Genome mining reveals the presence of a conserved gene cluster for the biosynthesis of ergot alkaloid precursors in the fungal family Arthrodermataceae

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Genome sequence analysis of different fungi of the family Arthrodermataceae revealed the presence of a gene cluster consisting of five genes with high sequence similarity to those involved in the early common steps of ergot alkaloid biosynthesis in Aspergillus fumigatus and Claviceps purpurea. To provide evidence that this cluster is involved in ergot alkaloid biosynthesis, the gene ARB_04646 of the fungus Arthroderma benhamiae was cloned into pQE60 and expressed in Escherichia coli. Enzyme assays with the soluble tetrameric His6-tagged protein proved unequivocally that the deduced gene product, here termed ChaDH, catalysed the oxidation of chanoclavine-I in the presence of NAD+, resulting in the formation of chanoclavine-I aldehyde. The enzyme product was unequivocally proven by NMR and MS analyses. Therefore, ChaDH functions as a chanoclavine-I dehydrogenase. Km values for chanoclavine-I and NAD+ were 0.09 and 0.36 mM, respectively. Turnover number was 0.76 s⁻¹.

INTRODUCTION

Ergot alkaloids are a complex family of indole derivatives which play an important role as pharmaceuticals. They are also toxins in contaminated food and feed (Schardl et al., 2006; Wallwey & Li, 2011). Ergot alkaloids are mainly produced by fungi of the families Trichocomaceae and Clavicipitaceae of Ascomycota (Gröcer & Floss, 1998; Schardl et al., 2006). Until now, the occurrence of ergot alkaloids in other fungal families such as Arthrodermataceae has not been reported. Based on their structures, ergot alkaloids can be divided into three groups (Wallwey & Li, 2011). Clavine-type alkaloids such as fumigaclavines are mainly found in Trichocomaceae, e.g. Aspergillus (A.) fumigatus and Penicillium commune, and the ergoamides and ergopeptines in Clavicipitaceae, e.g. Claviceps purpurea and Neotyphodium spp. (see Fig. 1). These ergot alkaloids have a tetracyclic ergoline ring as a characteristic structural feature (Flieger et al., 1997). Tricyclic precursors of these compounds, e.g. chanoclavine-I, are called secoergolenes or tricyclic seco derivatives due to their 6,7-seco-D-ring.

The first biosynthetic gene cluster of ergot alkaloids was identified in C. purpurea strain P1 by genomic walking (Tsai et al., 1995; Tuzynski et al., 1999). The complete cluster contains 14 genes which are coordinately induced under ergot alkaloid production conditions (Correia et al., 2003). Twelve homologous genes have been identified in the ryegrass pathogen Neotyphodium lolii Lp19 and its sexual counterpart Epichloë festucae (Panaccione et al., 2001). In A. fumigatus strain Af293, a gene cluster comprising 11 genes

Abbreviations: ChaDH, chanoclavine-I dehydrogenase; 4-DMAT, 4-dimethylallyltryptophan; 4-DMA-L-aborine, N-methyl-4-dimethylallyl-tryptophan; HR-ESI, high-resolution electrospray ionization; NOESY, nuclear overhauser effect spectroscopy.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of Arthroderma benhamiae CBS 112371 reported is ABSU01000001.1.

Eight supplementary figures are available with the online version of this paper.
was identified for the biosynthesis of fumigaclavine C (Unsöld & Li, 2005). *P. commune* produces the clavine-type alkaloid fumigaclavine A lacking the tert-prenyl moiety of fumigaclavine C at position C-2 of the indole ring. A putative gene cluster consisting of nine homologous genes from *A. fumigatus* was cloned and sequenced for the biosynthesis of fumigaclavine A in *P. commune* (Unsöld, 2006).

Comparison of the ergot alkaloid biosynthetic gene clusters from *C. purpurea*, *A. fumigatus* and *P. commune* revealed the presence of seven orthologous/homologous genes in the clusters, which were speculated to be responsible for the formation of the common tetracyclic ergoline scaffold (Unsöld & Li, 2005; Wallwey & Li, 2011). The functions of these genes have been proven in recent years by molecular genetic and biochemical approaches. Biosynthesis of the common ergoline ring begins with the prenylation of L-tryptophan to 4-dimethylallyltryptophan (4-DMAT) by a dimethylallyltryptophan synthase, e.g. FgaPT2 (Unsöld & Li, 2005). 4-DMAT is then converted to N-methyl-4-dimethylallyltryptophan (4-DMA-L-abrine) by an N-methyltransferase, e.g. FgaMT (Rigbers & Li, 2008). For the conversion of 4-DMA-L-abrine to chanoclavine-I, FgaOx1 as well as the catalase FgaCat are required in *A. fumigatus*, and at least CcsA (formerly known as EasE, the homologue of FgaOx1) in *C. purpurea* (Goetz et al., 2011; Lorenz et al., 2010). An alcohol dehydrogenase, e.g. FgaDH, catalyses the conversion of chanoclavine-I to chanoclavine-I aldehyde (Wallwey et al., 2010a). It has been demonstrated that chanoclavine-I aldehyde is the branch point, at least for the biosynthetic pathways of ergot alkaloids in *Claviceps* and in *Aspergillus* (see Fig. 1) (Cheng et al., 2010b). In *A. fumigatus*, chanoclavine-I aldehyde is converted to festuclavine by the festuclavine synthase FgaFS in the presence of the Old Yellow Enzyme FgaOx3 (Cheng et al., 2010a; Wallwey et al., 2010b). Festuclavine then serves as a precursor for fumigaclavines A, B and C (Wallwey & Li, 2011). In *C. purpurea*, chanoclavine-I aldehyde is converted to agroclavine by the agroclavine synthase EasG in the presence of GSH (Matuschek et al., 2011), which is then converted via elymoclavine to D-lysergic acid, the acidic component of ergoamides and ergopeptines (Haarmann et al., 2006).

