of sexual stimulation (see Fig. 5). Nevertheless, for enzyme isolation trisporoid-stimulated mycelia, grown on solid medium, were used.

The crude protein extract from such cultures was analyzed for 4-dihydrotrisporin dehydrogenase activity in renatured SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels. For activity staining, two different substrates were used, 4-dihydromethyltrisporate C and 4-dihydrotrisporin B (Fig. 2). 4-Dihydrotrisporin dehydrogenase has an apparent molecular mass of approximately 26 kDa in SDS gels and is able to convert both substrates. It shows, however, a preference for 4-dihydrotrisporin B (Fig. 2, lane 1). With 4-dihydromethyltrisporate as substrate (Fig. 2, lane 2), a prominent band with

**FIG. 2.** 4-Dihydrotrisporin dehydrogenase prefers 4-dihydrotrisporin over 4-dihydromethyltrisporate as substrate. Cytoplasmic protein fractions of *M. mucedo* (−), sexually stimulated for 8 h with a trisporoid preparation from mated cultures of *Blakeslea trispora*, were separated by SDS-PAGE on a 15% polyacrylamide gel. SDS was removed and the proteins were allowed to refold in the presence of 1% Triton X-100. Staining for dehydrogenase activities was performed on single lanes using NADP as cofactor and either 4-dihydrotrisporin (lane 1) or 4-dihydromethyl trisporate (lane 2) as substrate. Lane m, molecular mass markers. TDH, 4-dihydromethyltrisporate dehydrogenase; TNDH, 4-dihydrotrisporin dehydrogenase.
mass spectrometry. Several peptide sequences were determined (Table 1). These sequence data were used to generate primers (Table 1). The primer pair TSP2-5f and TSP2-6r amplified several bands with lengths of 200 bp, 400 bp, 1,500 bp, 1,900 bp, and 2,100 bp. The 200- and 400-bp fragments were isolated from the gel, cloned in pTZ19R, and sequenced. The 1,348-bp sequence contained the complete gene encoding 4-dihydrotrisporin dehydrogenase (accession no. AM937248). Colinearity between the sequenced peptides, originating from the enzymatically active band, and the cloned DNA proves that the isolated single-copy gene encodes dihydrotrisporin dehydrogenase.

**DNA sequence analysis.** Sequence analysis of the cloned 1.3-kb HinP11 fragment revealed that four peptides, 1, 5, 6, and 12 (Table 1), are part of the protein encoded by the TSP2 gene. Several other peptide sequences had no counterpart in the DNA sequence and were ascribed to contaminations of the protein with other polypeptides. The TSP2 gene starts at position 127 of the nucleotide sequence. Bioinformatic results based on a Rossmann fold prediction revealed an NADP cofactor binding domain between amino acid positions 1 and 40 (19; http://www.ifm.liu.se/bioinfo). The open reading frame is disrupted by two short introns. The first one begins at position 322 and has a length of 61 bp. The second intron starts at position 484, has a length of 92 bp, and disrupts codon 99, for threonine, of the protein sequence. These predicted introns have been verified experimentally by comparing the sequence of a cloned TSP2 cDNA with the genomic sequence. A putative polyadenylation signal was found at positions 1030 to 1035. The complete protein consists of 240 amino acids and has a calculated molecular mass of 26 kDa. Protein sequence comparison in the NCBI database established the protein as a member of the short chain dehydrogenase superfamily. Apart from the corresponding genes in other zygomycetes, the highest similarity was found with the CsgA protein of *Myxococcus xanthus* (21). At the amino acid level CsgA shares approximately 34% identity with the zygomycetous TSP2 sequences. In the proteobacterium *Myxococcus xanthus*, the NADP-dependent short chain dehydrogenase CsgA is responsible for C signaling during fruiting body development and is part of a signal chain controlling a transcription factor of early fruit body development (3, 15).

