

A *ras-1^{bd}* Mauriceville strain for mapping mutations in Oak Ridge *ras-1^{bd}* strains

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We describe the construction of a *Neurospora crassa* Mauriceville strain carrying the *ras-1^{bd}* mutation marked by the bacterial hygromycin resistance gene, *hph* (new FGSC # 10156). This strain is valuable for mapping mutations in Oak Ridge strains that carry the *bd* mutation.

The *bd* mutation has been a benefit to circadian rhythm research as it slows down wild type growth rates and sharpens the conidial banding pattern on racetubes (Sargent et al., 1966; Bell-Pedersen et al., 2005). Recent work (Belden et al., 2006) has shown that the *bd* mutation lies in the *ras-1* gene. Mutations that affect circadian banding patterns are typically isolated in strains carrying the *ras-1^{bd}* allele. Mapping such mutations by the CAPS method (Jin et al., in press) requires that the mutant phenotype be followed in a cross to a wild type Mauriceville strain. Assuming non-linkage, only half of the progeny will be *bd*, and thus readily scorable. Insertion of the *ras-1^{bd}* allele into the Mauriceville parent renders all progeny useful for segregation analysis. Furthermore, marking *ras-1^{bd}* with the hygromycin resistance gene, *hph*, permits a simple drug resistance test to determine linkage to *ras-1*.

The strategy for gene replacement was similar to published split marker gene deletion strategies (Catlett et al., 2003; Colot et al., 2006). However, rather than deleting the wild type gene, we replaced it with the *bd* allele and inserted the *hph* gene in the 3'UTR at position + 225 from the stop codon (Figure 1).

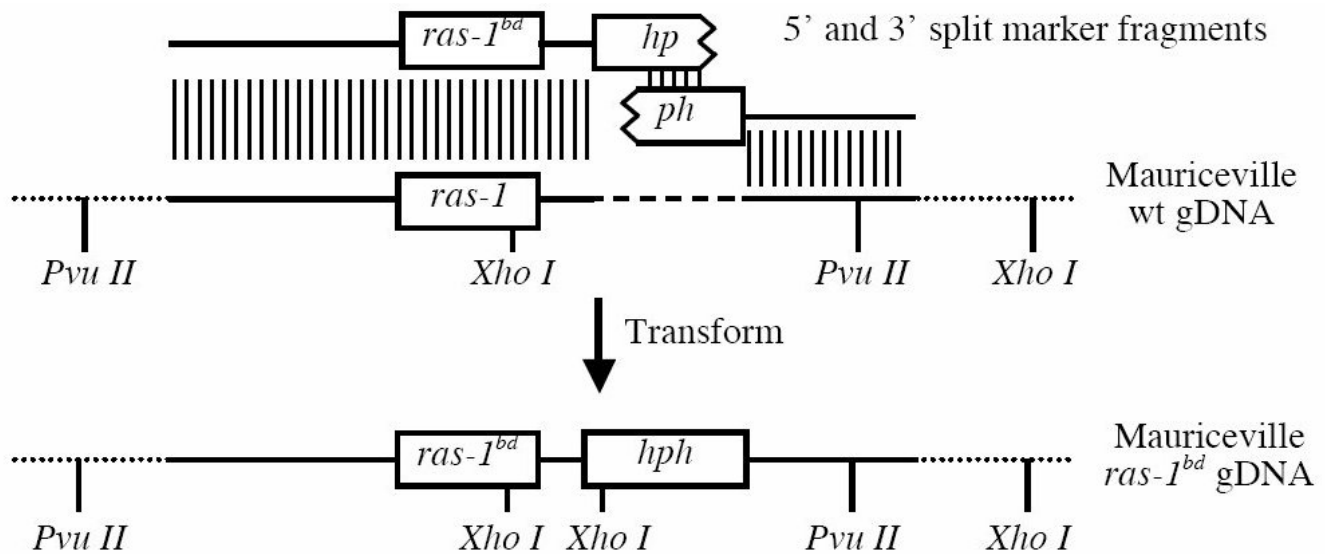


Figure 1. Schematic diagram of *ras-1* genetic manipulations. Regions of homology are indicated by vertical hatch marks; there are 763 bp of homology between the split *hph* fragments, 3223 bp of homology at the 5' end, and 2582 bp of homology at the 3' end. *Pvu II* and *Xho I* sites are marked to show the differences between the wild type (wt) genomic (g) locus and the proper *ras-1^{bd}-hph* integrant. The dashed line indicates a gap where the *hph* gene is integrated and the dotted lines indicate flanking genomic DNA that remains unchanged.

