A 7-dimethylallyltryptophan synthase from *Aspergillus fumigatus*: overproduction, purification and biochemical characterization

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A putative prenyltransferase gene, *Afu3g12930*, was identified in the genome sequence of *Aspergillus fumigatus*. EAL92290, encoded by *Afu3g12930*, consists of 472 aa, with a molecular mass of about 53 kDa. The coding sequence of *Afu3g12930* was cloned in pQE60, and overexpressed in *Escherichia coli*. The soluble His 6 -fusion protein was purified to apparent homogeneity, and characterized biochemically. The enzyme was found to catalyse the prenylation of Trp at the C-7 position of the indole moiety, in the presence of dimethylallyl diphosphate (DMAPP); therefore, it functions as a 7-dimethylallyltryptophan synthase (7-DMATS). The structure of the enzymic product was elucidated by NMR and MS analysis. 

**INTRODUCTION**

Like bacteria and plants, fungi produce pharmacologically important agents (Keller *et al.*, 2005), for example: the immunosuppressive cyclosporin A is obtained from *Tolypocladium inflatum* (Thali, 1995); statins are produced by several fungi, including *Aspergillus terreus* (Manzoni & Rollini, 2002); penicillins are produced by *Penicillium chrysogenum* and *Aspergillus nidulans* (Brakhage *et al.*, 2004); and ergot alkaloids from *Claviceps purpurea* show diverse pharmacological activities (Schardl *et al.*, 2006). Secondary metabolites from fungi are usually active as mycotoxins and virulence factors (Bennett & Klich, 2005; Rementeria *et al.*, 2005) that could be involved in the pathogenic development of fungal infections. *Aspergillus fumigatus*, a filamentous fungal saprophyte found ubiquitously in the environment, is responsible for allergic reactions and invasive aspergillosis, which is a life-threatening disease for immunocompromised patients (Denning *et al.*, 2003). It has been discussed that the virulence of *A. fumigatus* could be based on its numerous secondary metabolites, especially gliotoxin (Latgé, 1999), which is an epipolythiodioxopiperazine (ETP) derived from Phe and Ser (Gardiner & Howlett, 2005). However, the role of these compounds remains questionable, and needs to be investigated more thoroughly (Latgé, 1999). ETPs are characterized by the presence of an internal disulphide bridge (Gardiner *et al.*, 2005). The role of gliotoxin in the pathogenic development of *A. fumigatus* has been demonstrated by deleting the non-ribosomal peptide synthetase (NRPS) gene glIP from the gliotoxin biosynthetic gene cluster (Cramer *et al.*, 2006a; Kupfahl *et al.*, 2006), and it was clearly demonstrated that gliotoxin production was blocked in the resulting mutants; however, the abolition of gliotoxin production had no effect on the development of invasive aspergillosis by the mutants.
(Cramer et al., 2006a; Kupahl et al., 2006). This proved that gliotoxin is not, or at least not alone, responsible for the pathogenic development in A. fumigatus. Therefore, it would be useful to identify genetic information of further secondary metabolites, which could function as virulence factors.

From the genome sequence of A. fumigatus A293, at least 26 biosynthetic gene clusters for secondary metabolites have been identified (Nierman et al., 2005), including the gene clusters of fumitremorgin B and fumigaclavine C, as well as that of gliotoxin (Cramer et al., 2006b; Gardiner & Howlett, 2005; Grundmann & Li, 2005; Maiya et al., 2006; Unsöld & Li, 2005). One putative gene cluster has been proposed for the biosynthesis of an additional unknown ETP derivative (Cramer et al., 2006b). This putative gene cluster contains three genes showing significant sequence similarity to gliC, gliP and gliM of the gliotoxin cluster of A. fumigatus (Gardiner & Howlett, 2005). Homologues of these genes, i.e. sirC, sirP and sirM, have also been found in the gene cluster of sirodesmin, which is an ETP derivative from the phytopathogenic fungus Leptosphaeria maculans (Gardiner et al., 2004). The gene cluster of sirodesmin contains a putative prenyltransferase gene, sirD, which has been proposed to be responsible for the O-prenylation of L-Tyr or cyclo-L-Tyr-L-Ser (Gardiner et al., 2004, 2005). A putative prenyltransferase gene, Afu3g12930, has also been found in the cluster from A. fumigatus, and the protein that it encodes shares a sequence identity of 34% with SirD at the amino acid level; this is higher than the sequence similarity to other fungal prenyltransferases, or other entries in the databases, with the exception of its orthologues in Neosartorya fischeri and A. terreus (see Discussion). Based on this sequence similarity, it could be speculated that EAL92290, encoded by Afu3g12930, would catalyse the transfer reaction of a prenyl moiety to Tyr or its derivatives. To prove the function of Afu3g12930, we cloned and overexpressed this gene in Escherichia coli, and carried out biochemical investigation using the purified fusion protein. Our results showed, interestingly, that Afu3g12930 encodes a second dimethylallyltryptophan synthase (DMATS), termed 7-DMATS, in A. fumigatus, and this enzyme catalyses the prenylation of Trp instead of Tyr. The first DMATS (FgaPT2) identified in A. fumigatus is involved in the biosynthesis of fumigaclavine C; in contrast to this, 7-DMATS catalyses the prenylation of Trp at the C-7 position, instead of at the C-4 position, of the indole ring.