**Time course experiments.** To study the transcription of TSP2 and the enzyme activity of the 4-dihydrotrisporin dehydrogenase, we analyzed mRNA as well as protein extracts from samples harvested over a developmental time course for *M. mucedo* (−) and (+) (Fig. 5A). At the first stage (50 h), mycelia of both mating types cover the petri dish in essentially a single hyphal layer. At this stage sexual stimulation with trisporic acid has no effect on the formation of sexually deter-
mined hyphae (zygophores). At the second stage (62 h) the mycelium covers the petri dish in multiple layers but has not yet developed aerial hyphae. At this stage sexual stimulation with trisporic acid induces zygophore differentiation in the (−) mating type. At the third stage (74 h), the mycelium bears aerial hyphae and the first sporangia appear; however, they are not yet darkly pigmented. At this stage, the (+) mating type produces zygophores after sexual stimulation, too. At stages four (86 h) and five (98 h) the mycelia carry succeedingly maturing, darkly pigmented sporangia.

Crude protein extracts were separated on a nondenaturing polyacrylamide gel, and the enzyme activity was assayed in the gel using 4-dihydromethyltrisporate as substrate (Fig. 5B). This substrate detects both enzymes, dihydrotrisporate as well as dihydrotisporin dehydrogenase. The (−) mating type of M. mucedo shows a strong difference between stimulated and nonstimulated mycelia at the enzyme activity level. Stimulated mycelia show a strong dehydrogenase activity in the upper part of the gel, which represents the 4-dihydromethyltrisporate dehydrogenase (28). This activity is low at the first stage, which is characterized by the absence of zygophores. In the lower part of the gels several activity bands were detected, the strongest of which was ascribed to the 4-dihydrotisporin dehydrogenase. It is active in stimulated as well as in nonstimulated mycelia of the (−) mating type from the second stage and in nonstimulated and, especially at the third stage, in stimulated mycelia. Neither 4-dihydromethyltrisporate dehydrogenase nor 4-dihydrotisporin dehydrogenase activity was detected in native gels of the (+) mating type of M. mucedo, irrespective of sexual stimulation (data not shown). In contrast to these findings, renaturation studies with SDS-containing gels revealed 4-dihydrotisporin dehydrogenase activities at all developmental stages and in both mating types irrespective of sexual stimulation (data not shown).

Northern hybridization analysis showed a TSP2 transcript at all developmental stages of M. mucedo (+) and in the later stages of M. mucedo (−) (Fig. 5C). The transcript was not detectable at the first stage in the (−) mating type. It seems that the amount of TSP2 transcript increased toward later stages of development in the (−) mating type. Thus, we conclude that expression of the TSP2 enzyme depends on the developmental stage of the mycelium in both mating types. Sexual stimulation with trisporic acid from mated cultures of Blakeslea trispora leads to formation of zygophores in both mating types and an increase in the protein activity level in the (−) mating type (Fig. 5B), but the formation of the TSP2 transcript is not affected. Dihydrotisporin dehydrogenase is modulated posttranslationally at the level of enzyme activity rather than by regulating transcription of the corresponding gene.

DISCUSSION

Recently, genome sequences of three zygomycetes, Phycomyces blakesleeanus, Rhizopus oryzae, and Mucor circinelloides, became available. Especially for the latter, the targeted construction of mutants by integration of DNA via homologous integration was shown (1). Recently, R. oryzae was efficiently manipulated by Agrobacterium tumefaciens-mediated transformation (22). Although genetic manipulation of Mucor mucedo in vitro is presently restricted to transient transformation by autonomously replicating plasmids, and although the genome has not yet been sequenced, it is nevertheless the best-suited organism to study sexual differentiation and the action of the trisporoid system. Exclusively in M. mucedo, zygophores, the first visibly differentiated sexual hyphae, can be unequivocally identified in culture. It is the only fungus where visible zygophores can be induced in individual mating types by addition of trisporoids on plates. It is also the only organism to study sexual differentiation, uses trisporic acid derivatives, different from other mucoralean fungi, and is also amenable to genetic manipulation. The physiology of sexual development in R. oryzae has not been investigated; the organism does not cross deliberately and the sequenced, human pathogenic strain does not at all, at least not in our hands. M. circinelloides also crosses with low efficiency and has no discernible zygophores, and the physiology of trisporic acid formation has not been studied. Thus, we decided to isolate and analyze the putative gene for dihydrotisporin dehydrogenase from the classical model organism for sexual differentia-
tion of zygomycetes, *M. mucedo*. In this organism, two other genes for trisporic acid biosynthesis, *TSP1* for dihydromethyltrisporate dehydrogenase and *TSP3* for carotene oxygenase, have been cloned. Presumably, *M. mucedo* will be the fungus for which the regulatory network between all genes and enzymes involved in trisporic acid formation can be elucidated with reasonable efforts. In the long run, a system for constructing targeted insertion mutants in this organism needs to be developed. Due to the unequivocal identification of zygophores even in single cultures and its strict necessity for the complementation of mating types toward trisporic acid synthesis, it is the only organism in which clear phenotypes of mutants with defects early in pheromone synthesis can be expected.

