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Gene family encoding the major toxins of lethal Amanita mushrooms

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Amatoxins, the lethal constituents of poisonous mushrooms in the genus Amanita, are bicyclic octapeptides. Two genes in A. bisporigera, AMA1 and PHA1, directly encode α-amanitin, an amatoxin, and the related bicyclic heptapeptide phallacidin, a phallotoxin, indicating that these compounds are synthesized on ribosomes and not by nonribosomal peptide synthetases. α-Amanitin and phallacidin are synthesized as proproteins of 35 and 34 amino acids, respectively, from which they are predicted to be cleaved by a prolyl oligopeptidase. AMA1 and PHA1 are present in other toxic species of Amanita section Phalloideae but are absent from nontoxic species in other sections. The genomes of A. bisporigera and A. phalloides contain multiple sequences related to AMA1 and PHA1. The predicted protein products of this family of genes are characterized by a hypervariable "toxin" region capable of encoding a wide variety of peptides of 7–10 amino acids flanked by conserved sequences. Our results suggest that these fungi have a broad capacity to synthesize cyclic peptides on ribosomes.

Mushrooms in the genus Amanita section Phalloideae account for >90% of all fatal mushroom poisonings (1). The human LD₅₀ for α-amanitin (Fig. L4) is ~0.1 mg/kg, and one mature destroying angel (A. bisporigera, A. virosa, A. subiliciana and allied species) (Fig. 2A) or death cap (A. phalloides) (Fig. 2B) can contain a fatal dose of 10–12 mg (2). Only the carpophores (fruiting bodies) contain high concentrations of the toxins. Like other ectomycorrhizal basidiomycetes, species of Amanita grow slowly and do not form carpophores in culture (3). There are ~900–1,000 species of Amanita, but most do not produce amatoxins or phallotoxins, and some are edible (Fig. 2C) (4, 5).

The mammalian toxicity of amatoxins is because of active cellular uptake followed by inhibition of RNA polymerase II (6–9). The typical symptoms of amatoxin poisoning are gastrointestinal distress beginning 6–12 h after ingestion, a remission phase lasting 12–24 h, and progressive loss of liver function culminating in death within 3–5 days. One of the few effective treatments is liver transplantation (10).

In addition to amatoxins, several members of Amanita section Phalloideae produce bicyclic heptapeptides called phallotoxins (Fig. 1B). Although structurally related to amatoxins, phallotoxins have a different mode of action, which is the stabilization of F-actin (11). Phallotoxins are poisonous when administered parenterally, but not orally because of poor absorption.

The biosynthetic origin of the Amanita toxins has been unknown. Because of the difficulty of working with Amanita fungi in culture, we took a genomic approach to identify genes involved in the biosynthesis of the amatoxins and phallotoxins.

Results and Discussion

The genome of A. bisporigera, an amatoxin- and phallotoxin-producing species native to North America (Fig. 2A), was shotgun-sequenced to approximately two times the coverage of the genome (~70 MB total based on the known size of other homobasidiomycetes) (12) by a combination of automated Sanger sequencing and pyrosequencing (13). Because all known fungal cyclic peptides are biosynthesized by nonribosomal peptide synthetases (NRPSs) (14, 15), the genome survey sequences were first queried with known bacterial and fungal NRPSs. No evidence for any NRPS was found in A. bisporigera; the most closely related sequences were orthologs of aminoadipate reductase and acyl-CoA synthase, which are other members of the aminoaeryl-adenylating superfamily (15).

We then searched the A. bisporigera genome for DNA encoding amanitins’ amino acid sequences. Simplified to the unmodified 20 proteogenic amino acids (i.e., ignoring the hydroxyla-
tions and Trp-Cys cross-bridge) (Fig. 1), the sequence of the amatoxins is a cyclic permutation of either IWGIGCNP (H9251- and H9253-amanitins) or IWGICCDP (H9252- and H9255-amanitins). Nucleotide sequences that could encode the amino acid sequence of H9251-amanitin were found in the genome of A. bisporigera, and two specimens of A. franchetii (Mendocino County, CA).

