Utilization of the *Aspergillus nidulans pyrG* gene as a selectable marker for transformation and electroporation of *Neurospora crassa*

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We report the complementation of the *Neurospora crassa pyr-4* mutation with the *Aspergillus nidulans pyrG* gene. The use of the *pyrG* gene as a selectable marker for *N. crassa* transformation offers several advantages: a non toxic nutritional selection, the ability to put single-copy transformants through a sexual cross without the occurrence of RIP (Repeat-Induced Point mutation) by alleles of the *pyr-4* gene, and a relatively-high electroporation efficiency. These traits were exploited for targeted gene replacement in *N. crassa*. In addition, the high electroporation efficiency of *pyrG* achieved using supercoiled circular DNA renders it an ideal marker for random insertional mutagenesis studies in *N. crassa*.

The *A. nidulans pyrG* (Oakley *et al*., 1987 Gene **61**:385-399) and *N. crassa pyr-4* (Buxton and Radford 1983 Mol. Gen. Genet. **190**:403-405) genes encode orotidine 5'-phosphate carboxylase, the terminal enzyme in uridine 5'-phosphate biosynthesis. *N. crassa pyr-4* can complement the *pyrG89* mutation in *A. nidulans* (Waring *et al*., Gene **79**:119-130); however, it was not known if the *A. nidulans pyrG* would complement the *pyr-4* mutation. The inability to obtain non-leaky mutants at the *pyr-4* locus hampered this testing. We now have available a very tight *pyr-4* mutation in both mating types (FGSC 4030 & 4031) that allowed us to test for complementation of the *A. nidulans* gene in *N. crassa*. These mutants require uridine supplementation for growth.

Southern analysis under high-stringency conditions demonstrated that the *pyrG* DNA did not recognize any *N. crassa* genomic sequences in a wild type strain. This result is consistent with the very short regions of identity (maximum of 8 bp in a stretch; 46% identity overall) and numerous gaps observed after alignment of the DNA sequences of *pyrG* and *pyr-4* (corresponding to the amino acid coding region; data not shown). Taken together, these observations suggested that *pyrG* in single copy would not be subjected to RIP mutation by the presence of a *pyr-4* allele during a sexual cross in *N. crassa*.

We transformed 7-day old *pyr-4 A* conidia with supercoiled ppyrG plasmid DNA using the Vollmer and Yanofsky protocol (1986 Proc. Natl. Acad. Sci. USA **34**:573-577). The ppyrG plasmid consists of an *Eco*RI-*Nde*I fragment from pJR15 cloned into pUC19 (Gregory May, personal communication; Figure 1). The *N. crassa pyr-4* gene carried on the pRG1 plasmid (Waring *et al*., 1989 Gene **79**:119-130; generously provided by Gregory May) was used as a positive control. Transformants were selected for growth on minimal medium. The low transformation frequency obtained for both ppyrG and pRG1 (8 transformants/ ug DNA) was

attributed to a 30 minute spheroplasting step; in subsequent experiments, the spheroplasting time was reduced and the transformation frequency improved ten-fold.

The original transformation yielded stable transformants for both *pyrG* and *pyr-4* genes. The transformants were purified to homokaryons by repeated plating of single conidial isolates. Characterization of homokaryons included Southern analysis and examination of progeny from crosses to strains 73 *a* and *pyr-4 a*. Southern analysis was performed on nine *pyrG* transformants and two *pyr-4* transformants. We observed three types of integration events for *pyrG* transformants; 1) single ectopic integration (four transformants); 2) multiple ectopic integration (one transformant); and 3) integration of tandem repeats (three transformants). One transformant had no detectable *pyrG* DNA. Of the two *pyr-4* transformants, one contained a homologous integration and the other an ectopic integration event.

All single ectopic integration *pyrG* transformants produced *pyrG*+ progeny, indicating that *pyrG* was not mutated by RIP in *N. crassa*. When using the *pyr-4* mutant as a female it was necessary to supplement synthetic crossing medium with 20 mg/ml uridine (Perkins *et al*., 1982 Microbiol. Rev. **46**:426-570).

To test the utility of the *A. nidulans pyrG* gene as a selectable marker for electroporation (Vann, 1995 Fungal Genet. Newsl. **42A**:53; Ivey *et al*., 1996 Mol. Biol. Cell **7**:1283-1297), we electroporated 8-day old conidia from *N. crassa* strains *pyr-4A* and *pyr-4a* with ppyrG. Briefly, 40 ul of a solution containing 2.5 x 10^9 conidia/ml in 1 M sorbitol (10^8 conidia total) was mixed with 10 ul of supercoiled ppyrG plasmid (2 ug) in an electroporation cuvette. Electroporation coniditions were 25 mA, 25 W, 1500 V, 200 ohms and 71 F. After electroporation, 960 ul of 1M sorbitol was added to the electroporation cuvette. A substantial number of electroporants was obtained after plating only 5-10 ul of the 1 ml electroporation solution, with an efficiency of 3400 electroporants/10^8 conidia or 1700 electroporants/ ug DNA.

