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Transformation of *Aspergillus parasiticus* with a Homologous Gene (*pyrG*) Involved in Pyrimidine Biosynthesis

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The lack of efficient transformation methods for aflatoxigenic *Aspergillus parasiticus* has been a major constraint for the study of aflatoxin biosynthesis at the genetic level. A transformation system with efficiencies of 30 to 50 stable transformants per μg of DNA was developed for *A. parasiticus* by using the homologous *pyrG* gene. The *pyrG* gene from *A. parasiticus* was isolated by in situ plaque hybridization of a lambda genomic DNA library. Uridine auxotrophs of *A. parasiticus* ATCC 36537, a mutant blocked in aflatoxin biosynthesis, were isolated by selection on 5-fluoroorotic acid following nitrosoguanidine mutagenesis. Isolates with mutations in the *pyrG* gene resulting in elimination of orotidine monophosphate (OMP) decarboxylase activity were detected by assaying cell extracts for their ability to convert [^{14}C]OMP to [^{14}C]UMP. Transformation of *A. parasiticus pyrG* protoplasts with the homologous *pyrG* gene restored the fungal cells to prototrophy. Enzymatic analysis of cell extracts of transformant clones demonstrated that these extracts had the ability to convert [^{14}C]OMP to [^{14}C]UMP. Southern analysis of DNA purified from transformant clones indicated that both pUC19 vector sequences and *pyrG* sequences were integrated into the genome. The development of this *pyrG* transformation system should allow cloning of the aflatoxin-biosynthetic genes, which will be useful in studying the regulation of aflatoxin biosynthesis and may ultimately provide a means for controlling aflatoxin production in the field.

Aflatoxins are a group of fungal secondary metabolites that are produced by the filamentous fungi *Aspergillus parasiticus* and *Aspergillus flavus* and are known to be extremely powerful animal hepatocarcinogens (3, 24). They are commonly found as contaminants in corn, peanuts, and cottonseeds (18) and thus force strict inspection of these commodities due to lack of effective preharvest and storage control measures. Since their discovery in 1960, much has been learned about the synthesis of alflatoxins, including a proposed biosynthetic pathway (7, 27) and identification and purification of several enzymes (4). However, no information has been obtained yet regarding the molecular mechanisms which regulate aflatoxin production. This void is due mainly to inefficient methods of genetic manipulation in the aflatoxigenic fungi and the lack of efficient transformation systems.

Isolation of the biosynthetic aflatoxin genes should permit elucidation of the molecular control mechanisms involved and possibly lead to field approaches for preventing aflatoxin contamination. If the genetic regulation of aflatoxin biosynthesis is more fully understood, it may be possible to prevent synthesis of this toxin through repression of gene expression. Cloned aflatoxin-biosynthetic genes may also be useful for development of non-aflatoxigenic strains which could be used for competitive exclusion of aflatoxin-producing strains in the field without fear of reversion (8, 9, 15). In addition, obtaining the genes for aflatoxin biosynthesis may provide a direct and efficient method of purifying enzymes involved in the aflatoxin-biosynthetic pathway by allowing workers to make antibodies against peptides that are derived from the nucleotide sequence and can be used in affinity chromatography for isolation of the active enzyme. This approach may enable workers to overcome the difficulties associated with enzyme instability that are commonly encountered in isolation of the aflatoxin-biosynthetic enzymes (14).

We chose to develop a transformation system in *A. parasiticus* because of the availability of several stable mutants which have mutations that block unique conversion steps in the proposed scheme for aflatoxin biosynthesis. The intermediates which accumulate in these blocked mutants have been shown to be direct precursors of aflatoxin B_1 (2, 12, 13, 19, 20, 25). It is believed that these block points in the pathway are results of mutations in genes that encode either regulatory proteins or biosynthetic enzymes and that aflatoxin production can be restored by complementation of the aflatoxin-blocked mutants, using a functional gene contained within a cosmid library.

