FEMS Microbiology Letters 48 (1987) 305–310
Published by Elsevier

FEM 03001

A plasmid vector for simultaneous generation of *lacZ* protein fusions and *npt*-II operon fusions in vivo

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Received 2 August 1987
Accepted 11 August 1987

Key words: Gene fusion; *lacZ; npt*-II; *Escherichia coli*

1. SUMMARY

We have constructed a plasmid vector, pHLH1, that contains *lacZ* and *npt*-II genes flanked by ends of bacteriophage Mu. These sequences can be integrated into *Escherichia coli* chromosome by the Mu transposition system. The *lacZ* gene, deleted for its transcription and translation initiation signals, was positioned next to the 117 bp terminal fragment for the *s* end of Mu. The *npt*-II gene, deleted for its transcriptional signals, was positioned distal to *lacZ* in the same orientation. Transposition of this sequence to a target gene can create simultaneously a protein fusion to *lacZ* and an operon fusion to *npt*-II. To demonstrate the use of this vector, we isolated *lacZ npt*-II$^+$ fusions to *ompC*. These fusions exhibited the expected phenotypes and regulational properties of *ompC* fusions.

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2. INTRODUCTION

Fusion of marker or reporter genes (such as *lacZ*), encoding readily assayable products, to the genes of interest provide a powerful tool for the analysis of gene expression and function [1,2]. Two types of fusions can be generated: transcriptional or operon fusions in which an exogenous promoter is fused to the reporter gene that lacks its promoter but contains its translation initiation signals, and translational or protein fusions in which the reporter gene lacks both its transcription and translation start sites and is fused in frame to the coding sequence of the target gene.

Both types of fusions allow the study of transcriptional regulation of the target gene. The protein fusions have a number of additional applications. They can be used to analyze translation initiation and its control and have been valuable in assaying protein localization and targeting [3,4]. Furthermore, the hybrid protein can be used to raise antibodies, which in turn can be utilized to identify the product of the target gene [5].

The *lacZ* gene of *Escherichia coli* has been predominantly used as a reporter as it is amenable to genetic analysis and its gene product, β-galac-

tosidase, is easily assayed, and is active in both prokaryotes and eukaryotes as part of a hybrid protein [1,2,6]. Other reporter genes have also been employed as they can provide useful features over the *lacZ* system. Use of *phoA* encoding alkaline phosphatase avoids the lethality often observed with translational fusions of *lacZ* to genes coding for exported proteins, and it can be used to probe for export signals [7]. The gene for neomycinphosphotransferase II, *npt*-II, provides a good selectable phenotype (kanamycin resistance) and has been used to generate fusions both in bacteria [8,9] and eukaryotes [10,11].

Several in vivo techniques have been developed in bacteria to generate *lacZ* fusions based on transposition of the *lacZ* gene to the target gene sequence. Especially derivatives of phage Mu [12–16] have proven useful in generating fusions to a variety of genes in *E. coli*, and in other enterobacteria, such as *Salmonella typhimurium* [16–18], *Klebsiella pneumoniae* [19] and *Erwinia carotovora* [20]. Even the more recently described λ*plac*Mu vectors [21,22] employ the transposition system of phage Mu. In vivo fusion vectors have been also based on other bacterial transposons such asTn*3* [23], Tn*5* [7,9] and Tn*10* [24].

Here we describe the construction of a *lacZ* *npt*-II fusion vector pHLH1. This transposon vector can be used to generate simultaneously in vivo protein fusions to *lacZ* and operon fusions to *npt*-II.

3. MATERIALS AND METHODS

3.1. Strains, media and chemicals

The *E. coli* strain MC4100 has been described [12], as have the phages λpMu507 [25], λ*plac*Mu1 [21] and hy2 [26]. L medium and MacConkey indicator medium were as described previously [27]. Kanamycin (Kc) was added at 25 μg/ml and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XG) at 40 μg/ml. Restriction enzymes and T4 ligase came from Boehringer Mannheim GmbH and were used according to instructions provided by the manufacturer.

3.2. DNA techniques

Plasmid DNA was isolated as described previously [28]. General cloning procedures were according to Maniatis et al. [29].