**METHODS**

**Computer-assisted sequence analysis.** *GENEsher* (Softberry; www.softberry.com/berry.phtml) and the *DNASIS* software package (version 2.1; Hitachi Software Engineering) were used for intron prediction and sequence analysis, respectively. Sequence similarities were obtained by alignments of amino acid sequences using the BLAST 2 sequences program (release 2.9.9; http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

**Chemicals.** The trisammonium salt of dimethylallyl diphosphate (DAPP) was synthesized using methods analogous to those used for the synthesis of trisammonium geranyl diphosphate reported by Woodside et al. (1988). Geranyl diphosphate (GPP) was kindly provided by Wessjohann. The peptides used in this study were purchased from Bachem.

**Bacterial strains, plasmids and culture conditions.** pGEM-T and pQE60 were obtained from Promega and Qiagen, respectively. A UniZAP XR premade library of A. fumigatus strain B5233 (ATCC 13073) was purchased from Stratagene, and used to obtain phagemids as cDNA templates for PCR amplification. *E. coli* XL1 Blue MRF’ (Stratagene) was used for cloning and expression experiments, and it was grown in liquid Luria–Bertani (LB) medium, or on solid LB medium with 1.5% (w/v) agar, at 37 °C (Sambrook & Russell, 2001). Addition of 50 μg carbenicillin ml⁻¹ was used for selection of recombinant *E. coli* strains.

**DNA isolation, PCR amplification and cloning.** Standard procedures for DNA isolation and manipulation were performed, as described (Sambrook & Russell, 2001). PCR amplification was carried out on an iCycler from Bio-Rad. Using the Expand High Fidelity kit (Roche Diagnostics), a PCR fragment of 1425 bp, containing the entire coding sequence of Afu3g12930, was amplified from the cDNA library by using the primers 7-dmats-1 (5'-ACCCATGGGCA-TGGAGCGCCGAGAT-3') at the 5' end, and 7-dmats-2 (5'-TGCGATGTCGTCGACCCGAGGGA-3') at the 3' end of the gene. Bold letters represent mutations inserted to give the underlined restriction sites Ncol, located at the start codon in 7-dmats-1, and BglII, located at the predicted stop codon in 7-dmats-2. The PCR fragment was cloned into pGEM-T, resulting in plasmid pLW39, which has subsequently had its sequence confirmed (MWG-Biotech). To create the expression vector pLW40, pLW39 was digested with Ncol and BglII, and the resulting Ncol–BglII fragment of 1418 bp was ligated into pQE60, which had been digested with the same enzymes.