Fungi of the family Arthrodermataceae are important pathogenic dermatophytes and infect exclusively keratinized host structures. Natural hosts of *Arthroderma (Ar.) benhamiae* are guinea pigs, but the fungus also infects humans and is responsible for highly inflammatory cutaneous infections. The molecular basis of the pathogenicity of this fungal family is not yet clear. Therefore, genome sequence analysis, which was completed in 2009 by the *Arthroderma* Genome Sequencing Consortium, may provide information about basic pathogenicity-associated traits of these fungi (Burmester et al., 2011).

By blasting the genome sequences of different fungi with ergot alkaloid biosynthetic enzymes in *A. fumigatus*, we
found a putative gene cluster in fungi of the family Arthrodermataceae, e.g. *Arthroderma otae*, *Ar. benhamiae* and *Trichophyton verrucosum* (Fig. 2c). Analysis of this cluster revealed the presence of five putative genes, which share high similarity with those involved in the biosynthesis of ergot alkaloids in *A. fumigatus* and *C. purpurea* (see Fig. 2). Here, we report the identification of this gene cluster by bioinformatic analysis and biochemical characterization of the chanoclavine-I dehydrogenase. To the best of our knowledge, this is the first report about structural genes for ergot alkaloid biosynthesis of fungi belonging to the family Arthrodermataceae.

**METHODS**

**Chemicals.** NAD⁺ was obtained from Carl Roth (Karlsruhe, Germany). Chanoclavine-I was kindly provided by Professor E. Leistner (Bonn, Germany).

**Computer-assisted sequence analysis.** Sequence identities were obtained by alignments of amino acid sequences using the program DNASIS for Windows Ver. 2.1 (Hitachi Software Engineering).

**Bacterial strains, plasmids and cultural conditions for *Escherichia coli*.** pGEM-T Easy and pQE60 vectors were obtained from Promega and Qiagen, respectively. *E. coli* XL1 Blue MRF₉ (Stratagene) was used for cloning and expression experiments, and was grown in liquid or on solid Luria–Bertani medium with 1.5% (w/v) agar at 37 °C (Sambrook & Russell, 2001). Carbenicillin (50 µg ml⁻¹) was used for selection of recombinant *E. coli* strains.

**Fungal growth condition for DNA preparation.** *Ar. benhamiae* strain CBS 112371 (LAU23541) (Fumeaux et al., 2004) was cultivated at 200 r.p.m. for 48 h at 30 °C in Sabouraud 2% (w/v) glucose (SG; Merck). Mycelium was separated from the medium by filtration through Miracloth (Calbiochem). For isolation of genomic DNA, 0.2 g mycelium was ground under liquid nitrogen and subsequently incubated with 450 µl 50 mM Tris/HCl, pH 7.6, 3% (w/v) SDS and 50 mM EDTA for 1 h at 65 °C. Samples were incubated on ice for 5 min and then mixed thoroughly with 225 µl protein precipitation reagent (MasterPure Yeast DNA Purification kit; Epicentre Biotechnologies). The mixture was then centrifuged at 14 000 g for 10 min and the supernatant was precipitated with an equal volume of ice-cold 2-propanol for 10 min on ice. After centrifugation at 14 000 g and 4 °C for 10 min, the pellet was washed with 500 µl 70% (v/w) ethanol, followed by centrifugation at 14 000 g and 4 °C for 5 min. After air-drying, the DNA pellet was resolved in 50 µl bidistilled water.

**Expression analysis of the cluster genes ARB_04645–ARB_04649 by Northern blots.** For RNA isolation, *Ar. benhamiae* was cultivated in static culture on SG medium at 30 °C, keratin medium [KER, 10 g keratin l⁻¹ (Paninkret), 20 mM potassium phosphate, pH 5.5, 0.4 mM MgSO₄, 77 mM NaCl, 5 mM glucose] at 30 °C and with human keratinocytes (HA) at 37 °C, with 5% (v/v) CO₂. For the keratinocyte experiment, 3 x 10⁶ keratinocytes (HaCaT) (Boukamp et al., 1988) were seeded into cell culture flasks (75 cm²)