3.3. Assays

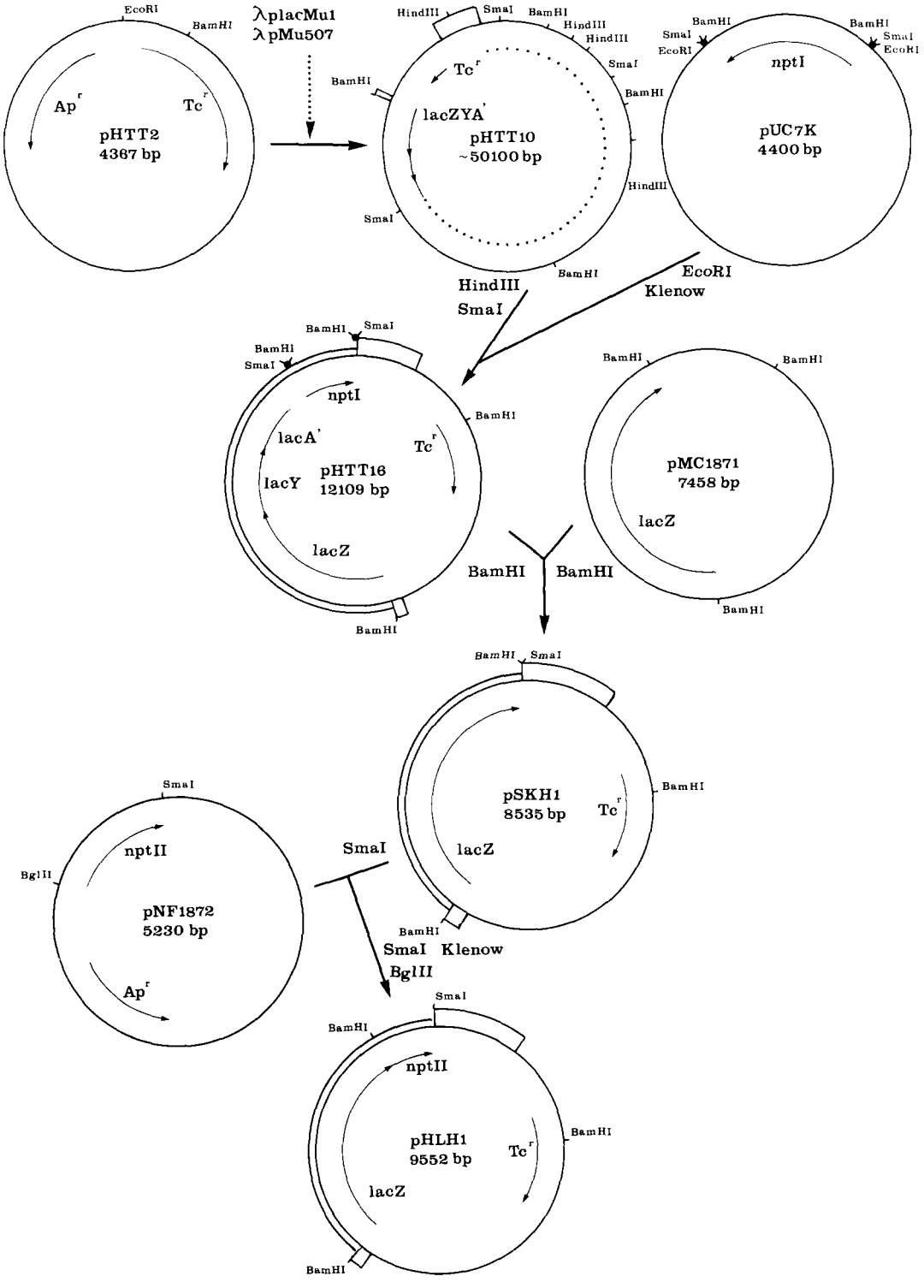
The activity of β-galactosidase was assayed as described by Miller [27] and that of neomycinphosphotransferase as by Van den Broeck et al. [10].

