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Recombinational Inactivation of the Gene Encoding Nitrate Reductase in *Aspergillus parasiticus*

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Functional disruption of the gene encoding nitrate reductase (*niaD*) in *Aspergillus parasiticus* was conducted by two strategies, one-step gene replacement and integrative disruption. Plasmid pPN-1, in which an internal DNA fragment of the *niaD* gene was replaced by a functional gene encoding orotidine monophosphate decarboxylase (*pyrG*), was constructed. Plasmid pPN-1 was introduced in linear form into *A. parasiticus* CS10 (*ver-1 wh-1 pyrG*) by transformation. Approximately 25% of the uridine prototrophic transformants (*pyrG*$^+$) were chlorate resistant (Chl^r), demonstrating their inability to utilize nitrate as a sole nitrogen source. The genetic block in nitrate utilization was confirmed to occur in the *niaD* gene by the absence of growth of the *A. parasiticus* CS10 transformants on medium containing nitrate as the sole nitrogen source and the ability to grow on several alternative nitrogen sources. Southern hybridization analysis of Chl^r transformants demonstrated that the resident *niaD* locus was replaced by the nonfunctional allele in pPN-1. To generate an integrative disruption vector (pSKPYRG), an internal fragment of the *niaD* gene was subcloned into a plasmid containing the *pyrG* gene as a selectable marker. Circular pSKPYRG was transformed into *A. parasiticus* CS10. Chl^r *pyrG*$^+$ transformants were screened for nitrate utilization and by Southern hybridization analysis. Integrative disruption of the genomic *niaD* gene occurred in less than 2% of the transformants. Three gene replacement disruption transformants and two integrative disruption transformants were tested for mitotic stability after growth under nonselective conditions. All five transformants were found to stably retain the Chl^r phenotype after growth on nonselective medium. These studies demonstrate that recombinational mutagenesis may be used effectively to stably disrupt a gene in *A. parasiticus*.

Aflatoxins are highly toxic and carcinogenic secondary metabolites produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus* (14, 16, 30). Aflatoxins are occasionally found in agricultural products such as peanuts, cottonseed, corn, and cassava (4). The occurrence of aflatoxin contamination in susceptible plants is highly influenced by several environmental factors, including insect or animal damage, drought prior to harvest, high humidity after harvest, and the maturity of plants at the time of fungal infection. It is often impractical or uneconomical to control these environmental factors, and this has led to increased efforts to develop alternative methods to reduce or eliminate aflatoxin contamination.

Recently, several bacteria and fungi, including nontoxigenic natural isolates and aflatoxin-blocked mutant strains of *A. parasiticus* and *A. flavus*, have been reported to be useful in controlling aflatoxin occurrence in corn, cotton, peanuts, and tree nuts (8–10, 15, 23, 33). Several potential problems are associated with the use of currently available fungal biocontrol strains: (i) aflatoxin-blocked mutants accumulate toxic intermediates, (ii) natural nontoxigenic isolates may produce aflatoxin under certain conditions, and (iii) nontoxigenic strains may mutate to toxigenicity during production of inoculum for biocontrol studies. In theory, disruption of genes early in the aflatoxin biosynthetic pathway will overcome these problems and generate genetically stable, safe, and effective biocontrol strains.

Gene disruption is a procedure in which a nonfunctional allele of a cloned gene is introduced into cells to disrupt or replace its wild-type chromosomal counterpart by homologous recombination (6, 25, 27, 31). Recent data on transformation of *A. parasiticus* with the homologous gene encoding nitrate reductase (*niaD*) or orotidine monophosphate decarboxylase (*pyrG*) suggested that transforming DNA can be integrated at homologous sequences in the genome at a relatively high frequency (18, 29).