A genomic survey sequence of A. bisporigera also predicted the peptide AWLVDCP, which matches phallacidin, one of the major phallotoxins (Fig. 1B). Inverse PCR using PvuI and SacI was used to isolate genomic fragments of 1.6 and 1.9 kb, respectively, covering the PHA1 gene. Two different classes of sequences were found, both of which could encode phallacidin. A cDNA for PHA1 was isolated by using the restriction enzyme PvuI and SacI by chance. Inverse PCR by using the restriction enzyme PvuI resulted in the isolation of a 2.5-kb fragment of flanking genomic DNA. An RNA blot probed with this DNA indicated that this region of the genome is transcribed into an mRNA of <400 nt (data not shown). PCR primers based on the genomic sequence were used to amplify a cDNA of ~380 bp by 3′ and 5′ rapid amplification of cDNA ends (RACE). Comparison of the cloned, polyadenylated cDNA to the genomic sequence indicated that the gene, AMA1, has three introns with conventional GT/AG intron borders. Two of the introns (53 and 59 nt in length) are in the 3′ untranslated region, and one intron (58 nt) interrupts the fourth from the last codon (Fig. 3A). The presence of these features indicates that AMA1 constitutes a true transcribed and processed gene. Assuming that translation starts at the first ATG downstream of the transcriptional start site, AMA1 encodes a proprotein of 35 amino acids (Fig. 3A).

AMA1 and PHA1 and their translation products are similar in overall size and sequence (Fig. 4). The translated regions upstream of the toxin sequences have 28 of 30 nt in common (93%), the regions downstream have 40 of 48 nt in common (83%), but the toxin regions have only 11 of 24 nt in common (46%). Thus, the proproteins of α-amanitin and phallacidin are composed of two domains, a variable toxin region flanked by conserved regions (Fig. 4).

Many secondary metabolites are limited in their taxonomic distribution, and most species of Amanita do not make amatoxins or phallotoxins. To test whether the lack of toxin production among other species of Amanita were because of absence of the encoding genes, a blot of genomic DNA from 12 species of Amanita was hybridized with AMA1 and PHA1. The species include four from section Phalloideae (this section contains all of Amanita).
the species that make amatoxins and phallotoxins), three from section *Validae* (the sister group to section *Phalloideae*), two from section *Amanita*, one from section *Caesarea*, and two from section *Vaginatae* (4, 5). All mushrooms were tested and confirmed by HPLC for the expected presence or absence of amatoxins and phallotoxins. All of the tested species that synthesize amatoxins and phallotoxins, but none of the nonproducers, hybridize to *AMA1* and *PHA1* (Fig. 5). This finding is consistent with *AMA1* and *PHA1* being responsible for amanitin and phallacidin biosynthesis and provides a molecular explanation for why *Amanita* species outside of section *Phalloideae* are not deadly poisonous. (Some of the *Amanita* species that do not make amatoxins or phallotoxins are edible, but others make different toxic compounds.)

The complex hybridization patterns shown in Fig. 5 indicate that *AMA1* and *PHA1* are members of gene families. Therefore, the conserved upstream and downstream amino acid sequences of *AMA1* and *PHA1* were used as queries to search for additional related sequences in the *A. bisporigera* genome. We thereby found at least 13 new, related complete or almost complete sequences (Fig. 6A) and another 10–15 sequences missing one end or the other (data not shown). All of these new sequences have an upstream conserved consensus sequence MSDINTALP (MSDIN, R, and P are invariant) and a downstream conserved consensus sequence CVGDDV (the first D is invariant). The putative toxin regions, which start immediately downstream of the invariant Pro residue and end after an invariant Pro residue, are hypervariable compared with the upstream and downstream sequences. The hypervariable regions contain 7–10 amino acids, and all 20 proteogenic amino acids are represented at least once.

To detect related genes in *A. phalloides*, which worldwide accounts for the majority of fatal mushroom poisonings, degenerate PCR primers were designed against the conserved upstream and downstream sequences of *AMA1* and *PHA1*. The predicted translations of four amplicons from *A. phalloides* and one from *A. ocreata* are shown in Fig. 6B. One of them (IWGIGCDP) matches the amino acid sequence of β-amanitin,
Small, modified, and biologically active peptides were previously identified from bacteria and several animals, including arachnids, snakes, cone snails, and amphibian skin (19–21). Like toxins, the animal peptides are synthesized as precursors (proproteins) by proteases that recognize basic amino acid residues whereas the animal peptides are processed from their respective precursors (Fig. 3–5). Fourth, although ribosomally synthesized cyclic peptides contain Pro, the last amino acid in the upstream conserved region is always Pro, and the predicted toxin sequences all have Pro as the last amino acid (Figs. 4 and 6).

