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Functional replacement of the ketosynthase domain of *FUM1* for the biosynthesis of fumonisins, a group of fungal reduced polyketides

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Abstract: The genetic manipulation of the biosynthesis of fungal reduced polyketides has been challenging due to the lack of knowledge on the biosynthetic mechanism, the difficulties in the detection of the acyclic, non-aromatic metabolites, and the complexity in genetically manipulating filamentous fungi. Fumonisins are a group of economically important mycotoxins that contaminate maize-based food and feed products worldwide. Fumonisins contain a linear dimethylated C_{18} chain that is synthesized by Fum1p, which is a single module polyketide synthase (PKS). Using a genetic system that allows the specific manipulation of PKS domains in filamentous fungus *Fusarium verticillioides*, we replaced the KS domain of fumonisin *FUM1* with the KS domain of T-toxin *PKS1* from *Cochliobolus heterostrophus*. Although PKS1 synthesizes different polyketides, the *F. verticillioides* strain carrying the chimeric PKS produced fumonisins. This represents the first successful domain swapping in PKSs for fungal reduced polyketides and suggests that KS domain alone may not be sufficient to control the product's structure. To further test if the whole fumonisin PKS could be functionally replaced by a PKS that has a similar domain architecture, we replaced entire *FUM1* with *PKS1*. This strain did not produce any fumonisin or new metabolites, suggesting that the intrinsic interactions between the intact PKS and downstream enzymes in the biosynthetic pathway may play a role in the control of fungal reduced polyketides.

Keywords: Biosynthesis, Fumonisin, Polyketide synthase, *Fusarium verticillioides*

Introduction

Fungal polyketide synthases (PKSs) are modular enzymes typically consisting of a single set of domains [*25*]. These domains function iteratively during the assembly of the polyketide chain, which is in contrast to bacterial modular PKSs (Type I). Typical bacterial modular PKSs function non-iteratively, and the number, order, and domain-composition of the modules dictate the length and reduction level of the polyketide product [*25*]. Although data from heterologous expression and in vitro assays of a number of fungal PKSs, for example 6-methylsalicylic acid synthase [*3*, *13*, *22*] and squalestatin tetraketide synthase [*8*], have shown that each of the single module fungal PKS contains the necessary information for synthesizing a distinct polyketide product, it is not clear how the single module enzymes determine the chain-length of products. The understanding of the biosynthetic mechanism is critical to the production of new industrially important fungal polyketide metabolites, such as the cholesterol-lowering drug lovastatin, via genetic manipulations of the biosynthetic genes.

Information regarding the biosynthetic mechanism for fungal polyketides has emerged in the recent years. Most of the studies were conducted with PKSs synthesizing fungal aromatic polyketides. The architecture of PKSs for aromatic polyketides, such as naphthopyrone synthase WA in *Aspergillus nidulans* [*11*], is very different from that for reduced polyketides, such as fumonisin Fum1p [*21*] and lovastatin LNKS [*15*]. Fungal PKSs for aromatic polyketides are characterized by having a thioesterase-like domain, Claisen cyclase (CYC) domain, at the *C*-terminus [*11*]. This domain has recently been shown to contribute to the chain length determination of fungal aromatic polyketides [*31*]. On the other hand, fungal PKSs for reduced polyketides do not have a CYC domain and therefore must require a discrete mechanism to release the polyketide with a specific chain-length from PKS.

We have been studying a group of fungal reduced polyketides, fumonisins, to understand the biosynthetic mech-

anism for fungal reduced polyketides [*5*, *10*, *29*, *33*, *34*]. Fumonisins are mycotoxins produced by *Fusarium verticillioides*, which is a widespread fungal contaminant of corn and maize-based food and feeds [*17*]. Fumonisins have a linear carbon backbone that is of polyketide origin (Fig. 1) [*21*]. Isotope feeding experiments have established that carbons 3–20 of fumonisins are derived from acetate and carbons 1–2 and the amino group on C-2 are from alanine [*4*, *6*]. The two methyl groups at C-12 and C-16 are derived from methionine [*19*]. The hydroxyl oxygen on C-5, C-10, C-14, and C-15 of fumonisin B_1 (FB_1) are derived from molecular oxygen, whereas the hydroxyl oxygen on C-3 is from the carbonyl group of acetate [*7*]. A cluster of 15 genes (*FUM*) for fumonisin biosynthesis has been cloned from *F. verticillioides* [*20*, *21*, *24*], among which is a PKS gene (*FUM1*). The disruption of *FUM1* led to the elimination of fumonisin production [*21*], showing that this gene is responsible for the biosynthesis of fumonisins.

FUM1 was predicted to code for a PKS with 7-domain (KS-AT-DH-MT-ER-KR-ACP) (Fig. 1) [*21*]. This organization is almost identical to several other PKSs for the biosynthesis of reduced polyketides, such as LDKS and LNKS for lovastatin in *A. terreus* [*15*] and PKS1 for T-toxins in *Cochliobolus heterostrophus* [*32*], although the polyketide chain-length of fumonisins, lovastatin, and T-toxins is substantially different from each other. The objectives of this study were to test whether a heterologous PKS is functionally interchangeable with *FUM1* in *F. verticillioides* and whether such a swapping would result in polyketide products with an altered chain-length. In light of the recent information regarding the chain-length determination by the heterodimeric ketosynthase-chain length factor (KS-CLF, also known as KS_α-KS_β) in the biosynthesis of bacterial aromatic polyketides [*14*, *28*], the outcomes from the fungal KS domain swapping experiments may shed light on the mechanism for the biosynthesis of fungal reduced polyketides.

Materials and methods

Materials and strains

Fusarium verticillioides wild-type strain A0149 was provided by Dr. David Gilchrist (University of California, Davis). YPD medium and V-8 juice agar were respectively used to produce mycelia and conidia [*21*], and cracked maize kernels (CMK) for fumonisin production [*7*, *21*].

