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Involvement of a Natural Fusion of a Cytochrome P450 and a Hydrolase in Mycophenolic Acid Biosynthesis

Bjarne Gram Hansen,* Ewelina Mnich, Kristian Fog Nielsen, Jakob Blæsbjerg Nielsen, Morten Thrane Nielsen, Uffe Hasbro Mortensen, Thomas Ostenfeld Larsen, and Kiran Raosaheb Patil*

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Mycophenolic acid (MPA) is a fungal secondary metabolite and the active component in several immunosuppressive pharmaceuticals. The gene cluster coding for the MPA biosynthetic pathway has recently been discovered in *Penicillium brevicompactum*, demonstrating that the first step is catalyzed by MpaC, a polyketide synthase producing 5-methylorsellinic acid (5-MOA). However, the biochemical role of the enzymes encoded by the remaining genes in the MPA gene cluster is still unknown. Based on bioinformatic analysis of the MPA gene cluster, we hypothesized that the step following 5-MOA production in the pathway is carried out by a natural fusion enzyme MpaDE, consisting of a cytochrome P450 (MpaD) in the N-terminal region and a hydrolase (MpaE) in the C-terminal region. We verified that the fusion gene is indeed expressed in *P. brevicompactum* by obtaining full-length sequence of the mpaDE cDNA prepared from the extracted RNA. Heterologous coexpression of mpaC and the fusion gene mpaDE in the MPA-nonproducer *Aspergillus nidulans* resulted in the production of 5,7-dihydroxy-4-methylphthalide (DHMP), the second intermediate in MPA biosynthesis. Analysis of the strain coexpressing mpaC and the mpaD part of mpaDE shows that the P450 catalyzes hydroxylation of 5-MOA to 4,6-dihydroxy-2-(hydroxymethyl)-3-methylbenzoic acid (DHMB). DHMB is then converted to DHMP, and our results suggest that the hydrolase domain aids this second step by acting as a lactone synthase that catalyzes the ring closure. Overall, the chimeric enzyme MpaDE provides insight into the genetic organization of the MPA biosynthesis pathway.

fungi are among the most elaborate chemical producers in nature, producing a range of secondary metabolites, including some that are mycotoxins, food additives, or pharmaceutical drugs. Among the latter is mycophenolic acid (MPA), which is the active component in several immunosuppressants. MPA has also been associated with antiviral, antifungal, antibacterial, and anti-tumor activities (11). Consequently, MPA biosynthesis has received considerable research interest, and a biosynthetic route has been established through chemical labeling and culture feeding studies (3). MPA is a meroterpenoid proposed to be derived from a nonreduced tetraketide moiety through a five-step process involving oxidation, lactonization, and condensation with a farnesyl residue, followed by oxidative cleavage of the terpene part and a final methylation step (Fig. 1A). However, the biosynthesis remained unelucidated at the genetic level until the recent discovery of a putative MPA biosynthetic cluster in *Penicillium brevicompactum* (23). The defined cluster contained eight putative open reading frames (ORFs), including mpaC, which encodes a polyketide synthase catalyzing the production of 5-methylorsellinic acid (5-MOA), the first step in MPA biosynthesis (9, 23). Furthermore, it was recently demonstrated that mpaF encodes an MPA-insensitive inosine-5′-monophosphate dehydrogenase (IMPDH) conferring self-resistance toward MPA (8, 10). These studies utilized *Aspergillus nidulans* as a fungus of choice for the heterologous expression of mpaC and mpaF. *A. nidulans* provides a good model system to study the biosynthesis of MPA, since it can produce polyketides and does not produce any of the intermediates in MPA biosynthesis. In addition, the genome has been sequenced, and in the recent years the molecular biology toolbox has been greatly expanded (9, 16, 17, 19).

In the present study, we set out to identify and characterize the enzyme(s) responsible for the conversion of 5-MOA to 5,7-dihydroxy-4-methylphthalide (DHMP), which are the first and second known intermediates in MPA biosynthesis. A bioinformatics study of the MPA biosynthetic cluster, followed by heterologous expression in *A. nidulans*, showed that the conversion of 5-MOA to DHMP is catalyzed by a natural fusion of MpaD, a cytochrome P450, and MpaE, a putative lactone synthase.

