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Genetic modification of an echinocandin B-producing strain of *Aspergillus nidulans* to produce mutants blocked in sterigmatocystin biosynthesis

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SUMMARY

The production of echinocandin B (ECB), a lipopolypeptide used for chemical manufacture of the anti-*Candida* agent Cilofungin™, was accomplished by fermentation using a strain of *Aspergillus nidulans*. In addition to ECB, this fermentation also produces a significant amount of sterigmatocystin (ST), a potent carcinogen structurally related to the aflatoxins. Mutants blocked in the ST biosynthetic pathway were created by genetic modification of the polyploid production strain C747. The following steps were involved: (i) reduction of the genotype to haploid by treatment with the spindle fiber poison methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate (MBC), using colony morphology, conidia size, and the ability to obtain 5-fluoro-orotic acid (5-FOA)-resistant mutants as criteria for ploidy; (ii) mutagenesis of a haploid isolate using UV irradiation; and (iii) screening of mutants for inability to produce ST by thin layer chromatography. Six mutants blocked in ST production were isolated. All six remained capable of producing ECB equivalent in quantity to the haploid strain C747-GR14. One of the mutants was shown to be the result of a chromosomal translocation.

INTRODUCTION

In *Aspergillus flavus* and *Aspergillus parasiticus*, sterigmatocystin has been identified as an intermediate in the biosynthetic pathway for aflatoxin B1 (Fig. 1). These mycotoxins exhibit similar biological properties due to their bisfuranoid structure, and are recognized as some of the most toxic and carcinogenic compounds known [4,8,25,28,31]. In other species of *Aspergillus*, including certain strains of *A. nidulans*, sterigmatocystin accumulates and there is no evidence of further conversion to aflatoxin B1 [12]. One such strain is *A. nidulans* A42355, which produces echinocandin B (Fig. 2). ECB is a lipopeptide which may be chemically modified to produce derivatives that are of pharmaceutical interest as therapeutic agents for treating fungal infections in man [2,3,11,13,14].

The yield of ECB produced in fermentations by *A. nidulans* A42355 has been improved considerably through selection of mutants with improved productivity and optimization of fermentation parameters. However, improvement in ECB production occurred simultaneously with an increase in sterigmatocystin production. For this reason, a program was initiated to isolate mutants incapable of sterigmatocystin production, while retaining high ECB productivity. Screening methods have been developed for aflatoxin B1-producing cultures which take advantage of either the high fluorescence or UV absorption of aflatoxin B1 [9,30]. Sterigmatocystin does not have these properties and screening for blocked mutants in strains which accumulate this compound have relied upon visualization of the highly colored intermediates in colonies growing on plates. However, some strains of *Aspergillus* produce a number of colorful pigments which can interfere with this detection method. Additionally, not all of the intermediates in the biosynthetic pathway are brightly colored and easily detectable thus mutants blocked at these steps would not be identifiable. We have developed a screening method based on thin layer chromatography (TLC) detection which can be used to obtain blocked mutants in organisms that accumulate sterigmatocystin. Initial attempts to isolate such a mutant directly from strain C747 in a screening program similar to the one described in this report were unsuccessful after evaluating 17 000 isolates obtained by mutagenesis of growing germlings using nitrosoguanidine (NTG). Analysis of the intermediate strains in the lineage of strain C747 for colony morphology, conidia size, ability to obtain 5-fluoro-orotic acid-resistant mutants, and level of resistance to the spindle fiber poison methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate (MBC) suggested the failure of the first screen may have been due to a polyploid genotype for the C747 strain.

Here, we describe the isolation of sterigmatocystin-blocked mutants using a multi-step process involving: haploidization of strain C747, selection of a haploid isolate, UV mutagenesis,

