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Characterization of Experimentally Induced, Nonaflatoxigenic Variant Strains of Aspergillus parasiticus

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Six previously isolated, nonaflatoxigenic variants of Aspergillus parasiticus, designated sec mutants, were characterized morphologically by electron microscopy, biochemically by biotransformation studies with an aflatoxin precursor, and genetically by Northern (RNA) hybridization analysis of aflatoxin biosynthetic gene transcripts. Scanning electron micrographs clearly demonstrated that compared with the parental sec+ forms, the variant sec forms had an abundance of vegetative mycelia, orders of magnitude reduced number of conidiophores and conidia, and abnormal metulae. Conidiospores were detected in sec cultures only at higher magnifications (×500), in contrast to the sec+ (wild-type) strain, in which abundant conidiospores (masking the vegetative mycelia) were observed even at lower magnifications (×300). All sec+ forms, but none of the sec forms, showed bioconversion of sterigmatocystin to aflatoxins. Northern blots probed with pathway genes demonstrated lack of expression of both the aflatoxin biosynthetic pathway structural (nor-1 and omiA) and regulatory (affR) genes in the sec forms; PCR and Southern hybridization analysis confirmed the presence of the genes in the sec genomes. Thus, the loss of aflatoxigenic capabilities in the sec form is correlated with alterations in the conidial morphology of the fungus, suggesting that the regulation of aflatoxin synthesis and conidiogenesis may be interlinked.

Allatoxins (AF) are highly carcinogenic decaketides produced by certain strains of Aspergillus flavus and A. parasiticus (12, 27). The AF biosynthetic pathway has been well studied, and a number of mutants that accumulate pigmented pathway intermediates are known (3, 4, 16).

In previous work, polyketide-producing A. parasiticus was developed as a model system to study strain degeneration in filamentous fungi, using mycelial pigmentation as an easily scored marker for the polyketide pathway (15). One wild-type and five genetically marked mutants, designated sec− for secondary metabolism positive, were subjected to a protocol of serial transfers. Variant strains, showing no detectable secondary metabolite production and designated sec mutants, were isolated after 5 to 12 transfers of nonsporulating mycelia. The sec forms were stable and grew as well as sec+ forms in liquid culture. However, in addition to their inability to make detectable levels of polyketide secondary metabolites, they exhibited reduced sporulation and altered colony morphology on solid media (15). Similar correlations between morphological changes and loss of virulence or secondary metabolite production have been commonly reported for filamentous fungi, but no satisfactory explanation has been found for most cases of strain degeneration. Proposed theories include chromosome instability, heterokaryosis, cytoplasmic inheritance, and the presence of transposable elements (14, 18).

The aim of this research was to characterize further the morphological, biochemical, and molecular profiles of the parental sec+ A. parasiticus strains with their derivative sec forms and to determine the reason for the loss of aflatoxigenic capability of the sec strains. In this report we present (i) scanning electron micrographs demonstrating morphological differences in the mycelia and conidiophores of sec+ and sec strains; (ii) data on the inability of sec forms to bioconvert a known AF pathway intermediate in feeding experiments; and (iii) Northern (RNA) blot analyses demonstrating the presence of AF pathway and regulatory gene transcripts in the sec+ forms and their absence in the sec forms.

MATERIALS AND METHODS

Fungal strains and culture conditions. One wild-type and five auxotrophic and spor color mutants of A. parasiticus used as parental sec− forms had the following genotypes: SU−1; wh-1 ver-1 avn-1; wh-1 nor-1 lys-6 ade-1; br-1 nor-1 lys-6 ade-1; wh-1 ver-1 lys-6 pdx-1; and br-1 pdx-1. The sec− mutant strains were originally derived from SU−1 (NRRL A-16,462) by UV light treatment, nirogossamine treatment, and/or genetic (parasexual cycle) recombination techniques (2, 5) and were obtained from J. W. Bennett’s culture collection for the present study. The geneologies of these sec− forms and the isolation of their respective sec derivatives have been described previously (15). All cultures were incubated at 30°C in the dark; liquid media were maintained on a gyratory shaker (New Brunswick Scientific model G76) operating at 150 rpm.

Media and chemicals. Stock cultures were maintained on complete medium made of potato dextrose agar (Difco) containing 0.5% yeast extract (Difco). Synthetic growth medium was modified by substituting sucrose for glucose but otherwise was formulated by the method of Adye and Matese (1). The low-sugar replacement medium (LSRM) was also formulated by the method of Adye and Matese (16). Fungal cultures were grown on YES medium (10) for isolation of DNA and RNA.