With respect to regulation of the trisporic acid system, the mating-type-specific reactions deserve special interest. The *TSP1* gene for the (−)-specific reaction from 4-dihydromethyltrisporate to methyltrisporate was the first one to be characterized (10). A second reaction, from the last common precursor, 4-dihydrotrisporin, to trisporin, is (−)-type specific, too. Because the chemical reactions are identical and the substrates at least similar, most authors have assumed that both activities might be accomplished by the same enzyme. Genetic analysis of mating-deficient *P. blakesleeanus* mutants indicated the existence of separate genes for these enzymatic activities (34). Alternatively, the catalytically active protein could still be the same for both reactions, while the discernible substrate specificity could be accomplished by an additional regulatory protein, coded for at a different locus. Thus, we decided to provide direct biochemical evidence, both at the enzymatic and the sequence level, for the existence of different proteins and their corresponding genes. In *M. mucedo*, we identified and purified a dehydrogenase distinct from 4-dihydromethyltrisporate dehydrogenase, converting 4-dihydrotrisporin to trisporin. We also cloned and sequenced the corresponding gene, which, following our established nomenclature with the amino acid sequence, CsgA is a short chain dehydrogenase too, but there is no clue with respect to its role in C signaling after specific proteolytic cleavage. According to the amino acid sequence, CsgA is a short chain dehydrogenase too, but there is no clue with respect to its substrate or to a role of the putative enzymatic activity in the catalysis (24); all of these are found in the 4-dihydrotrosporin dehydrogenase from *M. mucedo*.

By sequence comparison, putative *TSP2* homologues were found in the genomes of *M. circinelloides*, *R. oryzae*, and *P. blakesleeanus*. The degrees of identity in pairwise comparisons at the amino acid level are given in Table 2. As expected and in accordance with the phylogenetic distance between these species, *M. circinelloides* harbors the most similar relative (62% identity), whereas *P. blakesleeanus* shows only between 42 and 48% identity with the other *Mucorales*. Apart from the putative *TSP2* homologues in other mucoralean fungi, the CsgA gene from the myxobacterium *Myxococcus xanthus* was found to be the closest relative, with 31 to 35% sequence identity between CsgA and the mucoralean genes. Figure 6 shows an alignment of the *TSP2* genes in the sequenced zygomycetous genomes and the CsgA gene from *M. xanthus*. The CsgA protein is involved in the differentiation process toward aggregation and fruiting body formation, resides at the cell’s surface, and plays its role in C signaling after specific proteolytic cleavage. According to the amino acid sequence, CsgA is a short chain dehydrogenase too, but there is no clue with respect to its substrate or to a role of the putative enzymatic activity in the signaling function (33). Similarities with several other short chain dehydrogenases range around 20%. Only one dehydrogenase with a chemically comparable substrate, a mammalian 11-cis retinol dehydrogenase from *Bos taurus* (31), was found to have reasonable sequence similarities (21% identity).