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Fig. 2. Biosynthetic gene clusters of ergot alkaloids from different species. (a) Part of the ergot alkaloid gene cluster from *C. purpurea*; (b) fumigaclavine C gene cluster from *A. fumigatus* AF293; (c) putative ergot alkaloid gene cluster from three fungi of the family Arthrodermataceae. The corresponding orthologous genes are marked with the same frame and the fgaDH homologous genes are highlighted in black.
and pre-cultivated in Dulbecco’s modified Eagle’s medium (DMEM; Lonza) with 10% (v/v) fetal calf serum (FCS; Lonza) for 48 h at 37 °C, with 5% (v/v) CO2. Cells were washed with PBS (Lonza), infected with 2 x 10^7 conidia of Ar. benhamiae and further cultivated in DMEM without FCS under the same conditions. Total RNA was obtained from mycelia after 24 and 48 h of cultivation. Due to the slow growth, no RNA was obtained from KER medium after 24 h. RNA was isolated using the RiboPure Yeast kit (Ambion) according to the manufacturer’s instructions and used for Northern blot analysis. Ten micrograms of total RNA was loaded on each lane. Expression was followed by using standard Northern blot protocols and the Roche DIG system for detection. DIG-labelling of probes was achieved by PCR using the following primer pairs (each: 5'-3' direction): ARB_04645 (fwd: TCAACCTTGACATTGGTCC, rev: TTTGCAAACTTTAGCTGTCCT), ARB_04646 (fwd: ATATTGGCTATTACAGGC, rev: ATCCAGGAGCTGACGATGTA), ARB_04647 (fwd: AGAGGGCTCCTAGAAGCTGCTCA, rev: TTCGGGATCAAGAGCCTTTC), ARB_04648 (fwd: TGTGCTTGGCAGGTTGGTGT, rev: AAGTCAGAAATCCCACCTCCC), ARB_04649 (fwd: ATGGGCCAGAAGGACTCCTATG, rev: TCAGGTAGTCGGTCAAGTG), ARB_04650 (fwd: TGGTCTGGCAGGATTGGGTT, rev: AAGTCAGAAATCCCACCTCCC), ARB_04651 (fwd: ATGGGCTCTATCCAAATCCTC, rev: ATGCGAGATCTCTGGTCATCGA), ARB_04652 (fwd: ATGGCCAGAAGGACTCCTATG, rev: TCAGGTAGTCGGTCAAGTG), ARB_04653 (fwd: AGAGGGCTCCTAGAAGCTGCTCA, rev: TTCGGGATCAAGAGCCTTTC). PCR was carried out with Bioline BIOTAQ Red DNA Polymerase according to the manufacturer’s instructions by using a 40-cycle programme with an annealing temperature of 52 °C for 30 s.

**cDNA synthesis and PCR amplification for proof of the intron sequence.** cDNA was synthesized from Ar. benhamiae mycelium after 24 h cultivation on DMEM by using the cDNA synthesis kit (Bioline) according to the manufacturer’s instructions. The primers used were ORF-F (5'-ATGGGCTCCTAGAAGCTGCTCA, rev: TTCGGGATCAAGAGCCTTTC) and ORF-R (5'-TCAAGGATACAGGCGGCAC-3'), which leads to deletion of the predicted intron sequence. In the first round, a PCR fragment of 630 bp containing the coding sequence of the exon at the 5'-end and a PCR fragment of 228 bp containing the coding sequence of the exon at the 3'-end were amplified separately by using an Expand High Fidelity kit (Roche Diagnostics). The forward and reverse primers for the exon at the 5'-end were ChaDH_1 (5'-CACAAACACCTGAGCTGTCCTCCAAC-3') and ChaDH_2 (5'-CCAGCATCGGGCTCTTTGTAATCCAGGGGGAGGATATTAGCGTGCTG-3'). The forward and reverse primers for the exon at the 3'-end were ChaDH_3 (5'-AGGAGCTTCTTACGCTGTCCTCCAAC-3'), ARB_1 (5'-ATCAGGATACAGGCGGCAC-3') and ChaDH_4 rev (5'-ATCAGGATACAGGCGGCAC-3'). Bold letters represent mutations inserted in comparison with the original genomic sequence to give the italicized restriction sites NcoI, located at the start codon in ChaDH_1 forward and BamHI, located at the predicted stop codon in ChaDH_4 reverse. The underlined letters represent the overlapping region of the two PCR products, which is essential for fusion in the second PCR round. In the second round, the two PCR fragments were hybridized with the help of the overlapping sequence to get a single PCR fragment of 812 bp containing the entire coding sequence of the gene without intron, which was cloned into pGE-T Easy resulting in plasmid pCW17. pCW17 was subsequently sequenced (Eurofins MWG Operon) to confirm the sequence. To create the expression vector pCW18, pCW17 was digested with NcoI and BamHI and the resulting NcoI–BamHI DNA fragment of 794 bp was ligated into the NcoI–BamHI-digested pQE60.

**Overproduction and purification of His6-ChaDH.** For gene expression, E. coli XL1 Blue MRF cells harbouring the plasmid pCW18 were cultivated in 200 ml Erlenmeyer flasks containing 1000 ml liquid TB medium supplemented with carbenicillin (50 μg ml⁻¹) and grown at 37 °C to OD₆₀₀ 0.6. For induction, IPTG was added to a final concentration of 0.2 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, 300 mM NaCl, pH 8.0) at 2 ml (g wet weight)⁻¹. After addition of 1 mg lysosyme ml⁻¹ and incubation on ice for 30 min, cells were sonicated six times for 10 s each 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-tagged 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. To change the buffer, the protein fraction was passed through a PD-10 column (GE Healthcare), which had been equilibrated with 50 mM Tris/HCl and 15% (v/v) glycerol, pH 7.5. His₆-ChaDH was eluted with the same buffer and stored at −80 °C for enzyme assays.