Our goal for this work was to determine whether homologous recombination between transforming DNA and genomic DNA could be used to disrupt the function of a model gene (*niaD*) cloned from *A. parasiticus*. The *niaD* gene was chosen because simple positive (chlorate resistance) and negative (inability to utilize nitrate as the sole nitrogen source) selection protocols have been developed to analyze nitrate reductase function (12). The same strategy used for disruption of the *niaD* gene can now be applied to generate non-aflatoxin-producing strains for potential use as biocontrol agents. Disruption of genes associated with aflatoxin biosynthesis may also make it possible to establish whether aflatoxin or intermediates in the biosynthetic pathway play a role in fungal growth, morphogenesis, or survival in the environment.

MATERIALS AND METHODS

Strains and plasmids. Plasmid DNA used for transformation of *A. parasiticus* was propagated in *Escherichia coli* DH5α (GIBCO BRL, Life Technologies, Inc., Gaithersburg, Md.) and purified by CsCl gradient centrifugation of cell extracts prepared by alkaline lysis (22). The fungal strain used as the recipient in transformation, *A. parasiticus* CS10 (*ver-1 wh-1 pyrG*) (29), is a *pyrG* mutant strain derived from *A. parasiticus* ATCC 36537 (*ver-1 wh-1*) (3) which accumulates versicolorin A.

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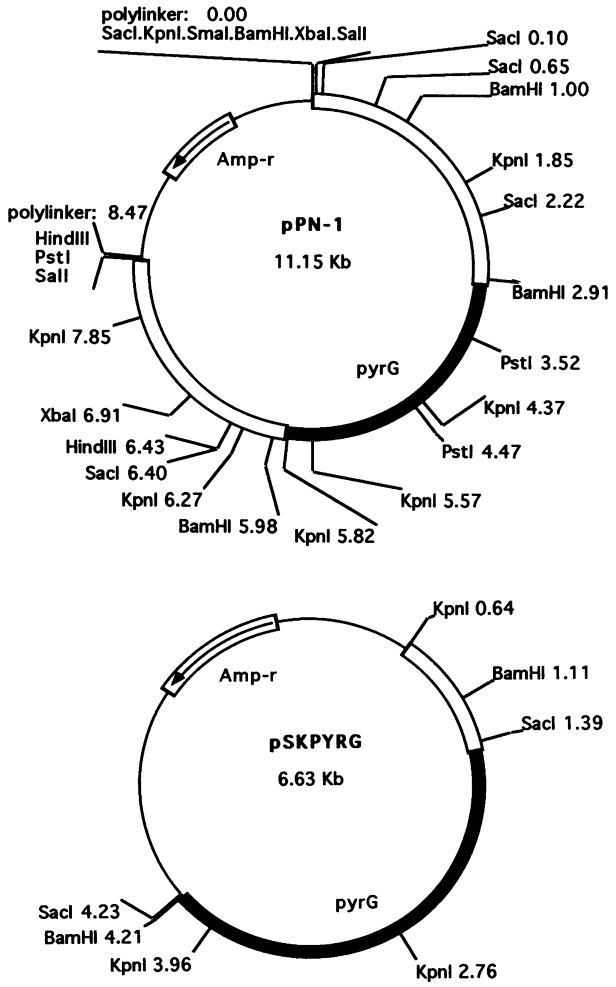

FIG. 1. Construction and restriction maps of pPN-1 and pSKPYRG. A 2.5-kb *Eco*RI fragment, which contains the majority of the *niaD* gene (open box) of *A. parasiticus*, was deleted from pSL82-1, a derivative of pSL82 (18), and replaced by a 2.85-kb *Bam*HI-*Sal*I fragment (solid bar) of pPG3J, containing the functional *pyrG* gene (29), to generate pPN-1. Plasmid pSKN1-82 was constructed by subcloning a 0.82-kb *Kpn*I-*Bgl*II internal fragment of the *niaD* gene into the *Kpn*I and *Bam*HI sites of pBluescriptII SK−. Plasmid pSKPYRG was then generated by subcloning the 2.85-kb *Bam*HI-*Sal*I *pyrG* fragment into the *Xba*I site (blunt-ended) of pSKN1-82.