The activities of the enzymes are regulated differentially. The *TSP1* gene is constitutively transcribed in both mating types of *M. mucedo*, irrespective of sexual stimulation, and the gene product, 4-dihydromethyltrisporate dehydrogenase, was detected in both mating types at the protein level. However, enzyme activity is exclusively found in stimulated mycelia of the (−) mating type and the activation is clearly regulated posttranslationally (28). The 4-dihydrotrosporin dehydrogenase transcript was detected in later developmental stages of (−)-type *M. mucedo*, in which sexual stimulation is possible. In contrast to the *TSP1* gene product, 4-dihydrotrosporin dehydrogenase is also active in nonstimulated mycelia of *M. mucedo* (−). Thus, 4-dihydrotrosporin dehydrogenase is able to convert its substrate very early and prior to zygophore induction. This is consistent with the general model of trisporoid synthesis, in which pheromone production precedes zygophore formation.

**TABLE 2. Amino acid sequence similarities between the *M. mucedo* TSP2 protein, the putative homologues from other mucoralean fungi, and the C-signaling protein CsgA from *Myxococcus xanthus***

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<th>Source of TSP2</th>
<th>% Amino acid sequence similarity with TSP2 (or CsgA) of:</th>
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<tr>
<td></td>
<td><em>M. mucedo</em></td>
</tr>
<tr>
<td><em>M. mucedo</em></td>
<td>100</td>
</tr>
<tr>
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<td><em>P. blakesleeanus</em></td>
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<td>100</td>
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<tr>
<td>CsgA</td>
<td></td>
</tr>
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</table>

"Source of TSP2 (or CsgA)

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Due to its early expression, the TSP2 gene product may function as a crucial part of the sensing system for initial recognition of potential mating partners.

In the (+) mating type of M. mucedo the TSP2 transcript was detected at all developmental stages, whereas no enzymatic activity was found on native gels. Similarly to the situation for the TSP1 gene, TSP2 is regulated at a posttranscriptional level. From comparative analysis of enzymatic activities in native and denaturing gels we found evidence for a small, (+)-type-specific protein that negatively regulates the primary translation product. Until now, the only evidence for considerable transcriptional control in trisporoid biosynthesis was provided for the carotene-cleaving oxygenase, the primary step in the pathway (9).

It has not yet been elucidated if the trisporic acid system or the differentiation program toward the formation of meiospores is regulated by a master system similar to the mating type loci in asco- or basidiomycetes. Provided zygomycetes strictly regulate the biosynthesis of those trisporoids that mediate partner recognition and the induction of the early developmental steps toward sexually determined hyphae, there is no strict necessity to assume a higher level of recognition. In particular, combinations of regulation events at different expression levels—the transcription level for TSP3, a posttranscriptional level for TSP2, and enzymatic regulation for TSP1—may provide a more efficient and versatile strategy for mating partner recognition in these fungi.

FIG. 6. Sequence alignment between the M. mucedo TSP2 gene and its putative homologues in the mucoralean fungi M. circinelloides, R. oryzae, and P. blakesleeanus and the bacterial C signal gene CsgA from Myxococcus xanthus. Amino acids in the lower line are shared among all zygomycetes; those positions that are also identical in the M. xanthus CsgA gene are underlined.
scriptional and a posttranslational level for TSP1, and posttranslationally for TSP2—are well-suited to warrant stringent regulation of sexual development, even if individual regulatory steps are not completely tight. Recently, however, database comparisons of various fungi have allowed identification of transcription factor genes belonging to the high-mobility group of regulatory proteins and thus resembling mating-type genes in the Dikarya group of Mycorota. These genes were mapped to the sex loci in \textit{P. blakesleeanus} (16). Transcription of a very promising HMG transcription factor was found to be induced during mating and in artificially produced strains heterozygous for mating type. Targets of these mating-type-associated transcription factors are not known, and it remains to be seen if they are correlated directly with the trisporic acid system or with subsequent steps in sexual development. They could play a role in regulating the posttranslational modification system that we have found for both the gene products of TSP1 and TSP2. In particular, the (+) type-specific protein of \textit{M. mucedo} that seems to be involved in downregulation of dihydrotirsporin dehydrogenase in this mating type is a good candidate for regulation at the transcription level.

ACKNOWLEDGMENTS

This work was supported by grants from Deutsche Forschungsgemeinschaft and by Fonds der Chemischen Industrie to J.W.

REFERENCES


SEX HORMONE PATHWAY OF \textit{MUCOR MUCEDO} 95

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