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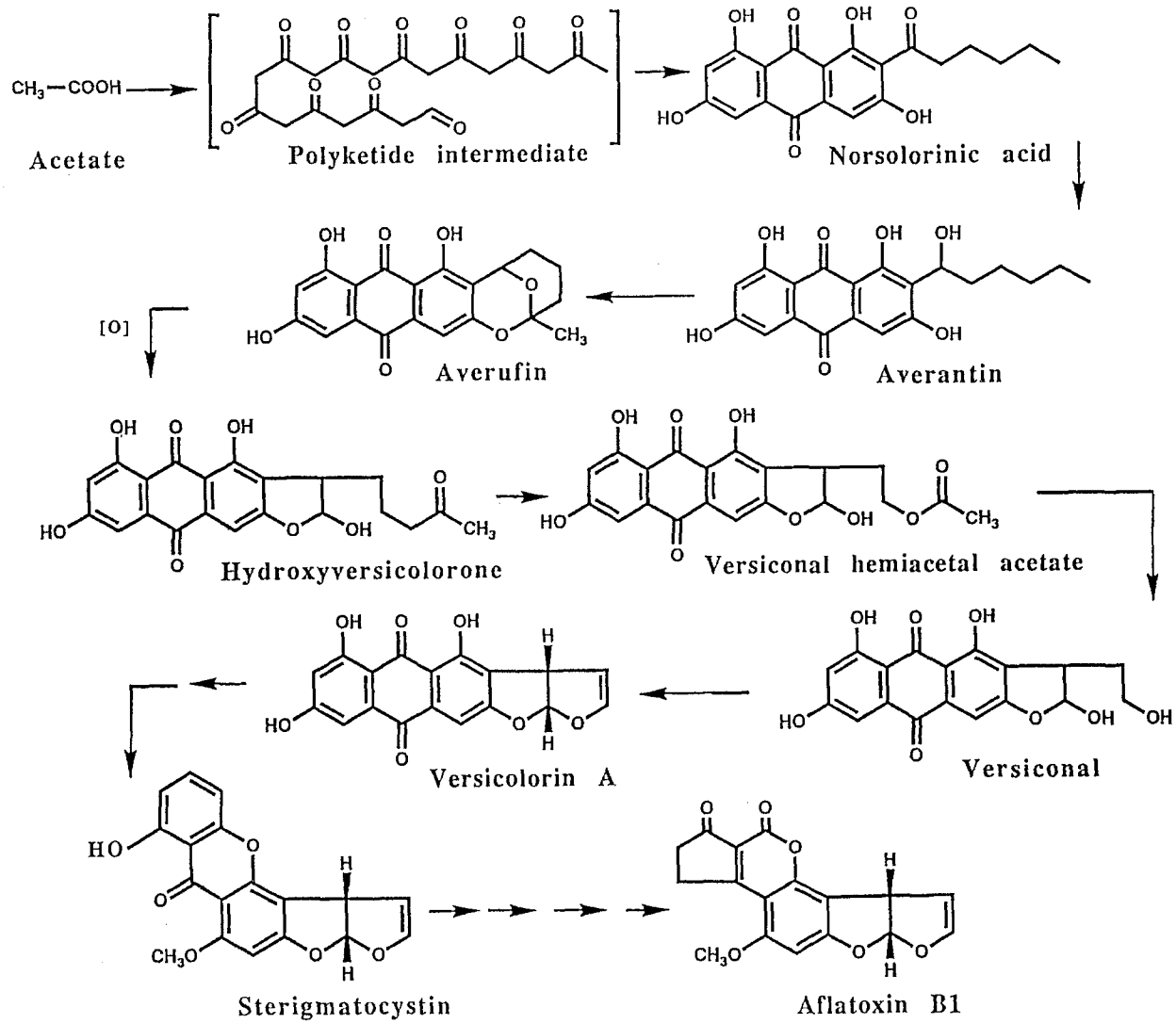

Fig. 1. Biosynthetic pathway for aflatoxin B1. Some intermediates are not shown.

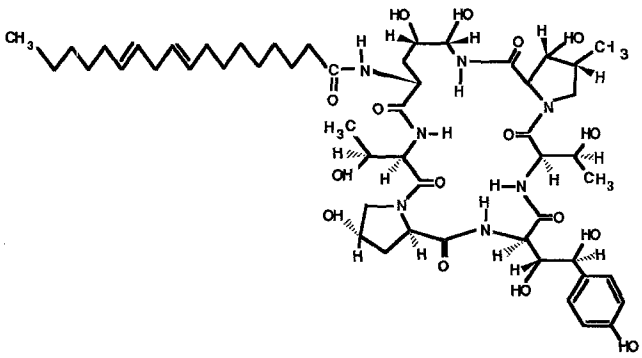

Fig. 2. The chemical structure of echinocandin B (ECB).

evaluation of isolates for sterigmatocystin synthesis by thin layer chromatography, evaluation of ECB productivity, and examination of the molecular karyotype of various mutants by CHEF gel analysis and southern hybridization.

MATERIALS AND METHODS

Strains used

The ECB-producing *Aspergillus* strain A42355.1 was a single colony isolate of the original soil isolate of *Aspergillus nidulans* (A42355, NRRL11440, ATCC 20600). Strain C747 was derived from strain A42355.1 by several rounds of UV or NTG mutagenesis and selection. Both strains were used as the controls for all experiments. *A. nidulans* strain FGSC4 and *Schizosaccharomyces pombe* were obtained from the Fungal Genetics Stock Center, Kansas City, Kansas, USA.

Fermentation conditions

ECB and sterigmatocystin production in various strains was determined under optimal production conditions for each strain. Isolated colonies of each strain were obtained following growth at 25 °C for 5 days on V8 agar plates (0.5% glucose, 0.2% yeast extract, 1.5% calcium carbonate, 20% V-8 juice, 2.0% agar). Cells from these colonies were used to start a

vegetative culture by inoculation into 10 ml of ECF-2 broth (2.25% powdered lexein, 2.1% soybean grits, 0.2% NZ-Amine A, 2.0% blackstrap molasses, 1.2% magnesium sulfate heptahydrate, 5.0% proflo oil, pH 7.0). This vegetative culture was grown at 25 °C with vigorous shaking for 1 day. A fermentation culture was started by inoculation of 10 ml ECF-2 medium with 0.5 or 1.0 ml of the vegetative culture, previously determined for each strain. The inoculated flasks were incubated at 25 °C with vigorous shaking for 5 days. Fermentation broths were extracted with 40 ml methanol and ECB and sterigmatocystin concentrations determined by HPLC as described below.

Determination of ECB concentration by HPLC

Methanol extracts of fermentation broths were allowed to settle overnight at 4 °C and then filtered through a 0.2-μm pore size cellulose acetate membrane. Analytical reverse phase HPLC was performed using a 15-cm C_{18} Nova-Pak column (Waters, Marlborough, MA, USA) at 25 °C. Injection volume was 20 μl. The mobile system employed was 45% to 54% acetonitrile in 0.04 M ammonium phosphate. The eluted peaks were detected by UV absorption at 225 nm. ECB concentrations were calculated by comparison of peak area with a standard curve.