Preparation and analysis of nucleic acids

Escherichia coli DH5-α strain was used as the host for general DNA preparation, and the pGEM-zf vector series from Promega (Madison, WI, USA) for cloning and DNA sequencing. Plasmid preparation and DNA extraction were carried out using Qiagen kits (Valencia, CA, USA), and all other manipulations were carried out according to standard methods [*23*]. Genomic DNA of *F. verticillioides* was prepared as described previously [*9*]. In experiments using PCR to quickly screen putative mutants, genomic DNA was mini-prepared from the mutants by using UltraClean™ Microbial DNA Isolation Kit (Mo Bio,

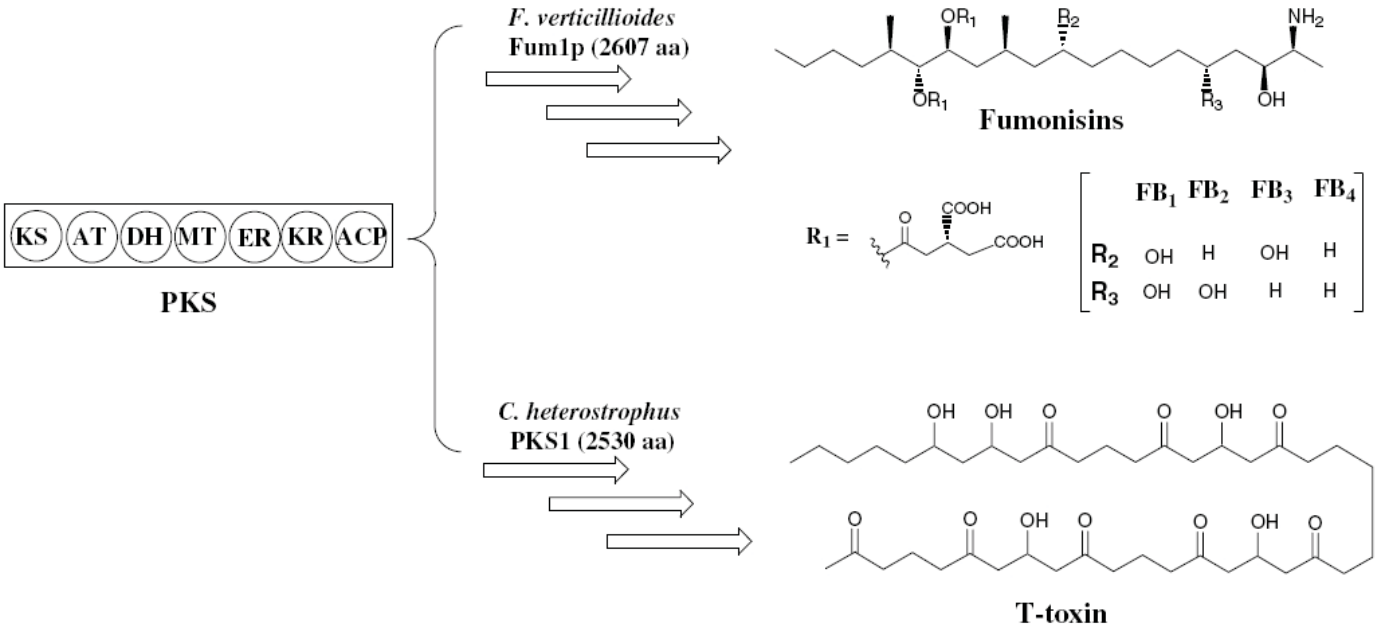

Figure 1 The domain organization of fumonisin Fum1p and T-toxin PKS1 and the chemical structure of the metabolites (only the best characterized C_{41} analog of T-toxins shown). Abbreviations: β-ketoacyl synthase (*KS*), acyltransferase (*AT*), dehydratase (*DH*), methyltransferase (*MT*), enoylreductase (*ER*), β-ketoacyl reductase (*KR*), and acyl carrier protein (*ACP*)

CA, USA). Southern hybridization was performed by using DIG DNA labeling and Detection Kit (Roche, Indianapolis, IN, USA) according to the manufacturer's manual. Approximately 10 μg of genomic DNA was used in the hybridizations, and Immobilon™–NY+ Transfer Membrane (Millipore, Bedford, MA, USA) was used to transfer the DNA. The probe-KS (1.3 kb, Fig. 3d, f) was amplified by PCR using primers tKS-F/tKS-R (Table 1) with *C. heterostrophus* clone pF5P1 [*32*] as template. The probe t1 (703 bp) and t2 (681 bp) in Fig. 5 were prepared using primers PKS1-1-F/PKS1-1-R and PKS1-2-F/PKS1-2-R (Table 1), respectively, with pF5P1 as template. The probe f1 (800 bp) and f3 (737 bp) in Fig. 5 were amplified by PCR using primers FUM1-1-F/FUM1-1-R and FUM1-3-F/FUM1-3-R (Table 1), respectively, with Cos6B [*21*] as template. RT-PCR was carried out using a kit from Invitrogen (Carlsbad, CA, USA). Total RNA was isolated from the cultures using the TRIzol method provided in the manufacturer's manual. The RNA was treated with Amp DNase I (Invitrogen) for 15 min at 25°C and used in the first-strand cDNA synthesis using SuperScript™ First-strand Synthesis System (Invitrogen). A reaction did not contain the reverse transcriptase was included as a control. A pair of primers, P1/P2 (Table 1), was used in the RT-PCR.

Construction of plasmid pZXC-lw92-KS for KS domain replacement

To construct the plasmid for replacing the KS domain of *FUM1*, the *Xho*I/*Sac*I fragment of pUCH2-8 containing the hygromycin resistance gene *HygB* (also known as *hph* or *hpt*) [*1*] was cloned into the same sites of pSP72 to form pUCH-SP. A 5.7 kb *Nsi*I fragment, which contains the 5′-noncoding region (1.1 kb) and the 5′-coding region of *FUM1* gene, was obtained from Cos6B [*21*] by *Nsi*I digestion. After purification from gel, this fragment was further digested with *Sma*I and *Bgl*II to yield a 4.3 kb fragment, which was cloned into *Sma*I/*Bgl*II sites of pUCH-SP to yield pUCH-fKS. A 1,280-bp fragment coding for the KS domain of T-toxin *PKS1* was amplified by PCR from *C. heterostrophus* clone pF5P1 [*32*] using primers tKS-F and tKS-R (Table 1). The fidelity of this PCR fragment was confirmed by DNA sequencing and it was then digested with *Sph*I/*Kpn*I. pUCH-fKS was also digested with *Sph*I/*Kpn*I, and the larger fragment was isolated and ligated with the *Sph*I/*Kpn*I digested PCR fragment to produce pZXC-1w92-KS (Fig. 3a). This construct contains the KS domain of T-toxin *PKS1* flanked by the upstream region (461 bp) and the downstream region (2,645 bp) of the KS domain of *FUM1*.

