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Deletion Analysis of *FUM* Genes Involved in Tricarballic Ester Formation during Fumonisin Biosynthesis

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Fumonisin is a carcinogenic mycotoxin produced by the maize ear rot pathogen *Gibberella moniliformis* (anamorph *Fusarium verticillioides*). These toxins consist of a linear polyketide-derived backbone substituted at various positions with an amine, one to four hydroxyl, two methyl, and two tricarballic ester functions. In this study, we generated and characterized deletion mutants of *G. moniliformis* for five genes, *FUM7*, *FUM10*, *FUM11*, *FUM14*, and *FUM16* in the fumonisin biosynthetic gene cluster. Functional analysis of mutants in four genes, predicted to encode unrelated proteins, affected formation of the tricarballic esters. *FUM7* deletion mutants produced a previously undescribed homologue of fumonisin B₁ with an alkene function in both tricarballic esters, *FUM10* and *FUM14* deletion mutants produced homologues of fumonisin B₃ and fumonisin B₄ that lack tricarballic ester functions, and *FUM11* deletion mutants produced fumonisins that lack one of the tricarballic ester functions. These phenotypes indicated specific roles for *FUM7*, *FUM10*, *FUM11*, and *FUM14* in fumonisin biosynthesis that are consistent with the predicted proteins encoded by each gene. Deletion of *FUM16* had no apparent effect on fumonisin production. The phenotypes of the deletion mutants provide further insight into the order of steps in fumonisin biosynthesis.

KEYWORDS: Fumonisin; *Gibberella moniliformis*; *Fusarium verticillioides*; *FUM* genes; mycotoxin; tricarballic ester

INTRODUCTION

Fumonisin is a polyketide-derived mycotoxin produced by the maize stalk and ear rot pathogen *Gibberella moniliformis* Wineland (anamorph *Fusarium verticillioides* (Sacc.) Nirenberg) (1). Fumonisin can disrupt sphingolipid biosynthesis by inhibiting the enzyme sphinganine *N*-acyltransferase (2–5). Because of the importance of sphingolipids in multiple cellular processes, the disruption of sphingolipid metabolism may be the mechanism by which fumonisins induce a variety of diseases in animals (4, 5). Pulmonary edema in pigs, leukoencephalomalacia in horses, and cancer and neural tube defects in rodents (2, 4, 6, 7) have been associated with the ingestion of fumonisin-contaminated maize. Fumonisin has also been implicated in human esophageal cancer (for review see ref 7).

Most field isolates of *G. moniliformis* produce predominantly four B-series fumonisins (B₁, B₂, B₃, and B₄, **Figure 1**) (8, 9). These mycotoxins are synthesized, at least in part, through the activity of enzymes encoded by the fumonisin biosynthetic (*FUM*) gene cluster. This cluster consists of 15 coregulated genes designated *FUM1*–*FUM3* (previously *FUM5*, *FUM12*, and *FUM9*, respectively (10–12)), *FUM6*–*FUM8*, *FUM10*, *FUM11*, and *FUM13*–*FUM19* that are located on chromosome I (11). The roles of some of the genes in the cluster have been

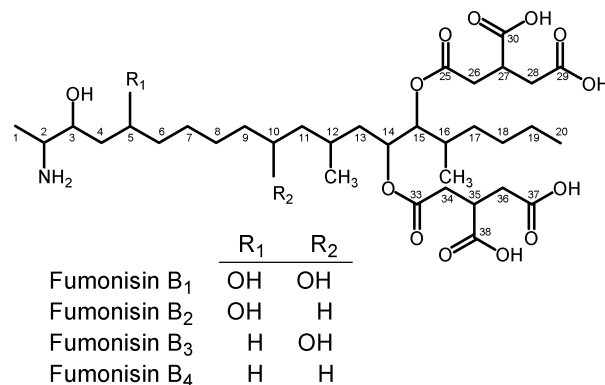


Figure 1. Major fumonisin homologues produced by wild-type strain M-3125.

confirmed by gene deletion analysis (10, 11, 13–15) and heterologous expression (16). Deletion of either *FUM1*, *FUM6*, or *FUM8* blocked accumulation of all fumonisins, indicating that these genes are required for fumonisin production (14, 15). Their exact roles in biosynthesis have been inferred by the similarity of their sequences to genes of known function and through analysis of deletion mutants. For example, analysis of fumonisin production in co-cultures of *FUM6* and *FUM8* deletion mutants provided details of the early steps of fumonisin biosynthesis (17). In contrast, deletion of either *FUM2*, *FUM3*, or *FUM13* led to the accumulation of less oxygenated fumo-