Based on the properties of the known proline-specific peptidases (28, 29), the prolyl oligopeptidase family (POP) (EC 3.4.21.26) is the most promising to be involved in the processing of the proproteins of the Amanita toxins. We identified sequences related to human POP (GenBank accession no. NP.0027717) in the genome survey sequences of A. bisporigera [Supporting information (SI Text)]. Orthologs of human POP also were found in every other basidiomycete for which whole genome sequences are available (Laccaria bicolor, Coprinus cinereus, Phanerochaete chrysosporium, Ustilago maydis, Sporobolomyces roseus, Puccinia graminis, and Cryptococcus neoformans) (see SI Text). A POP has been characterized from the mushroom Lyophyllum cinerscens (30). In contrast, orthologs of human POP are rare or nonexistent in fungi outside of the basidiomycetes. BLASTP (default parameters) identified no orthologs of human POP with a score >53 and E value <10\(^{-6}\) in any fungus outside of the basidiomycetes, except perhaps in the ascomycete Setosphaeria nodorum (SN0G11288; score = 166; E value = 3 \times 10^{-40}). Thus, it appears that at least one component of the biochemical machinery necessary for the biosynthesis of the Amanita toxins is both widespread in, and restricted to, the basidiomycetes.

The results presented here indicate that species of Amanita section Phalloideae synthesize their notoriously toxic cyclic peptides on ribosomes. Furthermore, these fungi have evolved a unique mechanism of combinatorial biosynthesis that endows them with the ability to biosynthesize a multitude of cyclic peptides. Further elucidation of the biosynthetic pathway of Amanita toxin biosynthesis could take advantage of the tractability of some basidiomycete fungi such as C. cinereus (31).

### Materials and Methods

Mushrooms were harvested from the wild in 2002, 2006, and 2007; frozen at \(-80^\circ\)C; and lyophilized. DNA was extracted from lyophilized fruiting bodies or cultures by using cetyltrimethylammonium bromide, phenol, and chloroform (32). RNA was extracted by using TRIzol (Invitrogen) (33).

PCR products were purified by using Wizard SV Gel and PCR Clean-Up System (Promega) and were cloned into TOPO pCR 4 (Invitrogen) for sequencing. For 3’ RACE, initial and nested primers from GeneRacer (Invitrogen) were used, and gene-specific primers were derived from the genomic sequence. Primer sequences may be found in SI Text.

Probe labeling. DNA blotting, and filter hybridization followed standard protocols (34, 35). DNA for blotting was cut with PstI and electrophoresed in 0.7% agarose. Hybridizations were performed overnight at 65°C in 4X \(\times\) SET, 0.1% sodium pyrophosphate, 0.2% SDS, 10% dextran sulfate, and 625 μg/ml heparin. SET (20X) is 3 M NaCl, 0.6 M Tris, and 0.04 M EDTA (pH 7.4). A 551-bp fragment of the A. bisporigera \(\beta\)-tubulin gene used as a control probe on DNA blots was amplified by PCR.

Variability in toxin content is known within species of Amanita (36, 37). All fungi analyzed for the presence of AMA1 and PHA1 (Fig. 5) were analyzed for amatoxins and phalloxins by established HPLC methods (32, 38). Standards of \(\alpha\)-amanitin, \(\beta\)-amanitin, phalloidin, and phallacidin were purchased from Sigma–Aldrich.

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Primers for inverse PCR for *AMA1* were CCATCTGGGGTATCGGTTGC and TTGGGATTGTGAGGTAGGTC, and for *PHA1* CGTCAACCGTGTCCTCCT and ACGCATGGCCAGTCT. For 3’ RACE, initial and nested primers from GeneRacer (Invitrogen) were used. For the other 3’ *AMA1* RACE primers, the initial primer was CCCATCGACAACCTAATCCAAGAC and the nested primer was CCTCTAAACCTCACTCTCCCAATG. For 5’ RACE of the *AMA1* cDNA, the initial primer was GCCCAAGGCGTAAACATCCCAACT and the nested primer was TATCGCCACTACTCTGGTCATA. For *PHA1*, the 3’ initial primer was GACCTGCTCTAAATCAACATA and the 3’ nested primer was ATCAATGCACCCGTCTCTCTCTG. The 5’ initial *PHA1* primer was CGGATCATTTACGTGGGTGTTTA and the 5’ nested primer was AACTTGCTTGACTAGTGATGAGAC.

