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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 *omtA*) and regulatory (*aflR*) 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.

Aflatoxins (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 spore 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, nitrosoguanidine 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 Mateles (1). The low-sugar replacement medium (LSRM) was also formulated by the method of Adye and Mateles (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 mm^2 to 1 cm^2) 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 petri plates. In a fume hood, a vial cap containing 4% osmium tetroxide 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) Next, segments (5 mm^2 to 1 cm^2) 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.

Biotransformation experiments. Flasks containing 100 ml of synthetic growth medium were inoculated with approximately 10^7 spores of the appropriate *sec*$^+$ 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 from which 1 g (wet weight) was added to 9.98 ml of LSRM. For biotransformation studies, 20 μg of sterigmatocystin (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 adding 20 μl of acetone without ST. In other controls, ST was added to the LSRM containing no mycelia or autoclaved mycelia.

Aflatoxin and anthraquinone 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 prescored 250-μm-thick silica gel G plates (20 by 20 cm; Analtech). The plates were developed with ether-methanol-water (96:3:1, by volume) for AF and ST (R_f = 0.44 for AFB_1 and 0.97 for ST) and toluene-ethyl acetate-acetic acid (50:30:4, by volume) for anthraquinones (R_f = 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.

The initial thin-layer chromatography, as described above, was conducted to obtain a visual estimate of the appropriate amount of extract to spot for densitometric readings, as recommended by the American Oil Chemists' Society (28). Prescored 250-μm-thick silica gel G plates were spotted, developed in appropriate solvent systems, and scanned for fluorescence with a Shimadzu model CSW 9-10 recording densitometer at an excitation wavelength of 360 nm for AF, 310 nm for norsolorinic acid, and 290 nm for averantin and versicolorin A. The quantities of these metabolites were calculated by comparison with areas under the peaks for standards run on the same plate. Quantities of standards and samples were also verified spectrophotometrically (Shimadzu UV-visible UV-160 spectrophotometer) with known extinction coefficients (8).

Northern blot analyses. Total RNA was prepared by the hot-phenol procedure (20) from mycelia grown for 48 h in YES medium. Upon quantitation, approximately 20 μg of total RNA was loaded per lane of a 1.2% formaldehyde–agarose gel, electrophoresed, and vacuum blotted to Zeta-probe (Bio-Rad) nylon membranes. The blots were baked at 80°C under vacuum and prehybridized for 6 h in hybridization buffer (50% formamide, 1 mM EDTA [pH 8.0], 7% sodium dodecyl sulfate [SDS], 0.25 M $NaPO_4$ [pH 7], 0.25 M NaCl, 0.15 mg of salmon sperm DNA per ml). Hybridization was carried out for 16 h with fresh hybridization buffers and the following ^{32}P labeled probes: (i) *nor-1* (a PCR-generated 488-bp fragment of the open reading frame, 647 bp after the translational start) (26), (ii) *omtA* (a full-length cDNA clone) (31), and (iii) *aflR* (a 1,300-bp *Eco*RI fragment from the C-terminal end of the full-length cDNA clone, not including the DNA encoding the Zn-finger region) (6, 7).

The blots were washed twice at 64°C for 5 min with 1 mM EDTA–40 mM $NaPO_4$ (pH 7)–5% SDS, and then once for 5 min with 1 mM EDTA–40 mM $NaPO_4$ (pH 7)–1% SDS. Exposure times with Kodak XRP films were 12 to 20 h at −80°C.

PCR AF biosynthetic genes. PCR was used to amplify genomic DNA regions corresponding to the *aflR* (6), *omtA* (31), and *nor-1* (26) genes from *sec*$^+$ and *sec* *A. parasiticus* strains. Approximately 250 ng of fungal total genomic DNA was amplified with the following oligonucleotide primer pairs (100 pmol each): *aflR* (5′-CCGATTTCTTGGCTGTCT-3′ and 5′-TCCTCATCCACACAATCC-3′), *omtA* (5′-GCAAGGACGCTACAATGTGCG-3′ and 5′-CTCAGCCGTTCTTC TGACA-3′), and *nor-1* (5′-ATGAACGGATCACTTAGCCA-3′ and 5′-AGTT GAGATCCATCCGTGT-3′). Reactions were performed with the GeneAmp PCR kit reagents (Perkin-Elmer, Foster City, Calif.), using the recommended concentrations of nucleotides, PCR buffer I, and AmpliTaq polymerase and a final $MgCl_2$ concentration of 3.5 mM. The thermocycler (no. PTC-100; MJ Research, Inc., Watertown, Mass.) temperature program parameters were as follows: 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min for one cycle; then the annealing temperature was increased to 58°C for one cycle and finally to 60°C for 35 cycles followed by a final extension at 72°C for 5 min. The size of the PCR products was confirmed by agarose gel electrophoresis with lambda *Hin*dIII and ϕX174 replicative-form *Hae*III DNA as size standards. The identity of the PCR products was confirmed by Southern blot analysis with the above-mentioned radiolabeled gene probes by the method of Yu et al. (31).