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nisins and provided insight into the exact function of these genes (10, 12, 13). For example, *FUM2* deletion strains produce only fumonisins B₂ and B₄, which lack the C-10 hydroxyl (the hydroxyl group at carbon atom 10) and indicate that the *FUM2*-encoded cytochrome P450 monooxygenase most likely catalyzes fumonisin C-10 hydroxylation (12). *FUM3* deletion mutants produce only fumonisins B₃ and B₄, which lack the C-5 hydroxyl and indicate that the *FUM3*-encoded dioxygenase most likely catalyzes fumonisin C-5 hydroxylation (10). *FUM13* deletion mutants produce 3-keto homologues of fumonisins B₃ and B₄ and indicate that the *FUM13*-encoded ketoreductase most likely catalyzes the reduction of the C-3 carbonyl to a C-3 hydroxyl (13). In contrast to *FUM2*, *FUM3*, and *FUM13*, deletion of *FUM17* and *FUM18* did not affect fumonisin production while deletion of *FUM19* subtly effected the ratio of fumonisin B₁ to B₃ produced (11). Deletion of *FUM15* had no observed effect on fumonisin production (18).

The functions for five other *FUM* genes, *FUM7*, *FUM10*, *FUM11*, *FUM14*, and *FUM16*, have been postulated on the basis of sequence similarity to genes of known function (11). The predicted *FUM10* and *FUM16* proteins have a high degree of similarity to acyl-CoA synthetases and either or both were predicted to catalyze the CoA activation of either the fumonisin polyketide or the tricarballic ester precursor (11). The predicted *FUM11* protein exhibits a high degree of similarity to mitochondrial membrane-bound tricarboxylate transporters and was proposed to be involved in transporting tricarboxylate precursors of the tricarballic esters to make them available for fumonisin production (11). The functions of the predicted proteins encoded by *FUM7* and *FUM14* in fumonisin biosynthesis was not obvious on the basis of their similarity to genes of known function; *FUM7* was predicted to encode a dehydrogenase and *FUM14* was predicted to encode a protein with similarity to the condensation domain of nonribosomal peptide synthetases (11). A detailed analysis of the function of the enzyme encoded by *FUM14* has been reported (19). The objective of this study was to determine the functions of *FUM7*, *FUM10*, *FUM11*, *FUM14*, and *FUM16* in fumonisin biosynthesis by analysis of individual gene deletion mutants.

MATERIALS AND METHODS

Strains and Media. *Gibberella moniliformis* strain M-3125 was used throughout this study (20). Other strains used in this study include M-5500 (*FUM1* mutant) (21) and the following deletion mutants: GfA3075 (*FUM6* deletion) (15), GfA3245 (*FUM8* deletion) (15), GMT9-206 (*FUM3* deletion) (10), and GfA2874 (*FUM2* deletion) (12). Strains were cultured on V8 juice agar medium for the production of conidia and in liquid GYEP (5% glucose, 0.1% yeast extract, 0.1% peptone) for production of mycelia for genomic DNA preparation and in cracked corn medium or liquid GYAM (15) for analysis of fumonisin production (15, 21).

***FUM* Gene Deletion Constructs, Transformation, and Southern Analysis.** Deletion constructs for *FUM7*, *FUM10*, *FUM11*, *FUM14*, and *FUM16* were engineered utilizing approximately 1-kb flanking regions both upstream and downstream of the coding region as described previously for deletions of other *FUM* genes (13, 22). Briefly, upstream and downstream regions were amplified by PCR and were subcloned into a single vector. Table 1 lists the primers used to amplify each region of each gene. The hygromycin B resistance gene (*HygB*) (23) was then inserted between the flanking regions. The resulting vectors, pF7KOH, pF10KOH, pF11KOH, pF14KOH, and pF16KOH, were transformed separately into strain M-3125 as previously described (14). Primary transformants were first screened for hygromycin resistance and then by PCR to determine whether double homologous recombination, and therefore gene deletion, had occurred. Briefly, PCR primers were designed to amplify fragments corresponding to wild-type *FUM*