The *Eco*RI restriction site of pUC19 was deleted by religating *Eco*RI-digested pUC19 by blunt-end ligation to yield pUC19-1. An 8.2-kb *Sal*I restriction fragment from pSL82 (18), which contains a complete copy of the *niaD* gene plus 5′- and 3′-flanking regions, was subcloned into the *Sal*I restriction site of pUC19-1 to produce pSL82-1. pPN-1, the plasmid construct used in gene replacement (Fig. 1), was constructed by deleting a 2.5-kb internal *Eco*RI fragment of *niaD* from pSL82-1 and replacing it with the functional *pyrG* gene contained on a 2.85-kb *Bam*HI-*Sal*I restriction fragment from plasmid pPG3J. pSKN1-82 was constructed by cloning a small internal restriction fragment of *niaD* (0.82-kb *Kpn*I-*Bgl*II) into the *Kpn*I and *Bam*HI restriction sites in pBluescriptII SK− (Stratagene, La Jolla, Calif.). pSKPYRG, used in integrative disruption (Fig. 1), was constructed by subcloning a 2.85-kb *Bam*HI-*Sal*I fragment containing the *pyrG* gene into pSKN1-82 at the *Xba*I site by blunt-end ligation.

Transformation of fungal protoplasts and analysis of transformant clones. Fungal protoplasts were transformed essentially as described by Oakley et al. (24), with minor modifications (29).

Fungal strains were maintained on potato dextrose agar (Difco, Detroit, Mich.). Frozen spore stocks of *A. parasiticus* CS10 were incubated to generate mycelia in yeast extract-sucrose liquid medium (YES; 2% yeast extract, 6% sucrose [pH 5.5]) supplemented with uridine (100 μg/ml). Uridine prototrophic transformants (*pyrG*$^+$) of *A. parasiticus* CS10 were isolated on Czapek-Dox agar (CZ agar; Difco) containing 1.69 g of sodium glutamate (Sigma Chemical Co.) per liter as the alternative nitrogen source (CZ agar also contains nitrate) and 0.6 M KCl as the osmotic stabilizer.

Transformants defective in nitrate assimilation could be identified by growth after 3 days (positive selection) on CZ agar supplemented with 470 mM potassium chlorate (17) as the selective agent and sodium L-glutamate (1.69 g/liter) as the alternative nitrogen source. Chlorate is an analog of nitrate and is generally thought to be reduced by nitrate reductase to the toxic compound chlorite or hypochlorite (1, 20). Cove (12), however, reports that chlorate resistance in *Aspergillus nidulans* is a complex phenomenon and can arise from mutations in several different genes. Not every loss-of-function mutation at these loci confers chlorate resistance, suggesting that factors other than reduction of chlorate may lead to chlorate resistance (11–13).

The resistance of *A. nidulans* to chlorate is reported to depend on defects in the nitrate permease gene (*crnA*), nitrate reductase structural gene (*niaD*), two genes involved in regulation of nitrogen metabolism (*nirA* and *areA*), or a gene (*cnx*) involved in the assembly of a molybdenum cofactor (11–13, 19, 32). Mutations in *niaD* can be distinguished from mutations at these other loci by growth on minimal salt medium (MM) (2, 26) supplemented with 470 mM $KClO_3$ plus one of the following nitrogen sources (in grams per liter): $NaNO_3$ (0.85), $NaNO_2$ (0.69), ammonium tartrate (1.84), or hypoxanthine (0.1). *niaD* mutants are unable to use nitrate but can grow on medium containing each of the other nitrogen sources, whereas mutants with mutations in the other genes involved in nitrogen metabolism have different growth patterns on these nitrogen sources (17, 19).

With *A. parasiticus*, it is important to select for chlorate resistance (Chl^r) after 3 days of growth because some colonies appearing after 6 days of growth on chlorate medium could also utilize nitrate as the sole nitrogen source. The explanation for these late-appearing colonies is not clear at this time. They may represent mutants deficient in nitrate uptake, which are moderately chlorate resistant but capable of growth on nitrate. A similar phenotype has been reported for some *A. nidulans* permease mutants (19, 32).