RESULTS AND DISCUSSION

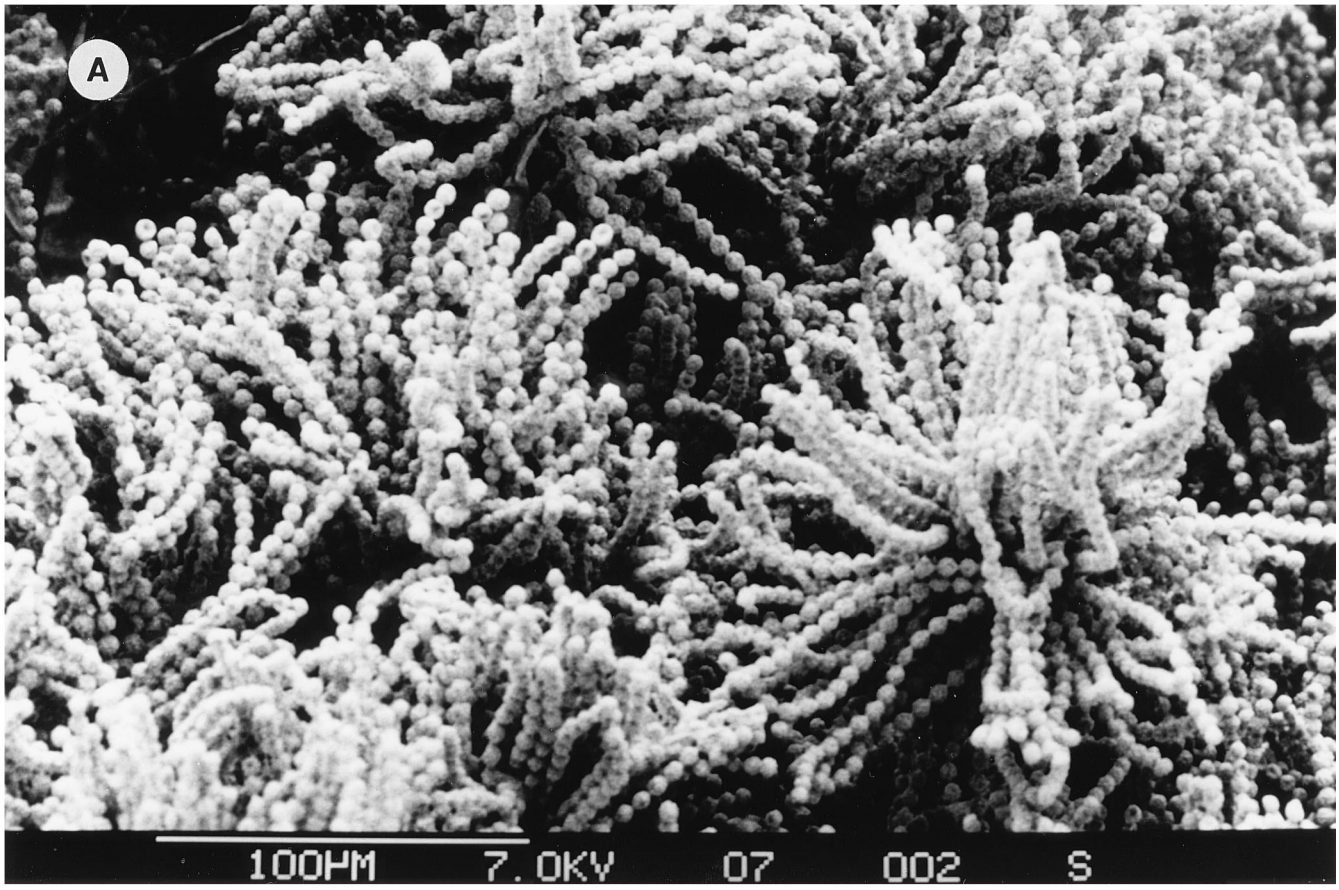
Representative *sec*$^+$ and *sec* strains of *A. parasiticus wh-1 ver-1 lys-6 pdx-1* were grown on complete medium for 5 days and subjected to scanning electron microscopic analysis, allowing adequate time for them to complete the asexual reproductive cycle (Fig. 1). The *sec*$^+$ form, at a magnification 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*$^+$ form showed normal, healthy conidiophores (Fig. 1C). Typically, the *sec*$^+$ 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*$^+$ 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*$^+$ 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 (*brlA*) mutants, a regulatory locus controlling conidiophore 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*$^+$ and *sec* mutants are shown in Table 1. Four of the parental *sec*$^+$ 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 AFB_1, whereas two blocked mutants (*wh-1 ver-1 avn-1* and *wh-1 ver-1 lys-6 pdx-1*) accumulated, respectively, the AF precursors averantin and versicolorin A. In biofeeding studies with the *sec*$^+$ strains, AF production increased threefold for *SU-1*, sixfold for *br-1 nor-1 lys-6 ade-1*, threefold 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 both these nonaflatoxigenic mutants, AFB_1 was produced upon addition of ST. In sharp contrast, no measurable levels of AFB_1 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 nontoxigenic strains isolated from