**MATERIALS AND METHODS**

**Strains and media.** The following strains were used in the present study: *P. brevicompactum* strain IBT23078 and *A. nidulans* strains NID210 (argB2 pyrG89 veA1 IS1::PgdA-mpaC-TrpC::argB), NID211 (argB2 pyrG89 veA1 IS1::PgdA-mpaC-TrpC::argB), NID410 (argB2 pyrG89 veA1 kuA ΔIS2::PgdA-mpaC-TrpC::AFpyrG), NID416 (argB2 pyrG89 veA1 IS1::PgdA-mpaC-TrpC::argB IS2::PgdA-mpaC-TrpC::AFpyrG), and NID944 (argB2 pyrG89 veA1 IS1::PgdA-mpaC-TrpC::AFpyrG), Strains NID210 and NID211 were constructed in a previous study (9).

*P. brevicompactum* was grown on Czapek yeast extract agar (CYA) at 25°C. CYA is composed of 5 g of yeast extract (Biokar Diagnostics, Beauvais, France)/liter, 15 g of agar/liter, 35 g of Czapek dox broth/liter, 10 mg of ZnSO4·7H2O/liter, and 5 mg of CuSO4·5H2O/liter. The pH levels were adjusted to 6.5 with NaOH/HCl. *A. nidulans* strains were grown on minimal medium (MM) containing 1% glucose, 10 mM NaNO3, 1× salt

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solution (5), and 2% agar for solid media or YES medium containing 20 g of yeast extract (Biokar)/liter, 150 g of sucrose/liter, 0.5 g of MgSO₄·H₂O and ZnSO₄·7H₂O/liter, 5 mg of CuSO₄·5H₂O/liter, and 2% agar (pH 6). The MM was supplemented with 10 mM uridine (Uri), 10 mM uracil (Ura), and 4 mM L-arginine (Arg) when necessary.

RNA purification and cDNA synthesis. Spores from *P. brevicompactum* IBT23078 were harvested and used to inoculate 200 ml of YES medium in 300 ml of shake flasks without baffles. *P. brevicompactum* was grown at 25°C and 150 rpm with shaking. After 48 h, the mycelium was harvested, and the RNA was purified using the fungal RNA purification miniprep kit (EZNA) according to the manufacturer's instructions. cDNA was synthesized from the RNA using a Finnzymes Phusion RT-PCR kit according to the manufacturer's instructions. The *mpaDE* transcript was amplified with the primer pair 657 and 660 (657, ATGAAGTCTTTGTCGCTAAC; 660, TTACTTCTGTCCTTCTATGG) and cloned into pJET1.2/blunt using a CloneJET PCR cloning kit (Fermentas) according to the manufacturer's instructions, resulting in pJet_mpaD_mpaE.

Plasmid construction. Amplification of DNA by PCR to produce DNA fragments suitable for USER cloning was performed in 30 PCR cycles using PfuX7 in 50 μl. USER cloning was performed as previously described (9,22), with minor modifications. The USER vectors were digested for 6 h with AsIISI and NcoI for the AsIISI/NcoI USER cassettes or with PacI for the PacI/NcoI USER cassettes and cloned into pJET1.2/blunt using a CloneJET PCR cloning kit (Fermentas) according to the manufacturer's instructions, resulting in pJet_mpaD_mpaE.

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tion of 1 ml of methanol-dichloromethane-ethyl acetate (1:2:3 [vol/vol/vol]), the vials were capped and subjected to ultrasonication for 60 min. The supernatants were transferred to clean vials, and the organic phase was evaporated under an N2 flow at 30°C. The residues were redissolved in 200 μl of acetonitrile-water (1:1 [vol/vol]) (with subjection to ultrasonication) and filtered through a 0.22-μm-pore-size PTFE syringe filter.

UHPLC-HRMS was performed on a maXis G3 quadrupole time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) ion source. The mass spectrometer was connected to an Ultimate 3000 UHPLC system (Dionex, Sunnyvale, CA) equipped with a diode-array detector scanning 200 to 700 nm. Separation of 1- to 3-μl samples were performed at 40°C on a Kinetex C18 column (100 by 2.1 mm [inner diameter], 2.6 μm; Phenomenex, Torrance, CA) using a linear-water-acetonitrile gradient (both buffered with 20 mM formic acid) at a flow of 0.4 ml min⁻¹ starting from 10% acetonitrile and increasing to 100% in 10 min, remaining at 100% acetonitrile for 3 min. Mass spectrometry (MS) analyses were performed in both ESI⁺ and ESI⁻ (separate runs) with a data acquisition range of m/z 100 to 1,000, and the MS was calibrated using sodium formate automatically infused prior to each analytical run, providing a mass accuracy of <1.5 ppm.