Scanning electron microscopy. Five-day-old samples were used for scanning electron microscopic observations. The following sample preparation technique based on previous work (19, 21–23, 29) was used to provide a more complete range of information for the fungal samples. (i) Mycelial segments (5 mm2 to 1 cm2) were cut and promptly placed in vials containing 3% glutaraldehyde in 0.05 M phosphate buffer (pH 6.8) at room temperature. Chemical fixation for 48 h was followed by dehydration in an ethanol series ending with absolute ethanol. The samples were critical-point dried from absolute ethanol in liquid carbon dioxide. (ii) Fungal cultures were placed in a VirTis model 20 SRC-X freeze-dryer and left overnight at a pressure of 30 millitorr (4 Pa). The plates were then removed and promptly placed in a desiccator until used for sampling. (iii) Portions of fungal cultures measuring several centimeters were cut and placed in quadrant metri plates. In a fume hood, a vial cap containing 4% ammonium tetramide in water was placed in an unoccupied quadrant of the plate. After being covered, the plate was sealed with Parafilm, and vapor fixation of the sample proceeded for 48 h. The fungal segments were placed in a desiccator. (iv) Nest, segments (5 mm2 to 1 cm2) were mounted on standard 0.5-in. (1.3-cm) Cambridge scanning electron microscopy stubs with double-stick adhesive tabs and coated with 20 to

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30 nm of 60-40 gold-palladium in a Technics Hummer II sputter coater. All samples were viewed in a Cambridge S-250 scanning electron microscope operating at 6 to 10 kV and a magnification range of 100 to 2,500.

**Bioretransformation experiments.** Flasks containing 100 ml of synthetic growth medium were inoculated with approximately 10⁸ colony-forming units of the appropriate strains or sec strains. The cultures were grown for 48 h at 30°C with constant shaking at 150 rpm, and the resultant mycelial pellets were harvested on cheesecloth and rinsed with LSRM. The mycelial pellets were weighed on sterile weighing paper (g wet weight) and was added to 9.98 ml of LSRM. For bioretransformation studies, 20 μg of steigmatocystin (ST) dissolved in 20 μl of acetone was added to the LSRM cultures, and incubation was continued for an additional 24 h at room temperature with constant shaking at 150 rpm. Controls consisted of 99.8 μl of acetone without ST. In other controls, ST was added to the LSRM containing no mycelia or autoclaved mycelia.

**Aflatoxin and anthraquione assays.** For extraction of secondary metabolites, 15 ml of acetone was added to each LSRM flask and the mycelia were allowed to equilibrate at room temperature overnight. The mycelial pellets were then separated from the liquid with a clean spatula, and the filtrate was decanted off in a separatory funnel. Methylene dichloride (10 ml) was added to each separatory funnel, and the contents were inverted 15 times. The lower, methylene dichloride-acetone layer was collected and air dried. The dried samples were then suspended in 1 ml of methylene dichloride, and thin-layer chromatography was carried out by spotting 10 μl of each sample and known standards on precised 250-μm-thick silica gel G plates (20 by 20 cm; Analytech). The plates were developed with ether-methanol-water (96:3:1, by volume) for AF and ST (R<sub>f</sub> = 0.44 for AFβ, and 0.97 for ST) and toluene-ethyl acetate-acetic acid (50:30:4, by volume) for anthraquinones (R<sub>f</sub> = 0.73 for norsolorinic acid, 0.59 for averantin, and 0.64 for versicolorin A). The plates were dried and viewed under long-wave UV light for fluorescent metabolites.

**Northern blot analyses.** Total RNA was prepared by the hot-phenol procedure (20) from mycelia grown for 48 h in YES medium. Upon quantification, approximately 20 μg of total RNA was loaded per lane of a 1.2% formaldehyde–agarose gel at pH 8.0. Total RNA was treated with RNase-free DNase I (21) and ethanol precipitated. Briefly, RNA (2 μg) was mixed with 2 μl of 4× denaturing gel loading buffer and heated at 90°C for 5 min. dawned in an eppendorf tube, and cooled on ice for 10 s. After that, 20 μl of 4× RNA loading dye was added. The mixture was heated at 70°C for 5 min, then the samples were loaded and subjected to electrophoresis in 1% agarose gel containing ethidium bromide at 60 W for 1 h. The gels were stained with ethidium bromide and photographed under UV transilluminator. The RNA was transferred to Hybond-N+ membrane (Amersham) by capillary transfer and hybridized with [32P]dCTP-labeled probe. The hybridization was performed at 65°C for 48 h. The membrane was washed for 10 min in 2× SSC, 0.1% SDS, at 50°C for 2× SSC, 0.1% SDS, at 45°C for 1× SSC, 0.1% SDS, at 2× SSC, 0.1% SDS, at 1× SSC, 1× SSC, and 2× SSC for 10 min at 45°C, 55°C, 65°C, and 75°C, respectively. After hybridization, the membrane was washed in 1× SSC, 0.1% SDS at 65°C for 10 min. The membrane was then exposed to X-ray film for autoradiography.