FIG. 1. Scanning electron micrographs of *A. parasiticus wh-1 ver-1 lys-6 pdx-1*. (A) *sec*$^+$ (magnification, ×300); (B) *sec* (magnification, ×500); (C) *sec*$^+$ (magnification, ×1,000); (D) *sec* (magnification, ×2,000).



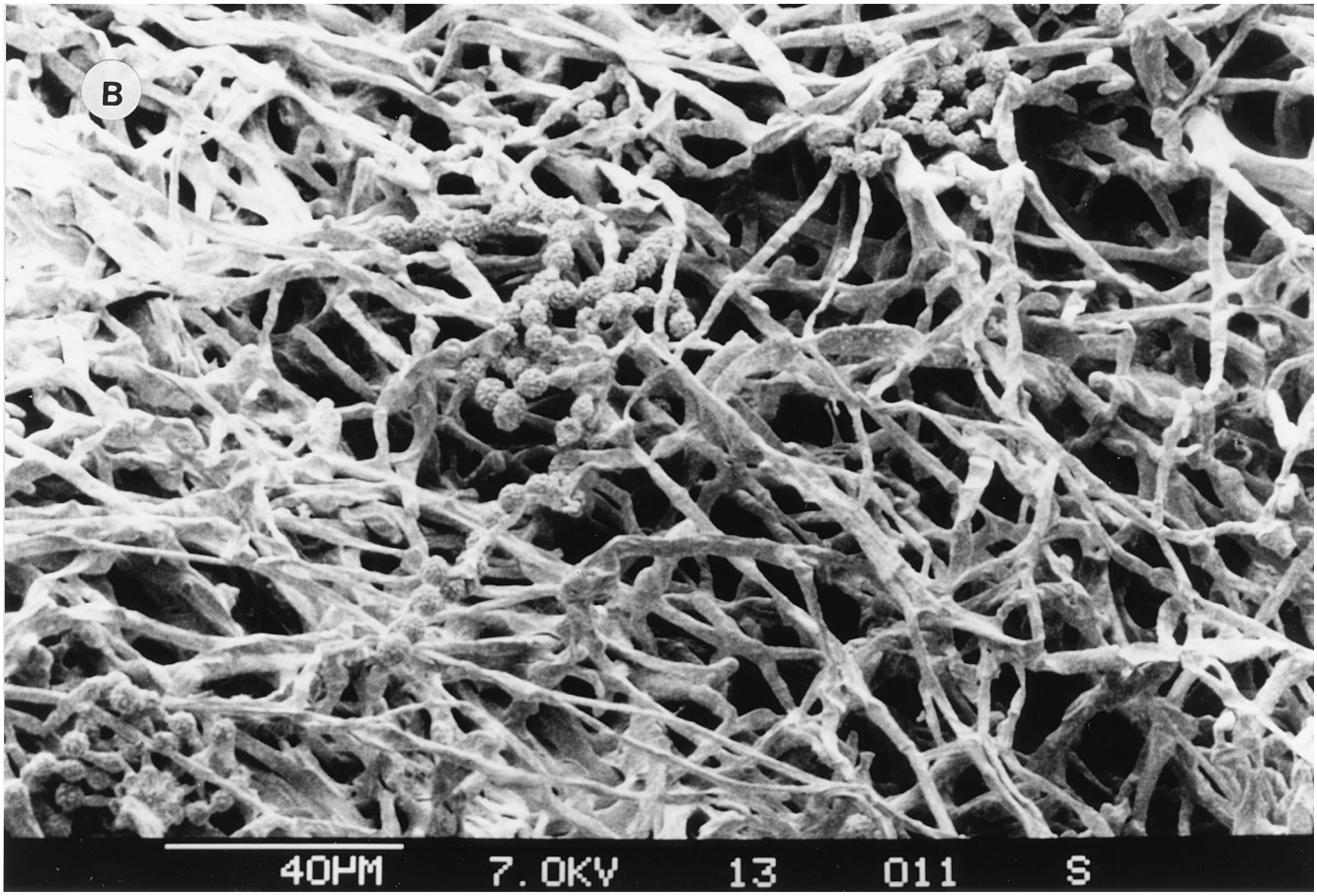
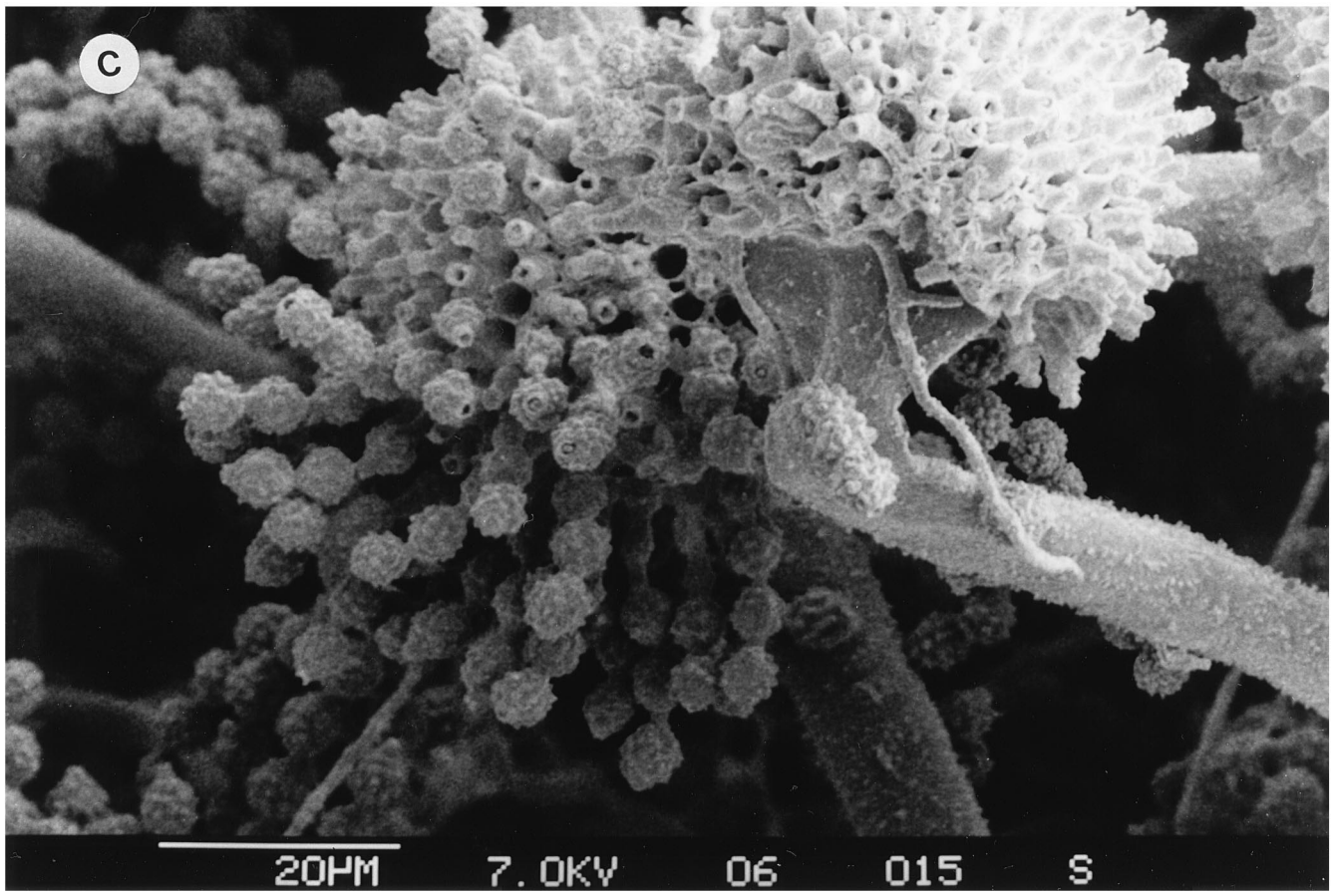


