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Selection of multiple disruption events in *Aspergillus fumigatus* using the orotidine-5′-decarboxylase gene, *pyrG*, as a unique transformation marker

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Abstract A 8.6-kb disruption cassette, referred to here as a *pyrG*-blaster and consisting of the *Aspergillus niger pyrG* gene flanked by a direct repeat that encodes the neomycin phosphotransferase of transposon Tn*5* was constructed. Following transformation of a uridine/uracil auxotrophic *pyrG* strain of *A. fumigatus*, genomic insertions of the *pyrG*-blaster were obtained either by targeted gene replacement at the *rodA* locus, resulting in the formation of hydrophilic spores, or by ectopic integration. In both cases, recombination between the two elements of the direct repeat could be selected in the presence of 5-fluoro-orotic acid and resulted in the excision of the *A. niger pyrG* gene, producing *A. fumigatus* uridine/uracil auxotrophs that retained their additional mutant phenotype because of the persistence of one of the two elements of the direct repeat at the site of insertion of the *pyrG*-blaster. Selection for uracil/uridine prototrophy can therefore be used again to disrupt another gene.

Key words *Aspergillus* · Transformation · *pyrG*-blaster · Infection

Introduction

In recent years, genetic transformation of the opportunistic fungal pathogen *Aspergillus fumigatus* has become possible, allowing the manipulation of the fungus at the genetic level and thus the assessment of the role of different proteins in pathogenesis (Tang et al. 1992; Monod et al. 1993). Transformation of *A. fumigatus* is integrative and relies on the use of dominant selection markers that encode resistance to various antibiotics [hygromycin (Punt et al. 1987; Tang et al. 1992; Monod et al. 1993); phleomycin (Austin et al. 1990; Jaton-Ogay et al. 1994; Smith et al. 1994)]. Other dominant selection markers including benomyl (Orbach et al. 1986; Seip et al. 1990) and phosphinothricin (Avalos et al. 1989), although they have not yet been tested, might also prove useful for transformation of *A. fumigatus*.

Because of the lack of a sexual cycle that prevents the characterization of auxotrophic mutants of *A. fumigatus*, transformation strategies that involve the conversion of an auxotrophic mutation to prototrophy have not been developed in *A. fumigatus*, whereas they are well established in many other fungal species including *Saccharomyces cerevisiae* (Guthrie and Fink 1991), *Candida albicans* (Kurtz et al. 1988), and several species of the *Aspergillus* group (Tilburn et al. 1983; van Hartingssveldt et al. 1987; Woloshuk et al. 1989; Skory et al. 1990). Complementation of uridine/uracil auxotrophs using the orotidine-5′-phosphate (OMP) decarboxylase-encoding gene has received much attention because of the properties that are associated with this enzyme: while mutants that lack the activity are auxotrophic for uridine and uracil, they become resistant to 5-fluoro-orotic acid (5-FOA) which is converted to the toxic intermediate 5-fluoro-UMP in prototrophs (Boeke et al. 1984). This provides a positive selection that has been applied successfully to the identification of uracil/uridine auxotrophs in various fungal species (van Hartingssveldt et al. 1987; Woloshuk et al. 1989; Skory et al. 1990) and to the development of new strategies for the manipulation of fungal genomes (Rothstein 1991; Sikorski and Boeke 1991). An example is the use of the so-called "ura-blaster" in gene-disruption experiments (Alani et al. 1987; Fonzi and Irwin 1993). Ura-blasters are tripartite cassettes that contain an OMP-decarboxylase gene flanked by two identical elements that form a direct repeat. After gene disruption, forced excision of the OMP-decarboxylase gene occurs in the presence of 5-FOA by recombination between the two elements of the direct repeat, yielding a strain that still has the mutation of interest because of the persistence of a single element of the direct repeat at the mutated locus. A consequence of this approach is the fact that the resulting strain has recovered its uridine/uracil auxotrophy,

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and can thus be subjected to another round of transformation using the same selection scheme.

The development of a transformation system using the OMP-decarboxylase gene as a selection marker in *A. fumigatus* might therefore provide a versatility that cannot be obtained with the currently available antibiotic resistance-based tansformation systems. Here, we report the identification of strains of *A. fumigatus* that have a mutation in the *pyrG* gene which encodes OMP-decarboxylase. Furthermore, we present the design of a disruption cassette analogous to the previously described ura-blasters (Alani et al. 1987; Fonzi and Irwin 1993) and referred to as a *pyrG*-blaster. A demonstration that the *pyrG*-blaster can be used to sequentially disrupt genes in the *A. fumigatus* genome is presented.