Methods involving transformation systems in which genetic complementation is used have proven to be effective methods for isolating desired genes in filamentous fungi (16, 22). Of particular interest was the ability to demonstrate complementation of uracil auxotrophic mutants of *A. flavus* with the *pyr-4* gene from *Aspergillus nidulans* (26). This heterologous transformation system is used to screen for complementation of aflatoxin-deficient mutants of *A. flavus* (23). A concerted study of aflatoxin-biosynthetic genes in *A. parasiticus* and *A. flavus* and related genes in other fungal species should provide interesting insights into the evolution and regulation of this metabolic pathway.

In this paper we describe the isolation and characterization of uridine auxotrophic mutants of *A. parasiticus* and the development of a transformation system in which we used the homologous *pyrG* gene, which was cloned from an *A. parasiticus* genomic library. This transformation system is the first essential step in cloning aflatoxin-biosynthetic genes, which may provide the tools to prevent preharvest contamination of commodities used in human and animal food.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. *Escherichia coli* HB101 [*hsdS20* (r_B^- m_b^-) *recA13 ara-14 proA2 lacY1 galK2*

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rpsL20 Sm^r *xyl-5 mtl-1 supE44*] was used for propagating plasmids. *A. parasiticus* ATCC 36537 (*afl* [versicolorin accumulating]), which was kindly provided by Joan Bennett, was the parent strain used to develop mutant strains with mutations in uridine synthesis. *A. nidulans* A722 (*pabaA1 pyr*G89 *fwA1 uaY9*) was obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, and was used as a negative control in enzymatic studies of *A. parasiticus pyr* mutants since the *pyrG* gene encoding orotidine monophosphate (OMP) decarboxylase activity has been mutated in this strain.

Plasmid pPL6 (kindly provided by Berl Oakley) is a 4.25-kb plasmid which contains the *pyrG* gene from *A. nidulans* FGSC4 (21) on a 1.5-kb DNA fragment cloned into the blunt-ended *Nde*I site of pUC19 (personal communication).

Fungal strains were maintained on potato dextrose agar or Czapek-Dox (CZ) agar, which was used as a defined medium. Coconut agar medium was used for detection of aflatoxins by visualization of blue fluorescence under UV light (10). CZ agar was supplemented with uridine (100 μg/ml) for growth of auxotrophs. Cultures were incubated at 32°C unless otherwise indicated.

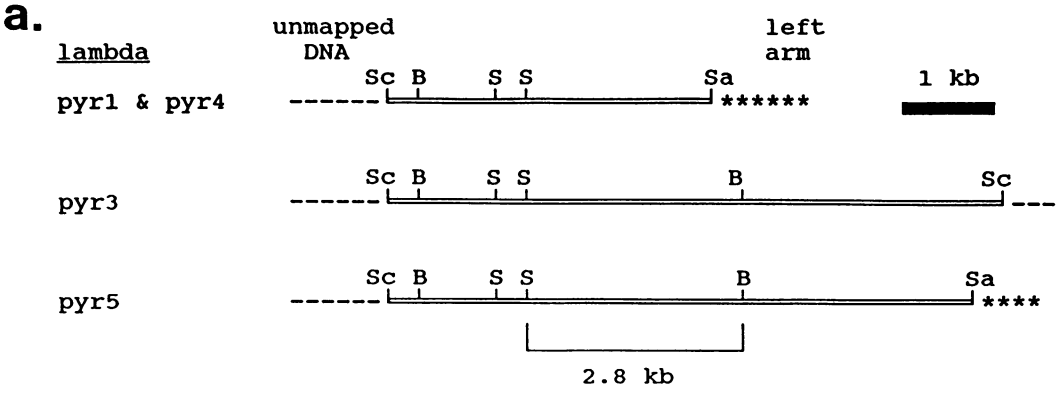
Chemicals. Chemicals whose supplier is not indicated below were purchased from Sigma Chemical Co. Potato dextrose agar and CZ agar were purchased from Difco Laboratories, Detroit, Mich.