Mitotic stability of disrupted transformant isolates. For the study of mitotic stability, five *pyrG*$^+$ Chl^r transformants were single-spore isolated. After the full vegetative growth cycle was completed on nonselective medium (PDA supplemented with uridine [100 μg/ml] and sodium glutamate [1.69 g/liter]), asexual spores were harvested. Appropriate dilutions of these spore preparations were inoculated again onto nonselective medium (CZ plus glutamate and uridine) and allowed to grow for 3 days. Individual colonies were then transferred onto selective growth medium (CZ plus glutamate and chlorate) and scored for growth and sporulation

after 3 days. As a control, spores from 100 colonies of *A. parasiticus* CS10 were patched onto selective (plus chlorate) and nonselective growth media, both containing uridine.

Chemicals and general procedures. Restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Enzyme digestion, agarose gel electrophoresis, and Southern hybridization analyses were performed according to standard procedures (22). Radiolabeled DNA probes were generated with a random-primer labeling kit (Boehringer Mannheim Biochemicals) with [α-^{32}P]dCTP (DuPont). High-molecular-weight genomic DNA was isolated from mycelia by the phenol-chloroform procedure of Cihlar and Sypherd (7) as modified by Horng et al. (18).

RESULTS

One-step gene replacement. Plasmid pPN-1 was digested with *Sma*I to generate a linear construct and used to transform *A. parasiticus* CS10. The frequency of transformation of *A. parasiticus* CS10 to *pyrG*$^+$ with linear pPN-1 (approximately 8 transformants per μg of DNA per 10^6 protoplasts) was similar to that of circular pPG3J, which contains the *pyrG* gene only (10 transformants per μg of DNA per 10^6 protoplasts). *pyrG*$^+$ transformants were then screened for defects in nitrate assimilation. Fifteen of 66 *pyrG*$^+$ transformants (23%) were Chl^r, and none of these 15 transformants could grow on CZ agar containing nitrate as the sole nitrogen source. This result indicated that these 15 isolates were nitrate-nonutilizing mutants. This experiment was repeated with similar results. Eight of 32 (25%) *pyrG*$^+$ transformants of *A. parasiticus* CS10 were Chl^r and could not grow on CZ agar. The 23 Chl^r transformants from two experiments were further characterized by replica plating to MM medium containing various nitrogen sources (see above). None of 23 Chl^r transformants could grow on $NaNO_3$ as the sole nitrogen source but could grow on $NaNO_2$, ammonium tartrate, or hypoxanthine.

Nine independent clones (designated 6, 13, 15, 17, 51, 57, 64, 67, and 69) defective in nitrate reductase function were analyzed by Southern hybridization analysis (see below).

Integrative disruption. Circular pSKPYRG was used to transform *A. parasiticus* CS10. In three independent experiments, the frequency of transformation of *A. parasiticus* CS10 with pSKPYRG to *pyrG*$^+$ was approximately 6 transformants per μg of DNA per 10^6 protoplasts. Only 4 of 238 *pyrG*$^+$ transformants (<2%) were Chl^r, and none of the 4 grew on CZ agar. These four Chl^r transformants could grow on $NaNO_2$, ammonium tartrate, or hypoxanthine. These results suggested that the *niaD* gene had been disrupted in these four isolates. Two of these four transformants (designated 8 and 174) were selected for Southern hybridization analysis. Two *pyrG*$^+$ Chl^s transformants (numbers 2 and 28) were also selected for Southern hybridization analysis.

Transformation of *A. parasiticus* CS10 protoplasts without DNA or with pPG3J was performed as controls. In the absence of transforming DNA, control protoplasts could not grow on CZ agar, indicating that no *pyrG*$^+$ revertants were generated. In addition, of 200 *pyrG*$^+$ clones obtained after transformation with pPG3J, no Chl^r colonies were observed after 3 days of growth, indicating that no spontaneous mutations occurred at the *niaD* locus.