**Overproduction and purification of His6-7-DMATS protein.** For gene expression, *E. coli* XL1 Blue MRF’ cells harbouring the plasmid pLW40 were cultivated in 300 ml Erlenmeyer flasks containing 100 ml liquid LB medium, supplemented with carbenicillin (50 μg ml⁻¹), and grown at 37 °C to an OD₆₀₀ of 0.6. For induction, IPTG was added to a final concentration of 0.8 mM, and the cells were cultivated for a further 16 h at 37 °C, before harvest. The bacterial cultures were centrifuged, and the pellets were resuspended in lysis buffer (10 mM imidazole, 50 mM NaH₂PO₄ and 300 mM NaCl, pH 8.0) at between 2 and 5 ml (g wet weight)⁻¹. After addition of 1 mg lysozyme ml⁻¹, and incubation on ice for 30 min, the cells were sonicated six times, for 10 s each time, at 200 W. To separate the cellular debris from the soluble proteins, the lysate was centrifuged at 14,000 g for 30 min at 4 °C. One-step purification of the recombinant His6-tag fusion protein by affinity chromatography with Ni-NTA agarose resin (Qiagen) was carried out according to the manufacturer’s instructions. The protein was eluted with 250 mM imidazole in 50 mM NaH₂PO₄ and 300 mM NaCl, pH 8.0. In order to change the buffer, the protein fraction was passed through a NAP-5 column (GE Healthcare), which had been equilibrated with 50 mM Tris/HCl and 15% (v/v) glycerol, pH 7.5. His₆-7-DMATS was eluted with the buffer described, and stored at −80 °C for enzyme assays.

**Protein analysis.** Proteins were analysed by SDS-PAGE, which was carried out according to the method of Laemmli (1970), and they were stained with Coomassie brilliant blue G-250.

**Assays for 7-DMATS activity.** All the enzyme assays contained 50 mM Tris/HCl, pH 7.5, 1.2–3.8% (v/v) glycerol, and 10 mM CaCl₂. They differed from each other by incubation volume and time, substrate concentration, and amount of recombinant 7-DMATS. The reaction mixtures were incubated at 37 °C, and the reactions were...
terminated by addition of 10 μL TCA (1.5 M) per 100 μL reaction volume. After removal of the protein by centrifugation (14,000 g, 10 min, 4 °C), the enzymic products were analysed on an HPLC system, as described below. The standard assays for determination of the substrate specificity (100 μL) contained 2 mM L-Trp or another aromatic substrate, 1 mM DMAPP and 2.5 μg purified 7-DMATS, and they were incubated for 45 min. Two independent assays were carried out for quantification. The assay for the isolation of the enzymic product for structural elucidation (5 ml) contained 400 μg purified 7-DMATS, and was incubated for 16 h. The reaction mixture was concentrated on a rotation evaporator at 35 °C to a volume of 750 μL, before injection of 100 μL into HPLC for isolation of 7-dimethylallyltryptophan (7-DMAT). The assays for determination of the kinetic parameters of L-Trp contained 1 mM DMAPP, 2.5 μg 7-DMATS, and L-Trp at a final concentration of 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0 or 5.0 mM, in a total volume of 100 μL. The incubation time was 45 min. For determination of the kinetic parameters of DMAPP, 2.5 μg 7-DMATS, 1 mM L-Trp, and DMAPP at a final concentration of 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.5 or 1.0 mM, were used.

**HPLC analysis and isolation of 7-DMAT.** Reaction mixtures of 7-DMATS were analysed on an Agilent HPLC Series 1100 by using an Eclipse XBD-C18 column (4.6 x 150 mm, 5 μm) at a flow rate of 1 mL min⁻¹. Water (solvent A) and acetonitrile (solvent B), each containing 0.5% (v/v) TFA, were used as solvents. A gradient was run from 10 to 100% solvent B over 30 min. After washing with 100% solvent B for 5 min, the column was equilibrated with 85% solvent A for 5 min. The substances were detected with a Photo Diode Array detector. 7-DMAT was isolated under the same conditions.

**Spectroscopic data.** A ¹H-NMR spectrum of 7-DMAT was taken on an Avance DRX 500 spectrometer (Bruker) using 0.1 M DCl in D₂O as the solvent. The solvent signal at 4.81 p.p.m. was used as reference. δ (p.p.m.): 7.53 (d, 7.6 Hz, H-4), 7.32 (s, H-2), 7.12 (t, 7.9 Hz, H-5), 7.10 (d, 8.2, H-6), 5.46 (br t, 7.0 Hz, H-2'), 4.39 (t, 6.1 Hz, H-11), 3.58 (d, 7.0 Hz, 2H-1'), 3.52 (dd, 15.5, 5.4 Hz, H-10), 3.43 (dd, 15.5, 7.0 Hz, H-10), 1.74 (s, 3H-5'), 1.72 (s, 3H-4').

