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Abstract

We have constructed 2 tn5-containing plasmids, pLH1 and pLH3, specialized towards mutagenesis of genes from fungi which are auxotrophic for arginine or sensitive to hygromycin (such as the filamentous fungi *Aspergillus nidulans* and *Magnaporthe grisea*.) These plasmids are also a useful means of integrating additional marker genes in the plasmid backbone.

Bacterial transposons containing markers for fungal gene disruption

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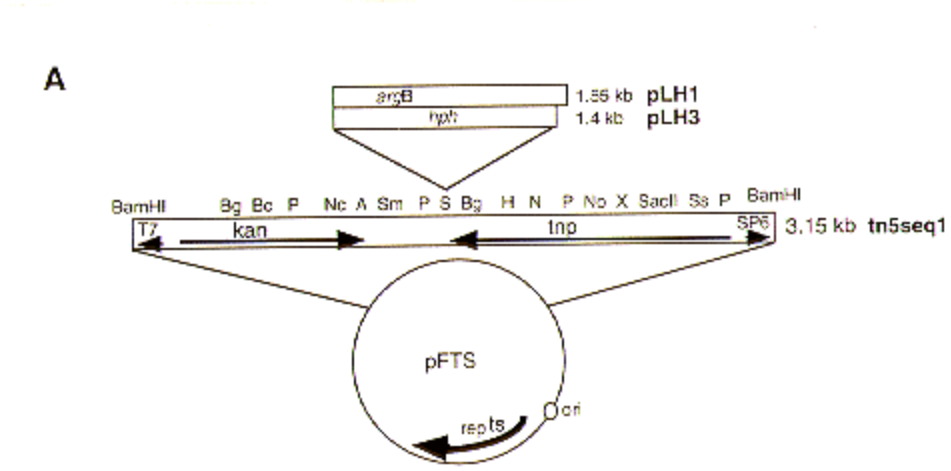
We have constructed 2 tn5-containing plasmids, pLH1 and pLH3, specialized towards mutagenesis of genes from fungi which are auxotrophic for arginine or sensitive to hygromycin (such as the filamentous fungi *Aspergillus nidulans* and *Magnaporthe grisea*.) These plasmids are also a useful means of integrating additional marker genes in the plasmid backbone.

Application of gene knock-out technology in filamentous fungi by homologous recombination generally requires construction of a disruption vector. This vector is composed of parts of the gene flanking an appropriate marker gene. To speed up the process of disruption vector construction, we have combined the transposing ability of tn5seq1 (Nag *et al*. 1988 Gene **64**:135-1145) and the thermosensitive *rep* gene from pFDX600 (Vogele *et al*. 1991 Nucl. Acids Res. **19**:4377-4385) as described (Urban *et al*. 1996 Mol. Gen. Genet. **250**:414-420) with the *argB* (Upshall *et al*. 1986 Mol. Gen. Genet *204*:349-354) gene marker for ornithine carbamoyltransferase (pLH1) and the hph (modified, from Carroll *et al*. 1994 Fungal Genet. Newsl. **41**:22-88) gene marker for hygromycin B phosphotransferase (pLH3). The *argB* and *hph* marker genes are functional in *A. nidulans* and *M. grisea*, respectively. Transposition events can be induced in *E. coli* cells harboring both the plasmid containing the gene to be disrupted and pLH1/3. Application of high selection pressure against the presence of pLH1/3 (restrictive temperature) and towards the transposition of tn5seq1 (high antibiotic concentration) allows identification of transposition into the target plasmid. Figure 1 outlines the procedure and the mutagenesis protocol is provided at the end of this article.

We show that the 6.1kb DNA fragment containing the *sepA* gene from *A. nidulans* strain A28 (*paba*A6 *bi*A1) cloned in *E. coli* (pLH5) (Harris *et al*. 1997 EMBO submitted) can be readily mutagenized by pLH1. We have made multiple disruption derivatives of pLH5 with the concomitant introduction of the *arg*B gene (Figure 2). Using a 3.8 kb pBR322-derived backbone, transpositions into the 6.1 kb *sepA* gene were observed at a frequency of 0.25. The exact position of the disruption can easily be determined, due to the presence of SP6 and T7 priming sites at the ends of the intervening tn5seq1/*arg*B cassette. Because the disruption is generated by an insertion, the entire *sepA* gene is preserved and provides the targeting sequence for later *in vivo* disruption of the *sepA* gene. Circular integration events of the *sepA*/tn5seq1 disruption plasmid in the A. nidulans strain ATW17 (*arg*B) have been verified (Figure 3). A parallel example to the integration of the *arg*B gene (as shown by pLH106) was verified by use of the tn5seq1/hph cassette from pLH3. In order to acquire an additional marker for selection, the *hph* marker has been transposed into the vector backbone of a plasmid later used for transformation of *M. grisea*. The function and presence of the *hph* gene in the *M. grisea* spores was confirmed (F. Tenjo, unpublished). pLH1 and pLH3 are available at the Fungal Genetics Stock Center.