Materials and methods

Strains and culture conditions. A. fumigatus strains used in this study are listed in Table 1. They were propagated at 37°C on YGA (0.5% Yeast extract, 2% glucose, 1.5% Bacto-agar), complete medium (Cove 1966), or minimal-medium with 0.5 mM sodium glutamate as a nitrogen source (Cove 1966). Uridine or uracil were added at a concentration of 5 mM when appropriate. Liquid cultures used for transformation or DNA preparation were grown in YG. Strains PAP105 [Δ(*lac-pro*) F′(*lacI*^{q}1 Δ(*lacZ*)*M15* pro$^+$ Tn*10*)] and DH5α {*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)U169 *deoR* [ϕ80*dlac* Δ(*lacZ*)*M15*]} of *Escherichia coli* were used for plasmid propagation. The β-lactam antibiotic carbenicillin (100 μg/ml), kanamycin (50 μg/ml) and tetracycline (15 μg/ml) were added to the growth medium when required.

Plasmids and DNA manipulations. pAB4-1, which carries the *A. niger pyrG* gene (van Hartingssveldt et al. 1987), pAF3 which carries a deleted version of the *A. fumigatus rodA* locus (Thau et al. 1994), and pHP45Ω-Km which carries the *neo* gene from transposon Tn*5* (Prentki and Krisch 1984), were kind gifts of C. Van den Hondel, S. Paris and H. Krisch, respectively. DNA manipulations were according to Sambrook et al. (1989) and Ausubel et al. (1992). Transformation of calcium-manganese-treated *E. coli* was as described (Hanahan et al. 1991). As a first step in the construction of pCDA14, two plasmids were produced by insertion of an adaptor containing a *Hpa*I restriction site (5′-GATCGTTAAC-3′) at one of the two *Bam*HI sites of pHP45Ω-Km. The resulting plasmids, pCDA7 and pCDA8, have a unique *Hpa*I site 3′ and 5′ of the *neo* gene respectively. A derivative of pCDA7, pCDA9*Xba*, was then constructed by inactivating the *Eco*RI site located 3′ of *neo* and then converting the *Bam*HI site located 5′ of *neo* into a *Xba*I site using the following adaptor: 5′-GATCTCTAGA-3′. pCDA10 was derived from pCDA8 by converting the *Bam*HI site 3′ of *neo* into an *Xba*I site as described above. pCDA14 was then obtained by subcloning the 2.3-kb *Eco*RI-*Xba*I fragment of pCDA10 and the 4.0-kb *Xba*I fragment of pAB4-1 into pCDA9*Xba* that had been cleaved at the *Xba*I and *Eco*RI sites located 5′ of *neo*.

pBLSN$^+$ is a derivative of Bluescript™ II SK$^+$ (Stratagene) whose *Kpn*I site has been converted into a *Not*I site by insertion of the following adaptor: 5′-GCGGCCGCGTAC-3′. Blue/white selection is maintained in pBLSN$^+$. pCDA15 was constructed by subcloning the 3.7-kb *Apa*I-*Eco*RV fragment of pAF3 into *Apa*I/*Eco*RV-digested pBLSN$^+$. pCDA16 was then obtained by subcloning the 8.6-kb *Hpa*I fragment of pCDA14 into the *Hin*dIII site of pCDA15 that had been filled in using the Klenow fragment of *E. coli* DNA polymerase I.

Isolation of A. fumigatus pyrG mutants. A suspension of freshly harvested spores of *A. fumigatus* strain CEA10 in water (2×10^7 sp/ml) was treated with 4-nitroquinoline-N-oxide as described (Harris et al. 1994). The mutagen was inactivated by the addition of 1 vol of 5% sodium thiosulphate and spores were plated at various dilutions on 5-FOA plates [minimal-medium agar plates containing 5 mM uridine, 5 mM uracil and 1 mg/ml of 5-fluoro-orotic acid (InterSpex Products Inc., Foster City, U.S.A.)]. Survival rates were estimated by plating non-mutagenized and mutagenized spores on minimal-medium agar plates containing uridine and uracil. 5-FOA-resistant colonies were observed after incubation at 37°C for 2 days. In order to limit the occurrence of multiple mutations, only mutants obtained from mutagenized spores with a survival rate above 75% were further tested for uridine and uracil requirement. Reversion of the uridine/uracil auxotrophy of selected mutants was tested by plating up to 5×10^8 spores on minimal-medium agar plates. Only mutants that did not show reversion were selected for further study.

