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Isolation and Characterization of Mutants Blocked in T-2 Toxin Biosynthesis

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Mutants of *Fusarium sporotrichioides* NRRL 3299 that were blocked or altered in the biosynthesis of the trichothecene T-2 toxin were generated by UV treatment and identified by a rapid screen in which monoclonal antibodies to T-2 were used. Three stable mutants were isolated and chemically characterized. Two mutants accumulated diacetoxyscirpenol, which suggests that they were defective in the step required for the addition of a hydroxyl group to the C-8 position in the trichothecene core structure. The third mutant appeared to be partially blocked at an early step or regulatory point in the pathway. This represents the first isolation of mutants in a trichothecene biosynthetic pathway.

The trichothecenes are a chemically related group of sesquiterpenoid secondary metabolites produced by certain species of *Fusarium*. T-2 toxin is the major trichothecene produced by *Fusarium sporotrichioides* NRRL 3299 (13). Like all trichothecenes, T-2 possesses a tetracyclic 12,13-epoxy-trichothec-9-ene core structure (Fig. 1). This core structure is formed from three molecules of mevalonate, which condense to form farnesyl PP_i (10). In a reaction catalyzed by trichodiene synthetase, farnesyl PP_i cyclizes to form trichodiene (4), the parent hydrocarbon of the trichothecenes (1, 2, 17). Among the remaining steps in the pathway to T-2 are six oxygenations which are catalyzed by molecular oxygen-dependent enzymes (6). Little else is known regarding the enzymology, biosynthetic pathway, or genetics of T-2 toxin production.

To study several aspects of trichothecene biogenesis, it would be useful to have mutants that are blocked or altered in T-2 synthesis. To date no such mutants are known (5).

The goal of this study was to devise a rapid screen for the identification of mutants that are unable to make T-2 toxin. The production of a monoclonal antibody to T-2 by Hunter and co-workers (8) has made such a goal feasible. This work describes the isolation and preliminary characterization of three UV-induced *F. sporotrichioides* mutants that were blocked or altered in the biosynthesis of T-2 toxin and identified by a rapid screen in which a monoclonal antibody to T-2 was used.

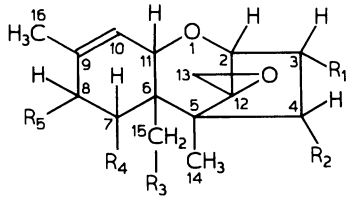
MATERIALS AND METHODS

Strains. A culture of the T-2 toxin-producing isolate *F. sporotrichioides* NRRL 3299 (ATCC 24043) was derived from a single spore (6). This strain was originally isolated from corn in France (5, 13).

Media and culture conditions. Solid medium cultures were grown under 20-W fluorescent tubes (F20 T12-CW; Cool White; General Electric Co., Schenectady, N.Y.) in a growth chamber programmed for an alternating 12-h, 25°C light/12-h, 20°C dark schedule. Strains were maintained on V-8 juice agar (16) slants at 4°C and as conidial suspensions in 10 to 15% glycerol at −70°C. Liquid cultures were inoculated at a starting density of 0.5×10^5 to 1×10^5 conidia per ml of 0.1% yeast extract (Difco Laboratories, Detroit, Mich.)–0.1% peptone (Difco)–50% glucose (19) (YEPD-5G medium), with conidia washed from strains grown on V-8 agar plates for 7 to 10 days. Liquid cultures were grown in Erlenmeyer flasks in a volume of medium equal to one-half the volume of the flasks for 6 to 7 days at 28°C on a rotary shaker operating at 180 to 200 rpm. The following were used for the isolation of mutants: M-100 minimal medium (10 g of glucose, 3 g of KNO_3, 20 g of agar, 62.5 ml of salt solution, 937.5 ml of deionized water [16]; salt solution [16 g of KH_2PO_4, 4 g of Na_2SO_4, 8 g of KCl, 2 g of $MgSO_4 \cdot 2H_2O$, 1 g of $CaCl_2$, 8 ml of trace elements solution, deionized water to 1 liter]; trace element solution [30 mg of H_3BO_3, 70 mg of $MnCl_2 \cdot 4H_2O$, 422 mg of $ZnSO_4 \cdot 7H_2O$, 20 mg of $Na_2MoO_4 \cdot 2H_2O$, 30 mg of $FeCl_3$, 200 mg of $CuSO_4 \cdot 5H_2O$, 500 ml of deionized water); M-100 supplemented with adenine (40 μg/ml), uracil (40 μg/ml), and 20 amino acids (40 μg/ml each); and 0.3% yeast extract, 1.0% peptone, 2.0% glucose, 2.0% agar, 0.05% (vol/vol) Triton X-100 (YEPD-2G-5T). Triton X-100, which restricts filamentous growth in *Nectria haematococca* (20), caused *F. sporotrichioides* NRRL 3299 conidia to grow as distinct, well-defined colonies.