Isolation of a haploid ECB-producing strain

A concentration gradient of MBC (0.1 μg ml^{-1} to 0.4 μg ml^{-1}) was prepared in a petri dish containing V8 agar medium and allowed to equilibrate overnight at room temperature. The gradient plate was inoculated with approximately 1×10^7 conidia from strain C747 and incubated at 30 °C for 5 days. Conidia and hyphae were removed at the leading edge of growth using a sterile inoculating loop and used to inoculate V8 agar medium without MBC to allow segregation. After growth for 5 days at 30 °C, conidia were harvested with 5 ml of 0.1% Tween-80 and filtered through a sterile C-fold paper towel. Dilutions were made of this concentrated conidial suspension and used to inoculate V8 agar plates without MBC. These plates were inoculated and incubated as above to obtain individual colonies.

Mutagenesis and analysis of 5-FOA resistance

Approximately 1×10^7 conidia of each strain were suspended in 5 ml of 0.1% Tween-80 and dispensed into a 150-mm diameter petri dish. These conidia were exposed to approximately 1100 J M^{-2} of 225 nm short wave UV light using a hand held UV lamp (Ultra-Violet Products, Inc., San Gabriel, CA, USA) at 5 cm distance for 90 s. This dose was determined to produce a reduction in viability of approximately 90% in previous experiments. Mutated conidia were plated onto modified Pontecorvo's minimal medium containing 1 mg 5-fluoro-orotic acid ml^{-1} [5,23]. Plates were incubated at 37 °C and colonies counted after 5 days.

Sterigmatocystin screen conditions

Conidia of strain C747-GR14, which were mutated to obtain 5-FOA resistant mutants, were also screened for a sterigmatocystin-blocked mutant. Dilutions of a conidial suspension of this strain were used to inoculate ECF-2 agar plates containing 0.1% Triton-X100 to reduce colony size. Plates were incubated at 37 °C for 3 days. Colony sections were transferred to a 96-well microtiter dish by stabbing each colony with a sterile straw and extruding the plug into separate wells of the dish. Methanol (150 μl) was added to each well to extract sterigmatocystin from the colony plug. Thin layer chromatography plates (TLC, Whatman LK5-D, Whatman Paper Ltd, Maidstone, UK) were spotted with 30 μl of this methanol extract. The use of the LK5-D plates allowed rapid sample application to the plate without regard for spot size using a standard pipetman. The TLC plates were developed 6–10 cm in dichloromethane : cyclohexane : methanol (90:9:1) and sterigmatocystin was visualized using short wave UV light after dipping plates in ethanol containing 10% aluminium chloride. The sterigmatocystin was easily recognized as a bright yellow fluorescing band. Blocked mutants were verified three times by repeating growth and analysis as before. Although TLC is often a tedious and time consuming analytical method, screening individual isolates for sterigmatocystin production as described was easily carried out at a rate of 500 isolates per day by one person.

Sterigmatocystin analysis of static cultures

Conidia of blocked mutants were used to inoculate 100 ml sterile ECF-2 liquid medium. Cultures were incubated at 25 °C for 12 days without shaking. The mycelial mat was removed from the surface of the broth and washed three times with 50 ml sterile distilled water. The wet mycelial mats were weighed and resuspended in 10 ml methanol. Samples were mixed vigorously and filtered through a 0.45-μm pore size filter. Samples (50 μl) were analyzed for sterigmatocystin by HPLC as described previously [19].

CHEF gel analysis

Chromosomal DNA preparations of *Aspergillus* DNA were prepared according to the method described by Brody and Carbon with minor modifications [7]. Agarose plugs containing the embedded chromosomal DNA were inserted into the wells of a 0.9% Seakem Gold™ agarose gel (FMC) and sealed with molten agarose. Pulsed field gel electrophoresis was carried out in 0.5× TBE buffer in a DRII apparatus (Bio-Rad, Richmond, CA, USA) maintained at 12 °C. Electric power was applied at 45 V with a 45-min pulse interval for 140 h, followed by 60 V with a 25-min pulse interval for 152 h. The gel was stained with ethidium bromide for 30 min and destained for 1 h prior to photographing.

Southern hybridization

The probe used was plasmid PNK10 [33] which includes a ca. 6.6-kb insert of *A. nidulans* DNA containing the *verA* gene labeled with α-P^{32} dATP. Radiolabeling and hybridization were carried out using standard methods [24].

RESULTS AND DISCUSSION

Isolation of a haploid segregant of strain C747

A mutation/random screening program has been carried out for a number of years at Eli Lilly and Company in an attempt

to isolate mutants of *Aspergillus nidulans* with an increased capacity for ECB production. Analysis of ECB yields in fermentations of the different strains isolated from mutagenesis revealed a significant increase in ECB production occurred in developing strain C747. We believe this increase may have been due, in part, to an increase in ploidy during the development of this strain as a result of mutagenesis of growing germlings with NTG [15]. Determining ploidy of these strains was difficult as no genetic markers, i.e. auxotrophies, were present. However, diploid strains of *Aspergillus* are known to have a variety of different characteristics as compared to haploid strains. These characteristics include differences in colony morphology, conidia diameter, altered resistance to the spindle fiber poison benomyl, and mutation frequency of various markers [27,32]. Comparison of these characteristics of strain C747 with the original soil isolate A42355.1 indicated an increase in ploidy occurred during the mutation/screening program. An increase in ploidy is suggested by: an increase in the size of conidia for strain C747 (Fig. 3), a change in the colony morphology for strain C747 including slower growth and poor conidiation (Fig. 4), a decrease in the number of mutants resistant to the compound 5-FOA following UV mutagenesis for strain C747 as compared to strain A42355.1, and a greater sensitivity to MBC (data not shown). These data collectively suggested an increase in ploidy occurred during development of the high ECB-yielding strain C747.