Table 1. Oligonucleotide Primers Used To Characterize *FUM* Gene Deletion Mutants

gene	primer name	sequence
A. Deletion Construction		
<i>FUM7</i>	rp499	GACACGCGTAGGTTTCATCTGGCCCGAATC
	rp500	GACGGCGCGCCCAACCTCAATTCCCTTCCC
	rp501	GACGGCGCGCCATGAGAACCATTGTTGCC
	rp502	GACGGCGCGCGGTGGTCTCATGGGACCT
<i>FUM10</i>	rp511	GACACGCGTCAAGGAAATGGCGCATAG
	rp512	GACGGCGCGCCCGTGTGATTGGGACGATTG
	rp513	GACGGCGCGCCTGAAGAAGCATATGCGCCATC
	rp514	GACCTCGAGTCGAGGACCGCGATTAGAGA
<i>FUM11</i>	rp515	AGCAGAATTCTGCAACCCATTTCGG
	rp516	GACGGCGCGCCAAGCAACGGAACCTAGCCGC
	rp517	GACGGCGCGCCTCGGCATCGAGAGATACAG
	rp518	GACAAGCTTTACAACTTGACCGCTCGAA
<i>FUM14</i>	rp532	GGACTCGAGGTAGCGGTAACTGACTGCATG
	rp533	CATGGCGCGCCAACATGGTCTTGGGAACTCG
	rp534	CATGGCGCGCCGATCCAGTCGTATCTCAGTCA
	rp535	GTAGCATGCGGACAAAGACTTTGATCTGTACA
<i>FUM16</i>	rp519	GACGGATCCTGGCTTCCATTACGACGAAAC
	rp520	GACGGCGCGCCACAGCGCTATACAACGGCT
	rp521	GACGGCGCGCCAGCTATCGGTTATCGGACCTG
	rp522	GACGGCGCGCGATATGCCAATGTGCGTGAA
B. Deletion Detection		
<i>FUM7</i>	rp603	GTTGCTCTGATCGAAGCACT
	rp304	GAGTTCGCGGAGTTTGCTTGG
	rp604	CCTGTCCAAAACGATATCCTG
	rp307	GAGGATGTCTGCACACAAG
<i>FUM10</i>	rp547	TGAATGGATGAGCCTCCT
	rp506	GACCTCGAGGGCAACAACTCCCTG
	rp548	TTGCTGATGACGATGGGA
	rp515	AGCAGAATTCTGCAACCCATTTCGG
<i>FUM11</i>	rp431	CATGGCGCGCCAAGAAGGGAGGACTCGAGTCT
	rp430	CGTGGATCCTGCCAGAGAATGCCGAACCT
	rp605	CATTACCTGGTATCACGCCG
	rp514	GACCTCGAGTCGAGGACCGCAGATTAGAGA
<i>FUM14</i>	rp529	CAAGTCGACTGGCCTATTGGAC
	rp609	GGAACAATCTCAACCATCTC
	rp606	GTATGATTGGTACCATACTG
	rp510	GACTCTAGAGGATGAACAACCTTCCCCG
<i>FUM16</i>	rp553	CACCAAGCGTATCGTATG
	rp554	TGCCGTGAGGTATAGTCTAC
	rp555	GTTCTTGGTCGCTAACGA
	rp556	CGGTGGCTAAGGTAATTAAG
<i>HygB</i>	rp250	CTGCTGCATTCCATTCCCATCGT
<i>HygB</i>	1098	ACCAAGCCTATGCCTACAGCATCC

genes (wild-type fragments) or fragments expected to result from replacement of the target *FUM* coding region with *HygB* (deletion fragments). Genomic DNA from transformants of each *FUM* gene was isolated and analyzed both for the presence of the deletion fragments and for the absence of the wild-type fragments. For each *FUM* gene analyzed in this study, two independently isolated deletion mutants, as determined by PCR, were selected for Southern blot analysis to confirm deletion of the targeted *FUM* gene and for LC-MS analysis to determine the effect of the *FUM* gene deletion on fumonisin production. Figure 2 shows an example of a Southern blot for *FUM11* deletion mutants. Genomic DNA was isolated, digested with *BspHI*, electrophoresed, and blotted to nylon membrane. A portion of the *FUM11* coding region that was deleted was labeled with ³²P using the RediprimeII kit (Amersham Pharmacia Biotech) and was used to hybridize the Southern blot.