3.4. Genetic techniques

The general genetic methods were according to [27]. Isolation of Kc^r gene fusions was as follows: A fresh overnight culture of strain MC4100 containing plasmid pHLH1 (see section 4) was infected with the helper phage λpMu507, that carries the Mu*AB* genes, at multiplicity of infection of 0.5–1.0. After 15 min adsorption, the cells were harvested and resuspended into fresh L-broth containing 20 mM sodium citrate and incubated at +37°C for 1.5 h to allow expression of the *npt*-II gene. The cells were subsequently plated on L medium containing Kc, incubated overnight at +37°C and the resulting Kc^r colonies were pooled. To obtain specific *lacZ* *npt*-II fusions to *ompC* encoding a major outer membrane protein

Fig. 1. Construction of pHLH1. The bacterial strain harboring pHTT2 (a derivative of pBR322 in which the *Hin*dIII site is removed by filling in) was infected with λp*lac*Mu1 and λpMu507. λp*lac*Mu1 insertions into the *bla*(β-lactamase) gene of pHTT2 were identified by selecting specialized transducing phages that carried the whole plasmid and transduced tetracycline (Tc) but not ampicillin (Ap) resistance. From one of these cointegrates, pHTT10, a *Sma*I-*Hin*dIII fragment containing *tet*, *lacZYA'* and both ends of Mu was isolated, filled in and ligated with isolated and filled-in *Eco*RI fragment containing the Kc^r *npt*-I gene of pUC7K. From the derived plasmid, pHTT16, the *Bam*HI fragment containing *lac* genes and Kc^r marker was replaced with the partial *Bam*HI fragment of pMC1871 containing *lacZ*, resulting in pSKH1. A *Sma*I-*Bgl*II fragment from pNF1872 carrying *npt*-II gene was inserted into the *Sma*I site of pSKH1. The *npt*-II gene carried on this fragment lacks the promoter but retains its ribosome binding site. pHLH1 thus created contains selectable marker *tet*, *lacZ* missing its 8 first codons and *npt*-II, flanked by the 117 bp long Mu *s* end and the 1006 bp long Mu *c* end.

Mu sequences are shown by a thick double line, genes transposing to the chromosome with a narrow double line. The single line indicates vector sequences, ... symbolizes λ sequences, and ∨ symbolizes a DNA-linker.


EcoRI
BamHI
Ap r
Tc r
pHTT2
4367 bp
λplacMu1
λpMu507
HindIII
SmaI
BamHI
HindIII
HindIII
SmaI
BamHI
Tc r
lacZYA'
pHTT10
~50100 bp
SmaI
BamHI
HindIII
BamHI
SmaI
EcoRI
nptI
pUC7K
4400 bp
HindIII
SmaI
EcoRI
Klenow
BamHI
SmaI
BamHI
SmaI
nptI
lacA'
Tc r
lacY
pHTT16
12109 bp
lacZ
BamHI
BamHI
BamHI
pMC1871
7458 bp
lacZ
BamHI
BamHI
BamHI
BamHI
SmaI
pSKH1
8535 bp
Tc r
lacZ
BamHI
BamHI
SmaI
nptII
BglII
pNF1872
5230 bp
Ap r
SmaI
SmaI
Klenow
BglII
SmaI
BamHI
nptII
pHLH1
9552 bp
Tc r
lacZ
BamHI
BamHI

OmpC [30], insertion pools were selected by the OmpC-specific phage hy2 [26]. The pools were grown to mid-exponential phase in L-broth containing Kc. Aliquots of these cultures were plated on L agar containing Kc and spread with 10^9 hy2 particles to obtain hy2-resistant insertion mutants.

4. RESULTS AND DISCUSSION

4.1. Construction of the fusion vector pHLH1

Our purpose was to construct an in vivo fusion vector that could be used to generate one-step translational fusions to *lacZ* and transcriptional fusions to *npt*-II. Thus, both translational and transcriptional controls of the target gene could be assayed with the same fusions. Furthermore, the *npt*-II gene provides a selectable Kc-resistant phenotype to the target gene that would facilitate the isolation and genetic analysis of the fusions. The transposition system of bacteriophage Mu was employed to generate the fusions. The constructed pHLH1 vector contains 117 bp of the *s* end and 1006 bp of the *c* end of bacteriophage Mu [12–14]. This transposon, Mu*d*(*lacZ npt*-II), cannot transpose by itself since there is no transposase gene. As the transposase activity is provided in trans, maximal stability of the fusions is ensured.