Isolation of *pyr* mutant strains. A total of 2×10^8 conidiospores from versicolorin-A-accumulating strains *A. parasiticus* ATCC 36537 were suspended in 1 ml of Tris maleate buffer (50 mM Tris maleate [pH 7.3], 35 mM $NaNO_3$, 7 mM KCl, 4 mM $MgSO_4$, 65 μM $FeSO_4$) and treated with 170 ng of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml for 1.75 h at room temperature. Conidia were washed, suspended in potato dextrose broth containing 2.4 mg of uridine per ml, and grown in a stationary culture at 30°C for 2 h before they were inoculated onto potato dextrose agar containing 2 mg of 5-fluoroorotic acid (FOA) per ml and a high concentration of uridine (2.4 mg/ml). FOA is lethal to uridine prototrophs of *A. parasiticus*, presumably due to the enzymatic synthesis of the toxic intermediate 5-fluoro-UMP (5). The enzymes orotate phosphoribosyltransferase and OMP decarboxylase are involved in the conversion of orotate to OMP and in the conversion of OMP to UMP, respectively. Mutations in either the *pyrF* gene (orotate phosphoribosyltransferase) or the *pyrG* gene (OMP decarboxylase) resulting in a loss of enzymatic activity confer resistance to FOA by preventing the production of 5-fluoro-UMP. Colonies that were resistant to FOA were also tested for stable uridine auxotrophy by serially replica plating them onto CZ agar with and without uridine.

Enzymatic analysis of *pyr* mutants. Since FOA selects for either *pyrF* or *pyrG* mutant strains, enzyme analysis with radiolabeled substrates was used to determine the ability of auxotrophic isolates to convert orotic acid to OMP (*pyrF*) and the ability of isolates to convert OMP to UMP (*pyrG*), thereby differentiating between the two mutant types. The protocol which we used was a modification of the method of Diez et al. (11). A total of 10^7 conidia from uridine auxotrophs were inoculated into 100 ml of potato dextrose broth containing uridine and were grown for 48 h with vigorous shaking at 30°C. Mycelium was harvested by filtration, frozen in liquid nitrogen, and ground to a fine powder in a precooled mortar and pestle. Powdered mycelium was suspended in enzyme buffer (25 mM Tris chloride [pH 8.0], 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol) at a concentration of 1 g of mycelium per ml of buffer. This suspension was centrifuged at 4°C in a microcentrifuge for 2 min at 16,000 × *g*. The supernatant was transferred to a new tube and centrifuged under the same conditions for 15 min. The supernatant was removed, and 8.5-μl portions were dispensed into two prechilled microcentrifuge tubes. The remaining supernatant was used to confirm that the protein concentration was between 0.5 and 1.0 mg of protein per ml by using the Bradford technique (6). The standard curve for the Bradford method was determined by using bovine serum albumin dissolved in enzyme buffer (see above) since both Tris chloride and 2-mercaptoethanol can affect the results of the protein determination.

[6-^{14}C]OMP was prepared as described by Diez et al. (11), using the enzymatic conversion of [6-^{14}C]orotate (50 mCi/mmol; ICN) with commercial yeast OMP pyrophosphorylase (orotate phosphoribosyltransferase [Sigma]) and 5-phosphoribosyl-1-pyrophosphate. To the first tube containing the crude cell enzyme extract, 3.5 μl of prepared OMP was added; this was followed by the addition of 0.5 μl of 0.2 M sodium arsenate. To the second tube, 2.5 μl of buffer containing 1.5 mM [6-^{14}C]orotate, 25 mM Tris chloride (pH 8.0), and 8 mM $MgCl_2$, as well as 1 μl of 5-phosphoribosyl-1-pyrophosphate (5 mg/ml; made fresh in chilled water), were added. Both tubes were incubated for 2 h at 30°C; then the proteins were precipitated by adding 6 μl of methanol, and the preparations were stored overnight at −20°C. The tubes were centrifuged for 3 min at 16,000 × *g* to remove the precipitated proteins, and 5 μl of each supernatant was spotted onto a polyethyleneimine-cellulose thin-layer chromatography (TLC) plate (20 by 20 cm; Sigma). Visual standards (observed under UV light) and ^{14}C-labeled standards of orotic acid, OMP, and UMP were used as controls for determining the location and identity of each compound in enzyme reactions. Samples were separated by using 0.75 M Tris chloride (pH 8.0) as the solvent, and the dried TLC plates were then analyzed by autoradiography by exposing the plates to Kodak XAR5 film for 48 h at −70°C.