Positive and negative electrospray ionization (ESI) mass spectra were obtained with a ThermoFinnigan TSQ Quantum. The mass spectrometer was coupled with an Agilent HPLC series 1100 equipped with an RS18-column (2 x 250 mm, 5 μm). For separation, the column was run with 10% solvent B (CH₃OH) in solvent A (H₂O) [each containing 0.1% (v/v) HCOOH] for 5 min, followed by a gradient from 10 to 100% solvent B over 30 min. After washing with 100% solvent B for 10 min, the column was equilibrated with 10% solvent B for 10 min. The flow rate was 0.2 ml min⁻¹.

**MS data of 7-DMAT.** Positive ESI-MS: [M + H]⁺: m/z 273.1; ms² of [M + H⁺]: m/z (%,): 272.9 (5), 256.0 (100), 240.8 (9), 214.0 (64), 212.0 (24), 199.9 (25), 156.0 (6). Negative ESI-MS: [M−H]⁻: m/z 271.2; ms² of [M−H⁻]: m/z (%,): 272.1 (100), 227.3 (40), 210.3 (65); 184.3 (94).

**MS data of the prenylated H-L-Trp-Gly-OH.** Positive ESI-MS: [M + H]⁺: m/z 330.2; ms² of [M + H⁺]: m/z (%): 313.0 (25), 271.1 (34), 257.0 (41), 244.9 (16), 238.0 (47), 227.0 (7), 212.0 (100), 198.1 (25), 156.1 (11). Negative ESI-MS: [M−H]⁻: m/z 328.4; ms² of [M−H⁻]: m/z (%): 328.2 (100), 284.3 (31), 225.3 (6), 198.2 (10), 131.1 (39).

**MS data of the prenylated cyclo-L-Trp-L-Gly.** Positive ESI-MS: [M + H]⁺: m/z 312.2; ms² of [M + H⁺]: m/z (%,): 256.0 (9), 198.1 (100). Negative ESI-MS: [M−H]⁻: m/z 310.6; ms² of [M−H⁻]: m/z (%): 310.2 (31), 113.1 (100).

**RESULTS**

**Sequence analysis of the putative prenyltransferase gene Afu3g12930 from A. fumigatus**

The nucleotide sequence of the genomic DNA from A. fumigatus Af293 is available at GenBank under accession number AAHF01000002.1, and the genomic sequence of the putative prenyltransferase gene Afu3g12930 spans bp 640 080–641 551, and consists of two exons of 1301 and 118 bp, respectively, interrupted by an intron of 53 bp; this was confirmed by sequencing the PCR products amplified from the cDNA of A. fumigatus strain B5233 (data not shown; GenBank accession no. EF539173). The predicted gene product of Afu3g12930 is EAL92290, which comprises 472 aa, and has a calculated molecular mass of 53 kDa. EAL92290 (7-DMATS) shows significant sequence similarity to aromatic prenyltransferases from various fungi. For example, by using the BLAST 2 sequences program, we found that 7-DMATS shares a sequence identity of 34 % with SirD from L. maculans (Gardiner & Howlett, 2005) at the amino acid level, 31 % with FgaPT2 (DMATS) from A. fumigatus (Unso¨ld & Li, 2005), 28 % with DMATS from C. purpurea and Claviceps fusiformis (Tsai et al., 1995; Tuyns et al., 1999), and 28 % with FtmPT1 (Grundmann & Li, 2005) and 26 % with FgaPT1 (Unso¨ld & Li, 2006), which are both from A. fumigatus. SirD is proposed to catalyse the O-prenylation of L-Tyr or cyclo-L-Tyr-L-Ser in the biosynthesis of sirodesmin (Gardiner et al., 2004). DMATS from different sources catalyse the prenylation of L-Trp at the C-4 position of the indole ring in the biosynthesis of ergot alkaloids (Li & Unso¨ld, 2006; Schardl et al., 2006). FtmPT1 and FgaPT1 have been found to catalyse the prenylation reactions at the C-2 position of the indole rings of brevianamide F and fumigaclavine A (Grundmann & Li, 2005; Unso¨ld & Li, 2006), respectively. Based on the high sequence similarity to SirD, we speculate that 7-DMATS could be responsible for a transfer reaction of a prenyl moiety to L-Tyr or its derivatives.