Aspergillus transformation. A. fumigatus protoplasts were prepared from germinating conidiospores grown for 5 h at 37°C in YG medium containing uridine and uracil, using Novozym 234 as described for *A. nidulans* (Osmani et al. 1987). Transformations were carried out as described and transformation mixtures were mixed with 3.5 ml of minimal medium containing 1% agar and 0.4 M $(NH_4)_2SO_4$ and 1 M sucrose as osmotic stabilizers, and then poured onto minimal-medium agar plates containing 0.4 M $(NH_4)_2SO_4$ and 1 M sucrose. In each transformation, 10^8 Novozym-treated spores were mixed with 5 μg of pAB4-1, *Hpa*I-digested pCDA14 or *Not*I-digested pCDA16. Transformants (2–10 per μg of transforming DNA) became visible after 2 days of incubation at 37°C and the RodA$^-$ phenotype could be scored after purified transformants had been incubated for 3 days at 37°C on complete medium.

Selection of A. niger pyrG excision in the presence of 5-fluoro-orotic acid. Freshly harvested spores from *A. fumigatus* strains carrying a genomic insertion of the *Hpa*I fragment of pCDA14 were plated at various densities on 5-FOA plates (see above); *pyrG* revertants were obtained after 2 days of incubation at 37°C. The use of high plate densities (>10^7 spores/plate) prevents the appearance of 5-FOA-resistant colonies. Determination of the frequency of *pyrG* loss was made by simultaneous plating on minimal-medium containing uridine and uracil.

Genomic DNA analysis. Genomic DNA of *A. fumigatus* strains was prepared according to Girardin et al. (1993) and digested with *Sal*I or *Eco*RI. DNA restriction fragments transferred onto nylon membranes (Hybond N$^+$, Amersham) were hybridized with the appropriate probes in 50% formamide, 5×SSC, 1% SDS, 5×Denhardt, 10% Dextran sulphate, for 16 h at 42°C. Final washes were performed at 65°C in 0.2×SSC. The 4.0-kb *Xba*I fragment of pAB4-1, the 3.7-kb *Not*I fragment of pCDA15, and the 2.3-kb *Hin*dIII fragment of pHP45Ω-Km were used to probe the *A. niger* and *A. fumigatus pyrG* genes, the *A. fumigatus rod*A gene, and the *neo* gene of transposon Tn*5*, respectively. In addition to the expected 6.7-kb fragment, the *rodA* probe detects a 4.8-kb fragment that is likely to be homologous

Table 1 *A. fumigatus* strains

Strain	Genotype	Origin
CEA10	Wild-type	CBS 144-89
CEA17	*pyrG*	This study
CEA17-16	*pyrG rodA::(neo-A.n.pyrG-neo)*[a]	This study
CEA17-16F1	*pyrG rodA::neo*[a]	This study
CEA17-14/1	*pyrG xxx::(neo-A.n.pyrG-neo)*[a,b]	This study
CEA17-14/1F	*pyrG xxx::neo*[a,b]	This study
CEA17-14/2	*pyrG yyy::(neo-A.n.pyrG-neo)*[a,b]	This study
CEA17-14/2F	*pyrG yyy::neo*[a,b]	This study

[a] *neo*: Tn*5* neomycin phosphotransferase gene; *A.n.pyrG*: *A niger* OMP decarboxylase gene
[b] *xxx* and *yyy* are arbitrary designations for random loci in the *A. fumigatus* genome

to the DNA region located 5′ of *rodA*, since it was not detected using a shorter probe (Thau et al. 1994). Labelling was performed using the Rediprime labelling kit (Amersham) in the presence of [α-^{32}P]dCTP (Amersham). Washed membranes were exposed to X-Omat films (Kodak).

Results

Isolation of a *A. fumigatus pyrG* mutant

Mutants of *Aspergillus* sp. that lack orotidine-5′-phosphate (OMP) decarboxylase activity are auxotrophic for uridine and uracil and resistant to 5-fluoro-orotic acid (5-FOA) (van Hartingssveldt et al. 1987; Woloshuk et al. 1989; Skory et al. 1990). Colonies able to grow in the presence of 5-FOA, uridine and uracil were selected after mutagenesis (see Materials and methods). Among 100 5-FOA-resistant colonies, 79 were found to be uridine/uracil auxotrophs. Most of these clones were similar in size, morphology and sporulation to the wild-type strain when supplemented with 5 mM uridine and uracil.