Construction of plasmid pUCH-LFUM1-PKS1-RFUM1 for intact PKS replacement

To construct the PKS gene replacement vector, pUCH-LFUM1-PKS1-RFUM1, a 1,096 bp DNA fragment (LFUM1) was amplified by PCR using primers of L-FUM1-F/L-FUM1-R (Table 1), and *F. verticillioides* clone Cos6B as template. This fragment is the upstream flanking region of *FUM1* gene. It was digested with *Not*I and *Spe*I and cloned into the same sites of pBlueScript SK(+) to yield pBSK-LFUM1. A 547 bp fragment (PKS1-N) was amplified by PCR using primers of PKS1-N-F/PKS1-N-R and *C. heterostrophus* clone pF5P1 as template. This fragment was digested with *Spe*I and *Eco*RI and cloned into pBSK-LFUM1 to produce pBSK-LFUM1-PKS1N. A 274 bp fragment (PKS1-C) was also amplified by PCR using primers of PKS1-C-F/PKS1-C-R and *C. heterostrophus* clone pF5P1 as template. It was digested with *Hind*III and *Apa*I and cloned into the same sites of pBSK-LFUM1-PKS1N to yield pBSK-LFUM1-PKS1N-C. pBSK-LFUM1-PKS1N-C

Table 1 Primers used in the experiments

Primers	Sequences
tKS-F	5′-TTGCTAAGCATGCTTTCTGAGGGCAGA-3′
tKS-R	5′-ACACTCTAAAGTTGTAGGTACC-3′
P1	5′-CTTGCGGCCGGTTCTAGC-3′
P2	5′-GTTGACGCTGACCCTAAG-3′
P3	5′-CAGTGTTATTGGAGACTC-3′
P4	5′-AACCCAGAATTCGTACATTGG-3′
L-FUM1-F	5′-GCATGCATGCGGCCGCCCCAAGCAATTTTGCAGA-
L-FUM1-R	5′-GACTAGTCGTTGATGATATCAGTAGCG-3′
PKS1-N-F	5′-CACTAGTATGACAGTTCGCGATTCTAAAAC-3′
PKS1-N-R	5′-AACCCAGAATTCGTACATTGG-3′
PKS1-C-F	5′-CAATCCTCAAGCTTTGATAGT-3′
PKS1-C-R	5′-TGAAAAGGGCCCTTAACCCCTATTATTTGT-3′
R-FUM1-F	5′-CCATAGGGGCCCACCTAGACACTGAGCTGG-3′
R-FUM1-R	5′-GGACCCTCGCATATGCAGCAGGTCACGGAAGCA-3
FUM1-1-F	5′-TCCAAGGTTTAGAGGTGAGTCTG-3′
FUM1-1-R	5′-CGAGAAGAAGATTAGGTATCGGG-3′
FUM1-3-F	5′-CAACTCCCTTCCTGAGATAAACA-3′
FUM1-3-R	5′-CTGTAGACCTTGCCTTCCAAAA-3′
PKS1-1-F	5′-ATTTCCAGTATCCAGCCTCTACC-3′
PKS1-1-R	5′-TCGTTTCCCATCTCTCTCATC-3′
PKS1-2-F	5′-GTGCTACTGCTTTGTGGATGTC-3′
PKS1-2-R	5′-CAGGAAGTGAAGTGTCAGGATG-3′

was then digested with *Not*I and *Apa*I, and the LFUM1-PKS1N-C fragment was cloned into pANT841 to yield pANT-LFUM1-PKS1N-C. To prepare the downstream flanking region (RFUM1) of *FUM1* gene, a fragment of 1,345 bp was amplified by PCR using primers of R-FUM1-F/R-FUM1-R and Cos6B as template. This fragment was digested with *Apa*I and *Nde*I and cloned into the same sites of pANT-LFUM1-PKS1N-C to produce pANT-LFUM1-PKS1NC-RFUM1. This construct contains the upstream (LFUM1) and downstream (RFUM1) flanking regions of *F. verticillioides FUM1* gene, as well as the *N*-terminus (PKS1N) and *C*-terminus (PKS1C) of *C. heterostrophus PKS1* gene. To prepare an intact *PKS1* gene in the construct, a 6,999 bp fragment was released from pF5P1 by *Eco*RI and *Hind*III digestion and cloned into the same sites of pANT-LFUM1-PKS1NC-RFUM1 to produce pANT-LFUM1-PKS1-RFUM1. This clone contains intact *PKS1* gene flanked by the upstream and downstream regions of *FUM1* gene, which is a cassette ready for gene replacement by homologous recombination. To introduce an antibiotic selection marker (*HygB*) for fungal transformants, pANT-LFUM1-PKS1-RFUM1 was digested with *Nde*I, followed by Klenow Fragment 3′-end fill-in. The DNA was further digested with *Not*I to release the whole gene replacement cassette. Finally, this cassette was cloned into *Not*I/*Sma*I sites of pUCH2-8 [*1*] to produce pUCH-LFUM1-PKS1-RFUM1 (Fig. 5a).

Transformation of *F. verticillioides* and screening for the mutants

The protocol used to isolate and transform protoplasts has been described previously [*34*]. The plasmid DNA (5 μg) was diluted with STC buffer (100 μl final) and mixed with protoplasts (100 μl), and transformation was mediated with PEG 8000 buffer (30% PEG 8000; 10 mM Tris–HCl, pH 8.0; 50 mM $CaCl_2$). Hygromycin-resistant colonies were selected on YPD plates containing hygromycin B (150 μg/ml, Calbiochem, La Jolla, CA, USA). The screening of mutants involves two stages, which was also described in the previous work [*34*]. The first stage is to select for gene disruption mutants resulted from a single crossover of the homologous regions, whereas the second stage is to select for domain-replaced mutants by screening for the loss of hygromycin B resistance.