Dry weight determinations. Mycelia from fractions (5 to 10 ml) of the liquid cultures were collected on preweighed filters (GF/C; Whatman, Inc., Clifton, N.J.) and stored at −20°C. Dry weights were measured after the filters were dried at 80°C for 22 h.

UV-mutagenesis and mutant screen. Microconidia were obtained for mutagenesis from V-8 agar plates that were incubated for 4 days following inoculation with approximately 2.5×10^6 freshly thawed conidia from frozen (−70°C) glycerol stocks. This procedure yielded primarily one- and two-celled microconidia, with approximately 65% of the total being one-celled. The microconidia were exposed to UV light (254 nm) as described by Avalos et al. (3), except that plating was done on YEPD-2G-5T agar. The UV-treated plates were immediately placed in the dark and incubated for 2 to 3 days in the growth chamber. To screen for T-2 toxin production, a portion of each surviving colony was transferred to a well in a 96-well microtiter plate (Falcon 3072; Becton Dickinson Labware, Oxnard, Calif.) containing 150 μl of YEPD-5G per well. The plates were sealed with Parafilm to reduce evaporation and were incubated at 28°C at 50 to 60 rpm on a minishaker (Dynatech Laboratories, Inc., Alexandria, Va.). After 4 to 5 days, the fungal mat was removed from each well with a toothpick to yield a cleared



	R_1	R_2	R_3	R_4	R_5
T-2 Toxin	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
Neosolaniol	OH	OAc	OAc	H	OH
Diacetoxyscirpenol	OH	OAc	OAc	H	H

FIG. 1. Structures of trichothecene 12,13-epoxy-trichothec-9-ene nucleus, T-2 toxin, NEO, and DAS. OAc, Acetate.

supernatant. The supernatants were stored at −20°C until the immunoassays were performed. For the immunoassays, each microtiter plate contained a blank control well (which yielded an A_{410} value for media only), a positive control well (which yielded an A_{410} value for a T-2 toxin standard solution containing 2×10^{-4} M T-2), and 94 test samples (culture filtrates). Colonies which produced test samples that yielded an A_{410} value either equal to that of the blank control or significantly higher than that of the T-2 control were scored as potential toxin mutants. To screen for auxotrophy, strains were tested for their ability to grow on M-100 minimal medium. Those that failed to grow were tested further for adenine, uracil, or amino acid growth requirements by standard methods (14).

CIEIAs. Competitive inhibition enzyme-linked immunoassays (CIEIAs) were performed with affinity-purified monoclonal antibody 15H6 as described by Hunter et al. (8, 9), except that methanol could be omitted from the phosphate-buffered saline–Tween 20 solution without consequence. A fraction of the culture supernatant, the T-2 standard solution, or the control medium (50 μl) was mixed in polystyrene microtiter plates (Falcon 3190) with 50 μl of 15H6 monoclonal antibody diluted to 5 μg/ml in phosphate-buffered saline containing 0.05% Tween 20. Following incubation for 1 h at room temperature, fractions (50 μl) of these samples were transferred to the wells of round-bottom polyvinyl microtiter plates (Dynatech) coated with T-2–bovine serum albumin (10 μg/ml in 0.1 M Tris hydrochloride [pH 8.2]; Sigma Chemical Co., St. Louis, Mo.) by overnight incubation at 4°C. After 30 min of incubation at room temperature, the wells were washed five times with phosphate-buffered saline–0.05% Tween 20 and successively incubated with rabbit anti-mouse kappa light chain antiserum (ICN Immunochemicals, Elkhart, Ind.), goat anti-rabbit immunoglobulin G-alkaline phosphate conjugate (Sigma), and enzyme substrate (*p*-nitrophenylphosphate; Sigma) as described previously (9). Assays were measured at 410 nm on an automatic microtiter plate reader (Dynatech). The 15H6 monoclonal antibody was a generous gift from K. W. Hunter, Uniformed Services University School of Medicine, Bethesda, Md.