5-FOA resistance results from mutation in the genes encoding either orotate phosphoribosyl transferase, *pyrF*, in *Aspergillus* sp., or OMP decarboxylase, *pyrG*, in *Aspergillus* sp. (Boeke et al. 1984). Complementation of a *pyrG* mutation is easily achieved by transforming a mutant strain with the *pyrG* gene from another fungal species (Ballance and Turner 1985; Woloshuk et al. 1989). Three 5-FOA-resistant *A. fumigatus* strains were therefore tested by transformation with pAB4-1, a pBR322 derivative carrying the *A. niger pyrG* gene (van Hartingssveldt et al. 1987). Uracil/uridine prototrophs were recovered in all three cases demonstrating that these three mutant strains carry a *pyrG* mutation. Southern analysis using the *A. niger pyrG* gene as a probe for the *A. fumigatus pyrG* locus or else a moderately repetitive sequence element of the *A. fumigatus* genome (Girardin et al. 1993) indicated the absence of any genomic rearrangement in all three mutants (data not shown). One of these strains, CEA17, was selected for further study.

A "*pyrG*-blaster" for gene disruption experiments in *A. fumigatus*

The newly-constructed vector pCDA14 (Fig. 1) carries the *A. niger pyrG* gene flanked by a direct repeat derived from a region of transposon Tn*5* coding for neomycin phosphotransferase, which confers kanamycin resistance in *E. coli*. The 8.6-kb *pyrG*-blaster is easily recovered by digestion of pCDA14 with *Hpa*I (Fig. 1).

Disruption of the *A. fumigatus rodA* gene using the *pyrG*-blaster

In order to test the functionality of the *pyrG*-blaster disruption cassette in transformation experiments and successive excision of the *pyrG* selection marker, we constructed

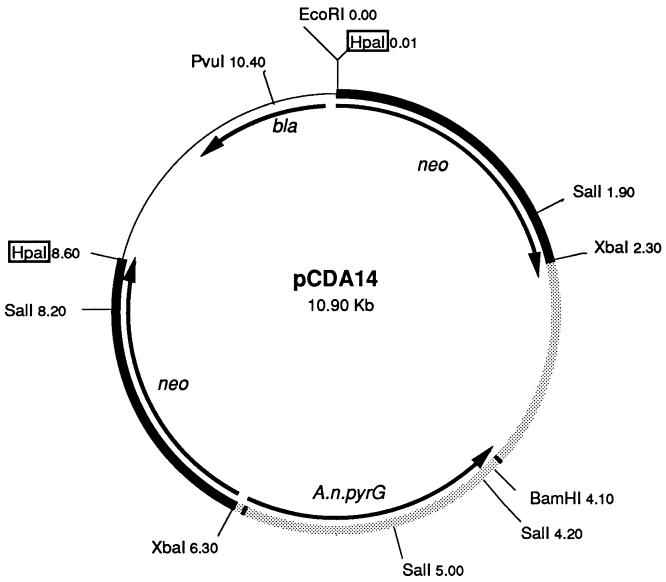
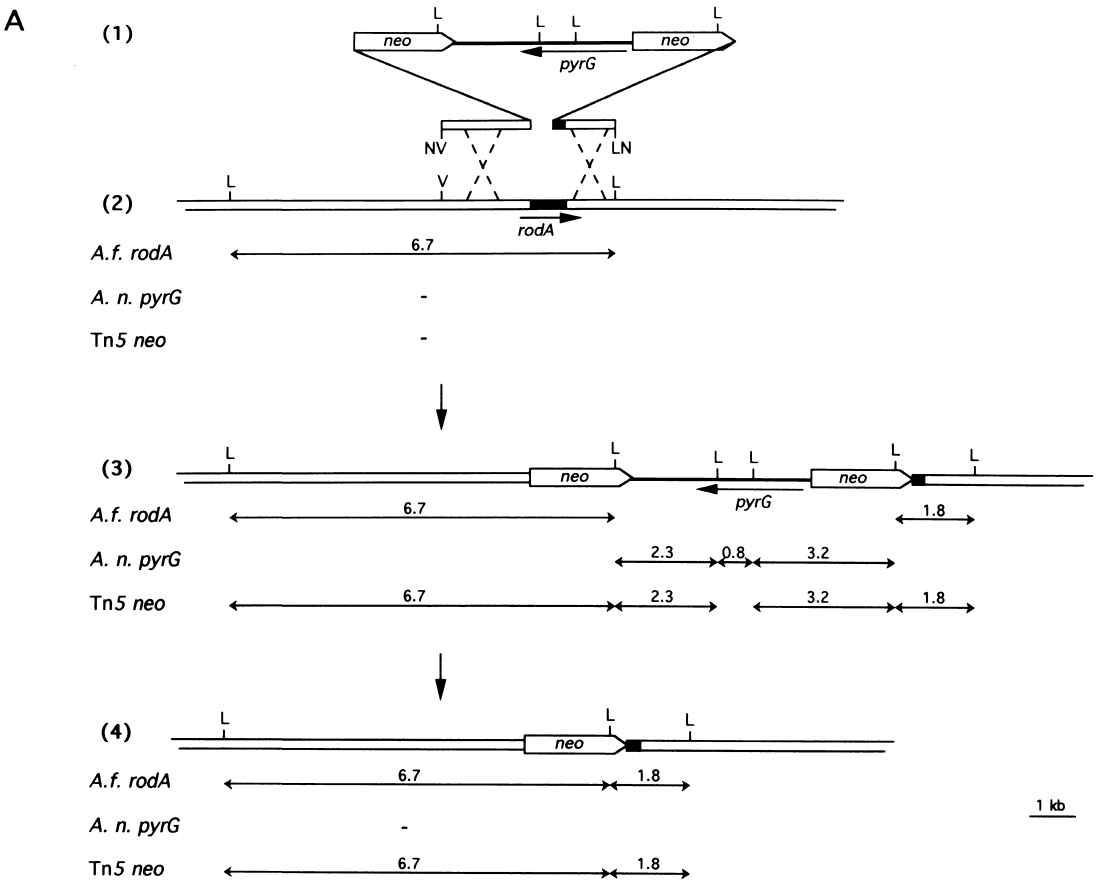