Degenerate primers for amplification of toxin genes from *A. phalloides* were ATGTCNGAYATYAYGCNACNCG (forward) and AAGGSYCTTCGCCGAGTGAGGAGWSKRKTGAC (reverse), in which W indicates A or T, S indicates C or G, K indicates G or T, R indicates A or G, and Y indicates T or C.

Primers to amplify a portion of the *A. bisporigera* tubulin gene were ACCTCCATCTCGTCCATACTCTCC and TGTTCGCCGACTGCTACTA.

**Sequences orthologous to human prolyl oligopeptidase (POP) in *A. bisporigera* and other fungi.** The following genome survey sequences were identified in the *A. bisporigera* genome (subject) by TBLASTN using human POP (GenBank NP_002717) as query:
>ECGK9LO02JKSHR R length=112
  Length = 112

  Score = 47.8 bits (112), Expect = 4e-06
  Identities = 20/33 (60%), Positives = 26/33 (78%)
  Frame = +3

  Query: 436 QTVQIFYPSKDGTKPMFIVHKKSIKLDGSHPA 468
     ++ Q+Y SKDGTK+PMFIV KS K DG+ PA
  Sbjct: 3 ESTQVWYESKDGTKVPMFIVRHKSTKFDTGAPA 101

>contig26093 length=206   numReads=6
  Length = 206

  Score = 41.2 bits (95), Expect = 3e-04
  Identities = 18/32 (56%), Positives = 23/32 (71%)
  Frame = +1

  Query: 440 IFYPSKDGTKPMFIVHKKSIKLDGSHPAFLY 471
     ++Y S DGTKIPMFIV K+ K +G+ PA  Y
  Sbjct: 109 VWYDSYDGTKPMFIVRHKNTKFNGTAPAIQY 204

>ECIMO1V02I2I05 S length=107
  Length = 107

  Score = 35.8 bits (81), Expect = 0.014
  Identities = 18/27 (66%), Positives = 21/27 (77%), Gaps = 1/27 (3%)
  Frame = +2

  Query: 546 KRLTINGGSNGGLLVAAAC-ANQRPDLF 571
     ++L I+GGSNGGLLV A    QRPDLF
  Sbjct: 26 EKLAISGGSNGGLLVGASRLTQRPDLF 106

>ECIMO1V01CKHE5 R length=94
  Length = 94

  Score = 35.4 bits (80), Expect = 0.019
  Identities = 16/27 (59%), Positives = 19/27 (70%)
  Frame = +2

  Query: 120 SDDGTVALRGYAFSEDGEYFAYGLSAS 146
    S DGT +L Y FS G+YFAYG+S S
  Sbjct: 2 SSDGTASLSMYDFSHCGRKFAYGISLS 82

>EEISCGG02IHTSV R length=106
  Length = 106

  Score = 33.1 bits (74), Expect = 0.093
  Identities = 14/20 (70%), Positives = 15/20 (75%)
  Frame = -2

  Query: 669 PLLIHVDTKAGHGAGKPTAK 688
    PLL+ VD KAGH GK T K
  Sbjct: 105 PLLLRVDKKAHGSGKSTEK 46
Orthologs of human POP in other Basidiomycetes are: Coprinus cinereus (GenBank CC1G_09936), Ustilago maydis (GenBank UM05288), Cryptococcus neoformans (GenBank XP_567311 and XP_567292), Laccaria bicolor (Lacbi1|303722), Phanerochaete chrysosporium (Phchr1|1293), Puccinia graminis (PGTG_14822.2), and Sporobolomyces roseus (Sporo1|33368).

The genome sequences of L. bicolor, P. chrysosporium, and S. roseus are available at http://genome.jgi-psf.org/. The genome sequence of P. graminis is available at http://www.broad.mit.edu/annotation/genome/puccinia_graminis.