**Cloning of Afu3g12930, and overproduction and purification of His₆-7-DMATS**

The coding region of Afu3g12930 was amplified using PCR from cDNA of A. fumigatus strain B3233 (available in the form of phagemids isolated from a cDNA library), and cloned into the cloning vector pGEM-T. For gene expression, the coding sequence of Afu3g12930 was released from pGEM-T, and cloned into the vector pQE60 (see Methods), resulting in the expression plasmid pLW40.

E. coli cells harbouring pLW40 were induced by 0.8 mM IPTG at 37 °C. His₆-7-DMATS was purified with Ni-NTA agarose to apparent homogeneity, as judged by SDS-PAGE (Fig. 1), and a protein yield of 5 mg purified His₆-7-DMATS per litre of culture was obtained. The observed molecular mass was 50 kDa, and this corresponded well to the calculated value of 54 kDa for His₆-7-DMATS.
Enzymic activity and substrate specificity of 7-DMATS

Initial attempts to detect the enzymic activity were carried out by incubations of 7-DMATS with L-Tyr and cyclo-L-Tyr-L-Ser in the presence of DMAPP or GPP. HPLC analysis was used to monitor the formation of an enzymic product. However, no product peak could be detected under different conditions, including various substrate concentrations and amounts of purified 7-DMATS, different metal ions with various concentrations, and different pH values and buffer compositions (data not shown). After the unsuccessful attempts, we decided to test other aromatic amino acids and their derivatives. HPLC analysis of the incubation mixture of L-Trp and DMAPP showed a significant product peak with a retention time of 12.9 min; this peak was absent in the reaction mixture containing heat-denatured enzyme (Fig. 2). Dependence of the product formation on the amount of protein was found up to 4 µg per 100 µl assay, and on a reaction time of up to 50 min. Product formation was strictly dependent on the presence of His6-7-DMATS, L-Trp and DMAPP. The enzyme 7-DMATS was found to be specific for DMAPP. Product formation was observed with DMAPP only, and not with GPP. In contrast, 7-DMATS showed relative flexible substrate specificity towards its aromatic substrates. D-Trp was accepted by 7-DMATS, with a relative activity of 15.5% of its enantiomer L-Trp. Linear and cyclic dipeptides were also substrates of 7-DMATS. Product formation was clearly observed when H-L-Trp-L-Gly-OH or cyclo-L-Trp-L-Gly was used instead of L-Trp in the incubation mixtures (Table 1). However, the linear tripeptide L-Trp-L-Trp-L-Trp was not accepted by 7-DMATS. No product formation was observed in incubation mixtures with L-Phe, or L-Phe-containing cyclic dipeptides (Table 1). These results demonstrated that
L-Trp was the best substrate for 7-DMATS, under our test conditions.

**Identification of the enzymic products**

For structural elucidation, the enzymic product of L-Trp was isolated on a preparative scale, and its structure was elucidated by NMR and MS analysis.

ESI-MS proved that the enzymic product is a prenylated derivative of Trp by detection of [M+H]$^+$ and [M−H]$^−$ at m/z 273 and m/z 271, respectively. Comparison of the $^1$H-NMR spectrum of the isolated compound with that of L-Trp revealed the presence of signals for a dimethylallyl moiety at 5.46 (br t, 7.0 Hz, H-2'), 3.58 (d, 7.0 Hz, 2H-1'), 1.74 (s, 3H-5') and 1.72 p.p.m. (s, 3H-4'). Correspondingly, signals for four protons instead of five protons were found in the region of the aromatic protons of the isolated compound, i.e. signals for three vicinal protons at 7.53 (d, 7.6 Hz), 7.12 (t, 7.9 Hz) and 7.10 p.p.m. (d, 8.2), and a singlet at 7.32 p.p.m. This indicated that the prenylation had taken place at the C-4 or C-7 position of the indole moiety. The spectrum of the isolated compound differed clearly from that of 4-DMAT for all of the protons, with the exception of the proton at C-2 (Unsöld & Li, 2005). 4-DMAT, the first pathway-specific intermediate in the biosynthesis of ergot alkaloids (Schardl et al., 2006; Unsöld & Li, 2005), is also formed from L-Trp by a prenyl-transfer reaction catalysed by DMATS, e.g. FgaPT2 from A. fumigatus (Unsöld & Li, 2005). The enzymic product of 7-DMATS showed a similar, but different, retention time to that of FgaPT2 on the HPLC chromatogram (Fig. 2).