Bg=*Bgl*II, Bc=*Bcl*I, P=*Pst*I, Nc=*Nco*I, A=*Ava*II, Sm=*Sma*I, S=*Sal*I, H=*Hin*dIII, N=*Nhe*I, No=*Not*I, X=*Xho*I, Ss=*Ssp*I. The *Bam*HI sites flank the transposing cassette. kan= kanamycin resistance gene, tnp =transposase, arg=ornithine carbamoyltransferase gene, hph=hygromycinB phosphotransferase gene

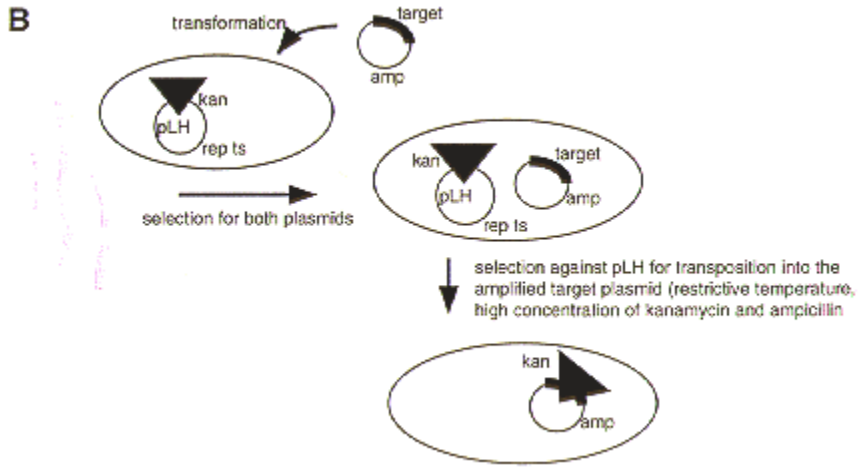


Figure 1. A. Construction of pLH1 and pLH3. pLH1: The 1.55 kb *arg*B gene was amplified with artificial *Xho*I overhangs and ligated to *Sal*I digested pFTS. pLH3: A 1.4 kb *Sal*I fragment containing the hph gene was ligated to *Sal*I digested pFTS. B. Transposon mutagenesis using pLH1 and pLH3. Competent *E. coli* cells containing pLH1 or pLH3 are transformed with the plasmid containing a gene to be disrupted. Transformants containing the pLH plasmid and the target plasmid are selected on medium containing ampicillin (selection for target plasmid) and kanamycin (selection for tn5seq1). The temperature and antibiotic concentration are raised to counterselect the pLH plasmid and promote transposition as well as the amplification of the target plasmid. Plasmids that are both kanamycin and ampicillin resistant are characterized and the exact site for tn5seq1 integration determined by restriction analysis (and/or sequencing).