Gas chromatographic analysis for trichothecene production. Each culture filtrate or whole culture was extracted twice with an equal volume of ethyl acetate. The two extracts were pooled and then passed through a charcoal column (Romer Laboratories, Inc., Washington, Mo.) that was prewashed with 20 ml of ethyl acetate. The eluent was combined with a subsequent wash (20 ml of ethyl acetate) and evaporated to dryness on a rotary evaporator. The residue was suspended in 1 ml of a mixture of toluene-acetone-methanol (2:1:1). A fraction equivalent to a 1- or 2-ml portion of the original sample was evaporated to dryness at 80°C under nitrogen, reacted with 100 to 200 μl of trimethyl silating reagent (Tri-Sil/TBT; Pierce Chemical Co., Rockford, Ill.) for 1 h at 80°C and brought to 1 ml with hexane. Analyses were conducted on a gas chromatograph (model SP7110; Spectra-Physics) as described previously (5a). Purified samples of T-2 toxin, diacetoxyscirpenol (DAS), and neosolaniol (NEO) standards were purchased from Sigma.

RESULTS

Detection of T-2 toxin in liquid culture filtrates by CIEIA. The utility of the T-2 monoclonal antibody in a CIEIA to quantitate T-2 toxin in culture filtrates was examined. When grown in liquid shake cultures, trichothecene-producing strains such as *F. sporotrichioides* NRRL 3299 secrete toxin into the medium. The results in Fig. 2 demonstrate that the amount of T-2 present in the culture filtrate from a liquid shake culture of strain NRRL 3299 can be accurately measured by the CIEIA when the culture filtrate is diluted. The T-2 content of the filtrate was determined to be 100 to 120 μg/ml. Gas chromatographic (GC) analysis of the same culture filtrate yielded identical results. Thus, T-2 production by liquid cultures can be determined with little sample preparation and with a minimal sample volume (50 μl) by the CIEIA method.

T-2 toxin production by cultures grown in microtiter plates. Conditions for growth and toxin production in microtiter plates were determined (Table 1). Efficient T-2 toxin production in YEPD-5G during 3 days of growth in microtiter wells required shaking. The addition of Triton X-100 to limit mycelial growth, and thereby to prevent potential cross contamination of the wells, was unnecessary. Cross contamination did not occur in its absence, and in fact, toxin levels were reduced in its presence. Thus, favorable conditions for toxin production in microtiter plates resulted from inoculation of YEPD-5G medium with either conidia or a portion of a colony, followed by incubation at 28°C for a minimum of 3 days with shaking.

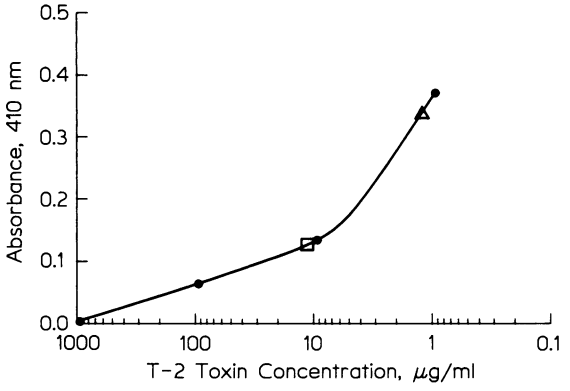


FIG. 2. Immunological detection of T-2 toxin in liquid shake culture filtrates of *F. sporotrichioides* NRRL 3299. YEPD-5G medium (150 ml in a 300-ml Erlenmeyer flask) was inoculated with 10^5 conidia per ml and incubated for 96 h at 28°C and 200 rpm. The culture filtrate was prepared by filtration through a filter (GF-C; Whatman) and assayed for T-2 as described in the text. Symbols: ●, T-2 standard curve from purified T-2; □, culture filtrate at 0.1× concentration; △, culture filtrate at 0.01× concentration. T-2 was prepared for the standard curve by dilution of a stock solution (2×10^{-2} in 100% methanol) with phosphate buffered saline containing 0.05% Tween 20.