Fig. 1 Restriction map of pCDA14, a pBR322 derivative that contains the 8.6-kb *pyrG*-blaster consisting of the *A. niger pyrG* gene (*A.n. pyrG*) flanked by direct repeats. Direct repeats that contain the neomycin phosphotransferase (*neo*) gene of transposon Tn*5*, conferring kanamycin resistance in *E. coli*, are shown as *solid boxes*. *A. niger* DNA is shown as a *dotted box*. pBR322-derived DNA is shown as a *thin line*. The transcriptional directions of *A.n. pyrG*, *neo*, and *bla* that confers carbenicillin resistance in *E. coli*, are indicated by *arrows*. The positions of important restriction sites, including the two *Hpa*I sites (*boxed*) that are used to release the *pyrG*-blaster from pCDA14, are indicated

A. fumigatus rodA mutants by gene replacement. Due to the lack of their conidial rodlet layer, these mutants produce hydrophilic spores that are easily wettable and form colonies that are much darker than wild-type, thereby providing a visual screen for transformation-mediated gene replacement at the *rodA* locus (Thau et al. 1994).

The 3.7-kb insert of pAF3 (Thau et al. 1994), carrying flanking genomic regions of the *rodA* gene, was first subcloned into pBLSN$^+$ (see Materials and methods) to yield pCDA15. The 8.6-kb *Hpa*I fragment of pCDA14 was then subcloned into the unique *Hin*dIII site of pCDA15 resulting in pCDA16. *Not*I digestion of pCDA16 produces a 12.3-kb fragment containing 2 kb of the *rodA* 5′ non-coding region and 1.7 kb of the *rodA* 3′ non-coding region separated by the *pyrG*-blaster (Fig. 2-A1). This fragment was used to transform protoplasts of CEA17 to uridine/uracil prototrophy. Seven transformants were recovered. Only one displayed the RodA$^-$ phenotype. Southern analysis of *Sal*I-digested genomic DNA of this mutant strain, CEA17-16, demonstrated that the RodA$^-$ phenotype resulted from the expected gene replacement at the *rodA* locus as shown in Fig. 2: the wild-type 6.7-kb *Sal*I fragment (Fig. 2-A2 and B) was converted into five *Sal*I fragments exhibiting different hybridization patterns with the *rodA*, *pyrG* and *neo* probes (Fig. 2-A3 and B). This result demonstrates that the *pyrG*-blaster can be used for gene replacement in *A. fumigatus*.