Fumonisin Analysis. To assess fumonisin production in deletion mutants, strains were cultured on cracked corn medium for 3 weeks and were extracted with acetonitrile:water (1:1) as previously described (14). The fumonisin content of culture extracts was determined by liquid chromatography–mass spectroscopy (LC-MS) as previously described (24). Liquid GYAM medium was also used to assay putative intermediates for their incorporation into the fumonisin biosynthetic pathway. Fumonisin and putative fumonisin biosynthetic intermediates produced by gene deletion mutants were dissolved in water and were added to

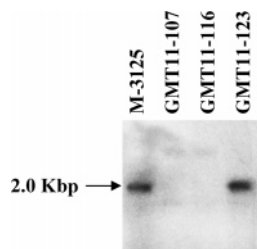


Figure 2. Southern analysis of *G. moniliformis* transformants with a deletion in *FUM11*. Genomic DNA was prepared from three transformants and from wild-type strain M-3125. Using a probe corresponding to the deleted portion of the gene, the absence of the *FUM11* coding region is illustrated in strains GMT11-107 and GMT11-116 compared to the wild-type strain M-3125 and a hygromycin resistant transformant in which the plasmid construct did not recombine at the *FUM11* locus.

20-mL liquid GYAM cultures of 5-day-old wild-type or mutant strains of *G. moniliformis* at a final concentration of 5 ng/ μ L. The cultures were incubated for a further 5 days, after which they were filtered through a 0.2- μ m Nalgene filter and culture filtrate analyzed by LC-MS. Purification of a fumonisin-like metabolite from *FUM7* deletion mutants was done by preparative high-performance liquid chromatography (HPLC) on C18 columns (Ranin Instrument Co, Woburn, MA) for analysis by 13 C NMR and hydrolysis. NMR spectra were obtained with a Bruker (Billerica, MA) Avance 400 spectrometer equipped with a 5-mm inverse broad-band Z-gradient probe (13 C NMR, 100 MHz, 1 H, 400 MHz).

RESULTS AND DISCUSSION

Phenotypes of *FUM* Deletion Mutants. When cultured on cracked corn, the two *FUM7* deletion mutants (GMT7-301 and GMT7-426) accumulate no fumonisin B₁, B₂, B₃, or B₄ but instead accumulate several metabolites with m/z 718 or m/z 702. These metabolites were not observed in culture of the wild-type parent strain M-3125. Fumonisin B₁ (m/z 722) less four hydrogen atoms would have an m/z of 718 while fumonisin B₂ and B₃ (m/z 706) less four hydrogen atoms would have an m/z of 702. One possible explanation for the loss of four hydrogen atoms could be the presence of two carbon–carbon double bonds instead of two carbon–carbon single bonds in each fumonisin-like metabolite. To determine whether the mass loss occurred within the tricarballylic moiety or within the fumonisin backbone, one of the metabolites with an m/z 718 was purified by HPLC and was subjected to potassium hydroxide-mediated hydrolysis, which removes the tricarballylic ester chains from the backbone. LC-MS analysis of the hydrolyzed product indicates that it was hydrolyzed fumonisin B₁, which has hydroxyl functions at C-14 and C-15 instead of tricarballylic esters. The generation of hydrolyzed fumonisin B₁ by hydrolysis suggests that the tricarballylic moiety of the m/z 718 metabolite contains the structural difference that accounts for the mass difference observed between the new metabolite and fumonisin B₁.

Preliminary 13 C nuclear magnetic resonance (NMR) analysis of the purified m/z 718 metabolite provided additional support to this hypothesis, however, the exact nature of the compounds is not clear. Broad shouldered peaks at the frequencies expected for the carbon atoms in the tricarballylic of the molecules indicate a complex mixture of more than one isomer present in the solution. Plattner et al. describe the carbon shift for propane-1,2,3-tricarboxylic acid and indicate that there is a pH-dependent equilibrium between the open-chain structure and a cyclic structure (25). Broad peaks in the NMR spectra indicate that the double bonds can be present between more than one pair of

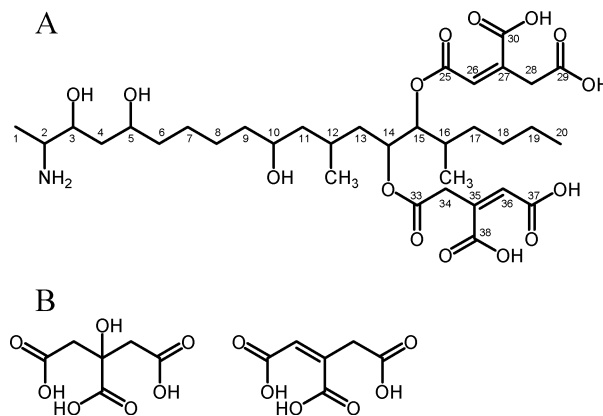


Figure 3. Panel A shows a predicted structure of the fumonisin-like compound, tetrahydro-fumonisin B₁, accumulated by *FUM7* deletion mutants cultured on cracked corn. One possible confirmation of the tricarballylic side chains is shown; however, the positions of each of the putative C–C double bonds have not been determined. If such double bonds are present in these side chains, it is equally likely that the bonds are between carbons 26 and 27 as it is possible they are between carbons 27 and 28 on each tricarballylic molecule, likewise for the double bonds on the tricarballylic ester on carbon 14, i.e., it is equally likely that the bonds are between carbons 35 and 36 as it is possible they are between carbons 34 and 35. Panel B shows the structures of two putative precursors of the tricarballylic esters of fumonisins, citrate on the right and *cis*-aconitate on the left.