The construction of the plasmid pHLH1 that carries the transposable Mu*d*(*lacZ npt*-II) fragment is summarized in Fig. 1. This plasmid contains *lacZ* missing its 8 first codons and *npt*-II missing its promoter but retaining its ribosome binding site, flanked by the 117 bp long Mu *s* end and the 1006 bp long Mu *c* end. As there are not transcription termination sites between *lacZ* and *npt*-II, they should be expressed as part of the same operon. An outline for generation of fusions with pHLH1 is presented in Fig. 2.

4.2. Isolation of ompC-lacZ-npt-*II fusions*

In order to test the ability of pHLH1 to create gene fusions to chromosomal genes of *E. coli*, we set out to select fusions to the *ompC* gene encoding OmpC, a major outer membrane protein. Fusion pools were generated as described in Section 3 and used to select for hy2-resistant insertion mutants. We obtained 14 potential *ompC-lacZ npt*-II$^+$ fusions that exhibited the expected phenotype LacZ$^+$, Kc^r, hy2^r. These fusions were transduced with P1 to a clean genetic background (MC4100) before further analysis. To ensure that the fusions isolated actually were located at *ompC*, they were mapped by P1 transduction using an *ompC*::Tn*10* donor strain. Out of the 14 candi-

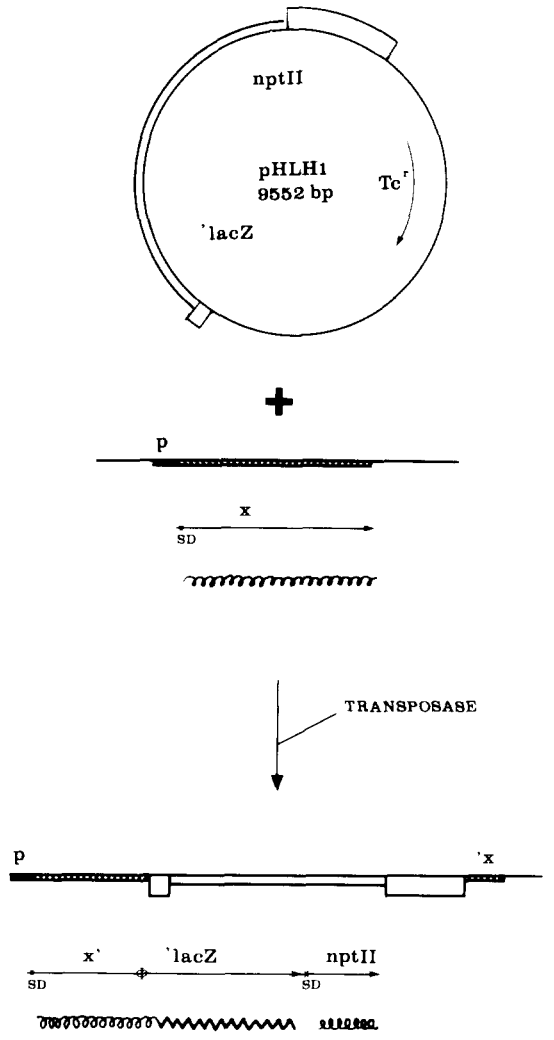


Fig. 2. Generation of fusions with pHLH1. When Mu*d*(*lacZ npt*-II) from pHLH1 is inserted into the gene x, a *lacZ* protein fusion and a *npt*-II operon fusion is formed. The transposon, Mu*d*(*lacZ npt*-II) is drawn as a double line, the thick parts of it symbolizing Mu *c* and *s* ends. Symbols: p, a promotor; x, mRNA of the gene into which the fusion is formed; SD, ribosome binding site; and ϕ, fusion joint. A prime denotes that a particular gene is not completely present. The helices and zig-zag lines indicate polypeptides encoded by the genes shown.