Therefore, the enzymic product of 7-DMATS was identified as 7-DMAT (Fig. 3). The enzymic products of H-L-Gly-L-Trp-OH and cyclo-L-Trp-L-Gly were unequivocally proven as prenylated derivatives by detection of [M+H]$^+$ at m/z 330.2 and 312.2, as well as (M−H)$^−$ at m/z 328.4 and 310.6, respectively.

**Table 1. Substrate specificity of 7-DMATS towards different aromatic substrates**

For incubation conditions, see Methods.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
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<tbody>
<tr>
<td>L-Trp</td>
<td>100.0 ± 1.5</td>
</tr>
<tr>
<td>D-Trp</td>
<td>15.5 ± 3.1</td>
</tr>
<tr>
<td>H-L-Trp-L-Gly-OH</td>
<td>10.9 ± 1.1</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Gly</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>H-L-Trp-L-Trp-L-Trp-OH</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>L-Tyr</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>cyclo-L-Tyr-L-Pro</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>cyclo-L-Tyr-L-Ser</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>L-Phe</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>cyclo-L-Phe-L-Phe</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>cyclo-L-Phe-L-His</td>
<td>&lt;0.3</td>
</tr>
</tbody>
</table>

**Biochemical properties and kinetic parameters of 7-DMATS**

Testing with different ions showed that metal ions, such as Mg$^{2+}$ or Mn$^{2+}$, are not essential for the prenyltransferase activity of 7-DMATS. In the presence of 10 mM EDTA, a chelating agent for divalent ions, a relative activity of 82% was observed, in comparison with the incubation mixture without additives. However, an increase in enzyme activity of up to 140% of that of the incubation without additives was detected in the presence of 10 mM Ca$^{2+}$. This finding is similar to those of some of the soluble aromatic prenyltransferases, e.g. CloQ from Streptomyces roseochromogenes (Pojer et al., 2003), LtxC from Lyngbya majuscule (Edwards & Gerwick, 2004), DMATS from C. purpurea (Gebler & Poulter, 1992), and FgaPT1, FgaPT2 and FtmPT1 from A. fumigatus (Grundmann & Li, 2005; Unsöld & Li, 2005, 2006).

The 7-DMATS reaction apparently followed Michaelis–Menten kinetics. By using Hanes–Woolf and Lineweaver–Burk plots, the $K_m$ values were determined as 137 μM for L-Trp, and 67 μM for DMAPP. The maximum reaction velocity observed with 7-DMATS was 0.21 μmol min$^{-1}$ mg$^{-1}$. These values are in the normal range of those of enzymes involved in the biosynthesis of secondary metabolites. Given that L-Trp was shown to be the best substrate in our test conditions (see above), it is very likely to be the natural substrate of 7-DMATS.

**DISCUSSION**

In this study, we cloned and overexpressed the putative prenyltransferase gene Afu3g12930 from the fungus A. fumigatus in a heterologous host, and proved its function by biochemical characterization of the overproduced enzyme. Investigation of substrate specificity showed that 39 of 53 compounds were accepted by 7-DMATS, with the highest conversion rate for L-Trp. $K_m$ values of six Trp derivatives, which had modifications to the side chain and the indole ring, were determined to be in the range of 190–460 μM, which is higher than that for L-Trp (137 μM). $K_m$ values of eight linear and cyclic Trp-containing dipeptides were found to be from 180 to 500 μM (Kremer & Li, unpublished data). This indicated that the natural substrate of 7-DMATS is very likely to be L-Trp. However, it cannot be excluded that an unidentified free or enzyme-bound
dipeptide containing a Trp moiety (see below for the discussion on the role of the NRPS) is the natural substrate of the identified enzyme. The enzymic product of 7-DMATS was unequivocally identified as 7-DMAT by NMR and MS analysis. This demonstrated clearly that 7-DMATS functions as a dimethylallyltryptophan synthase. Interestingly, 7-DMATS shares a sequence identity of only 31% at the amino acid level with another DMATS, FgaPT2 (Unsöld & Li, 2005), from the same fungus; this level of identity is slightly higher than the sequence similarities between 7-DMATS and other indole prenyltransferases from A. fumigatus, e.g. 7-DMATS shares a sequence identity of 28% with FtmPT1, which catalyses the prenylation of the cyclic dipeptide cyclo-L-Trp-L-Pro at the C-2 position of the indole moiety (Grundmann & Li, 2006). 7-DMATS accepted Trp, but not Tyr as a substrate. However, with the exception of its orthologues in N. fischeri NRRL181 and A. terreus NIH2624, with sequence identities of 95 and 82% (Table 2, see below) at the amino acid level, respectively, 7-DMATS showed the highest sequence similarity to SirD from the biosynthetic gene cluster of sirodesmin from L. maculans (Gardiner et al., 2004), i.e. a sequence identity of 34% at the amino acid level. SirD has been proposed to catalyse the O-prenylation of 1-Tyr, or its cyclic dipeptide, with Ser (Gardiner et al., 2005). It would be interesting to prove the function of this putative prenyltransferase biochemically.