A haploid isolate of strain C747 was required for isolation of sterigmatocystin non-producing mutants for two reasons. First, haploidy was necessary to insure a reasonable mutation rate at any given locus and, second, this haploid should be derived from a high ECB-producing strain to retain mutations responsible for increased ECB production not related to the increase in ploidy. This isolate was obtained by exposure of strain C747 to MBC, a compound similar to benomyl which is known to induce segregation in diploid strains of *Aspergillus* [16]. Growth of this strain on a plate containing a gradient of the spindle fiber poison MBC exhibited a marked decrease in survival at a concentration between 0.1 $\mu g\ ml^{-1}$ and

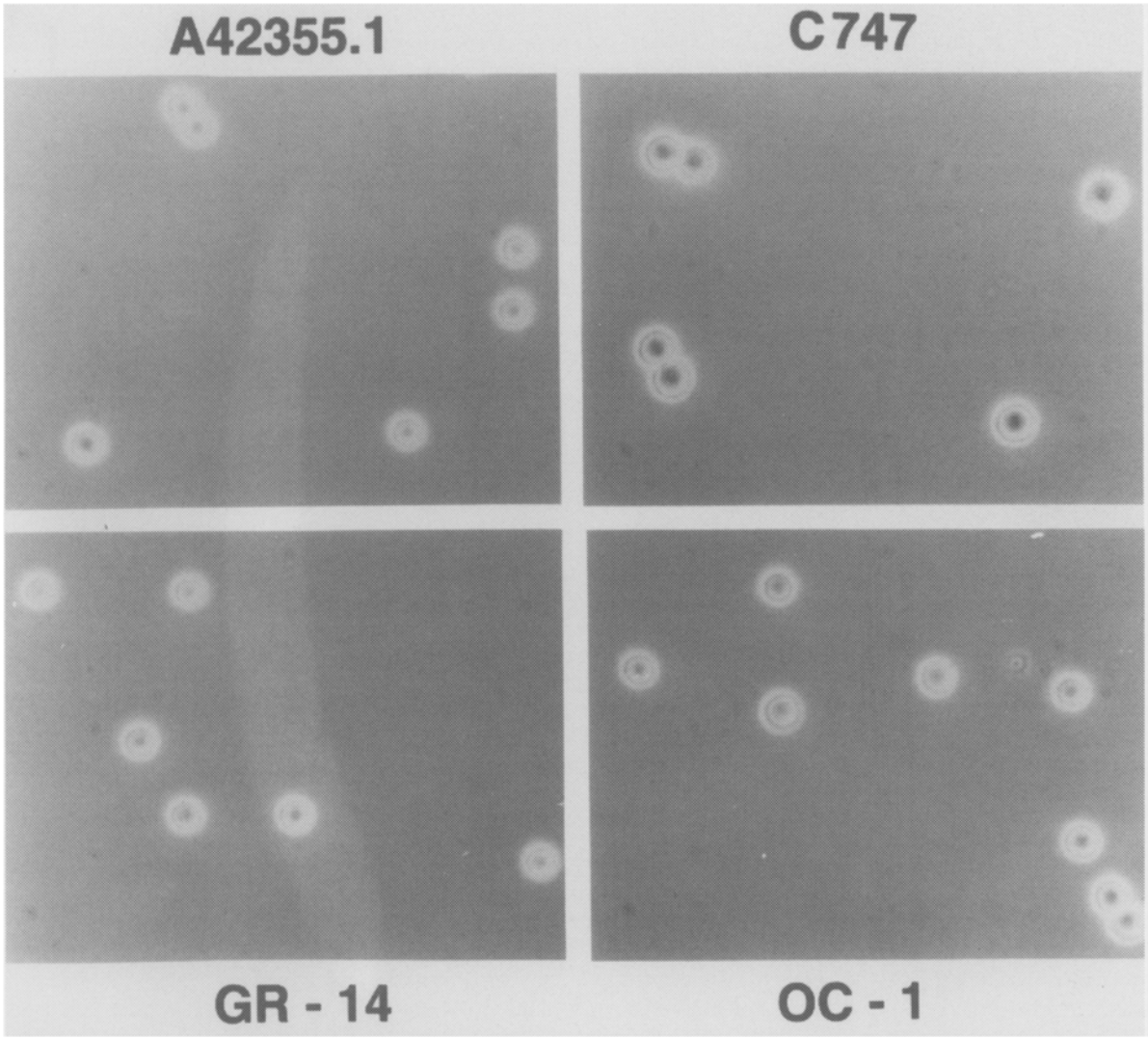

Fig. 3. Comparison of conidial diameter of different strains of *A. nidulans* at 1000× magnification. Top left, A42355.1; top right, C747; bottom left, C747-GR14; bottom right, OC-1.