Reference standards of nuclear magnetic resonance (NMR)-validated DHMP and 5-MOA (see below) and 3-methylorsellinic acid (Ambinter, Paris, France), as well as orsellenic acid (Apin Chemicals, Oxon, United Kingdom), were coanalyzed. 4,6-Dihydroxy-2-(hydroxymethyl)-3-methylbenzoic acid (DHMB) was tentatively identified in ESI⁻ with a matching accurate mass (<1 ppm accuracy, no other candidate isotopes and isotope patterns) and isotope pattern (SigmaFit better than 10), loss of CO₂ (diagnostic of a carboxylic acid), and UV absorptions at 210 nm (100%), 261 nm (30%), and 304 nm (25%). The retention time (1.61 min) compared to DHMP (2.78 min) fit well with the calculated LogD values of 0.60 and 1.5 ppm, respectively. The accurate masses (±0.01 ppm) for all target peaks were constructed for all extracts to exclude that the compounds were produced in various quantities or present in trace amounts in the medium or the wild-type strains. DHMB was detected as the [M-H]⁻ ions. The target compounds were identified by the whole UV/VIS spectrum, the retention time (±0.02 min), the accurate masses (±1.5 ppm), and the relative intensities of the [M+H]⁺, [M+Na]⁺, [M+H₂O]⁺, [M⁺], and [M-HCO₃]⁻ ions, as well as by their respective isotope patterns.

Structure verification of 5-MOA and DHMP. 5-MOA was isolated from a large-scale ethyl acetate extract prepared from 100 petri dishes with MM+Ura+Uri agar after cultivation of strain NID211 for 4 days at 37°C. Similarly, DHMP was isolated from a large-scale ethyl acetate extract prepared from 100 petri dishes with MM+Ura+Uri agar after cultivation of strain NID416 for 4 days at 37°C. The two compounds were isolated to give 15 mg of 5-methylorsellinic acid and 11 mg of DHMP. The NMR spectra were acquired on a Varian Unity Inova 500 MHz spectrometer equipped with a 5-mm probe and by using standard pulse sequences. The signals of the residual solvent protons and solvent carbons were used as internal references (δ₂H 2.50 ppm and δ_C 39.5 ppm for dimethyl sulfoxide). The ¹H NMR data (see Fig. S2 and S3 and Table S2 in the supplemental material) and the HSQC and HMBC data (see Table S2 in the supplemental material) of the two compounds were in agreement with the literature (6).

RESULTS AND DISCUSSION

Remote homology modeling: mpaD encodes a putative hydroxylase. Initial annotation of the eight proposed ORFs that constitute the putative MPA biosynthetic cluster in P. brevicompactum suggested the following candidates for the established biosynthetic reactions: (i) polyketide synthase (PKS), mpaC; (ii) cytochrome P450, mpaD; (iii) prenyl transferase, mpaa; (iv) oxidative cleaving, mpaH; (v) and finally O-methyltransferase, mpaG (23). However, the lactonization occurring at the second step in MPA biosynthesis is not a standard cytochrome P450 reaction, which prompted us to investigate this step in detail. In order to pinpoint what type of reaction MpaD catalyzes, we conducted a BLASTp search to identify homologs with known functions. The search identified putative homologs with >50% identity, indicating that orthologs are present in other filamentous fungi (data not shown). However, none of these orthologs had a known function. We therefore made a search using HHpred (26) in an attempt to identify remote homologs with known function. Subjection of MpaD as a query for HHpred analysis resulted in more than 10 hits crossing the recommended 95% score threshold (data not shown). Among the topmost hits were three cytochrome P450 enzymes, all known to catalyze hydroxylations. These hits included the *Rattus norvegicus* CYP24A1 catalyzing hydroxylation of 25-hydroxyvitamin D₃ (1), the human CYP2D6 catalyzing hydroxylation of debrisoquine (24), and P450 BM3 from *Bacillus megaterium* that catalyzes hydroxylation of several long-chain fatty acids (7). Multiple sequence alignments of MpaD with the three aforementioned CYP strains, as well as *Talaromyces stipitatus* TSTA_060710 and *Phaeosphaeria nodorum* SNOG_06679, revealed that the P450 heme signature motif and the EXXR motif of the P450 heme thiolate enzymes are conserved in MpaD (Fig. 2). Based on these predictions, MpaD was assigned as CYP631B5, TSTA_060710 was