Production and isolation of mutants. Mutants were induced by treating microconidia with a dose of UV light that produced 95% lethality; 1,008 survivors were isolated. A total of 25 auxotrophs, representing 2.5% of the survivors, were identified: 3 required adenine, 1 required lysine, 1 required methionine, 1 required glutamine, 1 required arginine, and 1 required both isoleucine and valine. The remaining 17 failed to grow on M-100 minimal medium supplemented with adenine, uracil, and a mixture of 20 amino acids and must therefore have some other nutritional requirement. As measured by the initial immunoassay, none of the five amino acid auxotrophs displayed a discernible reduction in T-2 toxin production. However, 16 of the remaining 19 auxotrophs did, including 2 that required adenine (data not shown). Finally, three strains, representing 0.3% of the survivors, proved to be prototrophic but were blocked in the production of T-2. These three potential trichothecene toxin mutants, designated Tox$^-$ mutants, were further examined.

Growth kinetics of Tox$^-$ mutants. The production of secondary metabolites in fungi often appears to be related to the growth rate and the onset of stationary growth. Consequently, maintenance of a wild-type growth pattern is critical for the evaluation of the effect of Tox$^-$ mutations on trichothecene biosynthesis. A direct comparison between the Tox$^-$ mutant strains MB1370, MB1716, and MB1755 and the parent demonstrated that each of these mutants retained a wild-type growth rate and yield in liquid shake cultures (Fig. 3).

GC analysis of trichothecene production in the Tox$^-$ mutants. GC analysis revealed that both strains MB1370 and MB1716 accumulated DAS (Fig. 4). However, while MB1716 was completely blocked and produced only DAS, MB1370 was partially blocked and continued to produce a small amount of T-2 toxin. These data suggest that MB1370 and MB1716 arose from independent mutational events and that both are defective in the addition of oxygen at C-8. MB1755 represents a different class of Tox$^-$ mutants. The GC data indicate that MB1755 produces greatly reduced levels of DAS, NEO, and T-2 compared with those of the wild-type parent and does not appear to accumulate other related metabolites. Further analysis by thin-layer chromatography and GC-tandem mass spectrometry showed that MB1755 fails to accumulate trichodiene or any other known trichothecene (M. N. Beremand and R. D. Plattner, unpublished data). These results suggest that MB1755 may be specifically altered in an early step of the T-2 biosynthetic pathway. It is equally possible that a regulatory mutation is involved which affects the extent to which the pathway is induced or the level at which the pathway functions. Based on the similarity between strains MB1370 and MB1716 and their dissimilarity to strain MB1755, these mutants have been given the following genotypic designations: *Tox1-1*$^-$ for MB1370, *Tox1-2*$^-$ for MB1716, and *Tox2-1*$^-$ for MB1755.

TABLE 1. T-2 toxin production by *F. sporotrichioides* NRRL 3299 grown in microtiter plates

Inoculum	Culture condition[a]		T-2 produced (μg/ml)
	Triton X-100	Shaking	
Colony	−	+	>100
Conidia	−	+	>100
Conidia	+	+	34
Conidia	−	−	<0.001
Conidia	+	−	<0.001

[a] Wells containing 150 μl of YEPD-5G medium with or without 0.01% Triton X-100 were inoculated in duplicate with either conidia (at a final density of 2×10^5/ml) or a small portion of a colony growing on a YEPD-2G-5T plate. Plates were incubated at 28°C for 3 days with or without shaking. CIEIAs were then performed on 50 μl of culture medium, as described in the text.