Divalent metal ions, such as Mg$^{2+}$ and Mn$^{2+}$, are not essential for the enzymatic reaction of 7-DMATS. However, like other indole prenyltransferases, such as FgaPT1, FgaPT2 and FtmPT1 mentioned above (Grundmann & Li, 2005; Unsöld & Li, 2005, 2006), as well as DMATS in the biosynthesis of ergot alkaloids from Claviceps (Lee et al., 1976), Ca$^{2+}$ enhanced the enzymatic reaction of 7-DMATS slightly. This finding, together with the sequence similarity, provided experimental data for the hypothesis that the indole prenyltransferases from fungi belong to the same group of enzymes, and that they have evolved directly from a common ancestor.

Afu3g12930 belongs to a putative biosynthetic gene cluster consisting of probably eight genes (Fig. 4, Table 2): two putative transcription factor genes Afu3g12940 and Afu3g12890, one putative transporter gene (Afu3g12900), one gene with unknown function (Afu3g12950), and four biosynthetic genes, i.e. the NRPS gene Afu3g12920, the prenyltransferase gene Afu3g12930 described in this study, a gene coding for a putative cytochrome P450 enzyme (Afu3g12960), and a putative methyltransferase gene Afu3g12910. With the exception of Afu3g12950, orthologous genes were also found in the genome sequence of N. fischeri NRRL181 and A. terreus NIH2624, with the same orientation and same relative position of the genes (GenBank entries) (Fig. 4). The gene products of the cluster from A. fumigatus share sequence identities of 92–96% at the amino acid level with their orthologues from N. fischeri, and 73–88% with the orthologues from A. terreus (Table 2), respectively. The gene Afu3g12950 in the gene cluster from A. fumigatus encodes a putative ubiquitin-conjugating enzyme. An orthologue of this gene was found in the gene cluster of N. fischeri, but not in the gene cluster of A. terreus. Therefore, it is questioned whether Afu3g12950 is essential for the biosynthesis of the compound encoded by this gene cluster.

The end product of the eight-gene cluster is unknown (Cramer et al., 2006b; Nierman et al., 2005). Deletion of the NRPS gene Afu3g12920 from the genome of A. fumigatus CEA17ΔakuB (Silva Ferreira et al., 2006) by T. Heinekamp and A. A. Brakhage (HKI, Jena, Germany), and HPLC analysis of the cultural extracts by our group, showed that no changes of the profile of the secondary metabolite production could be observed in the obtained mutants, in comparison with that of the wild-type (data not shown). This indicated that one or more genes of this cluster were not expressed, or were expressed at very low levels, so that no product of this cluster accumulated in the