Transformation of fungal protoplasts. Plasmid DNA used for transformation was purified by CsCl density gradient centrifugation (21) and was suspended in TE (10 mM Tris chloride (pH 8.0), 1 mM EDTA) at concentrations of approximately 0.2 μg of DNA per μl. Linearized pPG3J DNA (10.8 kb), which was used for transformation, was prepared by *Eco*RI restriction endonuclease digestion (Fig. 1b). The polyethylene glycol transformation procedure described by Oakley et al. (22) was performed by using the modifications described below. Instead of using swollen conidia as suggested by Oakley et al., we grew conidiospores for 12 to 14 h in 2% yeast extract–6% sucrose medium (pH 5.5) supplemented with 100 μg of uridine per ml. Harvested mycelium was digested with Novozyme 234 (Sigma) and β-glucuronidase (Sigma) as described by Oakley et al., but Driselase was eliminated from the protoplasting solution and the enzymatic incubation time was decreased from 5 to 3 h. The protoplasts were separated from the mycelium by passing the cell suspension (using gravity) through a nylon mesh filter (pore size, 60 μm; Nytex; Tetko Corp., Switzerland), which allowed only protoplasts and small mycelial fragments to pass through. The protoplasts were then harvested, washed, and treated with DNA as described by Oakley et al., and then the protoplasts were spread directly onto CZ agar containing 0.6 M KCl. The protoplast preparations were always divided into two equal portions; one of these was treated with DNA (2 to 3 μg), and the other was left untreated to serve as a control.



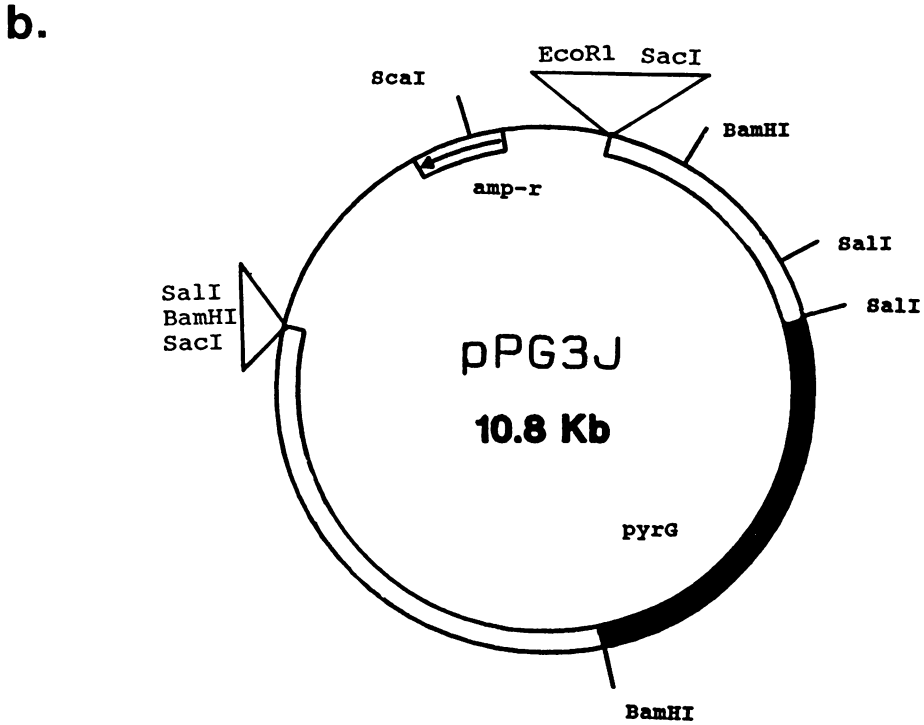


FIG. 1. (a) Restriction endonuclease analysis of inserted DNA fragments from four clones of an *A. parasiticus* NRRL 5862 lambda genomic DNA library which hybridized to the *A. nidulans* FGSC4 *pyrG* probe. The 2.8-kb *Sal*I-*Bam*HI fragment represents the region of probe binding. Restriction endonuclease sites: Sc, *Sac*I; B, *Bam*HI; S, *Sal*I; Sa, *Sau*3A. (b) Restriction endonuclease analysis of plasmid pPG3J (10.8 kb), which was used for transformation of uridine auxotrophs. The 8.1-kb *Sac*I fragment from lambda isolate pyr3 was ligated into the *Sac*I (*Sst*I) restriction endonuclease site of the polylinker region of pUC19. Only the enzymes listed on the restriction endonuclease map were tested. Additional sites in the polylinker region still remain. The solid box indicates the 2.8-kb region from *A. parasiticus* which hybridized to the *pyrG* gene from *A. nidulans* FGSC4, while the open boxes indicate flanking DNA.

