Introduction of the AAL-Toxin Polyketide Synthase Gene *ALT1* into *FUM1*-disrupted *Fusarium verticillioides* Produces Metabolites with the Fumonisin Methylation Pattern

Yaoyao Li,[†,‡] Yuemao Shen,[‡] Xiangcheng Zhu,[†,§] and Liangcheng Du*[,†]

Department of Chemistry, University of Nebraska−Lincoln, Lincoln, Nebraska 68588, and School of Life Science, Xiamen University, Xiamen 361005, People's Republic of China

Received March 27, 2009

Fumonisins and AAL toxins are polyketide-derived mycotoxins produced by several important fungal pathogens of crop plants. The toxins contain a linear, dimethylated polyketide chain, but differ in chain length and methylation positions. Here, we have isolated the major metabolites from a fumonisin null mutant, *Fusarium verticillioides* strain 5777, that has been transformed with the AAL-toxin polyketide synthase gene (*ALT1*). The results showed that the metabolites maintained the chain length and methylation pattern of fumonisins. This suggests that the timing and regioselectivity of the methyltransferase of the *ALT1* PKS could be reprogrammed in this *Fusarium* transformant.

Fumonisins (**1**) are produced by the fungus *Fusarium verticillioides* (synonym *F. moniliforme*, teleomorph *Gibberella moniliformis*, synonym *G. fujikuroi* mating population A), which is a widespread pathogen of corn and contaminates maize-based food and feed worldwide.[1,2] Fumonisins, together with AAL toxins (**2**) that are produced by the tomato pathogen *Alternaria alternata* f. sp. *Lycopersici*,[3−5] are called sphinganine-analogue mycotoxins (SAMT) due to their structural similarity to sphinganine, which is the backbone precursor of sphingolipids.[6] SAMT are competitive inhibitors of sphinganine *N*-acetyltransferase (ceramide synthase).[1,7] The inhibition of this enzyme leads to various diseases in animals and humans, as ceramides and sphingolipids are ubiquitous constituents of eukaryotic cells and involved in crucial signal transduction of numerous cellular processes.[1,8]

All SAMT contain a dimethylated linear carbon chain that is derived from a polyketide precursor.[9] The positions of the two methyl groups, which are derived from methionine,[10] relative to the methyl terminus of fumonisins are different from that of AAL toxins (Figure 1). The backbone of fumonisins is derived from a nonaketide precursor (C-3 to -20), whereas the backbone of AAL toxins is derived from an octaketide precursor (C-2 to -17). In addition, the two terminal carbons, C-1 and C-2, as well as the C-2 amino group, of fumonisins are derived from alanine.[11,12] In AAL toxins, the terminal C-1 and amino group are derived from glycine.[13] Although the carbon chain of SAMT contains several hydroxy groups (C-5, C-10, C-14, and C-15 hydroxy of FB_1), they are introduced after the polyketide chain is assembled and released from the polyketide synthase (PKS).[13,14] Therefore, the initially synthesized carbon chain is a highly reduced polyketide. The biosynthetic mechanism for these linear, highly reduced fungal polyketides is the least understood among all polyketide natural products.[15]