**Protein analysis and determination of molecular mass of active His₆-ChaDH.** Protein monomers were analysed by SDS-PAGE according to the method of Laemmli (1970) and stained with Coomassie brilliant blue R-250.

The molecular mass of the recombinant active His₆-ChaDH was determined by gel filtration on a HiLoad 16/60 Superdex 200 column (GE Healthcare), which had been equilibrated with 20 mM Tris/HCl buffer (pH 7.5) containing 150 mM NaCl. The column was calibrated with dextran blue 2000 (2000 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), carbonic anhydrase (29 kDa) and RNase A (13.7 kDa) (GE Healthcare). The protein was eluted with 20 mM Tris/HCl buffer (pH 7.5) containing 150 mM NaCl.

**Enzyme assays for ChaDH.** All of the enzyme assays contained 50 nM Tris/HCl, pH 7.5, and 0.5–1.5 % (v/v) glycerol. The reaction mixtures were incubated at 37 °C for different times and then extracted twice with two volumes of dichloromethane after adjusting to pH 9.0 with 1 M sodium hydroxide. The combined organic phase was evaporated to dryness and dissolved in 2 μl DMSO and 98 μl methanol. The enzyme products were analysed on an HPLC system as described below. Two independent assays were carried out routinely. Standard assays for determination of the enzyme activity contained 5 mM NAD⁺, 5 μg (420 nM) ChaDH and 1 mM chanoclavine-I. Assays for determination of the kinetic parameters (100 μM) of chanoclavine-I contained 10 mM NAD⁺, 1 μg (84 nM) ChaDH and chanoclavine-I at final concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 mM. For determination of the kinetic parameters of NAD⁺, 1 μg (84 nM) ChaDH, chanoclavine-I at 2 mM and NAD⁺ at final concentrations of 0, 0.15, 0.3, 0.5, 0.75, 1.0, 1.5, 3.0, 5.0 and 10 mM were used. The incubation time was 10 min.

The reaction mixture of ChaDH for isolation of the enzyme product for structural elucidation (20 ml) was incubated with 2 mg (840 nM) of purified ChaDH, 5 mM NAD⁺ and 1 mM chanoclavine-I. After incubation for 3 h, the pH of the reaction mixture was adjusted to 9 with sodium carbonate and extracted twice with dichloromethane. The combined organic phase was concentrated on a rotation evaporator at 35 °C to dryness. The residue was dissolved in 400 μl methanol and centrifuged at 14 000 g for 30 min at 4 °C before injection into the HPLC system for isolation of chanoclavine-I aldehyde.
HPLC conditions for analysis of incubation mixtures with ChaDH and isolation of the enzyme product. Reaction mixtures of ChaDH were analysed on an Agilent HPLC Series 1200 by using a Multispher 120 RP18 column (4 × 250 mm, 5 μm; CS-Chromatographic Service) at a flow rate of 1 ml min⁻¹. Water (solvent A) and acetonitrile (solvent B), each containing 0.5% (v/v) trifluoroacetic acid, were used as solvents. The substances were detected with a Photo Diode Array detector. The assays were analysed by beginning with 25% solvent B for 8 min and then with a gradient from 25 to 35% solvent B over 20 min. After washing with 100% solvent B for 5 min, the column was equilibrated with 25% solvent B for 5 min.

The remaining substrate chanoclavine-I and the enzyme product were isolated with the same method by using a Multispher 120 RP18 column (10 × 250 mm, 5 μm; CS-Chromatographic Service) at a flow rate of 2.5 ml min⁻¹. The collected fractions containing substrate or the enzyme product after HPLC separation were concentrated to dryness and dissolved in 5 ml diluted sodium carbonate (pH 9). The free bases were then extracted twice with chloroform. The combined organic phase was concentrated on a rotation evaporator at 35 °C to dryness and subjected to NMR and MS analyses. Approximately 1.3 mg chanoclavine-I aldehyde was obtained.

**Spectroscopic analysis.** NMR spectra were recorded at room temperature on a Bruker Avance 600 MHz spectrometer equipped with an inverse probe with z gradient. DQF-COSY (double-quantum-filtered correlation spectroscopy) and HSQC (heteronuclear single quantum coherence) spectra were recorded with standard methods (Berger & Braun, 2004). Gradient-selected NOESY (nuclear overhauser effect spectroscopy) experiments (Hwang & Shaka, 1992) were performed in phase-sensitive mode. Chemical shifts were referenced to CDCl₃. All spectra were processed with Bruker TOPSPIN 2.1.

**ESI-MS of the enzyme product.** Positive high-resolution electrospray ionization (HR-ESI) MS was carried out with an AutoSpec instrument (Micromass).