A
(1)
neo
neo
pyrG
NV
LN
(2)
rodA
A.f. rodA
6.7
A. n. pyrG
Tn5 neo
(3)
neo
neo
pyrG
A.f. rodA
6.7
1.8
A. n. pyrG
2.3
0.8
3.2
Tn5 neo
6.7
2.3
3.2
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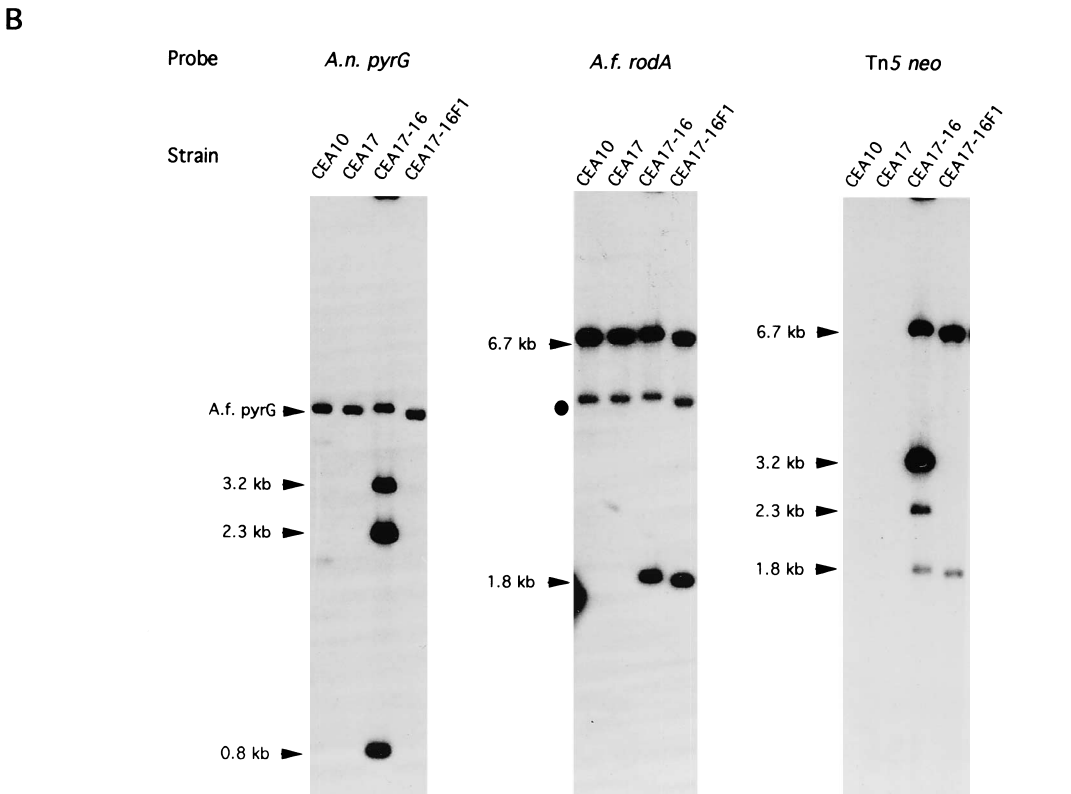
(4)
neo
A.f. rodA
6.7
1.8
A. n. pyrG
Tn5 neo
6.7
1.8
1 kb


B
Probe
A.n. pyrG
A.f. rodA
Tn5 neo
Strain
CEA10
CEA17
CEA17-16
CEA17-16F1
A.f. pyrG
3.2 kb
2.3 kb
0.8 kb
6.7 kb
1.8 kb
6.7 kb
3.2 kb
2.3 kb
1.8 kb

5-fluoro-orotic acid-mediated excision of the *pyrG*-blaster

Plating of conidiospores of strain CEA17-16 on minimal-medium containing 5-FOA, uridine, and uracil resulted in the appearance of uridine/uracil auxotrophs at a frequency of 4×10^{-4}. All of these auxotrophs retained their RodA$^-$ phenotype. In contrast, spontaneous uridine/uracil auxotrophs were obtained at a frequency of less than 10^{-7} from the wild-type strain, suggesting that the high level of reversion of the CEA17-16 strain was due to the insertion of the *pyrG*-blaster at the *rodA* locus. Southern analysis of *Sal*I-digested genomic DNA from six 5-FOA-resistant derivatives of strain CEA17-16 confirmed this hypothesis. As shown in Fig. 2 (panels A4 and B) for strain CEA17-16F1, the appearance of a PyrG$^-$ phenotype was correlated with the disappearance of three *Sal*I fragments of 3.2 kb, 2.3 kb and 0.8 kb corresponding to the *A. niger pyrG* gene and to one of the two elements of the direct repeat. Furthermore, the persistence of one *neo* gene at the disrupted *rodA* locus could be demonstrated by the similar hybridization profiles obtained with the *rod*A and *neo* probes (Fig. 2-A4 and B).