Isolation and analysis of genomic DNA from fungal cells. Genomic DNA was isolated from fungal mycelium by using a phenol-chloroform protocol for mammalian DNA isolation (1). Restriction enzymes were purchased from Bethesda Research Laboratories, Inc., and were used according to the instructions of the supplier. Southern hybridization analysis (21) was performed with [^{32}P]DNA probes that were generated by using the random primer procedure (Random Primed DNA Labeling Kit; Boehringer Mannheim Biochemicals). Nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) were hybridized with labeled probes (10^6 cpm/ml) overnight at 37°C in a solution containing 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate), 5× Denhardt solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 40% formamide, 0.1% sodium dodecyl sulfate, 5 mM EDTA, and 100 μg of denatured salmon sperm DNA per ml. Following hybridization, the filters were washed twice in 2× SSC–0.1% sodium dodecyl sulfate at 37°C for 20 min and then in 0.2× SSC–0.1% sodium dodecyl sulfate at 65°C for 20 min. The filters were exposed to Kodak XAR5 film for 20 h at −70°C with an intensifying screen (Lightning Plus; Dupont).

RESULTS

Isolation of the *pyrG* gene from *A. parasiticus* ATCC 36537. Southern analysis of *Eco*RI-digested genomic DNA from *A. parasiticus* NRRL 5862 revealed a single band when the DNA was hybridized to a 1.5-kb *Hin*dIII restriction endonuclease fragment containing the *pyrG* gene from *A. nidulans* FGSC4 (22). The *pyrG* gene from *A. parasiticus* NRRL 5862 was then isolated by in situ plaque hybridization (21) of a lambda genomic DNA library (17), using the *pyrG* gene from *A. nidulans* FGSC4 as a probe. We obtained four isolates (frequency, 2×10^{-4} clones per lambda plaque) that contained DNA insertions which hybridized strongly to the *A. nidulans pyrG* probe (Fig. 1a). An 8.1-kb *Sac*I fragment which hybridized to the *A. nidulans* probe was purified from one representative phage isolate (lambda pyr3) which appeared to contain the hybridizing regions within the middle region of the *A. parasiticus* DNA insertion. This *Sac*I (*Sst*I) fragment was then subcloned into pUC19 to facilitate restriction endonuclease analysis. The resulting 10.8-kb plasmid was designated pPG3J (Fig. 1b). All of the phage isolates contained overlapping DNA fragments, with the region hybridizing to the *pyrG* probe located on a common 2.8-kb *Sal*I-*Bam*HI fragment (Fig. 1a).

Isolation of uridine auxotrophs from *A. parasiticus* ATCC 36537. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis of *A. parasiticus* ATCC 36537 resulted in a conidiospore survival rate of 66% compared with nonmutagenized conidiospores when the conidiospores were inoculated onto potato dextrose agar. Inoculation of 1.3×10^8 viable mutagenized spores onto potato dextrose agar supplemented with FOA and uridine resulted in FOA-resistant (FOA^r) colonies that arose at a frequency of 3.5×10^{-7} CFU per viable conidiospore; 22% of these FOA^r isolates were determined to be stable uridine auxotrophs after serial inoculation onto CZ medium supplemented with uridine. All 10 strains used in further analyses were morphologically indistinguishable from the original parent strain, *A. parasiticus* ATCC 36537, and exhibited no detectable blue fluorescence when they were grown on coconut agar medium, suggesting that there was little or no aflatoxin production.