Analysis of metabolites

The single-conidia derived wild type strain or mutants were inoculated in CMK medium (25 g) and allowed to grow at 25°C in dark for 4 weeks as described previously [*5*, *10*]. The CMK cultures were extracted with water/acetonitrile (1:1, v/v). The extracts were filtered and subjected to HPLC-ELSD or LC-ESMS analysis [*5*]. A HPLC system (ProStar, Model 210, Varian, Walnut Creek, CA, USA) coupled to an evaporative light-scattering detector (ELSD2000, Alltech, Deerfield, IL, USA) was used to analyze the extracts. The column was Alltima C18LL (5 μ, 250 × 4.6 mm i.d., Alltech, Deerfield, IL, USA), and the experimental conditions were the same as described previously [*5*]. A RFC_{18} Vydac MassSpec column (5 μ, 250 × 1 mm i.d., Vydac™) was used in the LC-ESMS analysis. All positive electrospray spectra were acquired using a Q-Tof Mass spectrometer (Micromass, Manchester, UK).

Results

Generation of the KS-replaced mutants

Two restriction enzyme sites, *Sph*I and *Kpn*I, are conveniently located at the upstream and downstream boundary, respectively, of the *FUM1* KS domain (fKS). The boundaries were defined by Pileup comparison (GCG Program) of sequences from bacterial type I PKSs and fungal PKSs and following the domain definition in previous studies [*2*, *27*]. The sequence of fKS shows an identity/similarity of 41.8%/58.2% to the KS domain (tKS) of T-toxin *PKS1* (Fig. 2) [*32*]. A *Kpn*I site is also found at the downstream boundary of tKS domain. This site is located at the same position as the *Kpn*I site of *FUM1* in the Pileup comparison. These features facilitated the replacement of fKS by tKS. The approach for the replacement is homologous recombination. The pSP72-based construct (pZXC-1w92-KS) contains the hygromycin resistance gene *HygB* from pUCH2-8 [*1*]. The upstream and downstream homologous regions of the construct were directly cloned from cosmid Cos6B [*21*], which contains the *FUM* gene cluster, and the tKS domain was directly inserted into the *Sph*I/*Kpn*I sites of the construct. The fidelity of the replaced domain and the fusion regions between *FUM1* and tKS were confirmed by DNA sequencing. Thus, a region of 362 amino acid residues (from number 56 to 417) of Fum1p (AAD43562) was replaced with a region of 366 residues (from number 38 to 403) of PKS1 (AAB08104) (Fig. 2).

Using the two-stage screening strategy, we obtained approximately three to seven hygromycin-resistant colonies using 5 μg of plasmid DNA mixed with 5×10^7 protoplasts. PCR using a pair of primers binding to the *HygB* gene confirmed that all colonies contained the *HygB* gene (data not shown). One clone (L5) was confirmed by Southern blot to result from a homologous recombination, and the rest resulted from random insertions (data not shown). This single crossover event occurred at the fKS downstream homologous region (2,645 bp in length on plasmid ZXC-1w92-KS, Fig. 3a, b). For the second stage screening, L5 strain was cultured for five generations without hygromycin in the medium to encourage the second crossover in the homologous regions, either at the upstream to KS to produce domain-replaced mutants or at the downstream to KS to revert to the wild-type. Approximately 600 colonies were picked and individually inoculated on grid-lined plates, one copy on YPD and one on YPD/Hygromycin plates. Among the 600 colonies, four (K1, 2, 3, 4) lost the ability to grow on YPD/Hygromycin plates.

We first used PCR to identify the KS-replaced mutants. Two pairs of primers (P1/P2 and P3/P4, Table 1 and Fig. 3a) were used for PCR. For each pair of primers, one primer

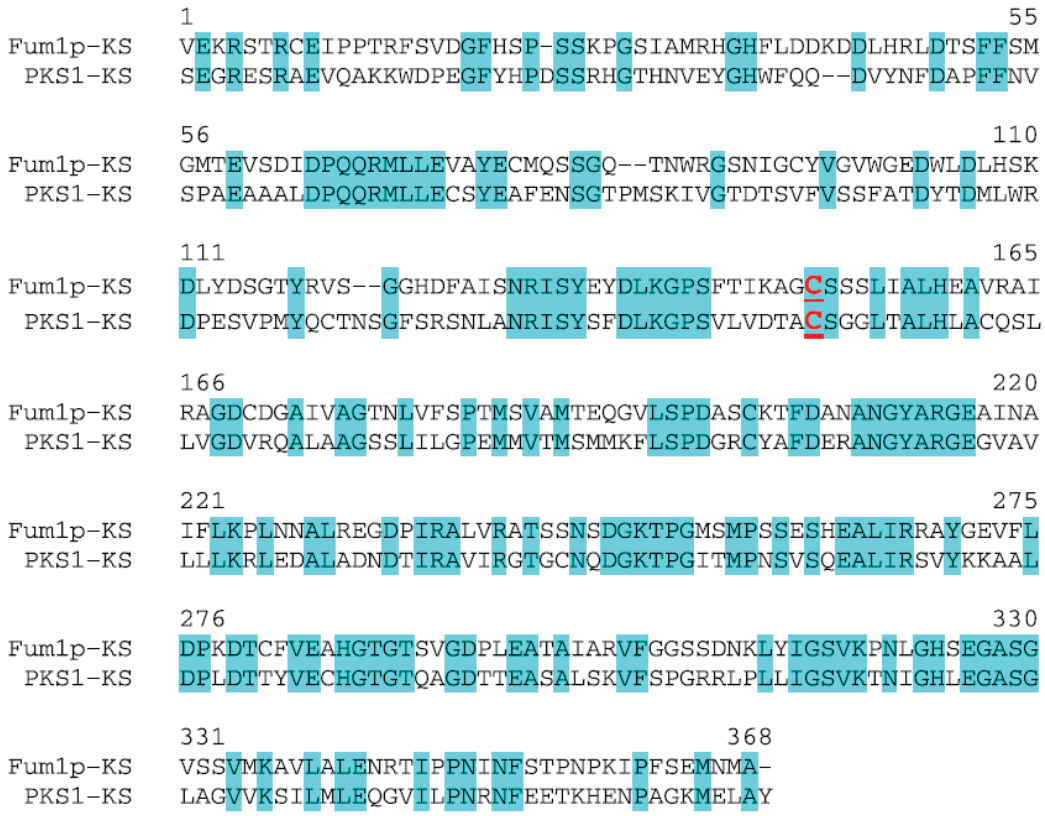

Figure 2 Sequence comparison of the KS domain of Fum1p and T-toxin PKS1. The identical residues are highlighted, and the conserved catalytic site cysteine residue is underlined and in a **bold face letter**.