adjacent carbon atoms as well as the result of cyclization of the molecule (25). A more detailed NMR analysis of this fraction is required to fully characterize this compound, and experiments are underway. The chemical shifts for all other carbons in the NMR spectra were identical to fumonisin B₁. Taken together, these results support the structure in **Figure 3**, referred to as tetrahydro-fumonisin B₁, which includes a carbon–carbon double bond in the esters attached to C-14 and C-15.

The *FUM10* (GMT10-206 and GMT10-213) and *FUM14* (GMT14-106 and GMT14-109) deletion mutants did not accumulate the wild-type complement of fumonisins B₁, B₂, B₃, and B₄ but instead accumulated two metabolites with retention times, masses, and mass spectra identical to hydrolyzed fumonisin B₃ and hydrolyzed fumonisin B₄ when cultured on cracked corn. Analysis of similar *FUM14* deletion mutants has been reported previously (19). Hydrolyzed fumonisins B₃ and B₄ are identical in structure to fumonisins B₃ and B₄ except that they have hydroxyl functions at C-14 and C-15 instead of tricarballylic ester functions.

The *FUM11* deletion mutants (GMT11-107 and GMT11-116) synthesized the wild-type complement of fumonisins, as well as a significant quantity of metabolites with retention times, masses, and mass spectra consistent with half-hydrolyzed and keto half-hydrolyzed homologues of fumonisins B₁, B₂, B₃, and B₄ when cultured on cracked corn. The half-hydrolyzed fumonisin homologues are identical to B-series fumonisins except that they lack one of the tricarballylic side chains and are detectable only at very low levels in culture filtrates of the wild-type strain. In half-hydrolyzed homologues the hydroxyl function is at either C-14 or C-15 instead of a tricarballylic ester, while keto half-hydrolyzed homologues have a carbonyl function at either C-14 or C-15 instead of a tricarballylic ester. We measured the amounts of fumonisin B₁, half-hydrolyzed fumonisin B₁, and keto half-hydrolyzed fumonisin B₁ in the wild-type strain and the two *FUM11* deletion mutants. In the wild-

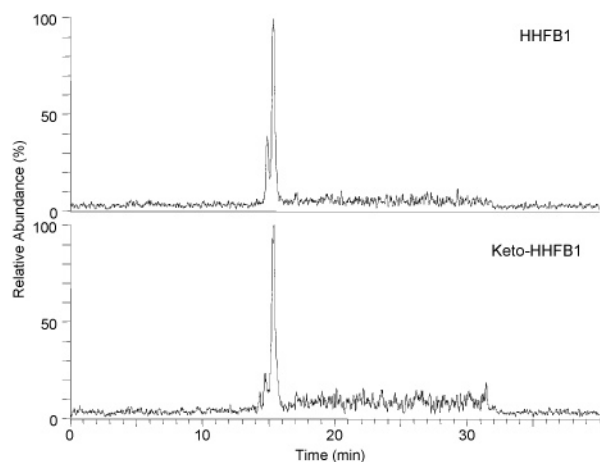


Figure 4. LC-MS analysis of extracts from *FUM11* deletion mutant GMT11-116. Top panel shows the peak at 15.35 min with an m/z of 564 corresponding to half-hydrolyzed fumonisin B₁. The bottom panel shows the peak at 15.41 min with an m/z of 562 corresponding to keto half-hydrolyzed fumonisin B₁.

type strain, half-hydrolyzed fumonisin B₁ and keto half-hydrolyzed fumonisin B₁ constitute only 1.2% and 2.1%, respectively, of fumonisin B₁ produced. However, in the *FUM11* deletion mutant GMT11-107, half-hydrolyzed fumonisin B₁ and keto half-hydrolyzed fumonisin B₁ constitute 15% and 6% of fumonisin B₁, respectively, while strain GMT11-116 produced 26% and 10% of fumonisin B₁, respectively. **Figure 4** shows the LC-MS analysis of extracts from a *FUM11* deletion mutant.