FIG. 1—*Continued.*



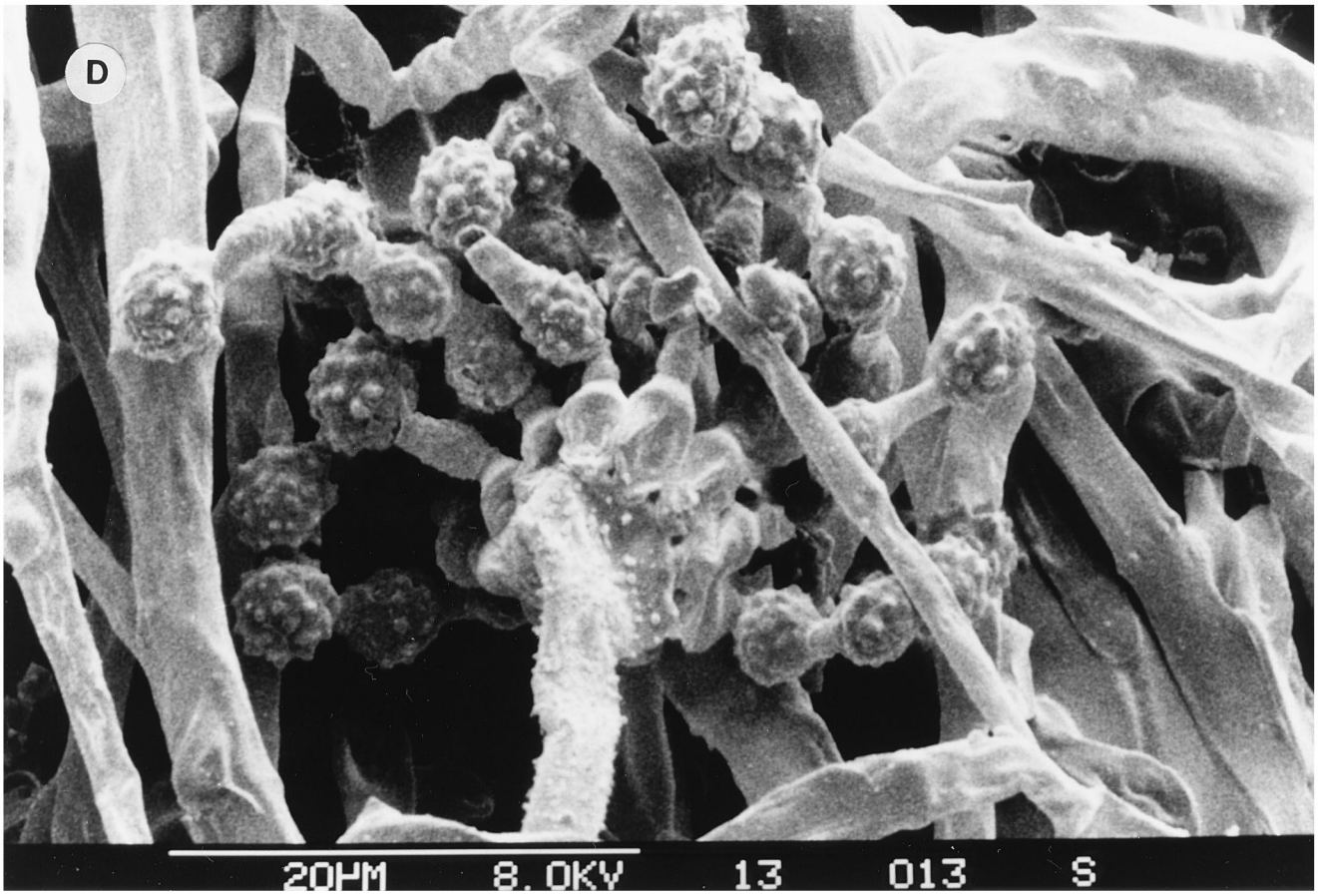


FIG. 1—*Continued.*

TABLE 1. Anthraquinones, ST, and AFB_1 recovered from *A. parasiticus sec*$^+$ and *sec* strains with or without ST

Strain	Amt of metabolite recovered (μg/g of mycelia)[a]					
	Anthraquinones[b]		ST		AFB_1	
	−ST	+ST	−ST	+ST	−ST	+ST
SU-1						
sec$^+$	ND	ND	ND[c]	Trace	3.7	11.1
sec	ND	ND	ND	6.7	ND	ND
br-1 nor-1 lys-6 ade-1						
sec$^+$	Trace (NOR)	Trace (NOR)	ND	Trace	2.8	17
sec	ND	ND	ND	6.9	ND	ND
wh-1 ver-1 lys-6 pdx-1						
sec$^+$	0.7 (VER)	1.4 (VER)	ND	Trace	ND	13.6
sec	ND	ND	ND	14.2	ND	ND
wh-1 ver-1 avn-1						
sec$^+$	180.8 (AVN)	209.2 (AVN)	ND	Trace	ND	9.5
sec	ND	ND	ND	10.7	ND	ND
br-1 pdx-1						
sec$^+$	ND	ND	ND	Trace	3.0	8.9
sec	ND	ND	ND	10.8	ND	ND
wh-1 nor-1 lys-6 ade-1						
sec$^+$	Trace (NOR)	Trace (NOR)	ND	Trace	1.2	8.8
sec	ND	ND	ND	9.0	ND	ND
Controls (no mycelia)	ND	ND	ND	13.3	ND	ND

[a] 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.
[b] Anthraquinones quantitated were norsolorinic acid (NOR), versicolorin A (VER), and averantin (AVN).
[c] ND, none detected.