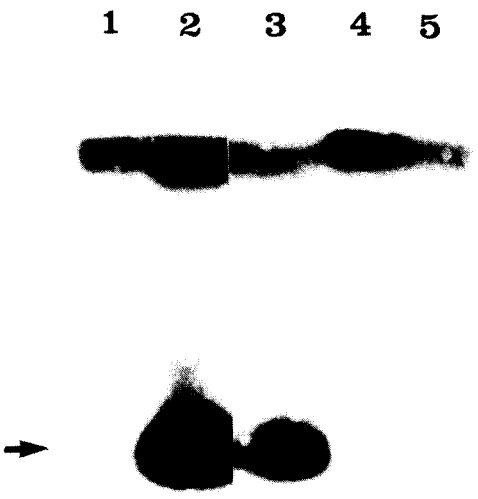


Fig. 3. Assay of neomycinphosphotransferase activity of *ompC-lacZ npt*-II fusions. The activities were assayed from sonicated cells essentially as described previously [10]. The strains analyzed were: Lane 1, MC4100; Lane 2, MH150 (MC4100 *ompC*::Tn*5*); Lane 3, HLH17 (φ(*ompC-lacZ npt-II*$^+$)); Lane 4, HLH170 (*ompC*::Tn*10* transductant of HLH17); Lane 5, HLH171 (*ompR*::Tn*10* transductant of HLH17). The arrow points to the neomycinphosphotransferase activity.

dates 10 were thus verified to contain fusions at the *ompC* locus.

Fusions to *ompC* should obey the normal regulatory properties of *ompC*. This was tested by taking advantage of the fact that the *ompR* gene product is a regulatory protein required for the expression of both porin genes *ompC* and *ompF* [31]. When an *ompR*::Tn*10* mutation was introduced to the fusion strains, both *lacZ* and *npt*-II expression were strongly reduced in all strains (Table 1, Fig. 3), suggesting that the fusions were to the *ompC* gene.

4.3. Frequence of fusions and fusion stability

The frequence of Kc^r fusion colonies was usually 10^{-6}, which is high enough to prepare a fusion pool easily. The frequence of *lacZ* fusion colonies was nearly the same. The frequence of *lacZ* fusions dropped more rapidly than the frequence of Kc^r fusions with decreasing m.o.i., suggesting that the actual number of insertions to the host chromosome is higher than can be calculated from the observed fusion frequencies. Also the killing effect of the helper phage λpMu507 made the evaluation of actual fusion frequencies difficult. By using a host strain that contains a lysogenized λ, we obtained a fusion frequence of 10^{-5} with m.o.i. 1.0.

As pHLH1 is not able to generate fusions without the transposase activity provided in trans the fusions are highly stable. All fusions could be transduced with P1 into other strains and the regulation of *ompC* could be studied without any complications. We have also isolated fusions with pHLH1 to other genes besides *ompC* and have

Table 1

Regulation of *ompC-lacZ npt*-II fusions

Strain	Relevant genotype	β-Galactosidase [a] activity	Neomycin [a] phospho-transferase	Growth on medium containing Kc (μg/ml)			
				15	50	75	100
MC4100	Δ (*argF-lac*)	7	0	–	–	–	–
MC4100/pHLH1		34	0	–	–	–	–
MH225	MC4100φ (*ompC-lacZ*)	1435	nt	–	–	–	–
HLH17	φ (*ompC-lacZnpt-II*$^+$)	1023	95.5	+	+	–	–
HLH171	HLH17 *ompR*::Tn*10*	11	0	–	–	–	–

[a] Activity of β-galactosidase was assayed as described [27] and that of neomycinphosphotransferase as in [10] (see also Fig. 3). The latter activity is given as percentage of that of a Tn*5*-containing strain MH150 [31]. The strain MH225 [31] was included as a control for β-galactosidase activity.

Growth with Kc was tested on plates with Kc concentrations in μg/ml as indicated.

nt = not tested.

not observed any secondary transpositions. The stability of these fusions should be particularly useful for selection of mutants altering the regulation of the target gene.

Structural analysis of the fusions made by pHLH1 can be facilitated with the in vivo cloning methods described previously [32]. The host specifity of the described fusion technique could be widened by introducing genes *lamB* and *nusA* to pHLH1. When these genes are provided λ can function at least in *Salmonella typhimurium*, consequently construction of gene fusions in other Gram-negative bacteria should become possible [33].

ACKNOWLEDGEMENTS

We thank Jill Thorngren for typing the manuscript.

Grants from the Neste Foundation, the Foundation for Biotechnical and Industrial Fermentation Research and the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 156) are gratefully acknowledged. This research was supported by the Academy of Finland and the Swedish Medical Research Council (project 07487).

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