Like *FUM10*, *FUM16* is predicted to encode an acyl-CoA synthetase and therefore could be involved in activation of the tricarboxylic acid molecules with CoA (11). However, in contrast to the *FUM10* deletion mutants as well as the *FUM7*, *FUM11*, and *FUM14* deletion mutants, the *FUM16* (GMT16-215 and GMT16-230) deletion mutants did not exhibit altered fumonisin production relative to the wild-type progenitor strain M-3125 when cultured on cracked corn. While *FUM10* is required for tricarballic ester formation, *FUM16* is not.

Intermediate Study. Metabolites produced by the *FUM* deletion mutants generated in this study may be fumonisin biosynthetic intermediates or end points of the truncated fumonisin biosynthetic pathway. To test the hypothesis that tetrahydro-fumonisin B₁ is an intermediate in the fumonisin biosynthetic pathway, the purified tetrahydro-fumonisin B₁ was added to liquid cultures of *FUM3*, *FUM6*, and *FUM8* deletion mutants and a naturally occurring fumonisin nonproducer with a point mutation in *FUM1* (12, 21). We predicted that if tetrahydro-fumonisin B₁ is a fumonisin biosynthetic intermediate it would be metabolized to fumonisin B₁ or other fumonisins. After 5 days of incubation, LC-MS analysis of culture filtrates indicated that none of the tetrahydro-fumonisin B₁ was converted to fumonisin B₁ (data not shown) and remained in the culture filtrate. In contrast, cultures of the mutants were able to convert fumonisin B₃ to fumonisin B₁ indicating that *FUM3* was still functional in these strains. These results indicate that tetrahydro-fumonisin B₁ is not an intermediate in the pathway but instead is a shunt or end product produced only after the deletion of *FUM7*.

Here, we have described the phenotypes of deletion mutants for five *FUM* genes, *FUM7*, *FUM10*, *FUM11*, *FUM14*, and *FUM16*. Our data indicate that the activities of enzymes encoded by four *FUM* genes are involved in the formation of the tricarballic ester functions at C-14 and C-15 of the fumonisin backbone.

FUM10 and *FUM14* deletion mutants had the same fumonisin production phenotype; they produce hydrolyzed fumonisins B₃ and B₄ that both lack tricarballic ester functions. The predicted functions of the proteins encoded by *FUM10* and *FUM14*, on the basis of their similarity to genes of known function, together with the mutant phenotype suggest roles for both genes in fumonisin biosynthesis. *FUM10* is predicted to encode an acyl-CoA synthetase (11), and we propose that the *FUM10* protein catalyzes the CoA activation of tricarballic acid or a tricarballic acid precursor. The protein encoded by *FUM14* has homology to nonribosomal peptide synthetase condensation domains (11), which catalyze peptide bond formation (19). The function of *FUM14* has been demonstrated by expression and isolation of the *FUM14* protein in *Escherichia coli* and in vitro esterification of hydrolyzed fumonisin B₁ (19). On the basis of these observations, we propose that the protein encoded by *FUM14* catalyzes the esterification of the CoA-activated tricarballic acids to the fumonisin backbone. The accumulation of only hydrolyzed forms of fumonisins lacking the C-5 hydroxyl group (hydrolyzed fumonisins B₃ and B₄) in the *FUM10* and *FUM14* deletion mutants rather than hydrolyzed B₁ suggests that esterification of the tricarballic acid must occur before the hydroxylation of C-5. This is consistent with previous work that indicated that C-5 hydroxylation is the last step in the fumonisin biosynthetic pathway (13, 26).

FUM7 deletion mutants did not produce typical B-series fumonisins but instead produced multiple metabolites that are four mass units less than fumonisin B₁ or B₂/B₃. Analysis of one of the metabolites with m/z 718 indicated that it was identical to fumonisin B₁ except that each tricarballic ester contained an alkene function. On the basis of these results, we propose that they all have an alkene function in both tricarballic esters and refer to them as tetrahydro-fumonisin (Figure 3). These results also suggest that the protein encoded by *FUM7* is involved in reducing the alkene function and, further, that prior to their esterification to the fumonisin backbone the tricarballic acid precursors have an alkene function. On the basis of its predicted sequence, the *FUM7* dehydrogenase belongs to the iron-containing alcohol dehydrogenases protein family. Another member of this class of enzymes is maleylacetate reductase, which catalyzes the reversible reduction of an alkene to an alkane (27). Thus, there is precedence for this class of iron-containing alcohol dehydrogenases catalyzing alkene reductions. Different positions of the alkene function within the tricarballic ester or different isomeric arrangements of the alkene could explain why *FUM7* mutants produce multiple metabolites with m/z 718 or 702 rather than a single homologue for fumonisin B₁, B₂, and B₃. Further analyses of each metabolite are required to determine whether this is the case. Similarly, detailed analysis of the enzyme encoded by *FUM7* will be useful for clarifying the steps in the pathway for which it is responsible.