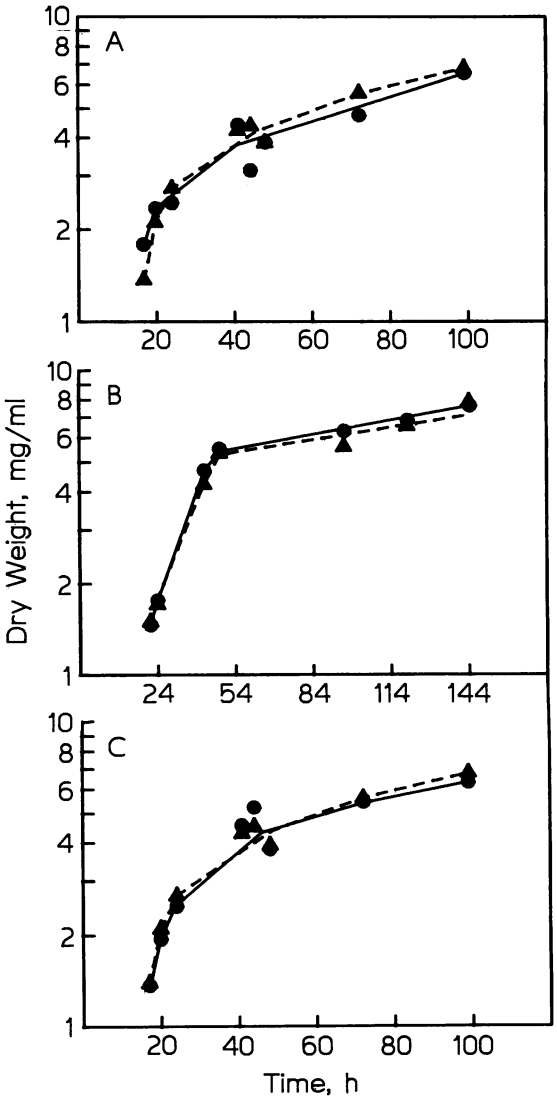


FIG. 3. Comparison of cell growth between the Tox$^-$ mutant strains and the wild-type parent *F. sporotrichioides* NRRL 3299. (A) MB1370; (B) MB1716; (C) MB1755. Symbols: ●, mutant strain; ▲, parent strain. The MB1370, MB1755, and corresponding NRRL 3299 liquid cultures were inoculated with 7.0×10^4 conidia per ml that were washed from 10-day-old cultures growing on V-8 agar plates; the MB1716 and corresponding strain NRRL 3299 liquid cultures were inoculated with 3.5×10^4 conidia obtained from 5-day-old cultures grown on V-8 agar plates. All cultures were incubated at 200 rpm and 28°C. Fractions were removed at regular intervals and processed for dry weight determinations, as described in the text.

DISCUSSION

In this study, three classes of mutants were isolated following UV irradiation of *F. sporotrichioides* NRRL 3299. Survivors were tested for both T-2 production and auxotrophy. The first class of mutants (composed of 16 strains) were auxotrophs with reduced T-2 levels. Mutants

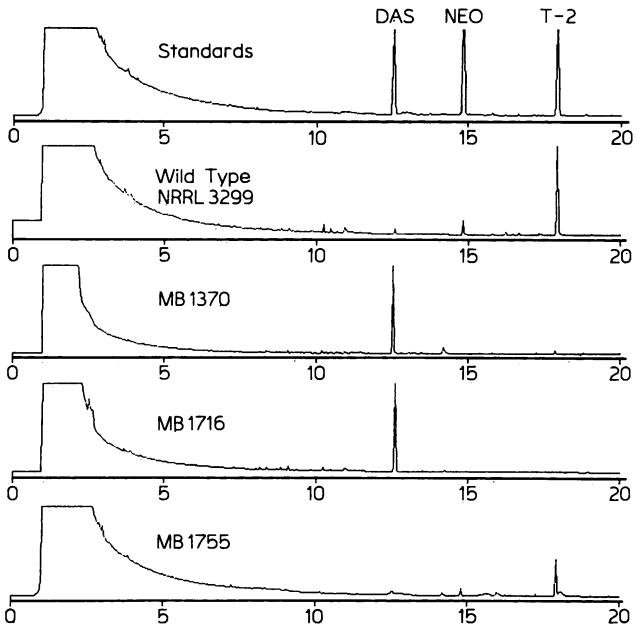


FIG. 4. GC analysis of trichothecenes produced by Tox$^-$ mutants and the wild-type parent *F. sporotrichioide* NRRL 3299. YEPD-5G liquid medium (25 ml) was inoculated with 5×10^4 conidia per ml and incubated at 28°C and 180 rpm for 8 days. Samples were prepared and assayed as described in the text. Retention times (in minutes) are indicated.