FIG 2 Multiple sequence alignment of conserved domains in MpaDE and selected orthologs. The P450 heme signature motif, the EXXR motif of P450 heme thiolate enzymes, and the Zn-hydroxylase motif HXHXDH are boxed. Conserved residues are written in red in the alignment. MpaD orthologs: PDB code 2iij (*Bacillus megaterium* P450 BM3), PDB code 3k9v (*Rattus norvegicus* CYP24A1), PDB code 29dg (*Homo sapiens* CYP2D6), TSTA_060710 (*Talaromyces stipitatus* TSTA_060710), SNOG_06679 (*Phaeosphaeria nodorum* SNOG_06679), MpaE orthologs: PDB code 3hda (*Bacillus thuringiensis* lactone hydrolase), PDB code 2r2d (Agrobacterium tumefaciens lactone hydrolase), PDB code 1sm1 (*Stenotrophomonas maltophilia* lactone hydrolase), TSTA_060680 (*Talaromyces stipitatus* TSTA_060680), SNOG_06681 (*Phaeosphaeria nodorum* SNOG_06681).
assigned as CYP631B4, and SNOG_06679 was assigned as CYP631C2 by D. R. Nelson of the P450 Nomenclature Committee (Department of Molecular Sciences, University of Tennessee). The output from HHpred indicated that MpaD is very likely to catalyze a hydroxylation reaction, and we hypothesized that the target is the methyl group in ortho position to the carboxylic acid group on 5-MOA (Fig. 1B). Lactonization then yields DHMP. We considered it unlikely that this reaction is also catalyzed by MpaD and turned our attention toward the remaining unassigned two putative ORFs in the gene cluster, mpaB and mpaE, annotated as encoding a protein of unknown function and a zinc-dependent hydrolase, respectively. Since the lactone formation from hydroxylated 5-MOA to DHMP requires what resemble a reverse hydrolysis, we turned our attention to mpaE.

Remote homology modeling: mpaE encodes a putative lactone synthase. MpaE was subjected to bioinformatic analysis as described for MpaD with similar results. The highest-scoring hits from HHpred for MpaE were acyl homoserine lactone (AHL) lactonases from Stenotrophomonas maltophilia (29), Agrobacterium tumefaciens (15), and Bacillus thuringiensis (14). This homology indicates that MpaE has the same structure as enzymes cleaving a lactone bond. Furthermore, multiple sequence alignments confirmed that the signature sequence HXHXDH, which is completely conserved and essential for activity in all Zn-dependent hydrolases, is present in MpaE (Fig. 2). We hypothesized that MpaE catalyzes the reverse reaction, i.e., the formation of a lactone through dehydration and is thereby a lactone synthase. The predicted activity of MpaD and MpaE would in combination result in the conversion of 5-MOA to the second intermediate DHMP in MPA biosynthesis in fungi (3).

mpaD and mpaE is a single gene that encodes a fusion protein. We decided to undertake a heterologous expression approach to investigate our hypothesis. We have previously used such an approach successfully for expressing the MPA PKS in the MPA-nonproducer fungus A. nidulans (9). The putative ORFs of mpaD and mpaE are located in tandem within the defined MPA cluster and were annotated as separate ORFs (23) using GenScan software (4). GenScan, however, is based on invertebrate sequences and, to be more certain that we would clone the correct and full sequences of the genes, we decided to confirm the proposed annotations with two additional algorithms, Fgenesh (25) and Augustus (27). Both of these programs have been trained on fungal sequences. Surprisingly, predictions from both of these two programs suggested that mpaD and mpaE are a single gene that encodes a single polypeptide (data not shown), hereafter named mpaDE. Curiously, the MpaD homologs P450 BM3 and CYP505A1 (12) are natural fusion proteins as well, unlike almost every other known cytochrome P450s. In P450 BM3 and CYP505A1, the CYP domain is fused with an electron-donating domain. To establish whether mpaD and mpaE are transcribed as two separate or one fused ORF, RNA was extracted from P. breviscompactum under MPA producing conditions. PCR performed on the corresponding cDNA yielded a 2,562-bp product corresponding to the mpaDE fusion. mpaDE is therefore indeed a natural fusion gene containing six introns (GenBank number BK008023). An alignment of the full-length mpaDE with the previously identified homlogs can be found in Fig. S4 in the supplemental material.

