Transformation of *Aspergillus nidulans* with the hygromycin-resistance gene, *hph*

(Recombinant DNA; plasmid vectors; filamentous fungi; hygromycin phosphotransferase; integration)

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**SUMMARY**

*Aspergillus nidulans* strain G191 was transformed to hygromycin resistance using plasmid pDH25, which contains the bacterial hygromycin B phosphotransferase gene (*hph*) fused to promoter elements of the *A. nidulans trpC* gene. Southern hybridizations of transformants revealed multiple, integrated copies of the vector. A pleiotropic effect conferring increased hygromycin B sensitivity was found to be associated with the *A. nidulans pyrG89* allele. Plasmid pDH25 features a *Cla*I site immediately preceding the *hph* start codon thus permitting convenient replacement of the *trpC* sequences with other eukaryotic promoters.

**INTRODUCTION**

Fungal transformation systems based upon expression of dominant selectable markers have been intensively sought. An *A. nidulans* system based upon resistance to oligomycin has been reported by Ward et al. (1986). A mutated ATPase subunit 9 gene, which confers oligomycin resistance, is used as the selectable marker. Similarly, an altered B-tubulin, which confers benomyl resistance, forms the basis of a *Neurospora crassa* transformation system (Orbach et al., 1986; Volmer and Yanofsky, 1986).

Another approach has been to use bacterial phosphotransferases which confer resistance to aminoglycoside antibiotics, particularly Hyg. Fusions of fungal promoters with the *hph* gene have been used to transform *Saccharomyces cerevisiae* (Gritz and Davies, 1983) and, most recently, several filamentous fungi, e.g., *Cephalosporium acremonium* (Queener et al., 1985; P. Skatrud, personal communication), *Ustilago maydis* (J. Wang, D. Holden, and S.A.L., manuscript submitted), and *Cochiobolus heterostrophus* (O. Yoder, personal communication). We describe here a vector which carries a fusion of the transcriptional elements of the *A. nidulans trpC* gene to *hph* and its integration and expression in *A. nidulans*. 

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*Abbreviations:* DMSO, dimethylsulfoxide; bp, base pair(s); HPH, Hyg phosphotransferase; *hph*, gene coding for HPH; Hyg, hygromycin B; kb, 1000 bp; Pollk, Klenow (large) fragment of *E. coli* DNA polymerase I; *, resistant; TBE, 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA; YEG, 0.5% (w/v) yeast extract, 2% glucose.
MATERIALS AND METHODS

(a) Strains and plasmids

Escherichia coli 294 (ATCC31446) was used for routine plasmid propagation. The sources and genotypes of A. nidulans are listed in Table I. Plasmid pLG90, containing the hph gene, was obtained from L. Gritz (Gritz and Davies, 1983). Plasmids containing the A. nidulans trpC gene were isolated from a plasmid library using oligodeoxynucleotide probes to the published trpC sequence (Mullaney et al., 1985).

![Diagram of plasmid pDH25]

Fig. 1. Construction of plasmid pDH25. A ClaI site was introduced immediately preceding the A. nidulans trpC start codon by mutagenesis of phage M13. A Clai site was also introduced immediately preceding the hph test codon. The sequence preceding the start codon derived from ligation of the two introduced Clai sites is shown at the bottom. Plasmid pDH25 was put together from the following modules: a 1250-bp EcoRI-ClaI fragment comprising the promoter of the A. nidulans trpC gene, denoted P

(b) DNA manipulations

Plasmid preparations used in fungal transformations were isolated by the cleared lysate procedure (Clewell and Helinski, 1972) and purified on a Bio-gel A50 column. Small-scale preparations of plasmids were isolated as described by Birnboim and Doly (1979). Oligodeoxynucleotide mutagenesis was performed by the procedure of Zoller and Smith (1982). Oligodeoxynucleotides were provided by the Genentech Organic Synthesis Group. Subsequent to mutagenesis, sequences were confirmed by the dideoxy chain termination method (Sanger et al., 1977). Restriction enzymes and PolIk were purchased from commercial sources. E. coli transformation was performed by the calcium shock procedure (Cohen et al., 1972) with selection on LB plates supplemented with 50 µg/ml of carbenicillin. Construction of pDH25 is described in the legend to Fig. 1.

(c) Preparation of Aspergillus nidulans protoplasts

Conidia were incubated at 37°C in YEG. Approximately 1 × 10⁸ log-phase germlings were harvested by centrifugation, washed with YEG, and incubated at 30°C in 50% YEG containing 0.6 M KCl, 0.5% Novozym 234 (Novo Industries, Copenhagen, DK), 0.5% MgSO₄ • 7H₂O and 0.05% bovine serum albumin. After 90 min incubation, the digest was filtered through Miracloth (Calbiochem-Behring Corp., La Jolla, CA), washed by centrifugation and resuspended in 0.6 M KCl.