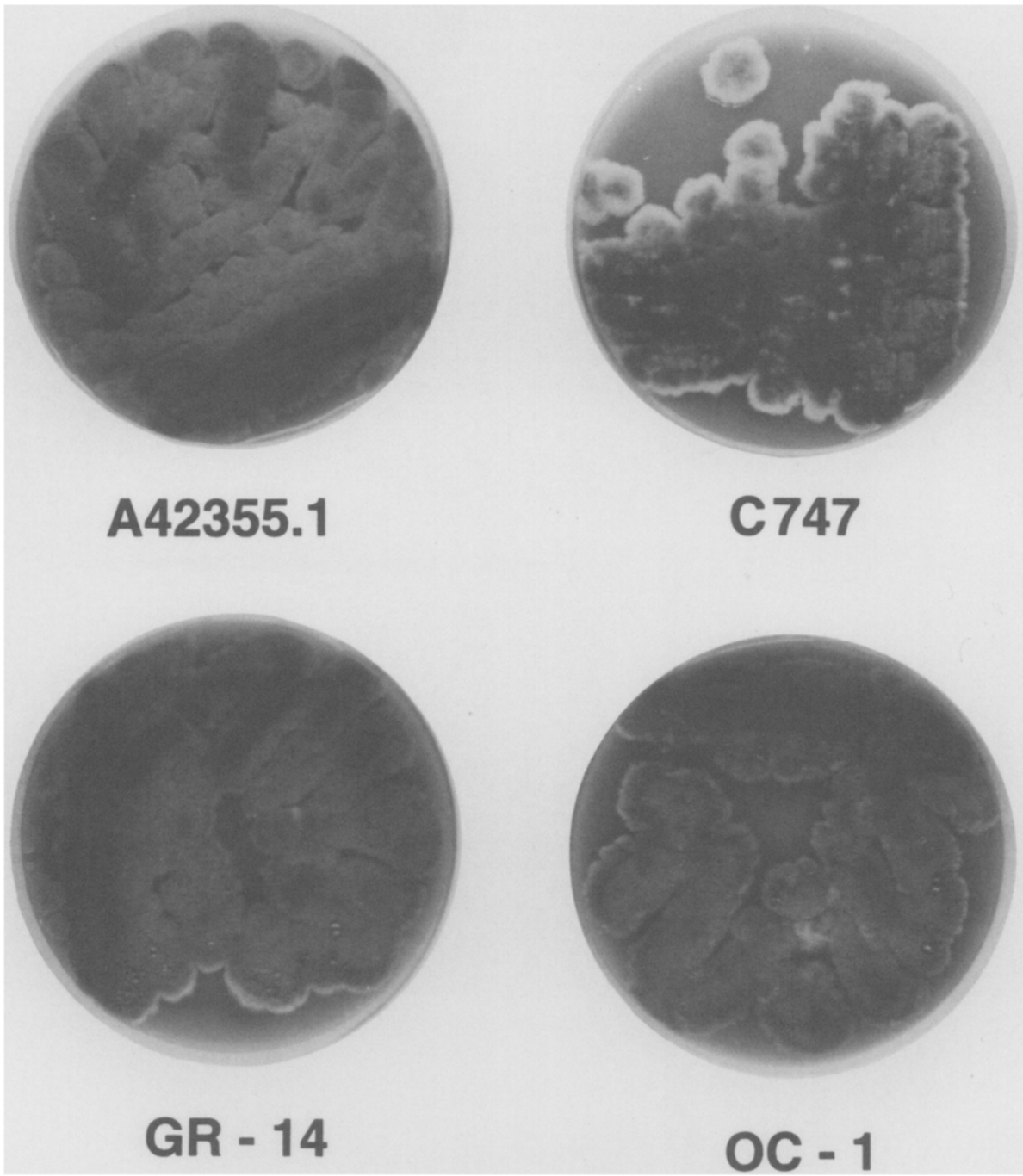

Fig. 4. Colony morphology of different strains of *A. nidulans* on V8 agar plates. Top left, A42355.1; top right, C747; bottom left, C747-GR14; bottom right, OC-1.

0.4 μg ml^{-1} (Fig. 5). Since haploids are normally more resistant to this compound than diploids, colonies were obtained from the leading edge of growth on this plate as described in Materials and Methods. Analysis of several isolates indicated that this procedure generated haploid clones as illustrated by one particular isolate, C747-GR14. Haploidy was indicated by the smaller conidia (Fig. 3), faster growth rate, more efficient conidiation (Fig. 4), and higher number of mutants obtained which were resistant to 5-FOA following mutation by UV light as compared to strain C747. Organisms which are normally sensitive to 5-FOA can become resistant as a result of mutations in the gene coding for orotidine decarboxylase (*pyr*G). Mutants resistant to 5-FOA were readily obtained for strains C747-GR14 and A42355.1. In contrast, strain C747 failed to produce any 5-FOA resistant mutants in three separate experiments providing further evidence for a state of polyploidy in strain C747. Based on the results of these experiments, strain C747-GR14 was considered to be haploid and mutants of this strain were subsequently screened for a mutant blocked in sterigmatocystin biosynthesis. Although we refer