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**Table 1.** Homologous proteins encoded by genes of the ergot alkaloid cluster of the fungal family Arthrodermataceae

<table>
<thead>
<tr>
<th>Strain</th>
<th>Protein</th>
<th>A.f, A. fumigatus A293; A.b, Ar. benhamiae CBS 112371; C.p, C. purpurea; T.v, T. verrucosum HKI 0517</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. purpurea</td>
<td>DMATS (448 aa)</td>
<td>EasF (344 aa)</td>
</tr>
<tr>
<td>A. fumigatus A293</td>
<td>FgaPT2 (451 aa)</td>
<td>FgaMT (339 aa)</td>
</tr>
<tr>
<td>Ar. otae CBS 113480</td>
<td>EEQ33236</td>
<td>EEQ33234</td>
</tr>
<tr>
<td></td>
<td>446 aa</td>
<td>340 aa</td>
</tr>
<tr>
<td></td>
<td>56% with C.p.</td>
<td>60% with C.p.</td>
</tr>
<tr>
<td></td>
<td>61% with A.f.</td>
<td>66% with A.f.</td>
</tr>
<tr>
<td></td>
<td>83% with A.b.</td>
<td>88% with A.b.</td>
</tr>
<tr>
<td></td>
<td>81% with T.v.</td>
<td>78% with T.v.</td>
</tr>
<tr>
<td>Ar. benhamiae CBS 112371</td>
<td>EFE37121</td>
<td>EFE37119</td>
</tr>
<tr>
<td></td>
<td>385 aa</td>
<td>340 aa</td>
</tr>
<tr>
<td></td>
<td>51% with C.p.</td>
<td>59% with C.p.</td>
</tr>
<tr>
<td></td>
<td>54% with A.f.</td>
<td>65% with A.f.</td>
</tr>
<tr>
<td></td>
<td>96% with T.v.</td>
<td>87% with T.v.</td>
</tr>
<tr>
<td>T. verrucosum HKI 0517</td>
<td>EFE43383</td>
<td>EFE43381</td>
</tr>
<tr>
<td></td>
<td>375 aa</td>
<td>382 aa</td>
</tr>
<tr>
<td></td>
<td>50% with C.p.</td>
<td>53% with C.p.</td>
</tr>
<tr>
<td></td>
<td>53% with A.f.</td>
<td>58% with A.f.</td>
</tr>
</tbody>
</table>

*The length of the coding sequence was corrected to 795 bp, corresponding to a polypeptide of 264 aa, which was actively overproduced in this study.

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**RESULTS**

**Identification of a putative biosynthetic gene cluster of ergot alkaloids by bioinformatic analysis**

As mentioned, seven homologous/orthologous genes had been identified previously in Trichocomaceae, namely fgaPT2, fgaMT, fgaOx1, fgaCat, fgaDH, fgaOx3 and fgaFS in *A. fumigatus*, and in Clavicipitaceae, namely dmaW, easF, ccsA, easC, easD, easA and easG in *C. purpurea* (Table 1). Blasting genome sequences with the deduced gene products of the ergot alkaloid gene cluster from *A. fumigatus* led to identification of five putative homologous/orthologous genes in fungi of the family Arthrodermataceae, e.g. *Ar. benhamiae*, *Ar. otae* and *T. verrucosum*. Bioinformatic analysis showed that the deduced amino acid sequences of these genes share high similarity with those shown to be involved in the common steps in the biosynthesis of ergot alkaloids in *A. fumigatus* and *C. purpurea*, i.e. from prenylation of tryptophan to oxidation of chanoclavine-I (Table 1 and Fig. 2). On the other hand, the homologous genes responsible for the conversion of chanoclavine-I aldehyde, i.e. fgaOx3 and fgaFS in *A. fumigatus* (Wallwey et al., 2010b) or easG in *C. purpurea* (Matuschek et al., 2011), were not found in arthrodermataceous fungi, and nor were further genes in the specific steps of the ergot alkaloid biosynthesis.

The five genes were found as clusters in the same order and orientation in all three arthrodermataceous fungi (Fig. 2c) and share sequence identity of 40–70% on the amino acid level with their homologues in *A. fumigatus* and *C. purpurea*.
Sequence analysis of the putative alcohol dehydrogenase gene ARB_04646 in *Ar. benhamiae*

The sequence of the putative alcohol dehydrogenase gene ARB_04646 spans base pairs 1957,951–1958,808 of ABSU01000001.1, contig01293.0 of the whole genome shotgun sequence of *Ar. benhamiae* CBS 112371. The deduced gene product (accession no. EFE37118) was predicted to consist of two exons of 598 and 143 bp, respectively, interrupted by an intron of 117 bp. The deduced gene product comprises 246 amino acids and has a calculated molecular mass of 26.2 kDa. EFE37118 showed significant sequence similarity to its orthologues in other arthrodermataceous fungi (see above) as well as in *A. fumigatus* and *C. purpurea*. By using the program DNASIS, EFE37118 was shown to share 64% sequence identity on the amino acid level with FgaDH from *A. fumigatus* and 60% with EasD from *C. purpurea*. FgaDH had been demonstrated to catalyse the oxidation of the alcohol group of chanoclavine-I to an aldehyde group (Wallwey et al., 2010a).

