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

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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, 2003; 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; Kupfahl 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 AF293, 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 similarity to other fungal prenyltransferases, or other entries in the databases, with the exception of its similarity to other fungal prenyltransferases, or other entries in the databases, with the exception of its similarity to other fungal prenyltransferases, or other entries in the databases, with the exception of its similarity to other fungal prenyltransferases, or other entries in the databases.

Addition of 50 μM carbenicillin ml⁻¹ was used for selection of recombinant E. coli strains.

**METHODS**

**Computer-assisted sequence analysis.** FGENESH (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/b2seq/wblast2.cgi).

**Chemicals.** The trimethylamine salt of dimethylallyl diphosphate (DMAPP) was synthesized using methods analogous to those used for the synthesis of trimethylamine geranyl diphosphate reported by Woodside et al. (1988). Geranyl diphosphate (GPP) was kindly provided by Wessler. 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 preemade 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'-CAACATGGCGACGAGATG-3') at the 5' end, and 7-dmats-2 (5'-TCGAGATGCTGTACACCGAGG-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 BgII, 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 BgII, and the resulting Ncol-BgII 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 liquid Lumeneyer 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 harvested at 14000 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
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 (Unsöld & Li, 2005), 28% with DMATS from C. purpurea and Claviceps fusiformis (Tsai et al., 1995; Tudznyski et al., 1999), and 28% with FtmPT1 (Grundmann & Li, 2005) and 26% with FgaPT1 (Unsöld & Li, 2006), which are both from A. fumigatus. SirD is proposed to catalyse the O-prenylation of L-1-Tyr or cyclo-L-1-Tyr-L-Ser in the biosynthesis of sirodesmin (Gardiner et al., 2004).

DMATS from different sources catalyse the prenylation of L-1-Tyr at the C-4 position of the indole ring in the biosynthesis of ergot alkaloids (Li & Unsöld, 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; Unsöld & 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-1-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-tagged 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
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>

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 ¹H-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).

Biochemical properties and kinetic parameters of 7-DMATS

Testing with different ions showed that metal ions, such as Mg²⁺ or Mn²⁺, 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 Ca²⁺. 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 majuscula (Edwards & Gerwick, 2004), DMATS from C. purpurea (Gebler & Poulter, 1992), and FgaPT1, FgaPT2 and FtmPT1 from A. fumigatus (Grundmann & Li, 2005; Unsöld & Li, 2005).

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
prenylation of L-Tyr, or its cyclic dipeptide, with Ser acid level. SirD has been proposed to catalyse the O-prenylation of the cyclic 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 cycl-o-L-Trp-L-Pro at the C-2 position of the indole moiety (Grundmann & Li, 2005), and 26 % with FgaPT1, which prenylates fumigavclavine A at the C-2 position of the indole moiety (Unsöld & 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-L-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 enzymic 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 enzymic 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 CEA17AkuB (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>Identity* to genes of</th>
<th>Putative function</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>N. fischeri</td>
<td>A. terreus</td>
</tr>
<tr>
<td>EAL92287</td>
<td>EAW21272 (92)</td>
<td>EAU31603 (85)</td>
</tr>
<tr>
<td>EAL92288</td>
<td>EAW21273 (95)</td>
<td>–</td>
</tr>
<tr>
<td>EAL92289</td>
<td>EAW21274 (96)</td>
<td>EAU31602 (84)</td>
</tr>
<tr>
<td>EAL92290 (7-DMATS)</td>
<td>EAW21275 (95)</td>
<td>EAU31601 (82)</td>
</tr>
<tr>
<td>EAL92291</td>
<td>EAW21276 (93)</td>
<td>EAU31600 (76)</td>
</tr>
<tr>
<td>EAL92292</td>
<td>EAW21277 (96)</td>
<td>EAU31599 (88)</td>
</tr>
<tr>
<td>EAL92293</td>
<td>EAW21278 (96)</td>
<td>EAU31598 (87)</td>
</tr>
<tr>
<td>EAL92294</td>
<td>EAW21279 (93)</td>
<td>EAU31597 (73)</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.
wild-type, and, therefore, no effect on secondary metabolite production could be observed after deletion of a gene from the cluster. This finding is in accordance with the results reported by Cramer et al. (2006b) that Afu3g12920 is not expressed, or is expressed at very low levels, under the conditions tested in their study. However, it can not be excluded that this cluster could be expressed in situ after inhalation of the fungus, and the secondary metabolite may then play a role as virulence factor.

Comparison with database entries revealed that the four biosynthesis enzymes EAL92287, EAL92290 (7-DMATS), EAL92291 and EAL92292 share sequence identities of 39, 34, 36 and 38% with SirC, SirD, SirP and SirM, respectively (Table 2), which have been identified in the gene cluster of sirodesmin, which is an ETP from L. maculans (Gardiner et al., 2004). Sequence identities of 29 and 34% were also observed between EAL92287 and GliC, EAL92291 and GliP, as well as EAL92293 and GliM, respectively (Table 2). GliP, GliC and GliM are members of the gene cluster of the ETP gliotoxin from A. fumigatus (Gardiner & Howlett, 2005). The sequence identities of EAL92294 to SirZ and GliZ were found to be 23 and 27%, respectively (Table 2). Therefore, the cluster discussed here is proposed to function in the biosynthesis of an ETP derivative (Cramer et al., 2006b; Nierman et al., 2005).

However, database and literature searches revealed that none of the known ETP derivatives contain a prenylated Trp moiety (Gardiner et al., 2005). Also, and more importantly, a homologue of the putative thioedoxin reductase, GliT/SirT, in the gene cluster of gliotoxin/sirodesmin, which is proposed to be responsible for the formation of the disulphide bond of the ETP derivatives (Gardiner et al., 2005; Gardiner & Howlett, 2005), is absent in the cluster. Therefore, it could be speculated that the cluster discussed here is not intact, or that the product of the gene cluster has a structure other than an ETP or a derivative thereof. Considering the fact that the cluster is present in the three fungi mentioned above, it seems that the latter is the most 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 trinodular architecture (A1-T1-C1-A2-T2-C2-T3) (Balibar & Walsh, 2006). This is in contrast to that of SirP of the sirodesmin cluster, and FmPS 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 C1 (Balibar & Walsh, 2006). The roles of the second condensation domain C2, 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 unmodified 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 diketopiperezine 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

Fig. 4. Putative unknown gene cluster from different Aspergillus strains. Top, A. fumigatus Af293; middle, N. fischeri (Aspergillus fischerianus) NRRL181; bottom, A. terreus NIH2624. Introns are not shown. The deduced protein names are given in parentheses.
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 N. fischeri or A. terreus, in which this gene cluster is expressed, would explore the natural role of the gene cluster.

ACKNOWLEDGEMENTS

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