with nutritional deficiencies frequently produce reduced levels of secondary metabolites. For example, in *Penicillium chrysogenum*, auxotrophs often suffer a reduction in penicillin yields (12). Similarly, auxotrophic mutants of *Claviceps* species are generally poor alkaloid producers; selection of high alkaloid producers from known auxotrophs is invariably accompanied by a reversion to prototrophy (15). Indeed, in this study 16 of the 19 T-2-reduced strains revealed by the CIEIA screen failed to grow on minimal medium. As in the cases cited above, auxotrophy may be pleiotrophic to the reduced T-2 toxin production that was observed in these 16 mutants. However, not all auxotrophs displayed a marked reduction in T-2 production under these growth conditions; nine other auxotrophs were obtained which, within the detection limits of the assay conditions, did not decrease the production of T-2. Finally, a third class of mutants (composed of three strains) was prototrophs which made little or no T-2. These mutants appeared to be specifically altered in their ability to synthesize T-2.

Chemical characterization of the three T-2-altered mutants revealed that two, MB1370 and MB1716, accumulated DAS; they were designated *Tox1-1*$^-$ and *Tox1-2*$^-$, respectively. It is not known yet whether these two mutations are allelic; however, both mutants apparently were altered in the ability to add a hydroxyl group to the C-8 position in the trichothecene core structure. The third mutant, MB1755, appeared to be partially blocked at an early step or regulatory point in the pathway and was designated *Tox2-1*$^-$. The isolation of these mutants suggests that at least some of the genes necessary for T-2 biosynthesis exist as single-copy genes and that they are not essential for growth.

The isolation of mutants that are affected in the production of secondary metabolites, such as the trichothecenes, is often difficult. It was not possible to employ the strategies commonly used for the isolation of conditionally lethal mutants because secondary metabolites, unlike primary metabolites, are not essential for growth. Secondary metabolism mutants traditionally have been obtained by time-consuming techniques. For example, for the isolation of *Claviceps purpurea* alkaloid mutants, it was required that each mutagenesis survivor be individually tested for alkaloid production in a liquid shake culture (11). The advent of convenient, sensitive immunoassays, however, offers the potential for the development of new strategies for the identification of mutants altered in the production of secondary metabolites. In this study, three mutants impaired in T-2 biosynthesis were isolated by means of a simple, accurate, and rapid screen based on an enzyme-linked immunoassay. This approach should be applicable for the isolation of mutants that are defective in the production of other metabolites.

The immunoassay for T-2 toxin in the mutant screen did not require extraction of culture filtrates. In a previous study (7), blood, urine, and milk samples similarly did not require any treatment prior to assay. The level of toxin produced by wild-type cells in liquid shake culture was sufficient (>90 μg/ml) to eliminate the necessity to concentrate the samples, and the assays could be performed with only 50 μl of the filtrate. Thus, potential mutants could be grown in microtiter wells, and large numbers of samples could be examined.

The properties of the 15H6 monoclonal antibody to T-2 toxin increased the utility of this method for screening toxin production mutants. This antibody is unable to recognize the common trichothecene core structure, and it requires the presence of an isovalerate group at C-8 for binding to occur (8). Thus, any lesion which blocks the pathway prior to the addition of isovalerate can be detected by use of this antibody.

The ability to obtain mutants altered in the synthesis of T-2 toxin allows the application of classical, biochemical, and molecular genetic approaches to the study of trichothecene production and biosynthesis. Many *Fusarium* species that produce trichothecenes are plant pathogens (18). Mutants blocked in trichothecene production can be used to determine the involvement of the trichothecenes in plant diseases. Biochemical studies involving blocked mutants are also being conducted, and they should contribute to the elucidation of both the T-2 toxin biosynthetic pathway and its regulatory controls. The isolated mutants described in this report are particularly well-suited to these studies because they have proven to be extremely stable (unpublished data). Finally, such mutants should be useful for the isolation of the genes that are necessary for T-2 production.

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