(d) Sensitivity to hygromycin

Approximately 1 × 10⁴ protoplasts of each A. nidulans strain were inoculated into modified Aspergillus minimal medium (Rowlands and Turner, 1973). Supplements included 1.2 M sorbitol, p-aminobenzoic acid (20 µg/ml), arginine (500 µg/ml), uridine (500 µg/ml), tryptophan (1 mg/ml), methionine (100 µg/ml), pyridoxine (20 µg/ml), and biotin (5 µg/ml). Plates also contained 0, 10, 50, 250, 500, 750, or 1000 µg Hyg/ml. Duplicate cultures were incubated at 37°C and radial diameters recorded after 5 days.
(e) Transformation of *A. nidulans*

Protoplasts of strain G191 were transformed as previously described (Ballance et al., 1983) except that protoplast fusion was accomplished using 25% polyethylene glycol 4000 containing 0.6 M KCl. Following transformation, fused protoplasts were concentrated by centrifugation and added to molten overlays of minimal media containing 1.2 M sorbitol, 500 µg/ml of uridine, 20 µg/ml of p-aminobenzoic acid, and 2.0% agar. Overlays were spread onto 15 ml of the same medium and incubated at room temperature. After 12 h, a second 10-ml overlay containing 12.25 mg Hyg in the same medium was spread over the first layer. Plates were incubated at 37°C. Hyg<sup>R</sup> colonies were transferred to minimal media containing 100 µg Hyg/ml.

(f) Southern hybridization analyses

Conidia of the parental strain, G191, and two Hyg<sup>R</sup> transformants were inoculated into 100 ml YEG amended with 20 µg/ml of p-aminobenzoic acid and 500 µg/ml of uridine. The relative stability of the transforming vector was assessed by inoculating parallel cultures; one containing 5 µg Hyg/ml and the other devoid of the drug. (Preliminary experiments had shown that although transformants could tolerate more than 1000 µg Hyg/ml in agar media, sensitivity was much increased in submerged culture such that 10 µg Hyg/ml effectively stopped all growth.) Cultures were harvested by filtration and the DNA was extracted and purified as described by Hynes et al. (1983).

RESULTS AND DISCUSSION

(a) Transformation procedure

Using the protocol described above, Hyg<sup>R</sup> colonies of strain G191 were clearly visible after five days incubation at 37°C. These presumptive transformants remained resistant even after growth on non-selective agar media. The procedure yielded transformation efficiencies similar to our transformations of G191 using the *pyr4* plasmid pDJB3 (Ballance et al., 1983), e.g., approximately five transformants per µg DNA. Attempts to transform *A. nidulans* with the yeast vector pLG90 (Gritz and Davies, 1983) or the *U. maydis* vector pHL1 (J. Wang, D. Holden, and S.L. Leong, manuscript submitted) were unsuccessful, as were the attempts to transform *S. cerevisiae* and *U. maydis* with pDH25.

Numerous variations in the selection protocol were assessed. The 12-h expression period was necessary. Longer periods (over 16 h) permitted

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**TABLE I**

<table>
<thead>
<tr>
<th>Strain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Source&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Genotype</th>
<th>Hygromycin B (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IRG&lt;sub&gt;90&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>(1) FGSC237</td>
<td>FGSC</td>
<td><em>pabA1</em>, <em>yA2</em>; <em>trpC801</em></td>
<td>750</td>
</tr>
<tr>
<td>(2) FGSC4</td>
<td>FGSC</td>
<td>wild type</td>
<td>875</td>
</tr>
<tr>
<td>(3) pDJB3-1</td>
<td>G191 transformant</td>
<td><em>pyrG89</em>, <em>pabA1</em>; <em>fxA1 waY9</em>:*pyr4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>500</td>
</tr>
<tr>
<td>(4) W-1</td>
<td>P. Węglenński</td>
<td><em>argB2</em>, <em>merH2</em>; <em>haA1</em></td>
<td>450</td>
</tr>
<tr>
<td>(5) GB20</td>
<td>G. May</td>
<td><em>pyrG89</em>, <em>pabA1</em>; <em>fxA1 waY9</em>, <em>benA22</em></td>
<td>200</td>
</tr>
<tr>
<td>(6) G191</td>
<td>D.J. Ballance</td>
<td><em>pyrG89</em>, <em>pabA1</em>; <em>fxA1 waY9</em></td>
<td>150</td>
</tr>
<tr>
<td>(7) GR5</td>
<td>G. May</td>
<td><em>pyrG89</em>, <em>wA3</em>; <em>pyroA4</em></td>
<td>150</td>
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<tr>
<td>(8) GR1</td>
<td>G. May</td>
<td><em>pyrG89</em>, <em>pabA1</em>, <em>yA2</em>; <em>waA9</em></td>
<td>125</td>
</tr>
</tbody>
</table>

<sup>a</sup> *Aspergillus nidulans* strains. pDJB3-1 is G191 transformed with pDJB3 as described by Ballance and Turner (1986). FGSC, Fungal Genetics Stock Center, St. Louis, MO. P. Węglenński, Dept. of Genetics, Warsaw University, 00-478 Warsaw (Poland).