The PKS gene for fumonisins (*FUM1*) and the PKS gene for AAL toxins (*ALT1*) share an identical domain organization, consisting of β-ketoacyl synthase (KS), acyltransferase (AT), dehydratase (DH), methyltransferase (MT), β-ketoacyl reductase (KR), enoylreductase (ER), and acyl carrier protein (ACP).[16] However, each PKS synthesizes a polyketide chain with a different length and a different methylation pattern (Figure 1). Although the MT domain is always present, the PKSs selectively transfer the methyl group, only at the second and fourth cycle of chain elongation for fumonisins and at the first and the third cycle of chain elongation for AAL toxins. In an attempt to understand the biosynthetic mechanism for these fungal polyketides, we had previously used *ALT1* to functionally complement fumonisin biosynthesis in a *F. verticillioides* mutant (strain 5777) with a disrupted *FUM1*.[17] The transformant produced fumonisin-like metabolites that contain an 18-carbon polyketide chain. The results show that the C16-synthesizing Alt1p is able to synthesize a C18 chain required for fumonisin biosynthesis in *F. verticillioides*. This is the first evidence that the same fungal PKS has the ability to make polyketide products with different lengths. The results also suggest that the PKS alone may not be sufficient to control the size of the polyketide chain. It is the chain-releasing enzyme that is more critical for producing a distinct product.[18] However, it remains unclear whether the fumonisin-like metabolites produced by this transformant have a "fumonisin-type" (**3**) or an "AAL-type" (**4**) methylation pattern, or both. As illustrated in Figure 1, the two methyl groups could be on C-12 and C-16 ("fumonisin-type") or on C-14 and C-18 ("AAL-type"), even though the total length of the polyketide chain is the same. In this study, we have obtained spectroscopic evidence to show that the major metabolites produced in this transformant retained the "fumonisin-type" methylation pattern. The data provide new insight into the biosynthetic mechanism for this economically important group of fungal polyketides.

High-performance liquid chromatography (HPLC) coupled to an evaporative light-scattering detector (ELSD2000) was used to screen the metabolites produced by the wild-type, *FUM1*-disrupted strain 5777 and transformant 5777-*ALT1*-4 of *F. verticillioides* that were grown on cracked maize kernels (CMK) medium.[17] The wild-type produced the typical B series fumonisins (FB_1, FB_2, FB_3, and FB_4), whereas strain 5777 did not produce any fumonisins. Transformant 5777-*ALT1*-4 produced a group of compounds that had similar retention times to the standard fumonisins on HPLC. ESIMS analysis confirmed that these compounds had $[M + H]^+$ signals identical to fumonisins.

To characterize these compounds, we made a large-scale (125 g of CMK) culture of transformant 5777-*ALT1*-4. The culture was extracted successively with CH_3CN/H_2O (1:1, v/v) and then subsequently solvent partitioned with EtOAc followed by CH_3OH. The CH_3OH extract was purified by repeated column chromatography (RP-18, Sephadex LH-20, and silica gel) to afford compounds **5** and **6** (see Supporting Information). Their structures were elucidated on the basis of spectroscopic data, including ^1H, ^{13}C,

* To whom correspondence should be addressed. E-mail: ldu@unlserve.unl.edu. Tel: 402-472-2998. Fax: 402-472-9402.
† University of Nebraska−Lincoln.
‡ Xiamen University.
§ Current address: Department of Chemical Engineering and Bioengineering, Zhejiang University, Hangzhou, 310027, China.