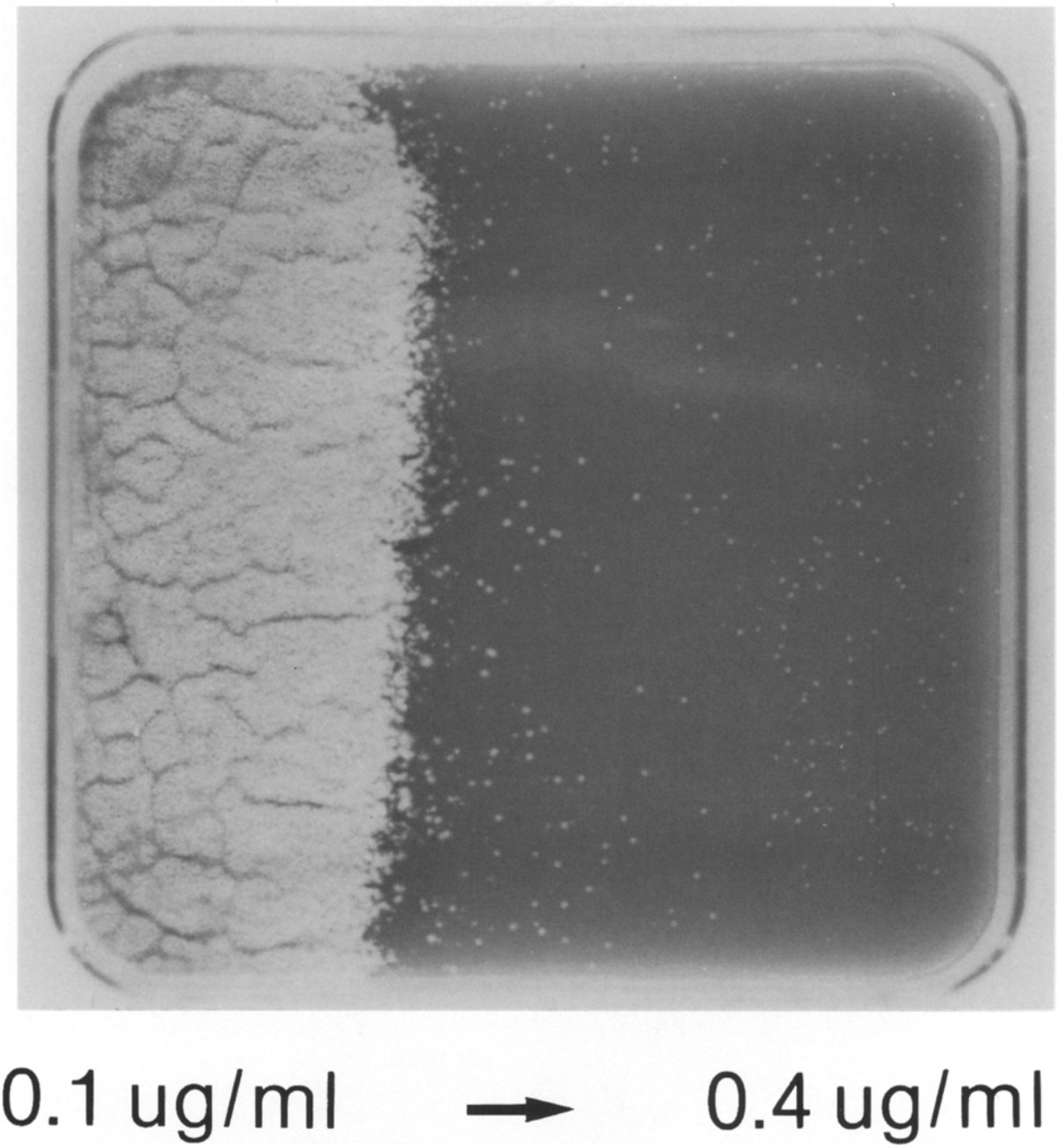

Fig. 5. Growth of strain C747 on an MBC gradient plate.

to the strains as polyploid or haploid in this report, it must be noted that the number of nuclei per conidia has not been determined and no genetic markers are available in these strains to positively determine the actual ploidy for each strain. Therefore, although available evidence suggested that strain C747-GR14 is haploid, we cannot completely rule out the possibility of aneuploidy.

Isolation of a sterigmatocystin non-producing mutant

Conidia mutagenized by UV light to obtain 5-FOA resistant mutants were also screened for mutants blocked in ST production. A typical analysis of 19 isolates is illustrated (Fig. 6). The arrow indicates the location of ST, which appeared as the only band fluorescing bright yellow. As little as 10 ng of ST could be detected in this system. Approximately 9000 isolates were screened and six mutant strains were isolated that were incapable of producing ST. These strains were designated TPB-19, OC-1, OC-2, 1329-4, 1851-2, and 0018-3. Since most of the intermediates in the aflatoxin biosynthetic pathway can be resolved by thin layer chromatography, this type of screen would also be suitable for isolating mutants blocked in each of the steps of the pathway.