Southern hybridization analysis. (i) One-step gene replacement. Southern hybridization analysis was performed on genomic DNAs isolated from *A. parasiticus* CS10 (control), *pyrG*$^+$ isolates transformed with pPG3J (control), and *pyrG*$^+$ *niaD* isolates transformed with linear pPN-1. Genomic DNA samples digested with *Sal*I were probed with a radiolabeled 0.93-kb *Sst*II fragment located within the region of the *niaD* fragment which was deleted in pPN-1. A single 8.2-kb DNA fragment hybridized to the *niaD* probe in genomic DNA purified from untransformed cells and *pyrG*$^+$ cells transformed with pPG3J, suggesting that the genomic copy of the *niaD* gene was unaffected by transformation. The *niaD* internal probe did not hybridize to any *Sal*I fragment in eight of nine randomly selected *pyrG*$^+$ niaD isolates transformed with pPN-1, indicating that this *niaD* fragment was deleted from the genome. A weakly hybridizing 8.2-kb DNA fragment was observed in the other isolate (isolate designated 51 in Fig. 2A), suggesting that it was a heterokaryon which had not been successfully resolved during clone isolation.

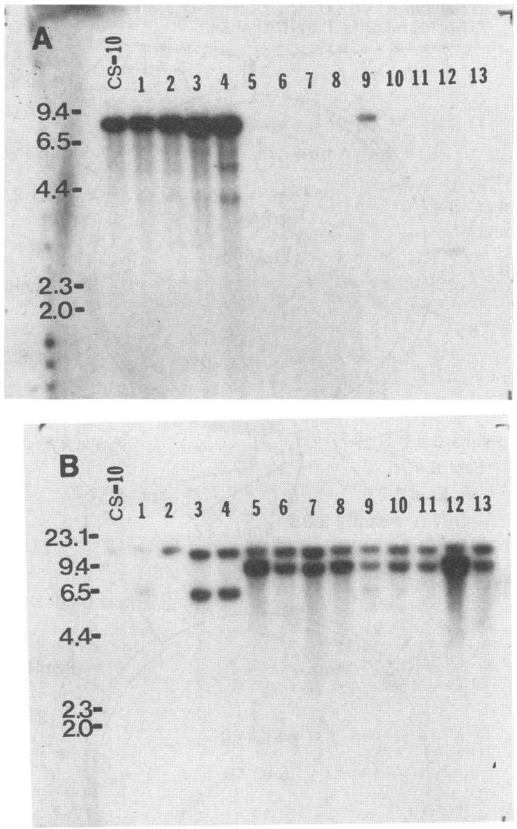

FIG. 2. Southern hybridization analysis of genomic DNAs from *A. parasiticus* CS10 (recipient) and its pPG3J and linear pPN-1 transformants. (A) Genomic DNAs were digested to completion with *Sal*I and hybridized with a radiolabeled internal *niaD* fragment. (B) Identical genomic DNA samples were hybridized with a radiolabeled 2.85-kb *Sal*I-*Bam*HI *pyrG* fragment from pPG3J. Lane CS10, genomic DNA from the untransformed *A. parasiticus* CS10; lanes 1 to 4, four *pyrG*$^+$ Chl^s transformants (isolates 0, 1, 2, and 6, respectively) of *A. parasiticus* CS10 transformed with pPG3J; lanes 5 to 13, nine *pyrG*$^+$ Chl^r transformants transformed with linear pPN-1 (isolates 6, 13, 15, 17, 51, 57, 64, 67, and 69, respectively). DNA size standards were *Hin*dIII-digested lambda DNA (in kilobases).