Having determined that *pyrG* was an efficient marker for electroporation, we next used this gene as a selectable marker for construction of a targeted gene replacement mutation in *N. crassa* (Baasiri *et al*., submitted). The gene subjected to mutation was *N. crassa gna-2*, which encodes a heterotrimeric G[[alpha]] subunit protein. The deletion construct (pRAB11) was made by replacing a *Stu*I-*Nco*I fragment containing most of the amino acid coding region of *gna-2* with the 2.3 kb *Eco*RI-*Nde*I fragment from ppyrG. The construct contained 2.4 kb and 1.26 kb of 5' and 3' flanking *N. crassa gna-2* DNA, respectively.

Electroporation was performed using 2 ug linearized (to increase the rate of double crossover events) pRAB11 and 10-day old *pyr-4 a* or *pyr-4 A* conidia. Approximately 450 electroporants were obtained/ ug linearized pRAB11 DNA. Genomic DNA was extracted from 47 *pyr-4 a* and 37 *pyr-4 A* electroporants. Southern analysis revealed that 2 of the *pyr-4 a* and 3 of the *pyr-4 A* electroporants possessed a band corresponding to targeted integration of the deletion construct (and no other ectopic copies of the electroporation construct), yielding a homologous recombination frequency of 5/84 or 6%. This frequency is similar to that obtained in making gene replacement mutations in other *N. crassa* genes using a comparable amount of homologous flanking DNA (Aronson *et al*., 1994 Mol. Gen. Genet. **242**:490-494; Ivey *et al*., 1996 Mol. Biol. Cell **7**:1283-1297). The five electroporants with the gene deletion were all heterokaryons. To

isolate homokaryotic mutants, each heterokaryotic electroporant was used as a male in a cross with a *N. crassa pyr-4* female of opposite mating type. All ascospore progeny which germinated and grew into colonies on minimal medium were homokaryons for the gene deletion as assessed by Southern analysis. The GenBank accession number for the *A. nidulans pyrG* gene is M19132. The ppyrG plasmid and *pyr-4* mutant strains used in these studies are available from the Fungal Genetics Stock Center.

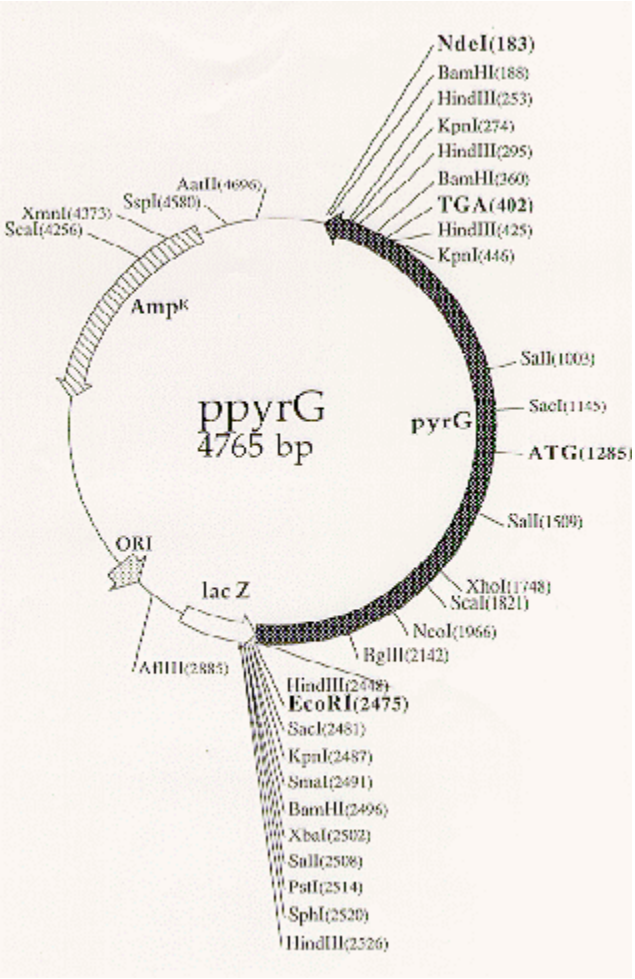

Figure 1. Map of the ppyrG plasmid.
The *A. nidulans pyrG* gene present on a 2.29 kb *Eco*RI-*Nde*I fragment from pJR15 was subcloned into the same sites in pUC19 to yield ppyrG (Gregory May, personal communication). All sites for the indicated restriction enzymes are shown. The bold ATG and TGA denote the *pyrG* translational start and stop codons, respectively.

[Return to the FGN 44 Table of Contents]

[Return to the FGSC home page]