binds to tKS and the other to the downstream or upstream of fKS on *FUM1*. Thus, the revertant (wild-type) may be distinguished from the domain replaced mutants. As expected, PCR amplified a 742 bp fragment from plasmid pZXC-1w92-KS (positive control) using primers P1/P2, but not from the wild-type strain (negative control) (Fig. 3d). Among the putative mutants, K1, K2, and K3 gave the expected band, whereas K4 did not give the band (Fig. 3d). The results suggest that K1, K2, and K3 contained the chimeric PKS gene and K4 did not contain this gene. K2 and K4 were also subjected to a second test by PCR using primers P3/P4. The plasmid and K2 gave the expected 687 bp, whereas K4 and the wild-type did not give any product (Fig. 3e). The fidelity of the PCR fragments was confirmed by DNA sequencing. Southern hybridization was conducted to further confirm the identity of these mutants. The genomic DNA and plasmid DNA were digested with *Eco*RI and probed with Dig-labeled tKS. The plasmid pZXC-1w92-KS was included as a control to produce two signals at 1.6 kb and 954 bp (Fig. 3f). The wild-type and K4 strain did not give any signal, confirming that K4 is a revertant. K2 gave two signals (7.8, 1.6 kb) expected for a KS-replaced mutant (Fig. 3f).

tKS domain was predicated to contain three small introns (74, 57, 41 bp) [*32*]. To test if the chimeric PKS could be transcripted in *F. verticillioides*, a RT-PCR was performed using primer P1 and P2. The primer site for P1 is located upstream to intron-2 (57 bp), and that for P2 is downstream to intron-3 (41 bp). If the PKS is correctly transcripted in the host, the expected RT-PCR product should be 98 bp shorter than the size of PCR product amplified from genomic DNA. Indeed, the RT-PCR data showed that a band of approximately 644 bp was amplified from RNA prepared from K2 mutant, which is approximately 98 bp smaller than the 742 bp product amplified from the control (Data not shown).

Metabolites in the KS-replaced mutants

The metabolites produced in the mutants as well as in the wild-type of *F. verticillioides* were analyzed by high performance liquid chromatography-evaporative light-scattering detection (HPLC-ELSD) and liquid chromatography-electrospray ionization mass spectrometry (LC-ESMS). The extracts from the wild-type gave one major peak at the retention time of 15.3 min (Fig. 4a) and a $[M + H]^+$ of 722.8 m/z, which is identical to standard FB_1. The yield of FB_1 as estimated from HPLC-ELSD was approximately 0.7–1.5 mg/g CMK medium. Three minor peaks appeared at 16.1, 16.8, and 18.2 min, which were coincident with standard FB_3, FB_2 , and FB_4, respectively (Fig. 4a). They gave a $[M + H]^+$ of 706.5, 706.3, and 690.4 m/z, respectively, which are the molecular mass expected for FB_3, FB_2, and FB_4. The L5 strain that resulted from a single crossover did not produce any fumonisins (data not shown). This result is expected because *FUM1* gene in this strain was disrupted by the plasmid insertion (Fig. 3b). K4 produced FB_1, FB_2, and FB_3 with a level similar to the wild-type (data not shown). This is consistent with the South-