MpaDE catalyzes the conversion of 5-MOA to DHMP. mpaDE was introduced into an A. nidulans strain (NID211) expressing MPA PKS (mpaC) and therefore capable of producing 5-MOA. In contrast to the reference strain NID211, this new strain (NID416) produced a compound eluting as a prominent peak at 2.9 min with the mass expected for DHMP (Fig. 3). The compound was purified and identified as DHMP by NMR (see Fig. S3 and Table S2 in the supplemental material) and by comparison with the published spectra. In addition, we note that, in contrast to NID211, the DHMP producing NID416 contained very little 5-MOA, which also points to that 5-MOA is converted by MpaDE (Fig. 3). We next addressed the question of whether the unique compound produced by NID416 is due to the mpaDE gene product converting 5-MOA to DHMP or whether it results from the conversion of an endogenous A. nidulans metabolite. We thus expressed mpaDE in an A. nidulans strain not producing 5-MOA. The resulting strain (NID410) did not produce detectable levels of DHMP. Finally, we constructed a strain NID944 where the mpaD part of mpaDE is expressed in a strain expressing mpaC. We found that strain NID944 contains a high amount of DHMB, whereas this metabolite was not detected in the other strains. This result conclusively shows that MpaD catalyzes the conversion of 5-MOA to DHMB. Interestingly, NID944 does produce DHMP, which shows that the conversion from DHMB can happen nonenzymatically or that A. nidulans has an endogenous enzyme that can
catalyze the lactonization. AN0028 and AN2639 do both have high structural similarity to MpaE, and in HHpred the top hits are the same as for MpaE (data not shown). Therefore, they are good candidates for catalyzing the lactone formation when a high concentration of DHMB is available. Taken together, these results show that the natural fusion protein MpaDE catalyzes the formation of DHMP in MPA biosynthesis, and the buildup of DHMB in NID944 supports that MpaD catalyzes the hydroxylation (Fig. 1B and see Fig. S5 in the supplemental material). The results also suggest that the lactonization step is potentially aided by MpaE.

MpaD and MpaE orthologs involved in lactonization in other fungi. To investigate whether the fusion of MpaDE is widespread in nature, we performed a BLASTP search. This search did not identify any orthologs in any of the organisms within the NCBI database. However, as previously mentioned, BLASTP analysis identified several orthologs of MpaD and MpaE as separate enzymes in a number of fungi. Interestingly, we noticed that in both Talaromyces stipitatus and Phaeosphaeria nodorum, orthologs of MpaD (TSTA 060710 and SNOG 06679) and MpaE (TSTA 060680 and SNOG 06681) are located very close to each other (Fig. 4). In addition, they are placed in the vicinity of a PKS. The two PKSs have ~40 and ~50% sequence identity, respectively, and the same domain architecture as MpaC (9) and AusA from A. nidulans that catalyzes the production of 3,5-dimethylorsellinic acid (20). For the remaining members of the P. brevicompactum MPA gene cluster, BLAST hits were found for MpaB and MpaG (see Table S1 in the supplemental material), although not in the vicinity of the corresponding PKSs. Based on the homology and genomic proximity, it can be hypothesized that the PKSs from T. stipitatus and P. nodorum are catalyzing the production of methylorsellinic acid and that the MpaD orthologs are involved in converting this methylorsellinic acid into a lactone. Furthermore, the role of MpaE identified here in the lactonization step of the MPA pathway adds to the list of diverse biochemical functionalities of the polyketide biosynthesis enzymes that belong to metallo-β-lactamase family (2, 13).

In conclusion, our results provide new insights into the biochemical and genetic organization of the MPA biosynthesis pathway. The fusion protein MpaDE may provide an advantage by increasing the rate of reaction due to the close proximity of the two catalytic domains and also by possibly decreasing the side-products from the pathway. As more fungal genomes and polyketide gene clusters get sequenced, it will be of interest to find the extent to which such fusion proteins play a role in shaping the molecular diversity and species-level specificity of fungal polyketides.

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FIG 4 Graphical representation of gene clusters from Talaromyces stipitatus and Phaeosphaeria nodorum with orthologs of MpaC, MpaD, and MpaE. The percent identity to MpaC, MpaD, and MpaE is indicated below each of the orthologs.