10.1021/np900193j CCC: $40.75
Published on Web 05/18/2009

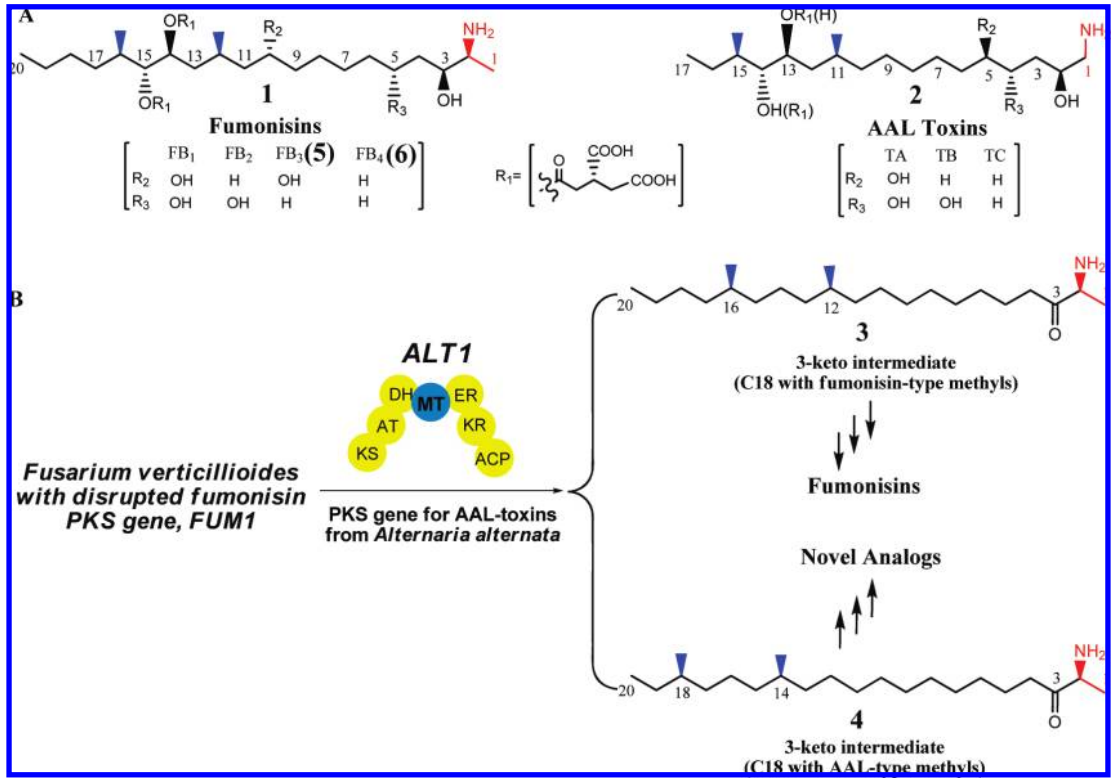

Figure 1. (A) Chemical structures of fumonisins (**1**) and AAL toxins (**2**). (B) Two possibilities of methylation in the metabolites produced by the *FUM1*-disrupted *F. verticillioides* strain 5777 that is transformed with the AAL-toxin PKS gene, *ALT1*. The upper structure shows the 3-keto intermediate with the "fumonisin-type" methylation (**3**) that leads to fumonisins, whereas the lower structure shows the 3-keto intermediate with the "AAL-type" methylation (**4**) that would lead to new analogues of fumonisins.

multiplicity-edited HSQC, HMBC, and $^1H-^1H$ COSY (Supporting Information).

Compound **5** was isolated as a colorless oil, with a mass of 706.5 $[M + H]^+$ as determined by ESIMS. The ^1H NMR spectrum showed four methyl signals, appearing as three doublets and one triplet (δ 0.82) (SI-Table 1). The ^{13}C NMR spectrum showed a nitrogen-connected methine at δ 53.6 (CH-2) and four oxygen-connected methines at δ 71.7 (CH-3), 70.1 (CH-10), 73.0 (CH-14), and 78.9 (CH-15). Both HMBC and $^1H,^1H-$COSY correlations as well as comparison to the literature NMR data for FB_3 readily established the structure of **5** as FB_3 (SI-Figure 2).[11,19] Compound **6** was obtained as a colorless oil, with a mass of 690.7 $[M + H]^+$. The NMR data verified **6** as FB_4.

These results demonstrated that the major metabolites produced by transformant 5777-*ALT1*-4 have a "fumonisin-type" (**3**) methylation pattern. It should be pointed out that the results do not exclude the existence of "AAL-type" methylation in the minor compounds. However, the fact that the AAL-toxin PKS is able to synthesize a polyketide with an altered methylation pattern suggests that the timing and regioselectivity of the methyltransferase domain of the PKS could be reprogrammed. In "fumonisin-type" (**3**), the methyltransferase activity is selectively activated during the second and fourth cycle of polyketide chain elongation. In "AAL-type" (**4**), the methyltransferase activity selectively functions during the first and third cycle of polyketide chain elongation. Our recent data have shown that the fumonisin polyketide chain-releasing enzyme Fum8p is critical to produce a distinct group of products.[9,18] In vitro characterization of the heterologously expressed Fum8p showed that this enzyme prefers an acyl chain with an 18-carbon length. This is consistent with the results obtained from the metabolites produced by transformant 5777-*ALT1*-4. All the metabolites are derived from a C_{18} precursor. The data also suggest that Fum8p preferably offloads a C_{18} chain with the "fumonisin-type" methylation. It is plausible that the interaction between chain-releasing enzyme and the chain-synthesizing enzyme may have led to the synthesis of a precursor with a proper methylation pattern that is releasable by Fum8p, although Alt1p in *A. alternata* synthesizes a polyketide with the "AAL-type" methylation. The results provide new insight into the biosynthetic mechanism for SAMT, in which the selectivity of the chain-releasing enzyme is probably the determining factor for the final product structure, and the PKS activity could be reprogrammed according to this selectivity.