Enzymatic analysis of *pyr* mutants. Separation of OMP, orotate, and UMP on TLC plates yielded average R_f values of 0.4, 0.6, and 0.7, respectively. In cell extracts of three *A. parasiticus* ATCC 36537 *pyr* isolates (strains CS2, CS3, and CS10), OMP was converted to UMP in trace amounts, suggesting that OMP decarboxylase activity was greatly impaired (Fig. 2a, lane a). Furthermore, [^{14}C]OMP accumulated when cell extracts of *A. parasiticus* CS2, CS3, and CS10 were incubated with [^{14}C]orotate plus 5-phosphoribosyl-1-pyrophosphate, indicating that there was complete conversion of orotate to OMP, and subsequent accumulation of OMP indicated that there was active orotate phosphoribosyltransferase activity (data not shown). *A. parasiticus* ATCC 36537, which was used as a positive control for functional *pyrF* and *pyrG* enzymatic activity, was able to convert both [^{14}C]OMP and [^{14}C]orotate to [^{14}C]UMP (data not shown). *A. nidulans* A722, which lacks OMP decarboxylase activity, was used as a negative control; this strain did not accumulate [^{14}C]UMP when it was incubated with [^{14}C]OMP (data not shown). The remaining 7 of the 10 *pyr* mutants tested (strains CS1 through CS10) converted [^{14}C]OMP to [^{14}C]UMP at levels comparable to the level found in the *A. parasiticus* ATCC 36537 control, suggesting that each had a functional OMP decarboxylase (data not shown). Our results strongly suggested that *A. parasiticus* uridine auxotrophs CS2, CS3, and CS10 were *pyrG* mutants which were deficient in OMP decarboxylase activity.

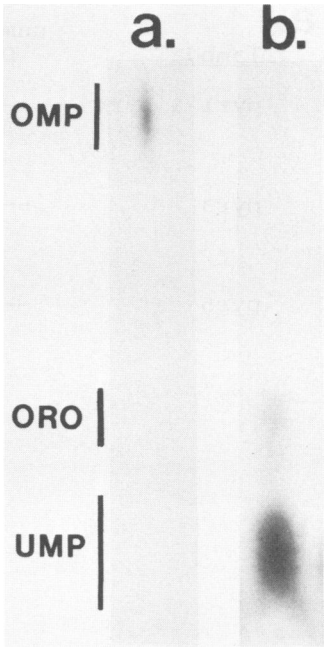


FIG. 2. Restoration of OMP activity in *pyrG* mutants after transformation. Cell extracts from *A. parasiticus* were assayed for the conversion of OMP to UMP. Extracts of *A. parasiticus* CS10 A (a uridine auxotrophic mutant, which was not transformed) (lane a) and *A. parasiticus* UT5 (strain CS10 transformed with pPG3J) (lane b) were incubated with [6-^{14}C]OMP and separated by TLC. The TLC plate was then exposed for 48 h to X-ray film. ORO, Orotate.

Transformation of *pyrG* mutants. Protoplast preparations of *A. parasiticus* typically yielded 1×10^6 to 3×10^6 protoplasts from 10^8 conidia, and the level of viability was approximately 25% when cells were grown under nonselective conditions (media supplemented with uridine). Transformation of 10^6 viable protoplasts of *A. parasiticus* ATCC 36537 *pyrG* isolate CS2 or CS10 with 2 μg of pPG3J resulted in 20 to 50 stable transformants per μg of DNA. Control protoplasts, which were not treated with DNA, showed no evidence of germination on CZ agar containing 0.6 M KCl as determined by microscopic examination. Both circular pPG3J and *Eco*RI-linearized pPG3J were tested and yielded approximately equal efficiencies. However, linearized plasmids resulted in significantly fewer abortive transformants (colonies which initially grew on selective medium but were unable to grow upon further subculturing). Transformant colonies typically appeared within 2 days but became readily distinguishable from abortive transformants at day 4 by their larger diameters (0.5 to 1.5 cm, compared with abortive colonies which were typically less than 0.2 cm in diameter) and by their abundant conidia formation. Transformant colonies transferred to CZ agar had colony morphology which was similar to that of *A. parasiticus* ATCC 36537.