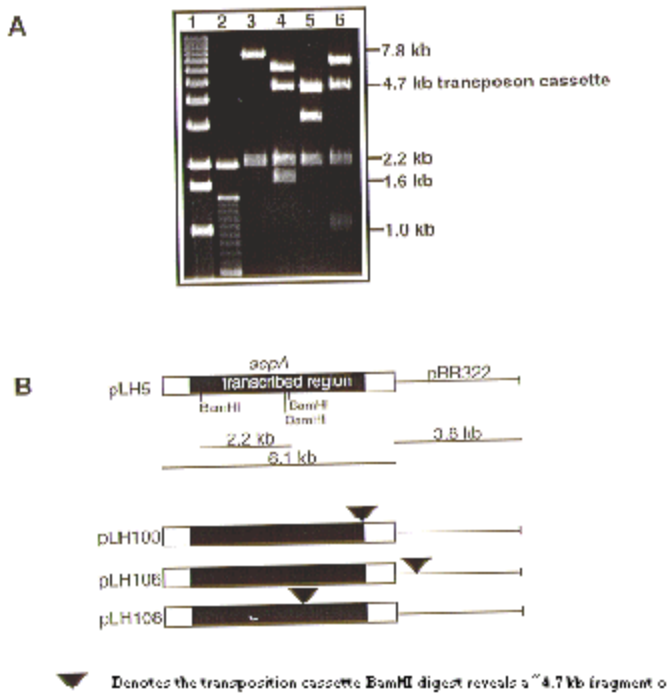


Figure 2. A. Examples of *Bam*HI restriction digests of pLH5 deletion derivatives. Lanes 1: 1 kb lambda ladder (GibcoBRL); 2: 100 bp ladder (GibcoBRL); 3: pLH5; 4: pLH100; 5: pLH106; 6: pLH108. B. Schematic presentation of pLH5 deletion derivatives. pLH5: intact *sepA* gene, no tn5seq1 transposition; pLH100: transposition in 3' end of *sepA*; pLH106: transposition in pBR322 vector backbone; pLH108: transposition in vital part of the *sepA* gene (see Figure 3).

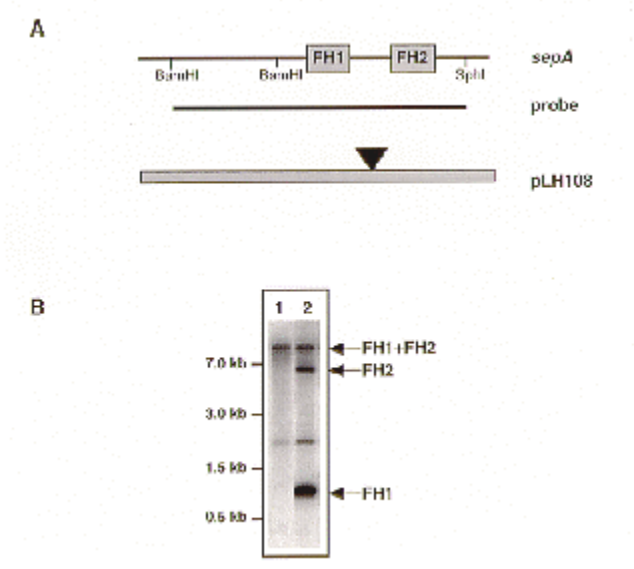


Figure 3. Circular integration of the *sepA*/tn5seq1 cassette at the *A. nidulans sepA* locus. A. *Bam*HI/*Sph*I restriction map of the *sepA* locus, the position of the *sepA* probe used in panel B,

and the disruption vector pLH108. B. Verification of disruption of the *sepA* locus by Southern analysis. *Bam*HI restriction digest of the parental *A. nidulans* strain ATW17 (lane 1), and a *sepA* integration event (lane 2). FH = formin homology.

Protocol: TRANSPOSON MUTAGENESIS USING PLH1 AND PLH3

ASPERGILLUS NIDULANS: USE PLH1 (CONTAINS THE *argB* gene)

MAGNAPORTHE GRISEA: USE PLH3 (CONTAINS THE *hph* gene)

1. Make competent *E. coli* strains LH1 or LH3 at 28 C (provided on agar, LH1 harbors pLH1,

LH3 harbors pLH3).

2. Transform your favorite plasmid (has to carry alternative (to kanamycin) drug resistance gene, i.e., ampicillin resistance) into competent *E. coli* strain LH1 or LH3. It is important that you are familiar with the *Bam*HI restriction map of your plasmid.

3. Incubate 1 hr in SOC at 28 C.

4. Plate on 1Xkan (25 mg/l), 1Xamp (50mg/l) plates (2XTY) (aliquots of 1 ul, 10 ul, and 100 ul).

5.Pick single colonies to ONC in liquid 4 ml (2XTY) cultures 1Xkan, 1Xamp, 28 C.

6. Plate aliquots of 0.4 ml on 10Xkan, 1Xamp plates (2XTY) incubate for 2 days at 43 C.

7. Pick large colonies, restreak onto 1Xkan, 1Xamp plates (2XTY).

8. ONC in liquid 4 ml (2XTY) cultures 1Xkan, 1Xamp, 37 C.

9. Miniprep colonies

10. Digest with *Bam*HI (gives an approximate 4.7 kb diagnostic band fr the pLH1 transposon and an approximate 4.55 kb diagnostic band for the pLH3 transposon). Map or sequence the insertion.

11. When appropriate disruption is verified, transform back into original host.

12. Select for arginine prototrophy (*A. nidulans*) or hygromycin resistance (*M. grisea*).

Common problems:

Sometimes the transposon cassette will integrate into the bacterial genome. This can be determined if the miniprepped plasmids from step 9 can transform kan/amp sensitive *E. coli* cells to resistance to these antibiotics.

We have noticed a tendency for nearly identical transposition site selection when using pUC and pBluescript plasmids as targets. This is only preferable if no gene desruption but a marker integration into the vector backbone is wanted.

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