Experimental Section

General Experimental Procedures. ESI mass spectra were recorded on a Finnigan mat LCQ at the Nebraska Center for Mass Spectrometry. NMR spectra were measured on a Bruker DRX Avance 300 and 500 spectrometers. Column chromatography: silica gel 60 (Geduran, 40−60 μm, EMD), Sephadex LH-20 (Amersham Biosciences), Sep-Pak Vac C-18 cartridge (Waters, Milford, MA). The metabolites were analyzed by a high-performance liquid chromatography (HPLC) system (Prostar-210, Varian, Walnut Creek, CA) coupled to an evaporative light-scattering detector (ELSD2000, Alltech, Deerfield, IL). The column was Alltima C18LL (5 μm, 250 × 4.6 mm i.d., Alltech, Deerfield, IL). Thin layer-chromatography (TLC) was performed on silica gel IB-F (Baker-flex, J. T. Baker).

Fungal Strains and Fermentation. *Fusarium verticillioides* A0149 (FGSC number 7600) was the wild-type strain. The *FUM1* mutant strain 5777 was obtained from Dr. Robert Proctor (U.S. Department of Agriculture, Peoria, IL). Transformants 5777-*ALT1*-4 and 5777-*ALT1*-5 were generated in our lab.[17] All strains were cultivated on cracked maize kernels (CMK) medium as described previously.[17]

Extraction and Isolation. The CMK culture of transformants 5777-*ALT1*-4 was extracted four times with 250 mL of CH_3CN/H_2O (1:1, v/v), and the crude extract was decanted and concentrated under reduced pressure and sequentially solvent partitioned into EtOAc-, CH_3OH-, and H_2O-soluble extracts. The CH_3OH extract (4.8 g) was loaded onto a 10 g C-18 cartridge (Sep-Pak Vac 35 cm^3; H_2O, 30, 50, 70% CH_3CN/H_2O, containing 0.3% formic acid). The 50% CH_3CN fraction was subjected to gel permeation chromatography (Sephadex LH-20; CH_3OH) followed by 10 g C-18 cartridge (30, 40, 50% CH_3CN/H_2O

containing 0.3% formic acid) to afford two fractions: Fr. 1 and Fr. 2. Fr. 1 (110 mg) was separated by silica gel column chromatography using 3:1 $CHCl_3/CH_3OH$ and further purified by repeated 1 g C-18 cartridge (30% CH_3CN/H_2O containing 0.3% formic acid) to yield **5** (11 mg). Fr. 2 (30 mg) was subjected to a 1 g C-18 cartridge (35% CH_3CN/H_2O containing 0.3% formic acid) followed by silica gel column chromatography using 6:1 $CHCl_3/CH_3OH$ to obtain **6** (4 mg).

Acknowledgment. This work was supported by NSF (MCB-0614916). We thank S. Basiaga, J. Dumais, R. Cerny, and K. Wulser for technical support. The *F. verticillioides* strain 5777 was obtained from R. Proctor. The research was carried out in facilities renovated with support from NIH (RR015468-01).

Supporting Information Available: Details of isolation of the two major metabolites from the transformant 5777-*ALT1*-4 and 1-D and 2-D NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP900193J