<sup>b</sup> Transformant contained the mutant allele *pyrG89* plus multiple copies of wild-type *N. crassa pyr4* gene.

<sup>c</sup> Concentration producing 50% Inhibition of Radial Growth. Average of two determinations.

<sup>d</sup> Maximum concentration tolerated. Average of two determinations.
significant background growth and shorter periods (less than 8 h) yielded no colonies. Diffusion methods (Ward et al., 1986; Orbach et al., 1986), which permit an expression period after plating, failed. The choice of osmotic stabilizer was important. Ionic osmotic buffers, e.g., KCl, MgSO₄, dramatically decreased drug sensitivity. The effect of Mg²⁺ and Ca²⁺ ions has been reported by Queener et al. (1985).

Substantial strain variability, with respect to Hyg sensitivity, was observed (Table I). Interestingly, the pyrG89 mutation appears to confer a pleiotropic effect of increasing sensitivity. Regardless of genetic background, pyrG89 was associated with increased sensitivity, and this sensitivity was reversed by transforming with the *N. crassa* pyr4 gene, i.e., pDJB3-1 (Table I). A similar pattern of sensitivity was observed on media lacking osmotic stabilizer. In practice, this phenomenon limits the usefulness of *hph* as a selectable marker within *A. nidulans*, although it is possible to recombine pyrG into other backgrounds by sexual or parasexual methods or by selection for 5-fluoro-orotic acid resistant strains (Boeke et al., 1984).

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**Fig. 2.** Southern hybridizations of DNA extracted from parental strain G191 (lane A), and transformants No. 1 (lanes B and C) and No. 2 (lanes D and E). DNA was purified from mycelia grown in YEG medium (lanes A, B, D) or YEG supplemented with 5 μg Hyg/ml (lanes C and E). Digestions were size-fractionated in a 0.5% agarose gel in 0.5 x TBE. Each lane was loaded with approx. 5 μg digest DNA. Four separate hybridizations were performed. (Panel a) Total genomic DNA was digested with *Eco*RI + *Cla*I and the corresponding vector fragment containing the trpC promoter was used as probe. (Panel b) DNA was digested with *Bam*HI and probed with the 712-bp *Bam*HI terminator fragment. (Panel c) DNA was digested with *Cla*I + *Bam*HI and probed with the 1-kb *hph* *Cla*I-*Bam*HI fragment. (Panel d) DNA was digested with *Hind*III and probed with the entire vector. There were no *Hind*III sites within pDH25. The positions of phage λ DNA fragments (kb) are marked. In all cases, Nytran DNA transfers were prepared as recommended by the manufacturer (Schleicher & Schuell, Keene, NH), and high-stringency hybridization conditions using heparin were employed (Singh and Jones, 1984). Vector fragments were electrotoluted from polyacrylamide gels, nick-translated, and incorporated at 5 x 10⁵ cpm/ml in hybridization buffer.
(b) Chromosomal integration of vector

Transformation involved chromosomal integration of the plasmid. Attempts to transform *E. coli* with total DNA from transformants have failed repeatedly suggesting that no free plasmid was present. Similarly, Southern hybridization analysis of undigested DNA failed to show any low-\(M\) hybridization characteristic of autonomous plasmids (not shown).

Southern transfers (Fig. 2) clearly show that multiple copies of vector integrated into the genome, and that transformant No. 2 had more copies than No. 1. Hybridization patterns for DNA isolated from cultures containing antibiotic vs. DNA from those without antibiotic were identical demonstrating that the integrated sequences were relatively stable (B vs. C; D vs. E; Fig. 2).

The integration mechanisms can be inferred from Southern hybridization analysis. For example, in the case of transformant No. 2, some integration events may have occurred by non-homologous recombination within the vector *trpC* promoter region. This is inferred from the presence of hybridization signals at 1.9 and 3.9 kb, in addition to the native promoter at 2.4 kb and the intact vector sequences at 1.3 kb (Fig. 2a; lanes D and E). Homologous recombination at the *trpC* promoter or recombination involving other vector regions (e.g., *trpC* terminator) would yield only the 1.3 and 2.4 kb bands. Using similar reasoning, some integration events involving non-homologous recombination or deletions within the vector *hph* gene may have occurred in transformant No. 1 (Fig. 2c; lanes B and C).

Tandem duplications are likely to have occurred. The *Hin* dIII digestion yielded bands larger than 25 kb when probed with pDH25, which contains no *Hin* dIII sites. In contrast, multiple, unlinked integration events should have yielded some lower-\(M\) bands.

(c) Conclusions

In addition to the transformation of *pyrG* *A. nidulans* strains, pDH25, or its derivatives, may be useful for other fungi. For example, the important plant pathogens *Fusarium oxysporum* (H.K. Kistler, personal communication), *Colletotrichum* sp. and *Cochiobolus* sp. (O. Yoder, personal communica-

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