**RESULTS AND DISCUSSION**

Representative sec<sup>+</sup> and sec strains of *A. parasiticus* wh-1 ver-1 lys-6 pdx-1 were grown on complete medium for 5 days and subjected to scanning electron microscopic analysis, allowing adequate time for them to complete the asexual reproductive cycle (Fig. 1). The sec<sup>+</sup> form, at a magnification of ×300, displayed heavy sporulation, with long chains of conidiospores masking the underlying conidiophores and mycelia (Fig. 1A). Even at a higher magnification (×500), the corresponding sec variant displayed only an extensive, intertwined network of largely vegetative hyphae with orders of magnitude reduction in conidiophore formation (Fig. 1B). At a higher magnification of ×1,000, the sec<sup>+</sup> form showed normal, healthy conidiophores (Fig. 1C). Typically, the sec<sup>+</sup> conidiophore on the right (Fig. 1C) displays a vesicle covered evenly with normal-sized metulae, whereas the other one on the left bears chains of healthy conidia. In sharp contrast, the sec conidiophore, at twice the magnification (×2,000) of the sec<sup>+</sup> strain, displays an altered morphology with reduced numbers of abnormal elongated metulae, giving rise to a very small number of conidiospores (Fig. 1D). Specifically, the sec conidiophore showed a smaller vesicle with eight club-shaped elongated metulae containing only about 20 spores arising from it. However, no apparent differences in the morphology or size of the individual conidiospores of sec<sup>+</sup> and sec strains were detected.

**Aspergillus conidiation has been best studied at both the descriptive and molecular levels in the model system *A. nidulans* and is believed to be similar in *A. parasiticus* (23, 25). It is interesting that some of the developmental abnormalities observed in our sec strains are similar to those described for bristle (brl1) mutants, a regulatory locus controlling conidio- phore development in *A. nidulans* (24).

The *A. parasiticus* sec forms were selected originally not for their aberrant morphology but for their inability to produce AF and/or pigmented polyketides. Therefore, we also examined the biosynthetic capacity of these strains through the use of biofeeding experiments. ST, a late intermediate in AF biosynthesis, is biotransformed into AF by blocked anthraquinone-accumulating mutants (11, 13). The results of biofeeding experiments with the sec<sup>+</sup> and sec strains are shown in Table 1. Four of the parental sec<sup>+</sup> strains (SU-1, br-1 nor-1 lys-6 ade-1, br-1 pdx-1, and wh-1 nor-1 lys-6 ade-1) made AFβ, whereas two blocked mutants (wh-1 ver-1 avn-1 and wh-1 ver-1 lys-6 ade-1) accumulated, respectively, the AF precursors averantin and versicolorin A. In biofeeding studies with the sec<sup>+</sup> strains, AF production increased threefold for SU-1, fivefold for br-1 nor-1 lys-6 ade-1, threelfold for br-1 pdx-1, and sevenfold for wh-1 nor-1 lys-1 ade-1 (Table 1). Similarly, averantin production increased about 1.2-fold for wh-1 ver-1 avn-1 while versicolorin A production increased 2-fold for the wh-1 ver-1 lys-6 pdx-1 mutant. In these both nonaflatoxigenic mutants, AFβ was produced upon addition of ST. In sharp contrast, no measurable levels of AFβ or any anthraquinone intermediates were detected in any of the sec strains with or without exogenous ST in biofeeding experiments. Recovery of ST after 24 h of incubation ranged from 33.5% SU-1 sec to 61.5% wh-1 ver-1 lys-6 pdx-1 sec. The controls consisting of media without any mycelia yielded about 67% recovery of ST (Table 1).