An identical set of *Sal*I-digested genomic DNAs were also hybridized to a 2.85-kb *Bam*HI-*Sal*I fragment containing the *pyrG* gene from pPG3J (Fig. 2B). One DNA fragment, approximately 10.0 kb in size, hybridized to the *pyrG* probe in all DNA samples, indicating that the chromosomal *pyrG* gene lay on this genomic DNA fragment. An additional

6.3-kb *Sal*I fragment hybridized to the *pyrG* probe in three of four *pyrG*$^+$ isolates transformed with pPG3J, suggesting that a single crossover between circular pPG3J and the chromosomal *pyrG* locus had occurred, resulting in two copies of *pyrG* separated by vector sequences.

Southern hybridization analyses were also performed on genomic DNA samples from the same three pPG3J transformants digested with *Sal*I plus *Sac*I. The sizes of the restriction fragments in this analysis were also consistent with a single crossover event at the chromosomal *pyrG* locus (data not shown). Although plasmid integration clearly did not occur at the *niaD* locus in these three transformants, we cannot conclusively rule out the possibility that the integration might have occurred at another location than within the *pyrG* gene. Only the 10.0-kb *Sal*I fragment was observed in the fourth pPG3J transformant, suggesting that the functional *pyrG* gene in pPG3J replaced the nonfunctional chromosomal *pyrG* allele of *A. parasiticus* CS10.

In clones transformed with the gene replacement vector pPN-1, the *pyrG* probe hybridized to an additional 8.2-kb *Sal*I DNA fragment, suggesting that the nonfunctional *niaD* gene construct containing the *pyrG* gene replaced the functional chromosomal *niaD* gene by a double crossover event in the flanking region of *niaD* adjacent to the cloned *pyrG*. Since the *pyrG* gene fragment (2.85 kb) used to replace the *niaD* internal fragment (2.5 kb) in pPN-1 was of approximately the same size, gene replacement by this vector would result in the generation of a *Sal*I restriction fragment of approximately 8.2 kb which now hybridized to *pyrG* and not to *niaD*. Southern hybridization analysis was repeated on these genomic DNA samples (pPN-1 transformants) which had been digested with *Sal*I plus *Sac*I and probed with *niaD* and *pyrG*. The hybridization patterns observed were also consistent with a gene replacement event (data not shown). When genomic DNAs from pPN-1 transformants were probed with pUC19 (data not shown), four of nine isolates (numbers 6, 17, 51, and 64) contained different-sized DNA fragments which hybridized to the pUC19 probe (but not to an *niaD* or *pyrG* probe), suggesting that pUC19 was capable of heterologous recombination independent of the remaining vector. The other five isolates did not hybridize to the pUC19 probe, indicating that pUC19 sequences were deleted during the double crossover gene replacement event.

(ii) Integrative gene disruption. Genomic DNAs from *A. parasiticus* NRRL 5862 (SU-1), the recipient strain *A. parasiticus* CS10, and several clones transformed with pPG3J or the integrative vector pSKPYRG were digested with *Sac*I and hybridized to a 2.85-kb *pyrG* gene probe (Fig. 3). A single *Sac*I DNA fragment hybridized to the *pyrG* probe, suggesting that gene replacement or gene conversion between the *pyrG* gene in plasmid pSKPYRG and the nonfunctional genomic *pyrG* allele occurred in one of two *pyrG*$^+$ Chl^s transformants. In the other clone, homologous integration of pSKPYRG occurred at the genomic *pyrG* locus, resulting in tandem copies of *pyrG* carried on 7.4-kb and 4.6-kb *Sac*I DNA fragments. For the two *pyrG*$^+$ Chl^r transformants (numbers 8 and 174) analyzed, an 8.2-kb *Sac*I DNA fragment and a 3.2-kb *Sac*I DNA fragment, respectively, hybridized to the *pyrG* probe. The sizes of these *Sac*I fragments were consistent with homologous integration of pSKPYRG at the chromosomal *niaD* allele in these clones.