Southern analysis of transformant colonies. Southern hybridization analysis of genomic DNAs purified from several transformant clones of *A. parasiticus* CS10 suggested that both *pyrG* and pUC19 plasmid sequences were integrated into the genome (Fig. 3). Transformant isolates 1 through 5 were transformed with linear pPG3J, while isolates 6 through 10 were transformed with circular pPG3J. A single band was observed with *Eco*RI-digested genomic DNA from nontrans-

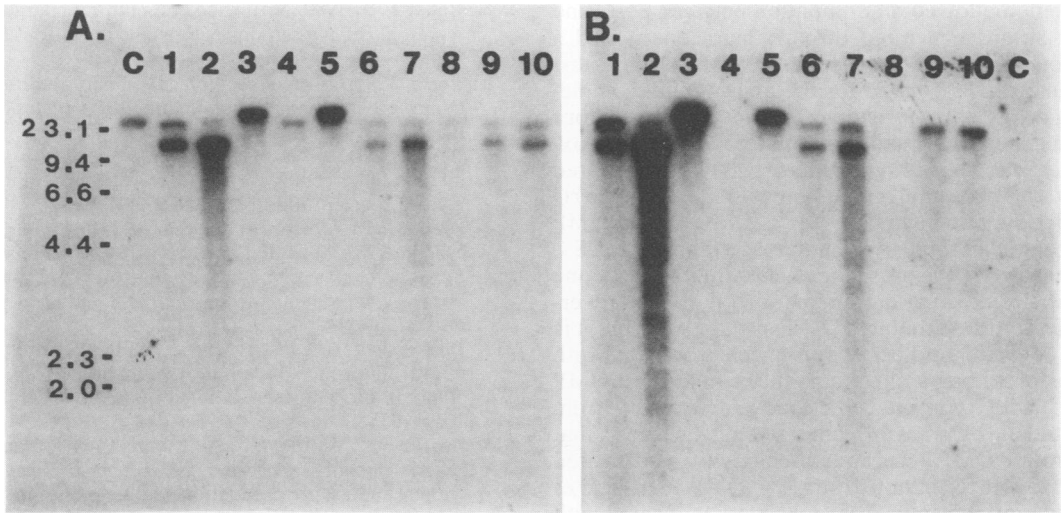


FIG. 3. Southern hybridization analysis of 10 *A. parasiticus pyrG* transformants. Genomic DNA from untransformed *A. parasiticus* CS10 (lanes c), five isolates transformed with linearized *Eco*RI pPG3J (lanes 1 through 5), and five additional isolates transformed with circular pPG3J (lanes 6 through 10) were digested with *Eco*RI and electrophoresed in a 0.8% agarose gel (8 μg of DNA per lane). DNA was blotted onto nitrocellulose and probed with a ^{32}P-labeled 2.8-kb *Sal*I-*Bam*HI *pyrG* fragment (A) and ^{32}P-labeled pUC19 (B). Blots were washed under high-stringency conditions (see text).

formed *A. parasiticus* CS10 when it was hybridized with the 2.8-kb *Sal*I-*Bam*HI *pyrG* fragment. No hybridizing fragment was observed in the same DNA when it was probed with pUC19, confirming the lack of sequence similarity with this plasmid. The hybridization patterns for transformant isolates 4 and 8 were the same as those for the untransformed cells when they were probed with *pyrG* and did not show any hybridization to pUC19 sequences, indicating that a double crossover recombination event may have occurred between the *pyrG* gene on the plasmid and the *pyrG* gene on the chromosome, leading to replacement of the homologous DNA. Protoplasts that were not treated with DNA did not grow on CZ (KCl) medium, implying that clones 4 and 8 were not revertants at the *pyrG* locus.

It was not possible to determine conclusively from the data whether gene replacement also occurred in addition to integration of the plasmid sequences in the remaining transformant clones. A single crossover and integration event of circular pPG3J into the *pyrG* locus was the most likely event for transformants 9 and 10. Such an event would have resulted in two different *Eco*RI fragments (approximately 23 and 11 kb), each containing *pyrG* sequences, but only the larger of the two would have contained pUC19 sequences. The Southern data for the remaining transformants suggested that there was integration of all or part of plasmid pPG3J into a site other than the *pyrG* locus.