**Similar precursor feeding experiments with *A. flavus* have been conducted with four nonaflotoxin strains isolated from**
FIG. 1—Continued.
nature and two strains isolated by repeated transfer on laboratory media (17). In these studies, the nontoxicogenic strains isolated by transfer experiments bioconverted both ST and O-methyl-ST into AF whereas the nontoxicogenic strains isolated from nature were biochemically similar to our sec strains and did not cause bioconversion. Other studies have demonstrated that some nontoxicogenic strains of *A. flavus* isolated from nature do convert precursors to AF or could have permeability problems in the uptake of ST. To investigate the former possibility, Northern hybridization analyses were performed with total RNA from representative sec and sec strains, using fragments of nor-1, omtA, and aflR as probes (Fig. 2). Abundant mRNA levels were detected in the sec control strains for all the three probes tested. In contrast, the sec strains showed no detectable levels of mRNA for any of the probes used. Furthermore, PCR products of the expected size were observed for the aflR (~500-bp), omtA (~1.6-kb), and nor-1 (~1-kb) genes in both the sec and sec strains. Southern hybridization of PCR products with their respective gene probes confirmed their homology to the pathway genes (data not shown). This demonstrated that the pathway genes were present in the sec strains. Therefore, the sec strains were unable to make any metabolic precursors of AF and were unable to carry out bioconversions owing to lack of production of the necessary enzymatic activities.

With respect to the AF biosynthetic pathway, to date only one regulatory gene (*aflR*), which positively induces the expression of the other AF pathway genes, has been cloned (6, 7, 30). No expression of *aflR* was detected in the sec forms, suggesting a regulatory abnormality leading to a loss of aflatoxigenic capability in these strains. The correlated morphological abnormalities of sec strains suggest the possibility of a global regulatory system, some pleiotropic elements of which may be involved both in AF production and one or more developmental processes, such as conidiation.

In summary, none of our experimentally induced sec strains produced detectable levels of decaketides in the AF biosynthetic pathway. This appears to be due to the lack of expression

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amt of metabolite recovered (μg/g of mycelia)</th>
<th>Anthraquinones&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ST</th>
<th>AFB&lt;sub&gt;1&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-ST</td>
<td>+ST</td>
<td>-ST</td>
<td>+ST</td>
</tr>
<tr>
<td>SU-1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Trace</td>
</tr>
<tr>
<td>sec&lt;sup&gt;+&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Trace</td>
</tr>
<tr>
<td>sec&lt;sup&gt;-&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>6.9</td>
</tr>
<tr>
<td>br-1 nor-1 lys-6 ade-1&lt;sup&gt;sec+&lt;/sup&gt;</td>
<td>Trace (NOR)</td>
<td>Trace (NOR)</td>
<td>ND</td>
<td>Trace</td>
</tr>
<tr>
<td>sec&lt;sup&gt;-&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Trace</td>
</tr>
<tr>
<td>wh-1 ver-1 lys-6 pdx-1&lt;sup&gt;sec+&lt;/sup&gt;</td>
<td>0.7 (VER)</td>
<td>1.4 (VER)</td>
<td>ND</td>
<td>Trace</td>
</tr>
<tr>
<td>sec&lt;sup&gt;-&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Trace</td>
</tr>
<tr>
<td>wh-1 ver-1 avn-1&lt;sup&gt;sec+&lt;/sup&gt;</td>
<td>180.8 (AVN)</td>
<td>209.2 (AVN)</td>
<td>ND</td>
<td>Trace</td>
</tr>
<tr>
<td>sec&lt;sup&gt;-&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>10.7</td>
</tr>
<tr>
<td>br-1 pdx-1&lt;sup&gt;sec+&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Trace</td>
</tr>
<tr>
<td>sec&lt;sup&gt;-&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>10.8</td>
</tr>
<tr>
<td>wh-1 nor-1 lys-6 ade-1&lt;sup&gt;sec+&lt;/sup&gt;</td>
<td>Trace (NOR)</td>
<td>Trace (NOR)</td>
<td>ND</td>
<td>Trace</td>
</tr>
<tr>
<td>sec&lt;sup&gt;-&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>9.0</td>
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<tr>
<td>Controls (no mycelia)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>13.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cultures with or without 20 μg of ST were incubated at 28°C for 24 h in LSRM formulated as described by Adye and Mateles (1). The metabolites were extracted and quantitated by TLC and densitometry by using the protocol outlined by Walker (28). The experiment was conducted in duplicate.

<sup>b</sup> Anthraquinones quantitated were norsolorinic acid (NOR), versicolorin A (VER), and averantin (AVN).

<sup>c</sup> ND, none detected.
of the pathway regulatory gene aflR and the resulting inability to bioconvert pathway intermediates to AF. In addition, all these nonaflatoxigenic strains displayed a pleiotropic phenotype that involved changes in conidiophore development and sporulation patterns. These isogenic sec+ and sec pairs remain an interesting model system for strain degeneration (15, 18). They may also be useful tools in providing an insight into the correlation between secondary metabolism and morphological development in filamentous fungi.

ACKNOWLEDGMENT

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REFERENCES