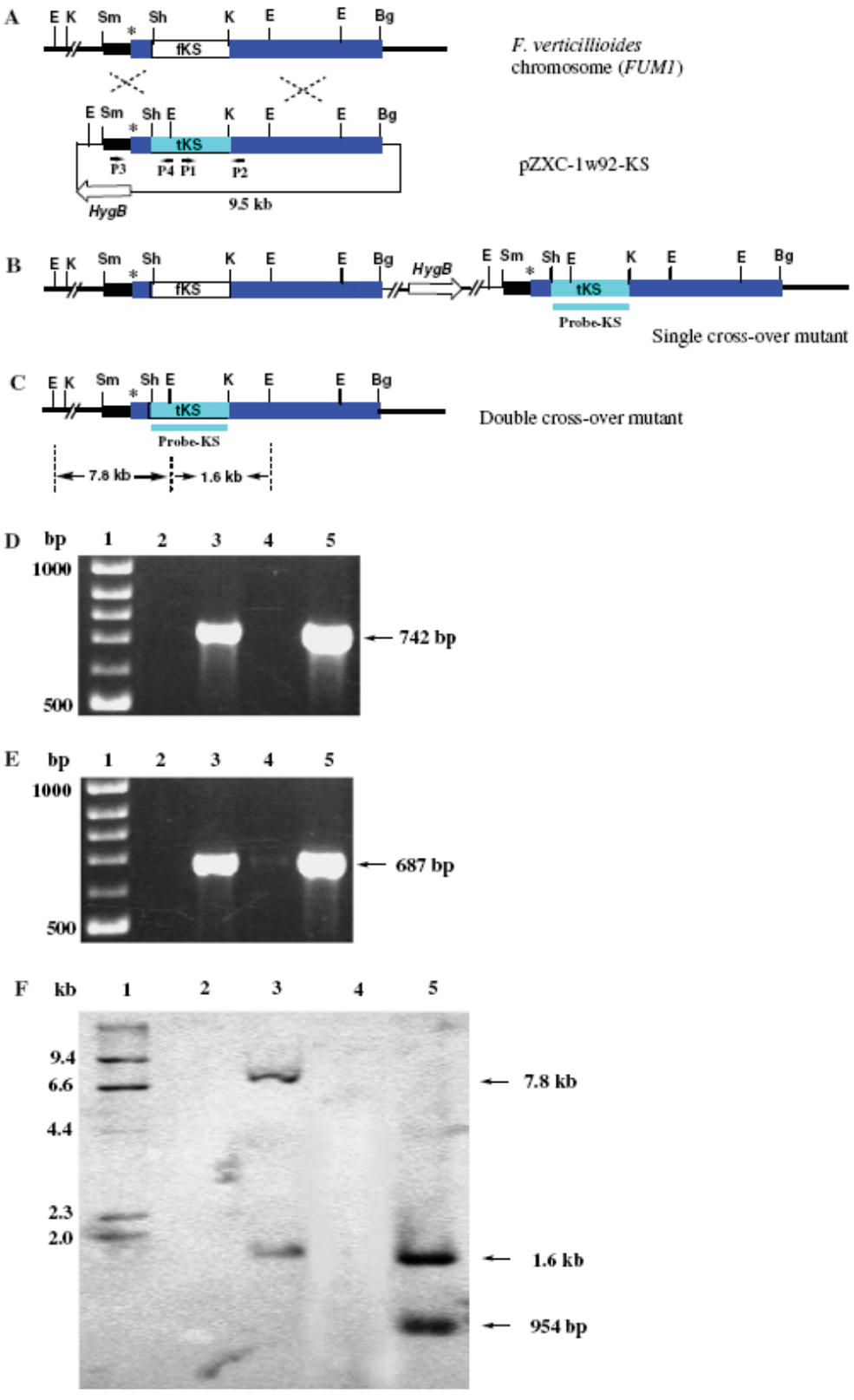

Figure 3 Screening and confirmation of KS domain replaced mutants. a Homologous recombination between *FUM1* on chromosome of *F. verticillioides* and the sequences on plasmid pZXC-1w92-KS. The *asterisk* indicates the position of the start codon of *FUM1*. The positions for PCR primer binding sites in the screening of the mutants are indicated by *small arrows*. Abbreviations: Bg: *Bgl*II; E: *Eco*RI; K: *Kpn*I; Sm: *Sma*I; Sh: *Sph*I; *HygB*, hygromycin B resistant gene. **b** Gene disruption mutants resulted from a single crossover at the downstream homologous region. The position of the tKS probe used for Southern hybridization is indicated by a *bar*. **c** KS domain replaced mutant resulted from a second crossover at the homologous regions. **d** and **e** Confirmation of the mutants by PCR using primers P1/P2 (**d**) and P3/P4 (**e**), respectively. Lane-1, size markers; lane-2, wild-type; lane-3, K2; lane-4, K4; lane-5, plasmid. **f** Confirmation of mutants by Southern hybridization. The DNA was digested with *Eco*RI and probed with a DIG-labeled KS domain of *PKS1* as shown in **c**. The sizes of hybridizing signals are indicated with *arrows*. Lane-1, size markers; lane-2, wild-type; lane-3, K2; lane-4, K4; lane-5, plasmid

ern hybridization and PCR results, which showed that K4 is a revertant. The KS-replaced mutant K2 produced a pattern of fumonisins similar to the wild-type (Fig. 4b). The peaks at 15.1, 15.7, and 16.2 min gave a $[M + H]^+$ of 722.9, 706.4, and 706.3 m/z, respectively (Fig. 4c, d). The results show that tKS domain is functional in the chimeric *FUM1* and that the fumonisin pathway could tolerate an individual domain swapping in the PKS.

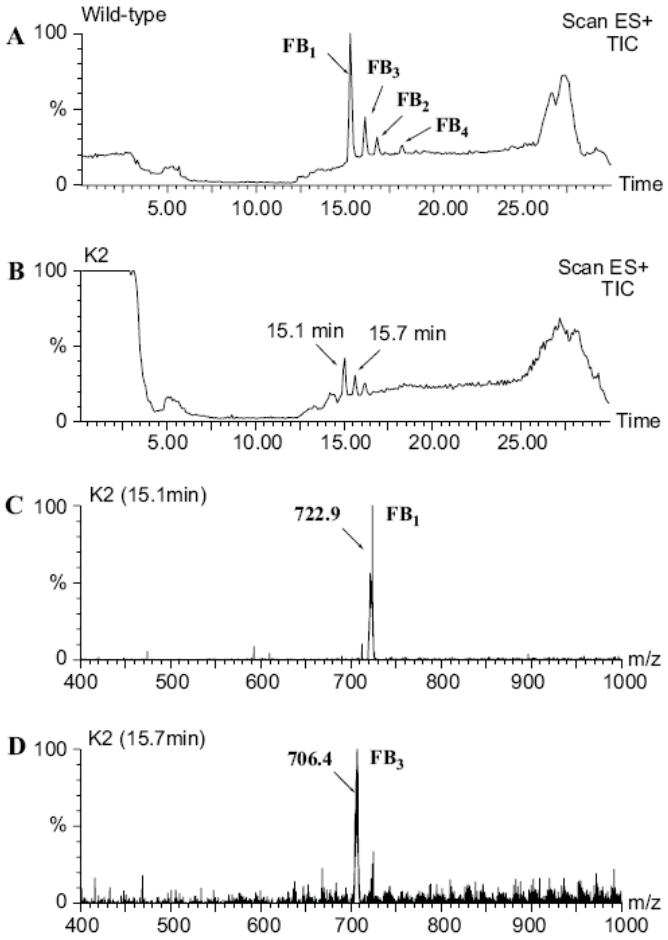

Figure 4 LC-ESMS analysis of metabolites isolated from the wild-type strain and the KS-replaced mutant. **a** LC of the extracts from the wild-type strain, with the peaks of fumonisins indicated. **b** LC of the extracts from K2 mutant, with the retention times of the major peaks indicated. **c** and **d** are the MS of the peak at 15.1 and 15.7 min, respectively, on the LC of K2 in **b**