Additional hybridizing bands observed in the genomic DNAs from transformant isolates 1 through 3 and 5 through 7 when they were probed with the *pyrG* fragment were also observed in the same apparent locations when they were hybridized to pUC19, indicating that both pUC19 and *pyrG* sequences were integrated into the genome. It should be mentioned that transformant isolates 3 and 5 may actually have resulted from integration of pPG3J sequences into the *pyrG* locus, resulting in a larger *Eco*RI band, although this was difficult to confirm since the intensity and size of this band prevented accurate interpretation. The multiple bands observed with transformant isolate 2 when it was probed with pUC19 may have resulted from integration and rearrangement of the pUC19 vector which occurred independently of the integration of *pyrG* DNA. The magnified intensities of the additional *pyrG* hybridizing bands for transformant isolates 1 through 3, 5, and 7 suggest that multiple copies of the plasmid may have been integrated since equal quantities of genomic DNA were in all of the lanes.

Enzymatic analysis of *pyrG* transformants. An enzymatic analysis with [^{14}C]OMP and [^{14}C]orotate substrates was performed by using cell extracts from five randomly isolated transformant colonies of *A. parasiticus* CS10. All of the transformants tested completely converted OMP to UMP, and none accumulated OMP when the transformants were incubated with orotic acid, which demonstrated that OMP decarboxylase activity had been restored (Fig. 2, lane b).

DISCUSSION

To our knowledge, in this paper we describe the first transformation protocol for *A. parasiticus* which has been developed. Several attempts to transform *A. parasiticus* ATCC 36537 *pyrG* mutants by using the heterologous gene from *A. nidulans* FGSC4 contained on circular pPL6 (22) were unsuccessful, and these unsuccessful attempts prompted the isolation of the homologous *pyrG* gene. The efficiencies obtained with the homologous *pyrG* gene were slightly higher than those obtained by Woloshuk et al., who developed a heterologous *pyrG* transformation system in *A. flavus* (26). Another transformation protocol for *A. parasiticus*, in which complementation of nitrate reductase mutants with the homologous *niaD* gene was used, was developed simultaneously in our laboratory (J. S. Horng, P. K. Chang, J. J. Pestka, and J. E. Linz, Mol. Gen. Genet., in press). Both systems have been used to construct cosmid genomic DNA libraries that facilitate the cloning of aflatoxin-biosynthetic genes by complementation of aflatoxin-blocked mutant strains of *A. parasiticus*.

In this study, we performed enzymatic analyses with cell extracts from *A. parasiticus pyr* mutants for two reasons.

First, this method allowed differentiation between *pyrG* and *pyrF* mutant strains generated through mutagenesis and a positive selection protocol, thus eliminating the need to test all uridine auxotrophs isolated for the ability to be complemented by the cloned *A. parasiticus pyrG* gene. Second, our procedure enabled confirmation of ablated OMP decarboxylase enzyme activity in *A. parasiticus* CS10 and restored enzyme activity after complementation with the cloned *pyrG* gene. Although slight conversion of [^{14}C]OMP to [^{14}C]UMP seemed to occur in all three *pyrG* mutants (strains CS2, CS3, and CS10), suggesting the presence of leaky mutations, none of the mutant strains grew on minimal medium. The reversion frequency for these mutants was less than 10^{-8}, but it is possible that reversion did occur during cell growth in broth cultures, thus providing a small amount of functional OMP decarboxylase activity in the cell extracts. The results from enzymatic studies of transformants strongly suggest that there was restored OMP decarboxylase activity, as indicated by increased UMP formation from [^{14}C]OMP and lack of OMP accumulation, when preparations were incubated with [^{14}C]orotate and compared with untransformed *A. parasiticus* CS10 controls.

A cosmid library of *A. parasiticus* NRRL 5862 genomic DNA was constructed by using the 2.8-kb *Sal*I-*Bam*HI *pyrG* fragment as a selectable marker and is currently being used in transformation studies with *A. parasiticus* ATCC 36537 with the hope that complementation of the blocked step in aflatoxin biosynthesis can be demonstrated. Concurrently, we are also developing *pyrG* mutants in other *A. parasiticus* aflatoxin-blocked mutants which will also be used in complementation studies.

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