Using the *Hpa*I fragment of pCDA14 for transformation of strain CEA17, we had obtained several transformants that carry a single copy of the *pyrG*-blaster integrated at a random position in their genome (data not shown). As observed with strain CEA17-16, 5-FOA-resistant derivatives of these strains were obtained at a relatively high frequency (Table 2) and were found to result from the excision of the *pyrG*-blaster by recombination between the two elements of the direct repeat, one of which is maintained at a random site in the genome (data not shown). Data presented in Table 2 show that 5-FOA-resistant derivatives of *pyrG*-blaster-transformed strains can occur at frequencies close to 10^{-4} in all cases tested, suggesting that excision of the *pyrG*-blaster is independent of its chromosomal location.

Table 2 Frequency of *pyrG* loss by different *A. fumigatus* strains

Strain	Growth medium for spore production[a]	Frequency of PyrG$^-$[b]
CEA10	–U	$<10^{-7}$
CEA17-16	–U	$4\times10^{-4}/1.8\times10^{-4}$[c]
CEA17-14/1	–U	2×10^{-5}
CEA17-14/1	+U	2×10^{-4}
CEA17-14/2	–U	8×10^{-6}
CEA17-14/2	+U	9×10^{-6}

[a] –U: complete medium; +U: complete medium + 5 mM uridine and uracil
[b] Ratio between the number of colonies obtained on minimal medium containing uridine, uracil and 5-FOA and those obtained on minimal medium containing uridine and uracil
[c] Two separate experiments were performed for strain CEA17-16

Table 3 Frequency of RodA$^-$ mutants among transformants of various *A. fumigatus* strains

Strain	PyrG$^+$ transformants	RodA$^-$ transformants	Frequency of RodA$^-$ transformants
G10[a]	(42)	4	11.9%
CEA17 (exp.1)	7	1	14.3%
CEA17 (exp.2)	33	16	48.5%
CEA17-14/1F	59	12	20.3%
CEA17-14/2F	49	9	18.4%

[a] Thau et al. 1994; primary transformants of strain G10 were scored for hygromycin resistance using plasmid pAF3*hph*

Multiple disruptions using the *pyrG*-blaster

In order to test if the *pyrG*-blaster could be used in successive rounds of transformation, we took advantage of strains CEA17-14/1F and CEA17-14/2F which were generated by random integration of the *pyrG*-blaster in the genome followed by 5-FOA-mediated excision of the *A. niger pyrG* gene (see previous section). These strains can therefore be used to monitor the influence of an integrated copy of one element of the direct repeat on the legitimate recombination of a disruption construct containing the *pyrG*-blaster. Strains CEA17, CEA17-14/1F and CEA17-14/2F were transformed using *Not*I-digested pCDA16 and PyrG$^+$ transformants were scored for the RodA$^-$ phenotype. Results presented in Table 3 show that the presence of an endogenous copy of the *neo* gene does not interfere with the integration of the disruption construct at its expected target, since similar levels of RodA$^-$ transformants have been obtained in all strains tested.

Discussion

We have identified *pyrG* strains of *A. fumigatus* and shown that they are efficiently transformed to uridine/uracil prototrophy using the *A. niger pyrG* gene, thus providing a

Fig. 2 **A** gene replacement at the *rodA* locus of *A. fumigatus* using the *pyrG*-blaster. *1* map of the *Not*I fragment of pCDA16 containing *rodA* disrupted by the *pyrG*-blaster; *2* genomic DNA of the CEA17 recipient strain; *3* genomic DNA of RodA$^-$ transformants following gene replacement at the *rodA* locus with sequences of the *Not*I fragment of pCDA16; *4* genomic DNA of RodA$^-$ strains obtained after 5-FOA-selected excision of the *A. niger pyrG* gene and of one direct repeat. *Open box*, *A. fumigatus* DNA; *solid box*, coding region of the *rodA* gene; *solid line*, *A. niger* DNA; *open-boxed arrows*, direct repeats with the *neo* gene of transposon Tn*5*. *Sal*I restriction fragments predicted to hybridize with the *A. fumigatus rodA* (*A.f. rodA*), *A. niger pyrG* (*A.n. pyrG*) and Tn*5 neo* probes respectively are shown as *arrows* with their expected size in kb. *L Sal*I; *V Eco*RV; *N Not*I. **B** Southern hybridization of *Sal*I-digested genomic DNAs of wild-type strain CEA10, *pyrG* strain CEA17, *pyrG rodA::(neo-A.n. pyrG-neo)* strain CEA17-16 and *pyrG rodA::neo* strain CEA17-16F1. Identical filters were probed with the *A. niger pyrG* (*A.n. pyrG*), *A. fumigatus rodA* (*A.f. rodA*) and Tn*5 neo* probes as described in Materials and methods. The 4.5-kb *Sal*I fragment carrying the *A. fumigatus pyrG* gene is detected by the *A.n. pyrG* probe as indicated (*A.f. pyrG*). An unexpected 4.8-kb fragment, detected by the *A.f. rodA* probe in all four strains, is shown by a *dot* (see Material and methods). Sizes of the hybridizing restriction fragments are indicated in kb