The production of tetrahydro-fumonisin by *FUM7* mutants raises the question, what is the substrate of the *FUM7* dehydrogenase? One possible substrate is *cis*-aconitate because of the similarity of its structure to the tricarballic ester of the putative alkene-containing tetrahydro-fumonisin (Figure 3). *cis*-Aconitate is an intermediate in the mitochondrial citric acid cycle and thus a primary metabolite that could be utilized in fumonisin biosynthesis. However, it is not clear how *cis*-aconitate could give rise to multiple isomers of tetrahydro-fumonisin. Thus, it is possible that some tricarboxylic acids other than *cis*-aconitate is the tricarballic ester precursor.

FUM11 deletion mutants produced the wild-type complement of fumonisins, however, they also produce elevated levels of

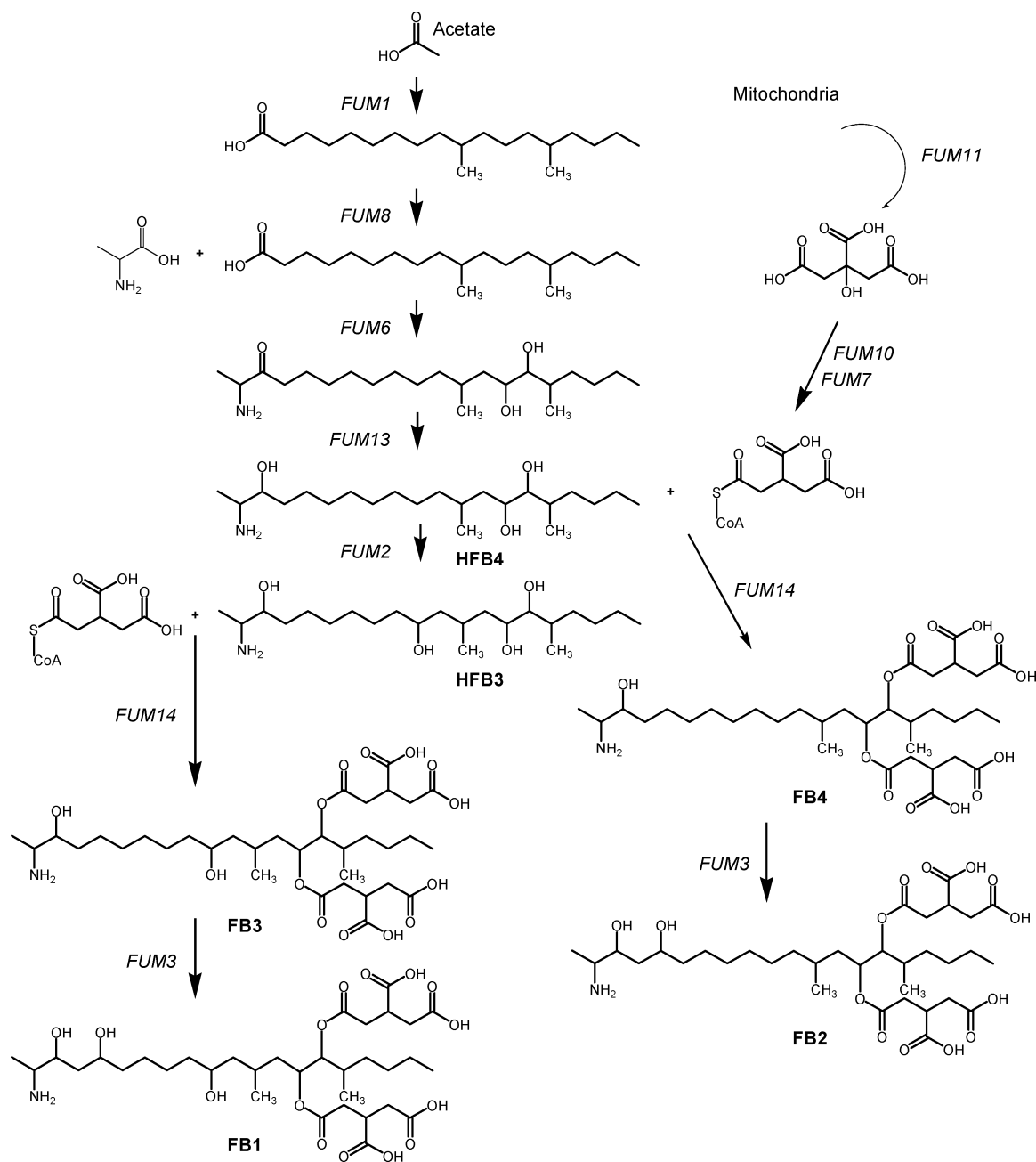


Figure 5. Proposed fumonisin biosynthetic pathway.

half-hydrolyzed and keto half-hydrolyzed fumonisins relative to the wild-type progenitor strain. The predicted *FUM11* protein shares similarity to tricarboxylate transporters suggesting that it might be involved in the transport of tricarboxylic acid precursors of the tricarballic esters from the inner mitochondria lumen, where they would be produced as a result of the citric acid cycle, to the cytoplasm where they would presumably be accessible to the fumonisin biosynthetic machinery (11). The production of the wild-type complement of fumonisins by *FUM11* deletion mutants indicates that *FUM11* is not essential for the procurement of the tricarballic acid precursor molecules. However, elevated production of half-hydrolyzed and keto half-hydrolyzed fumonisins suggests that the availability of tricarballic acid precursors could be limited and therefore that the protein encoded by *FUM11* may aid in making the tricarballic acid precursors available.

The types of fumonisins and fumonisin-like compounds that accumulate in *FUM* gene deletion mutants reported here shed

further light on the order of steps in the biosynthetic pathway. The inability of *F. verticillioides* to metabolize the purified tetrahydro-fumonisin B₁ suggests that this metabolite is not a fumonisin biosynthetic intermediate. This in turn suggests that the *FUM7* protein-catalyzed reduction of the carbon-carbon double bond in the tricarballic acid precursor occurs before esterification to the fumonisin backbone. *FUM7*, however, is not required for esterification to the fumonisin backbone.