Table 2. ORFs of the putative cluster in A. fumigatus Af293

<table>
<thead>
<tr>
<th>A. fumigatus ORF</th>
<th>N. fischeri Identity* to genes of</th>
<th>A. terreus</th>
<th>Gliotoxin cluster</th>
<th>Sirodesmin cluster</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAL92287</td>
<td>EAW21272 (92)</td>
<td>EAU31603</td>
<td>GliC (32)</td>
<td>SirC (39)</td>
<td>Cytochrome P450 monooxygenase</td>
</tr>
<tr>
<td>EAL92288</td>
<td>EAW21273 (95)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Ubiquitin conjugating enzyme</td>
</tr>
<tr>
<td>EAL92289</td>
<td>EAW21274 (96)</td>
<td>EAU31602</td>
<td>–</td>
<td>–</td>
<td>C-6 transcription factor</td>
</tr>
<tr>
<td>EAL92290 (7-DMATS)</td>
<td>EAW21275 (95)</td>
<td>EAU31601</td>
<td>GliP (29)</td>
<td>SirP (36)</td>
<td>NRPS containing A1-T1-C1-A2-T2-C2-T3</td>
</tr>
<tr>
<td>EAL92291</td>
<td>EAW21276 (93)</td>
<td>EAU31600</td>
<td>–</td>
<td>–</td>
<td>Transporter</td>
</tr>
<tr>
<td>EAL92292</td>
<td>EAW21277 (96)</td>
<td>EAU31599</td>
<td>GliM (34)</td>
<td>SirM (38)</td>
<td>Methyltransferase</td>
</tr>
<tr>
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<td>EAW21278 (96)</td>
<td>EAU31598</td>
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<td>–</td>
<td>Transporter</td>
</tr>
<tr>
<td>EAL92294</td>
<td>EAW21279 (93)</td>
<td>EAU31597</td>
<td>GliZ (27)</td>
<td>SirZ (23)</td>
<td>C-6 transcription factor</td>
</tr>
</tbody>
</table>

*Percentage similarity values of the A. fumigatus ORFs to those of N. fischeri, A. terreus, and the gliotoxin and sirodesmin clusters, are given in parentheses.
plausible explanation. The product of the gene cluster could be a derivative of a cyclic dipeptide containing 7-DMAT. It is plausible that l-Trp is converted to 7-DMAT, and that this is catalysed by the prenyltransferase 7-DMATS characterized in this study. 7-DMAT would then undergo the condensation with a second amino acid, which could not be predicted by the sequence analysis, to a prenylated (cyclic) dipeptide, under the catalysis of the putative NRPS EAL92291. Like GliP, EAL92291 has a trimodular architecture (A1-T1-C1-A2-T2-C2-T3) (Balibar & Walsh, 2006). This is in contrast to that of SirP of the sirodesmin cluster, and FtmPS for the formation of cyclo-l-Trp-l-Pro from the fumitremorgin cluster, as these do not contain the third thiolation domain T3 (Gardiner et al., 2004; Maiya et al., 2006). It has been proven by biochemical investigation that the A1 and A2 domains in GliP are responsible for the activation of l-Phe and l-Ser, respectively, which are then loaded onto the T1 and T2 domains of GliP, respectively. The linear enzyme-bond peptide l-Phe-l-Ser-T2 was formed under the catalysis of the condensation domain C2 (Balibar & Walsh, 2006). The roles of the second condensation domain C3, and the third thiolation domain T3, are unclear, although the results of mutational experiments have suggested that they are involved in the same process (Balibar & Walsh, 2006). It has been speculated that the subsequent reactions to gliotoxin, e.g. methylation or sulphur insertion, may occur while the linear dipeptide is still covalently bound to the NRPS GliP (Balibar & Walsh, 2006). The release of an intermediate in the biosynthesis could occur at some later step. This could also be the case for EAL92291. This means that an unmethylated cyclic dipeptide with the prenylated tryptophan is probably not involved in the biosynthesis of the unknown compound discussed here. The putative methyltransferase EAL92290 is probably responsible for the N-methylation of an enzyme-bound linear dipeptide or the diketopiperazine ring, similar to the proposed function of GliM and SirM in the biosynthesis of gliotoxin and sirodesmin (Gardiner & Howlett, 2005). The putative cytochrome P450 EAL92287 could be involved in the modification of the enzyme-bond dipeptide or a derivative thereof. The two regulators EAL92289 and EAL92294 are
probably involved in the regulation, and the putative transporter EAL92293 could be responsible for the efflux, of the product of the gene cluster from the fungus. Deletion of the genes from the genome of \textit{N. fischeri} or \textit{A. terreus}, in which this gene cluster is expressed, would explore the natural role of the gene cluster.

**ACKNOWLEDGEMENTS**

We thank Alexander Grundmann for the synthesis of DMAPP, and Inge Unsold for the prenyltransferase FgaPT2. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Priority Program SPP 1152: Evolution of metabolic diversity) to S.-M.L.

**REFERENCES**


Edited by: B. A. Horwitz