Mitotic stability of *niaD*-disrupted strains. Chl^r clones transformed with pPN-1 or pSKPYRG were tested for mitotic stability by allowing them to complete the full vegetative cycle on nonselective growth medium. Appropriate dilutions of the resultant spore stocks were inoculated onto nonselective medium. Individual colonies grown on nonselective medium were then transferred onto selective growth medium (see Materials and Methods) to test for chlorate resistance. In two separate experiments, all 700 individual clones from each of the three gene replacement transformants and two integrative disruption transformants tested stably retained the Chl^r phenotype. None of the 100 control *niaD*$^+$ *A. parasiticus* CS10 colonies were Chl^r after 3 days of growth.

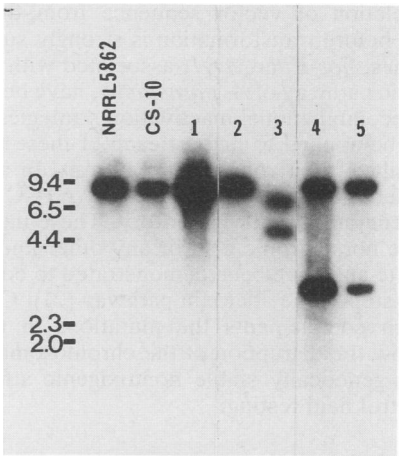

FIG. 3. Southern hybridization analysis of genomic DNAs from *A. parasiticus* NRRL 5862, *A. parasiticus* CS10 (recipient), and pPG3J and pSKPYRG transformants of *A. parasiticus* CS10. Genomic DNAs were digested to completion with *Sac*I and hybridized with a radiolabeled 2.85-kb *pyrG* probe. Lane NRRL 5862, genomic DNA from *A. parasiticus* NRRL 5862; lane CS10, genomic DNA from the untransformed *A. parasiticus* CS10; lane 1, *pyrG*$^+$ Chl^s transformant of *A. parasiticus* CS10 transformed with pPG3J; lanes 2 and 3, two *pyrG*$^+$ Chl^s transformants transformed with pSKPYRG (isolates 2 and 28, respectively); lanes 4 and 5, two *pyrG*$^+$ Chl^r transformants transformed with pSKPYRG (isolates 8 and 174, respectively). DNA size standards were *Hin*dIII-digested lambda DNA (in kilobases).

DISCUSSION

One-step gene replacement of the *niaD* gene was performed at a high frequency (25%) by introducing a linear plasmid construct, pPN-1, into *A. parasiticus* CS10. A circular plasmid, pSKPYRG, containing a truncated *niaD* gene fragment also disrupted the genomic *niaD* gene by a single-crossover integrative recombination event but at a lower frequency (<2%). The observed difference in disruption frequency is likely due in part to a difference in the sizes of DNA fragments which undergo homologous recombination. Since plasmid pSKPYRG carries a small (0.82-kb) *niaD* internal fragment, a single crossover or gene replacement at the 2.85-kb *pyrG* gene on pSKPYRG appeared to be the preferred event. In addition, the mitotic stability data suggest that genetically stable transformants can be generated effectively by either gene replacement or integrative disruption.

With linearized pPN-1, four of nine *niaD* mutant gene replacement transformants carried vector sequences in the genome, suggesting that pUC19 was capable of heterologous recombination. Since the absence of vector sequences (foreign DNA) would be highly desirable in potential biocontrol

strains, deletion of vector sequence from the disruption constructs before transformation is strongly suggested.

Two genes, *nor-1* and *ver-1*, associated with the aflatoxin biosynthetic pathway of *A. parasiticus*, have been cloned (5, 28). The recombinational inactivation strategies used in this study can be applied to disrupt each of these two genes to further analyze their role in the pathway. In addition, two mutants derived from *A. parasiticus* ATCC 24690 were recently generated in our laboratory. These mutants do not accumulate norsoloronic acid or any other known pathway intermediate and have been demonstrated to be blocked at a very early step in the aflatoxin pathway (21). Cloning of the gene which complements the mutations in these strains should allow the disruption of the chromosomal gene copy, generating genetically stable nontoxigenic strains suitable for biocontrol field testing.

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