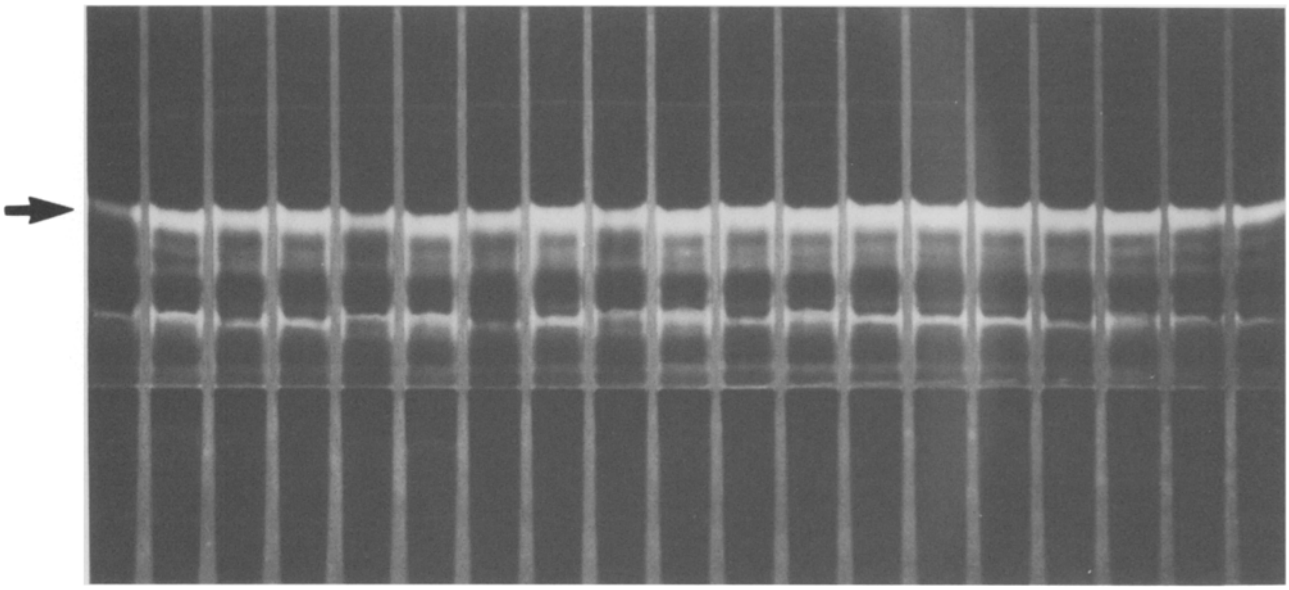

Fig. 6. TLC plate for sterigmatocystin screen. Each lane shows the ST production by an individual colony. The arrow identifies the location of ST, which is the only bright yellow fluorescing band visible.

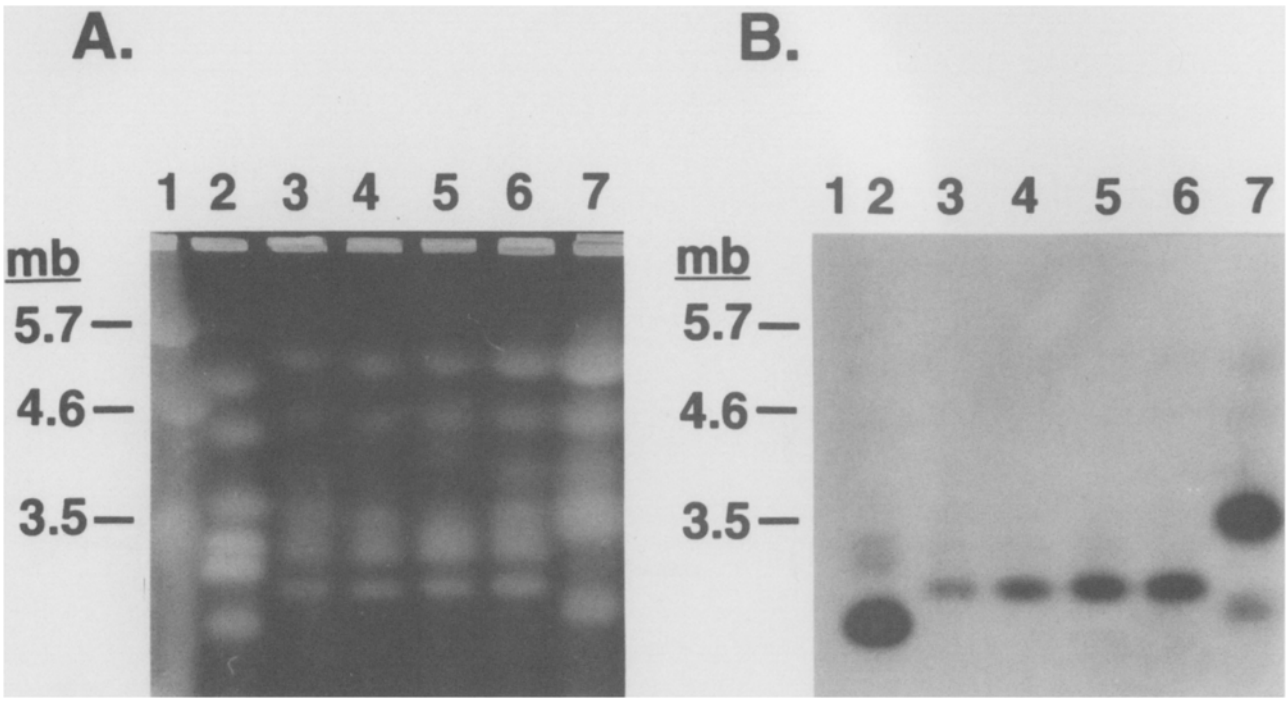

Fig. 7. Karyotype and hybridization analysis of different *A. nidulans* strains. (A) Photograph of ethidium bromide-stained CHEF gel. Lane 1, molecular size markers for *S. pombe*. Lane 2, strain FGSC4. Lane 3, strain A42355 original isolate. Lane 4, strain A42355.1. Lane 5, strain C747. Lane 6, strain C747-GR14. Lane 7, strain OC-1. (B) Autoradiograph of gel after hybridization with the plasmid pNK10 containing the *verA* gene.

Analysis of sterigmatocystin-blocked mutants

Strains isolated in the ST screen were analyzed for changes in growth characteristics, ECB production, and ST production. Comparison of growth characteristics for the ST-blocked mutants with the strains A42355.1, C747, and C747-GR14 indicated they were similar to the putative haploids C747-GR14 and A42355.1, as illustrated by isolate OC-1, with respect to colony morphology and conidia size (Figs 3 and 4). This data suggested no change in ploidy occurred as a result of the UV mutagenesis procedure used to isolate the ST-blocked mutants. The isolated strains retain the rapid growth rate, efficient conidiation, and reduced conidia size seen in the strain C747-GR14.