new transformation system for *A. fumigatus*. Furthermore, we have reported on the design of a tripartite disruption cassette, referred to as a "*pyrG*-blaster", and its application to the construction of multiple gene replacements in *A. fumigatus*. This cassette was successfully used to disrupt the *rodA* gene of *A. fumigatus* or random genes in the *A. fumigatus* genome. Recombination between the two elements of the direct repeat in the *pyrG*-blaster can occur at high frequency (10^{-4}) and is easily selected in the presence of 5-FOA. The resulting strains have the expected mutation with only one element maintained at the target gene, and are therefore susceptible to a new round of transformation using *pyrG* as a selection marker. Our results are similar to those obtained by others with "ura-blasters" in *S. cerevisiae* (Alani et al. 1987) and *C. albicans* (Fonzi and Irwin 1993): a frequency of recombination between the two elements of the direct repeat averaging 10^{-4}, a low level (if any) of interchromosomal recombination (likely to be reduced in our experiments because of the use of the *A. niger pyrG* gene), and efficient integration of disruption constructs at their target site in strains that already carry the *neo* gene. In contrast to results obtained in *S. cerevisiae* and *C. albicans* using ura-blasters (Alani et al. 1987; Fonzi and Irwin 1993), we could not demonstrate spontaneous excision of the *A. niger pyrG* gene during vegetative growth (data not shown) because of technical limitations (replica plating of a large number of colonies of *A. fumigatus* is not easily achieved). However, the similar frequency of loss of *A. niger pyrG* and the homokaryotic nature of the 5-FOA-resistant colonies suggest that the excision process precedes the first nuclear division that occurs during conidiospore germination in the presence of 5-FOA.

The availability of a *pyrG*-based transformation system in *A. fumigatus* provides new opportunities to study this human pathogen. In addition to the transformation vectors that have been developed for the examination of other *Aspergillus* species, the dual selection that can be applied to the *pyrG* gene will allow the development in *A. fumigatus* of strategies that have already been used to manipulate the genomes of lower eukaryotes such as *S. cerevisiae* and *C. albicans* and that could not be developed using antibiotic-based selection procedures. For instance pop-in/pop-out gene replacements that allow targeted mutagenesis (site-specific deletion) at the genomic level (Rothstein 1991) are now accessible and should facilitate the characterization of genes that participate in *A. fumigatus* pathogenicity and/or proliferation. It is now possible to construct multiply disrupted strains of *A. fumigatus* and other fungal species that are efficiently transformed by the *A. niger pyrG* gene. The method should be especially useful in the study of processes that involve numerous isozymes, e.g. chitin biosynthesis (Mellado et al. 1995) and proteolysis (Jaton-Ogay et al. 1994; Reichard et al. 1994).

Transformation of *A. fumigatus* frequently results in ectopic integrations of the transforming DNA (Tang et al. 1992; Monod et al. 1993; Paris et al. 1993; Jaton-Ogay et al. 1994; Smith et al. 1994; Thau et al. 1994). A similar phenomenon was observed during transformation with the *pyrG*-blaster and resulted, after 5-FOA-mediated excision of the *A. niger pyrG* gene and of one element of the direct repeat, in *A. fumigatus* strains that only carry a single insertion of the Tn*5 neo* gene at a random site in their genome. This new aproach to produce insertional mutants of *A. fumigatus* might prove useful since the resulting strains, in contrast to those generated by ectopic integration of a shuttle vector, do not carry bacterial sequences other than the *neo* gene and are therefore more suitable for further gene manipulation. The method might be improved by taking advantage of restriction enzyme-mediated integration (REMI) of the transforming DNA, a process that has been shown to increase transformation efficiency and single-copy integration in several fungal species (Schiestl and Petes 1991; Shi et al. 1995). Furthermore, DNA regions flanking an insertion that causes a specific phenotype can be recovered, together with the Tn*5 neo* gene, on the basis of the kanamycin resistance conferred in *E. coli*. The applicability of this strategy, as well as its advantage over transformation-mediated mutagenesis with plasmids carrying an *E. coli* replication origin and selection marker, remain to be evaluated.

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