To provide evidence that the identified gene cluster is in fact responsible for the biosynthesis of ergot alkaloids or their precursors, we initially tried to express the coding region of ARB_04646 as predicted by the genome annotation mentioned above. The sequence was amplified by fusion PCR and cloned into pQE60. No protein was overproduced after expression in *E. coli*. After this unsuccessful attempt, we again analysed the exon and intron structure of ARB_04646. Amino acid alignments of sequences from different fungi mentioned above showed that EFE37118 in *Ar. benhamiae* is 18 or 19 amino acids shorter than its orthologues in other fungi. The corresponding position was found as a gap before the second exon sequence. Therefore, we concluded that the second exon is longer than that predicted by genome annotation. According to our analysis, ARB_04646 consists of two exons of 598 and 197 bp, respectively, interrupted by an intron of 63 bp. This was later confirmed by amplification and sequencing of PCR products from cDNA with two primer pairs (see Fig. S1 available with the online version of this paper). The deduced gene product of the coding region comprises 264 amino acids and has a calculated molecular mass of 28.2 kDa, which corresponds well to FgaDH (EAL94099) from *A. fumigatus* and EasD (CAB39316) from *C. purpurea*, both with 261 amino acids. To distinguish the correct gene sequence from the automatically annotated ORF based on genome sequencing, we use the term ChaDH (chanoclavine-I dehydrogenase) for the enzyme in this study. By using the program DNASIS, ChaDH was shown to share sequence identities of 69% on the amino acid level with FgaDH and of 62% with EasD. Sequence identities of ChaDH to EEQ33233 from *Ar. otae* and EFE43380 from *T. verrucosum* were 89%, slightly higher than the values obtained for the protein deduced from genome annotation.

**Cloning and expression of the coding region of ARB_04646 as well as purification of His<sub>6</sub>-ChaDH**

The newly predicted coding region of ARB_04646 was amplified by a two-round fusion PCR from genomic DNA of *Ar. benhamiae* CBS 112371 and cloned into the expression vector pQE60, resulting in the expression plasmid pCW18 (see Methods). Gene expression with pCW18 in *E. coli* was induced by 0.2 mM IPTG at 37°C for 16 h. Protein overproduction was clearly observed after induction (Fig. 3). ChaDH was subsequently purified by Ni-NTA agarose to apparent homogeneity as judged by SDS-PAGE and a protein yield of 3 mg purified His<sub>6</sub>-tagged ChaDH per litre of culture was obtained. The observed molecular mass was 30 kDa and corresponded well to the calculated value of 29.5 kDa for His<sub>6</sub>-ChaDH (Fig. 3).

**Enzyme activity of ChaDH and identification of the enzyme product**

To establish the enzyme activity, the purified enzyme was incubated with chanoclavine-I in the presence of 5 mM NAD<sup>+</sup> and the reaction mixture was analysed by HPLC. As shown in Fig. 4, product formation was clearly detected in the incubation mixture (Fig. 4a), similar to that of FgaDH with chanoclavine-I and NAD<sup>+</sup> (Fig. 4c). The product peak with a retention time of 10.6 min was absent in a control assay of chanoclavine-I and NAD<sup>+</sup> with previously heat-denatured ChaDH by boiling for 30 min (Fig. 4b), demonstrating its dependence on the presence of active enzyme. Product formation was also strictly dependent on the presence of NAD<sup>+</sup>. No detectable enzyme product was obtained with NADP<sup>+</sup>, FMN (flavin mononucleotide) or FAD (flavin adenine dinucleotide) instead of NAD<sup>+</sup> as proton acceptor (data not shown). A linear dependence of

![Fig. 3. Analysis of the overproduction and purification of His<sub>6</sub>-ChaDH.](http://mic.sgmjournals.org)

The proteins were separated on a 12% SDS-PAGE gel and stained with Coomassie brilliant blue R-250. Lanes: 1, total protein before induction; 2, total protein after induction; 3, soluble protein after induction; 4, purified His<sub>6</sub>-ChaDH; 5, molecular mass standard.
product formation was observed with the amount of protein up to 1.5 μg per 100 μl assay and with incubation time up to 12 min (see Figs S2 and S3).

For structural elucidation of the enzyme product, an enzyme assay was carried out on a volume of 20 ml (see Methods). A conversion rate of 28 % was achieved after incubation for 2 h. The enzyme product was isolated by HPLC using water and acetonitrile containing 0.5 % (v/v) trifluoroacetic acid as solvents. For better comparison with the data in the literature, both the enzyme product and chanoclavine-I were converted to their free base form after HPLC isolation by treatment of the collected fractions with sodium carbonate. Spectroscopic methods were chosen for structural elucidation of the enzyme product. Assignments of 1H-NMR signals given in Table 2 were also confirmed by DQF-COSY and HSQC measurements (see Figs S5 and S6).

Comparison of the 1H-NMR signals of the enzyme product with those of the substrate revealed unambiguously the disappearance of the signal for CH2OH of the substrate at 4.15 p.p.m. and the presence of a signal for an aldehyde proton at 9.48 p.p.m. in the spectrum of the enzyme product (Fig. S4). This showed that oxidation of chanoclavine-I to its aldehyde had taken place. NOESY spectra verified the cis-configuration of the aldehyde group and the olefinic methine proton at position 9 (Fig. S7). The NMR data obtained corresponded well to those of chanoclavine-I aldehyde reported previously (Wallwey et al., 2010a). The structure of the enzyme product was also confirmed by detection of the [M + 1]+ ion at m/z 255.1451 (calculated value 255.1497) in the HR-ESI-MS spectrum. These results showed that ChaDH catalysed the conversion of chanoclavine-I to chanoclavine-I aldehyde in the presence of NAD+ and therefore functions as a chanoclavine-I dehydrogenase.