Mutants deficient in ST biosynthesis were further charac-

terized for ability to produce ECB. This ability was of significant importance because inability of the isolates to produce ST could be due to a reduction or block of all secondary metabolic processes. If this were the case, it is likely that such a mutation would significantly lower or block production of ECB as well. ECB production by these strains is summarized in Table 1. All six ST-blocked mutants isolated have been tested for ECB production. Each produced as much ECB as strain C747-GR14. These data indicate the mutations that caused an absence of ST formation was not due to a mutation which abolished secondary metabolism in general. It should be noted that strain C747-GR14 and the ST-blocked mutants obtained are not capable of producing as much ECB as strain C747, presumably as a result of the reduction in ploidy. An increase in the ploidy of the strain by, for example, polyethylene glycol induced fusion, may restore ECB production to yields equivalent to strain C747. Although we have not carried out this type of experiment, numerous examples already exist for selection of strains with increased ploidy [1,17,18,20,21,26].

Analysis of ST production in the mutant strains was performed under various growth conditions. This strategy was important because the isolated strains may contain mutations in regions involved with regulation of ST production and, thus, may only remain blocked during growth under specific conditions. All strains isolated which failed to produce ST under screening conditions were tested for sterigmatocystin production under fermentation conditions, using a 10% and a 5% inoculum as well as static growth conditions. Results are summarized in Table 2. Two inoculum amounts were tested in fermentation studies due to a difference in growth rate between strain C747 and the isolated haploid mutants. Analysis of ST production in these mutants demonstrated that only isolates OC-1 and OC-2 failed to produce any detectable ST under all conditions tested. These isolates were likely the result of a mutation in one of the genes in the biosynthetic pathway unique to ST production. The remaining isolates were not completely blocked in ST production under all conditions tested although a significant reduction in ST production was evident. Of particular interest was isolate 1851-2. Although this mutant produced a significantly low amount of ST during dynamic growth conditions, it was capable of producing an amount of ST almost equivalent to the control under static growth conditions. These data indicated the reduction of ST production in this mutant was probably the result of a mutation in either a regulatory or dispensable gene. Since isolates OC-1 and OC-2 appear to be completely blocked under all conditions tested, these mutants were probably the result of loss of function of unique and indispensable genes.

TABLE 1

ECB production by mutants blocked in ST biosynthesis

Strain	ECB yield (%)[a]
A42355.1	30
C747	100
GR-14	28
TPB-19	21
OC-1	21
OC-2	22
1329-4	28
1851-2	28
0018-3	26

[a] ECB yield is relative to the amount produced by strain C747. The average error is approximately 1.5%.

TABLE 2

Analysis of blocked mutants for ST production

Strain	Sterigmatocystin level		
	Fermentation culture[a]		Static culture[b]
	10% inoc.	5% inoc.	
A42355.1	97.5	62.0	588
C747	ND[c]	38.0	6979
GR-14	ND[c]	59.0	548
TPB-19	2.1	15.0	111
OC-1	<0.5	<0.5	<75
OC-2	<0.5	<0.5	<75
1329-4	1.1	<0.5	<75
1851-2	ND[c]	0.6	471
0018-3	ND[c]	1.6	<75

[a] $\mu g\ ml^{-1}$ of ST in fermentation broth culture. Detection limit = $0.5\ \mu g\ ml^{-1}$.
[b] ng ST g^{-1} mycelia (wet weight). Detection limit = 75 ng g^{-1}.
[c] ND, not done.

Molecular karyotypes were determined for *A. nidulans* A42355 and mutants derived from it by pulsed field gel electrophoresis (Fig. 7(A)). The karyotype of FGSC4 was significantly different as compared to that of A42355. Furthermore, mutants derived from A42355 exhibited what appear to be mutagenesis-induced alterations in karyotype. Genomic architectural rearrangements appeared to range from size alterations of chromosomes (in particular note the smallest chromosome of strain OC-1) to apparent chromosomal loss (note the absence of the ca. 3 Mb chromosome). A fragment of DNA approximately 10.5 kb in length, containing the *verA* gene of the ST biosynthetic pathway (from strain FGSC4) was used as a hybridization probe to search for ST biosynthetic pathway anomalies. The *ver*A gene probe hybridized to the smallest chromosome of all strains analyzed. However, it hybridized to two chromosomes in strain OC-1, the smallest chromosome and a larger novel one of approximately 3.5 Mb. The radioactive signal from the smallest chromosome was relatively weak as compared to the signal from the larger hybridizing chromosome in OC-1. Additional hybridization studies suggested that probes containing the region downstream of the *verA* gene hybridized only to the 3.5 Mb chromosome, while probes containing the upstream region hybridized to the smallest chromosome in OC-1 (personal communication, N. Keller). These data suggested that the block in ST production was due to a chromosomal translocation that occurred within or near the *verA* gene or perhaps within a cluster of genes involved in

ST biosynthesis. HPLC analysis of fermentation extracts from strain OC-1 revealed significant accumulation of norsolorinic acid, averantin, versiconal acetate, and versiconal hemiacetal acetate while no accumulation of versicolorin A or sterigmatocystin was evident.

The screen for sterigmatocystin-blocked mutants described in this report may have other industrial applications, since a number of *Aspergillus* species are used commercially and may produce either sterigmatocystin or other related compounds [6,10,22,29]. This type of screen may also prove useful in efforts to isolate mutants blocked at various enzymatic steps in the aflatoxin biosynthetic pathway, as part of a program to accomplish the molecular cloning of structural genes. Such genes would potentially be useful, both for determining whether *Aspergillus* strains are genetically capable of synthesizing aflatoxins, or for the genetic engineering of strains that are irreversibly blocked in aflatoxin synthesis by gene disruption.

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