The replacement fragments were generated in two steps. The first step involved amplification of *ras-1^{bd}* genomic regions from the Oak Ridge *bd* strain (FGSC 1858). The 5' region was amplified using primers *ras1 F1* and *ras1-hyg R1*, while the 3' region was amplified using primers *hyg-ras1 F2* and *ras1 R2*. The second step was to generate hybrid pieces that contained a portion of the bacterial *hph* gene fused to either 5' or 3' regions of *ras-1^{bd}* (Figure 1). The 5' split marker fragment was generated by amplification of the 5' region of *ras-1^{bd}* and the complete *hph* gene, with primers *ras1 F1* and *YG2*. The 3' split marker fragment was generated by amplification of the 3' region of *ras-1^{bd}* and the complete *hph* gene, with primers *HY2* and *ras1 R2*. The complete *hph* gene was obtained by amplification of pBP15 (a gift from D. Ebbole) with primers *M13F2* and *M13R2*. The pBP15 plasmid contains a 1.4kb *HpaI PtpC-hph* fragment from pCB1003 (Carroll et al., 1994) subcloned into the *EcoRV* site of pBluescript II SK

(Stratagene, TX). The sequences for all primers used are shown in Table 1, and the genotypes of strains are listed in Table 2.

Table 1

Name of primer	Sequence (5' to 3')
ras1 F1	TGCATGCTGTGCCGCCTAAC
ras1-hyg R1*	actggccgctggtttacaacgtcgtgacTAAGGTATGGGCGTGGCCGT
hyg-ras1 F2*	ggctatagctgtttcctgtgtgaaattgGCTCACGTACGGTGACCAGT
ras1 R2	TATGGTGGAGCTACCGACAG
HY2	GTTGGTCAAGACCAATGCGGAGCA
YG2	CGACAGCGTCTCCGACCTGATG
M13F2	GTCACGACGTTGTAAAACGACGGCCAGT
M13R2	CAATTTACACAGGAAACAGCTATGACC

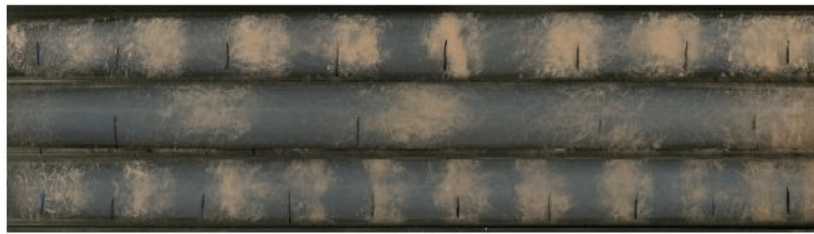
* lower case sequence corresponds to *hph* homology regions.

Table 2

Name of strain	Genotype
FGSC 1858	Oak Ridge <i>ras-1^{bd} mat A⁺</i>
FGSC 2225	Mauriceville-1c <i>mat A⁺</i>
FGSC 10156 (FGSC 2225 tfm10)	Mauriceville-1c <i>ras-1^{bd} mat A⁺</i>

The 5' and 3' split marker products were co-transformed by electroporation into the wild type Mauriceville-1-c *mat A⁺* strain (FGSC 2225) (Colot et al., 2006). We restricted this work to the *mat A⁺* strain since the *mat a⁺* Mauriceville-1d strain (FGSC 2226) cannot be used as a crossing partner with an Oak Ridge strain of opposite mating type for CAPS mapping. This is because the Mauriceville-1d strain is an independent isolate that possesses primarily Oak Ridge-type polymorphisms (Jin et al., in press). Several (~50) hygromycin resistant colonies grew, and conidia from 12 of these were extruded through a 5µm filter (Millex-SV, Millipore) to obtain homokaryotic micro-conidia (Ebbole and Sachs, 1990). Each isolate was placed on racetubes for growth rate determination and period determination of the conidiation rhythm. Seven out of twelve transformants had growth rates and periods consistent with replacement of the wild type *ras-1* gene with the *bd* allele; growth rates were reduced from the wild type Mauriceville level of ~7.8 cm/day to ~2.7 cm/day, and their conidial bands became more focused, permitting period measurement (~22.4 hours). One of these transformants (tfm10) was grown and conidia were extruded through a 5µm filter a second time to ensure isolation of a homokaryon. Figure 2 shows the race tube phenotype of the doubly purified tfm10 compared to Oak Ridge *bd* (FGSC1858) and Mauriceville wild type (FGSC 2225) strains. Thus, tfm10 is hygromycin resistant and carries the *bd* allele of *ras-1*.