Biochemical properties and kinetic parameters of ChaDH

By using size exclusion chromatography, the molecular mass of the recombinant active His6-ChaDH was determined to be 128 kDa. This indicates that ChaDH acts as a homotetramer, which was also observed for its orthologue FgaDH from A. fumigatus (Wallwey et al., 2010a). The ChaDH reaction apparently followed Michaelis–Menten
Table 2. $^1$H-NMR data for chanoclavine-I and chanoclavine-I aldehyde

The spectra were taken at 600 MHz in CDCl$_3$. The solvent signal (7.24 p.p.m.) was used as reference. See Fig. 1 for numbering of the carbons. br, Broad; s, singlet; d, doublet; t, triplet; td, triplet of doublet; dd, doublet of doublet.

<table>
<thead>
<tr>
<th>Proton</th>
<th>Chanoclavine-I  $\delta$ (p.p.m.), multiplicity (J; Hz)</th>
<th>Chanoclavine-I aldehyde $\delta$ (p.p.m.), multiplicity (J; Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH-1</td>
<td>7.98, br s</td>
<td>8.01, br s</td>
</tr>
<tr>
<td>H-2</td>
<td>6.92, br s</td>
<td>6.96, br s</td>
</tr>
<tr>
<td>H-4a</td>
<td>2.85, dd (14.9, 9.1)</td>
<td>2.89, dd (15.3, 7.2)</td>
</tr>
<tr>
<td>H-4b</td>
<td>3.32, dd (15.0, 4.1)</td>
<td>3.27, dd (15.3, 4.0)</td>
</tr>
<tr>
<td>H-5</td>
<td>3.01, td (8.7, 4.2)</td>
<td>3.15, td (6.9, 4.2)</td>
</tr>
<tr>
<td>H-6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-7</td>
<td>4.15, s</td>
<td>9.48, s</td>
</tr>
<tr>
<td>H-9</td>
<td>5.44, d (9.8)</td>
<td>6.49, d (10.1)</td>
</tr>
<tr>
<td>H-10</td>
<td>4.02, brt (9.1)</td>
<td>3.31, dd (10.0, 6.7)</td>
</tr>
<tr>
<td>H-12</td>
<td>6.74, d (7.1)</td>
<td>6.71, d (7.1)</td>
</tr>
<tr>
<td>H-13</td>
<td>7.12, t (7.6)</td>
<td>7.14, t (7.6)</td>
</tr>
<tr>
<td>H-14</td>
<td>7.19, d (8.1)</td>
<td>7.25, d (7.5)</td>
</tr>
<tr>
<td>H$_3$-17</td>
<td>1.88, br s</td>
<td>2.00, d (0.9)</td>
</tr>
<tr>
<td>H$_3$-18</td>
<td>2.56, s</td>
<td>2.52, s</td>
</tr>
</tbody>
</table>

kinetics. The $K_M$ values were determined from a Hanes–Woolf plot at 90 mM for chanoclavine-I and at 0.36 mM for NAD$^+$. The maximum reaction velocity observed with ChaDH was 385 nmol min$^{-1}$ mg$^{-1}$, corresponding to a turnover number of 0.76 s$^{-1}$. These $K_M$ values are about one-third of those previously obtained for FgaDH (Wallwey et al., 2010a), indicating a higher affinity of ChaDH to its substrates.

To test the dependence of the ChaDH reaction on metal ions, chanoclavine-I was incubated with ChaDH and NAD$^+$ in the presence of EDTA or different metal ions at final concentrations of 5 mM. Incubation without metal ions or EDTA was used as a control. Addition of Mg$^{2+}$, Ca$^{2+}$, K$^+$ or Na$^+$ to the reaction mixtures did not change the enzyme activity significantly in comparison with that of the control assay without additives. Even in the presence of EDTA, only a slight reduction of enzyme activity was observed and 71 % of activity of the control assay was recovered. Addition of Ni$^{2+}$, Mn$^{2+}$ and Co$^{2+}$ resulted in activity reduction to 73, 35 and 31 % of that of the control assay, respectively. Almost no product formation was found with Fe$^{2+}$, Zn$^{2+}$ or Cu$^{2+}$ as additives.

**Expression analysis of the putative cluster genes by Northern blotting**

To study the expression of cluster genes, RNA was isolated from five different Ar. benhamiae cultures. Analysis of the isolated RNA by gel electrophoresis confirmed the amount and high quality of the RNA used for Northern blotting (Fig. 5a). Signals were clearly detected for the constitutively expressed actin and hydrophobin genes, which were used as positive controls, under all of the five culture conditions (Fig. 5b, c). By contrast, no signal was detected for the five cluster genes under the same conditions, i.e. exposure time of 5 min (data not shown). Even after prolongation of the exposure time to 60 min, only very weak signals were observed (Fig. 5d–h). Detection of several bands under this condition could indicate unspecific hybridization. Results of RT-PCR for chaDH (ARB_04646) with gene-specific primers confirmed its low expression (Fig. S1). The yield of amplified DNA from cDNA was very low, although low annealing temperature and additional PCR cycles (40) were applied. Due to a generally observed degradation of RNA when isolated from older mycelia, it was not possible to investigate the expression of the cluster genes at later time points, e.g. after cultivation for 96 h (data not shown).