Figure 5 shows a proposed fumonisin biosynthetic pathway. Some of the steps in the pathway are based on previously published data (12–17). The results of the current study provide evidence for the following additional steps in the proposed fumonisin biosynthetic pathway. The predicted *FUM11* tricarboxylate transporter makes a tricarboxylic acid precursor available for fumonisin biosynthesis. The exact nature of the precursor is not completely clear from this work, but candidates include citrate or *cis*-aconitate (**Figure 3**) (19). If the precursor is citrate, the *FUM7* dehydrogenase could remove the C-3

hydroxyl of citrate to form tricarballic acid either before or after the CoA activation by the *FUM10* acyl CoA synthetase and *FUM14* catalyzed esterification of CoA-activated tricarballic acid to the C-14 and C-15 hydroxyls of the fumonisin backbone. Without *FUM7*, chemical dehydration of the hydroxyl group of citric acid could yield a mixture of products available for CoA activation and esterification resulting in the complex mixture of esters that are observed in the *FUM7* deletion mutant culture extract. Alternatively, if the precursor is *cis*-aconitate, *FUM7* may function to reduce the double bond. In this alternate proposal, feeding studies with tetrahydro-fumonisin B₁ suggests that *FUM7* cannot function on the tricarballic ester and must therefore act before the *FUM14*-mediated esterification. The fumonisin phenotype of the *FUM7* deletion mutants leaves opportunity for future experiments to describe the fine details of this portion of the fumonisin biosynthetic pathway. Additional studies involving isolation of the gene products and cell-free reactions or producing additional transgenic strains with multiple gene deletions will be needed to determine the exact precursor and order of the steps in the formation of the tricarballic esters.

Mycotoxin contamination of food and feed products remains a problem. Understanding the biochemistry and genetics of secondary metabolism provides an avenue for determining how these toxins are produced and may lead to a better understanding of why they are produced. We have exploited the tools of molecular biology to dissect the fumonisin biosynthetic pathway in *G. moniliformis*. Analysis of *FUM7*, *FUM10*, *FUM11*, *FUM14*, and *FUM16* deletion mutants in this study has led to a better understanding of the biochemical reactions required for formation of fumonisins. Further clarification of the fumonisin biosynthetic pathway requires analysis of the activities of enzymes encoded by *FUM* genes (16, 19, 28).

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