To verify that recombination occurred as expected at the *ras-1* locus, and that extra copies of the inserted DNA were not present, we performed Southern blot analysis on the doubly purified tfm10 (Figure 3). As expected, digestion with *Pvu II* and hybridization with a 5' *ras-1* probe yielded a 5144 bp signal in the wild type Mauriceville strain (FGSC 2225), and a larger 6804 bp signal in tfm10. Digestion with *Xho I* and hybridization with a *hph* probe yielded no signal in the wild type Mauriceville strain, and a unique 5655 bp signal in tfm10. Thus, the doubly filtered tfm10 has recombined without additional ectopic insertion. We have deposited this transformant to the Fungal Genetics Stock Center, *Neurospora* strain FGSC 10156.



Strain	Growth Rate (cm/day)	Period (hrs)
1858	3.6	22.1
2225	7.8	ND
2225 tfm10	2.7	22.4

Figure 2. Racetube phenotype. Racetubes containing 1 x Vogels Medium N, 0.1% glucose, 0.17% arginine, and 1.5% agar were inoculated with conidia from the indicated strains and grown for one day at 30°C in the light. The position of the growth front was marked, and tubes were then shifted to constant darkness at 25°C. The growth front was marked subsequently every 24 hours (vertical black lines). Controls include the wild type Mauriceville strain (FGSC 2225) and an Oak Ridge *bd* strain (FGSC 1858).

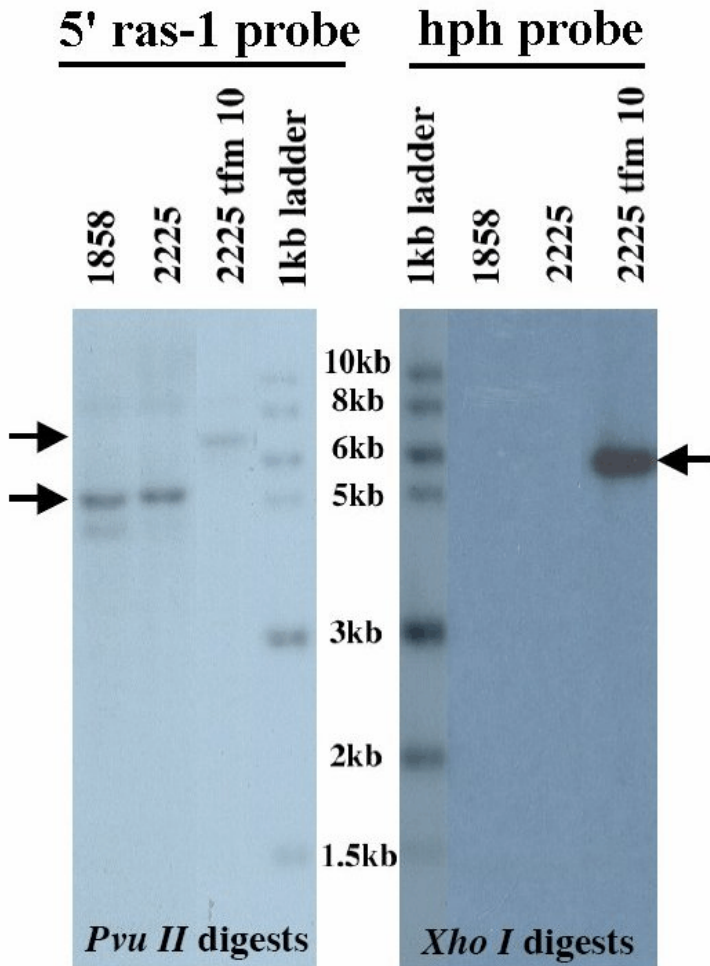


Figure 3. Southern blot analysis of *Neurospora* strains. Genomic DNA from the indicated strains was digested with *Pvu II* and probed with a labeled 5' *ras-1* fragment or digested with *Xho I* and probed with a labeled *hph* fragment. Blots were hybridized overnight, washed, and exposed to film for 16 hours.

We are currently using this new *ras-1^{bd}* Mauriceville strain to genetically map a mutation isolated in the Oakridge *bd* background that permits circadian conidiation rhythms to occur in constant light. This strain will also permit rapid determination of linkage to

ras-1 using a simple hygromycin resistance test, and will allow mapping of any mutation in Oak Ridge strains that depends on the *ras-1^{bd}* allele. Investigators studying genetic interactions with the *RAS* pathway may find this strain useful. Lastly, the methods described to create the knock-in *ras-1^{bd}* allele are applicable for generating strains carrying point mutations in any *Neurospora* gene of interest, and in particular for investigating the function of essential genes.

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