**DISCUSSION**

In this study, we noted the presence of an ergot alkaloid cluster consisting of five genes with high similarity to those in A. fumigatus and C. purpurea in three fungi of the family Arthrodermataceae, i.e. Ar. benhamiae, Ar. otae and T. verrucosum. The family Arthrodermataceae belongs to the same subclass Eurotiomycetidae of the class Eurotomyctetes as the family Trichocomaceae, whereas the family Clavicipitaceae is a member of the class Sordariomycetes. The neighbour-joining tree based on the partial $b$-tubulin gene clearly shows three different clades for these families (Fig. S8).

Based on sequence analysis and comparison with ergot alkaloid biosynthesis enzymes, the deduced products of the five homologous genes could be assigned to the common steps in the conversion of tryptophan to chanoclavine-I aldehyde (Fig. 1). Taking the gene products of Ar. benhamiae as examples, EFE37121 can be predicted to...
catalyse the prenylation of tryptophan at position C-4 of the indole ring, resulting in the formation of 4-DMAT, which would be converted to 4-DMA-L-abrine by the putative methyltransferase EFE37119. Formation of chanoclavine-I would be catalysed by EFE37120 and EFE37117. Oxidation of chanoclavine-I by EFE37118 (ChaDH) led to formation of its aldehyde, as demonstrated in this study. Similar roles in the biosynthesis of chanoclavine-I aldehyde in *Ar. otae* and *T. verrucosum* are also expected for the orthologues identified in these strains.

To the best of our knowledge, this is the first report about the presence of genes for ergot alkaloid biosynthesis in Arthrodermataceae. Analysis of the genomic region of the five-gene cluster in *Ar. benhamiae*, *Ar. otae* and *T. verrucosum* did not indicate the existence of genes involved in the conversion of chanoclavine-I aldehyde, such as fgaOx3 and fgaFS in *A. fumigatus* (Wallwey et al., 2010b) or easG in *C. purpurea* (Matuschek et al., 2011). Furthermore, the genes in the vicinity of the five cluster genes have no significant similarity to known secondary metabolism genes. Therefore, it can be speculated that the cluster consists of just five genes. However, it cannot be excluded that the genes responsible for the further conversion of chanoclavine-I aldehyde have been translocated to another region of the genome, as observed for genes involved in cephalosporin biosynthesis in *Acremonium chrysogenum* (Gutiérrez et al., 1992). Thus, the end product of the gene cluster of arthrodermataceous fungi is probably the ergot alkaloid precursor chanoclavine-I aldehyde or an unknown derivative thereof (Fig. 1). HPLC and TLC analyses of extracts from *Ar. benhamiae* cultures with different media showed no detectable amounts of chanoclavine-I aldehyde or other violet spots after spraying with van Urk reagent.
which is used for detection of ergot alkaloids. The results of the Northern blot suggest that the genes in this cluster were not or only poorly expressed, at least under the culture conditions used in this study. This is not surprising, as most of the fungal biosynthesis gene clusters are silent under standard laboratory conditions (Brakhage & Schroeckh, 2011). However, in vivo it is also possible that chanoclavine-I aldehyde is further metabolized to unknown compounds, which could not be determined in this study due to the absence of gene expression.

The results presented in this work expand our knowledge about the distribution of ergot alkaloids in fungi. As mentioned in the Introduction, ergot alkaloids were found in only two fungal families, Trichocomaceae and Clavicipitaceae, prior to this work. Each family produces predominantly particular types of ergot alkaloids, i.e. clavine-type alkaloids by members of Trichocomaceae and ergoamides and/or ergopeptides by fungi of the family Clavicipitaceae. Identification of the ergot alkaloid gene cluster in Arthrodermataceae provides evidence for the presence of ergot alkaloids, or at least of the genetic information for their biosynthesis, in a third family. It seems that fungi of the family Arthrodermataceae have the genetic potential to produce ergot alkaloid-related substances with only three rings or a strongly modified fourth ring. This could represent a structural feature characteristic of this natural product group in this fungal family.

The availability of the genome sequences of bacteria and fungi, which has increased tremendously in recent years, has accelerated the identification of genes involved in the biosynthesis of secondary metabolites. In this study, we demonstrated that genetic information for secondary metabolites can be identified easily by genome mining, even though the genes are not expressed and therefore no secondary metabolites are detectable. The essential conditions for gene expression in their natural hosts are usually unknown and often difficult to predict. However, expression of an identified gene cluster could be achieved by co-cultivation, promoter exchange or overexpression of transcription factors (Bergmann et al., 2010; Brakhage & Schroeckh, 2011). Identification of gene clusters is a prerequisite for optimization of gene expression and production of new secondary metabolites. Furthermore, recombinant enzymes can be obtained in substantial amounts by heterologous gene expression and protein purification, as described in this study. The proteins obtained can then be used as catalysts for chemoenzymatic synthesis.

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REFERENCES


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