Generation of the intact *FUM1*-replaced mutants and analysis of metabolites

To further test if the entire fumonisin PKS could be functionally replaced by a PKS that has similar domain architecture, we replace the intact fumonisin *FUM1* in *F. verticillioides* with T-toxin *PKS1* from *C. heterostrophus* [*32*]. A plasmid, pUCH-LFUM1-PKS1-RFUM1, was constructed to contain the intact *PKS1* gene flanked by the upstream (1,096 bp) and downstream (1,345 bp) non-coding regions of *FUM1* (Fig. 5a). Using the two-stage screening strategy, we first obtained 58 hygromycin-resistant colonies. PCR analyses of these putative transformants indicated that five resulted from homologous recombination, with four from the upstream homologous region and one at the downstream (Fig. 5b, c). The single cross-over mutants were then used to select for double cross-over mutants. We obtained 43 colonies lost the hygromycin resistance. PCR analyses of these putative double crossover mutants indicated that two contained an intact *PKS1* at the place of *FUM1* in *F. verticillioides* (Fig. 5d) and the rest were revertants. The identity of the mutants was confirmed by Southern hybridization (Fig. 5e, f). Four probes were used in the Southern analysis. When the mixed t1 and t2 probes were used, mutant D4-1 gave two signals of 5.8 and 7.5 kb (Fig. 5e). The result confirms that D4-1 contains *PKS1* in the place of *FUM1* in *F. verticillioides*. As controls, the wild-type or a revertant (D4-5) did not give any signal, whereas the plasmid pUCH-LFUM1-PKS1-RFUM1 produced the expected 2.9 and 9.0 kb signals (Fig. 5e). When the mixed f3 and f1 probes were used, mutant D4-1 gave no signal (Fig. 5f). The wild-type or a revertant (D4-5) gave the expected 2.2 and 4.8 kb signals, whereas the plasmid pUCH-LFUM1-PKS1-RFUM1 gave no signal (Fig. 5f). Together, the results show that D4-1 is a *F. verticillioides* mutant in which entire *FUM1* gene has been replaced by *PKS1* gene.

The metabolites produced in the *FUM1*-replaced mutant as well as in the revertants of *F. verticillioides* were analyzed by HPLC-ELSD and LC-MS. The revertants produced a level and pattern of fumonisins similar to that of the wild-type (data not shown). However, the *FUM1*-replaced mutant did not produce any fumonisin or new analogs under the experimental conditions (data not shown). Extensive LC-MS search of the extracts from this mutant did not reveal any fumonisin-like or T-toxin-like metabolite.

Discussion

The genetic manipulation of PKS domains has proven to be a powerful way to elucidating the biosynthetic mechanism of bacterial polyketides [*12*, *25*]. However, this strategy is not directly applicable to fungal modular PKSs, because only a single set of domains is present in the fungal enzymes. In this study, we exploited this strategy by using two fungal PKSs that share similar domain architecture but synthesize different carbon chains. This approach has been used to study the biosynthesis of fungal aromatic polyketides in the recent years. For example, Watanabe and Ebizuka [*30*] constructed a chimeric PKS composed of domains from wA gene in *A. nidulans* [*18*] and pks1 gene in *Colletotrichum langenarium* [*26*]. These two genes code for PKSs synthesizing a heptaketide and a pentaketide, respectively. However, the chimeric PKS synthesized a novel hexaketide, showing the catalytic flexibility of the fungal PKS domains [*30*]. The genetic manipulation of domains, however, has not been conducted in the studies of fungal reduced polyketides due to the difficulties in the analysis of the acyclic, non-aromatic metabolites, as well as the challenge in genetically manipulating the filamentous fungi. We recently developed a genetic system for *F. verticillioides* that enables a specific manipulation of the domains of *FUM1* [*34*]. In this study, we tested if a heterologous PKS and its KS domain could function in a chimeric *FUM1* in this fungus.

FUM1 is the only PKS gene found in the 15-gene cluster (*FUM*) responsible for the biosynthesis of fumonisins in *F. verticillioides* [*20*]. The deduced protein (Fum1p) was pre-