nature and two strains isolated by repeated transfer on laboratory media (17). In these studies, the nontoxigenic strains isolated by transfer experiments biotransformed both ST and *O*-methyl-ST into AF whereas the nontoxigenic strains isolated from nature were biochemically similar to our *sec* strains and did not cause biotransformation. Other studies have demonstrated that some nontoxigenic strains of *A. flavus* isolated from nature do convert precursors to AF in biofeeding studies (9). Both the *sec* strains of *A. parasiticus* and the wild-type nontoxigenic strains of *A. flavus* either lack the enzymatic activity to bioconvert ST 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

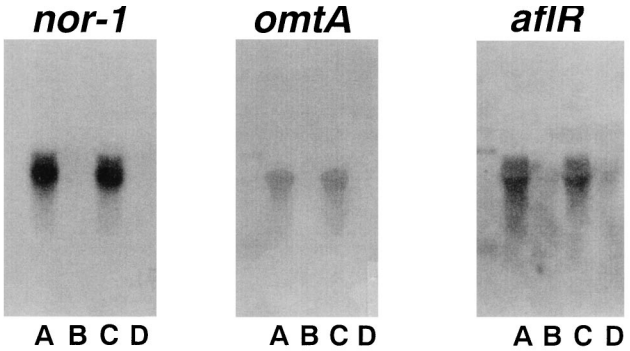


FIG. 2. Northern blot analysis of AF biosynthetic pathway transcripts from *sec*$^+$ and *sec* strains of *A. parasiticus*. Total RNA from 48-h cultures of YES-grown fungal mycelia was electrophoresed, transferred to nylon membranes, and hybridized with radiolabeled fragments of the *nor-1*, *omtA*, or *aflR* gene. RNAs analyzed are as follows: lane A, *SU-1 sec*$^+$; lane B, *SU-1 sec;* lane C, *wh-1 ver-1 lys-6 pdx-1 sec*$^+$; lane D, *wh-1 ver-1 lys-6*, *pdx-1 sec.*

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.

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