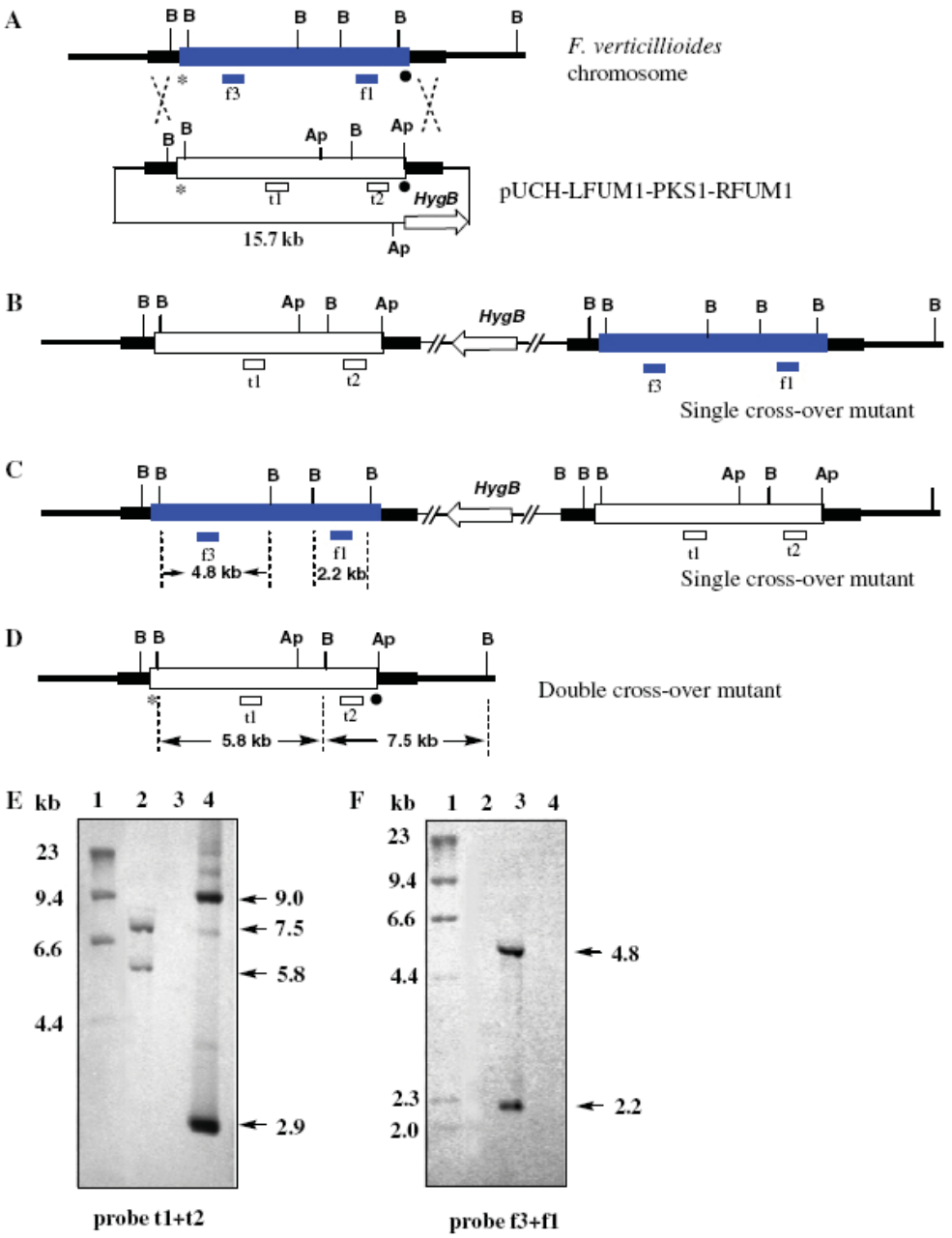

Figure 5 Screening and confirmation of the whole PKS replaced mutants. a Homologous recombination between *FUM1* on chromosome of *F. verticillioides* and the sequences on plasmid pUCH-LFUM1-PKS1-RFUM1. The *asterisk* and *filled circle* indicate the position of the start and stop codon of the PKS genes, respectively. The positions of probes for Southern hybridizations are indicated by *small bars*. Abbreviations: Ap: *Apa*I; B: *Bam*HI; *HygB*, hygromycin B resistant gene. **b** and **c** Gene disruption mutants resulted from a single crossover at the upstream homologous region (**b**) or at the downstream homologous region (**c**). **d** The whole PKS replaced mutant resulted from a second crossover at the homologous regions. The expected sizes of hybridizing signals are indicated with *arrows*. **e** and **f** Confirmation of mutants by Southern hybridization using probes t1 + t2 (**e**) or probes f3 + f1 (**f**). The DNA was digested with *Bam*HI and probed with DIG-labeled probes. Lane-1, size markers; lane-2, D4-1 (double crossover mutant); lane-3, D4-5 (revertant); and lane-4, plasmid (digested with *Apa*I)

dicted to contain a single module, KS-AT-DH-MT-ER-KR-ACP [*21*]. Fum1p shares sequence similarity to many modular PKSs, with PKS1 for T-toxins showing the highest similarity (44.1% similarity and 34.2% identity for the whole amino acid sequence). *PKS1* is a gene involved in the biosynthesis of T-toxins in *C. heterostrophus* [*32*]. T-toxins are a family of mycotoxins with carbon chain lengths varying from C_{35} to C_{45}, most of which have not been well characterized structurally, except the most abundant (50%) C_{41} analog (Fig. 1) [*16*]. When the KS domain of *FUM1* was replaced with the KS domain of *PKS1*, the mutant strain produced fumonisins. The result has two interesting implications. First, the result shows that the KS domain of PKS1 can function in the context of Fum1p. Although many domains of bacterial PKSs have been successfully replaced [*12*, *25*], this is the first example of functional domain replacement in a PKS synthesizing a fungal reduced polyketide. The result suggests that, like the non-iterative bacterial PKSs, the domains of iterative fungal PKSs may be functionally manipulated, although these domains need to iteratively function in the biosynthesis of fungal polyketides. The level of products in the mutant was approximately 4% of that from the wild-type, suggesting that the hybrid enzyme may not be as active as the native form, probably due to sub-optimal interactions between the heterologous KS domain and the other domains in the chimeric enzyme or between the chimeric enzyme and the other biosynthetic enzymes in the fumonisin pathway. Second, the KS domain alone may not be sufficient to control the chain length of polyketide products. Among the domains of

PKS, KS is the domain to catalyze the formation of new carbon–carbon bonds during the polyketide chain elongation. In bacterial type II PKS, the heterodimeric complex of ketosynthase and chain-length factor controls the chain length of aromatic polyketides [*14*, *28*]. Here, our data show that a switch of the KS domain did not lead to a new polyketide product. This suggests that the single module PKS for fungal reduced polyketides may have some yet-to-be-characterized factors/features to control the structure of products. Under the experimental conditions used, no other metabolite, except fumonisins, was detected in the KS-replaced mutant. However, this does not exclude the possibility that the chimeric PKS may be producing other polyketides of different chain length, but these linear, fatty acid-like products may be metabolized by other pathways and, hence, not detected. This phenomenon has been observed in a previous study of other *FUM* mutants [*5*].

Since the fungal PKS appears flexible in the single domain replacement, we further tested if a replacement of the entire PKS could be tolerated. A mutant strain of *F. verticillioides* was generated, in which the entire *FUM1* was replaced by *PKS1*. In order to keep a proper transcription and translation of the heterologous PKS gene in the new host, we only replaced the coding region of *FUM1* and did not make any changes to the upstream and downstream non-coding regions of *FUM1*. Indeed, RT-PCR results showed that the gene was expressed in the host (F. Yu and L. Du, unpublished data). Extensive HPLC-ELSD and LC-MS analyses showed that the mutant did not produce any fumonisin-like or T-toxin-like metabolite. Fungal PKSs for reduced polyketides do not contain a thioesterase/cyclase domain [*11*] and the release of the covalently attached polyketide intermediate from the PKS must rely on a mechanism different from the thioesterase/cyclase-dependent hydrolysis. In the biosynthesis of fumonisins, Fum8p, an α-oxoamine synthase, has been proposed to carry out the polyketide chain release by a nucleophilic attack of the α carbon of alanine on the carbonyl carbon of polyketide acyl-*S*-PKS [*5*, *20*]. This step not only releases the polyketide chain but also adds the two terminal carbons and the amino group of fumonisins [*5*, *29*]. Thus, the substrate specificity of Fum8p could be important for the production of a distinct product. We are currently undertaking both biochemical and genetic approaches to test this possibility.

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