Technical Tips Online, Vol. 2, 1997

A suicide plasmid (pJR*lacZ*ins) for targeted integration of non-native genes into the chromosome of *Escherichia coli*

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Keywords: Microbiology, Cloning & Sequencing

▼A number of genes are simply not suitable for cloning using common plasmid systems. In particular, the characterization of phenotype expression sometimes fails because of unpredicted stability or lethal phenotype owing to multicopy intermediates (e.g. membrane-, lipo- or regulatory proteins) (Ref. 1). To address this problem, we constructed an expression–delivery system that allows the targeted integration of one copy of a non-native gene into a defined site on the chromosome of *Escherichia coli*. For this purpose, a R6K origin-derived plasmid, which depends on replication factor *pir* (Ref. 2, 3, 4, 5), was used and reconstructed to contain a unique blunt cloning site within the *lacZ* gene. *Lac*$^+$ derivatives of *E. coli* not expressing the *pir* replication factor are therefore suitable recipients and will integrate the new constructed plasmid pJR*lacZ*ins into the *lac* operon of the chromosome. We tested this prediction and subcloned a blunt-ended DNA fragment containing the *hel* gene into pJR*lacZ*ins. The *hel* gene encodes the *e*(P4) outer membrane lipoprotein, derived from *Haemophilus influenzae* type b, and was found to be necessary for aerobic growth in *H. influenzae* (Ref. 6). *e*(P4) was suspected to promote hemin uptake across the outer membrane of *E. coli* when present in multiple copies on pACYC184 plasmids. Therefore, we wanted to utilize the pJR*lacZ*ins system to investigate whether a single copy of *hel* would be sufficient to allow hemin uptake in *E. coli*.

pJR*lacZ*ins

pJR*lacZ*ins is a hybrid plasmid consisting of reconstructed plasmid pMD35 (Ref. 5) (pGP704 derivative, encoding *lacZY'* with unique *Eco*RV site) and plasmid pCVD422 (Ref. 4) (1.8 kb fragment encoding *sacB*). The plasmid has been reconstructed to contain a unique *Eco*RV cleavage site for blunt-end ligation within the *lacZ* gene, and an intact R6K origin. The plasmid also contains the *sacB* gene which provides the negative selectable marker levansucrase. Plasmid pJR*lacZ*ins (Fig. 1) confers ampicillin resistance (amp^r, 50 μg/ml as single copy and 100 μg/ml in *pir*$^+$ strains). Transformation of pJR*lacZ*ins containing a DNA fragment within the *Eco*RV site into a pir$^-$*Lac*$^+$ *E. coli* strain will result in recombination of pJR*lacZ*ins into the *lac* operon of the chromosome which can be selected by amp^r and screened for a *Lac*$^-$ or *lacZ*$^-$ phenotype (Fig. 1). Subsequent selection for growth in the presence of sucrose will provoke reciprocal recombination (through sequence homology with merodiploid *lacZY'* sequences), which eventually will cause the deletion of the plasmid background (amp^s). However, clones that have maintained their insert within the chromosomal *lacZ* loci can be isolated by subsequent screening for a *Lac*$^-$ or *lacZ*$^-$ phenotype (Fig. 1).

Protocol

Cloning and integration

To subclone the *hel* gene into pJR*lacZ*ins, we obtained a 1 kb *Bam*HI DNA fragment encoding the *hel* gene from plasmid pJRP4 (Ref. 6). This fragment was blunt-ended by the fill-in reaction of the large DNA polymerase α-subunit [Klenow (Life Technologies, GIBCO-BRL)] and subsequently ligated into an *Eco*RV-digested pJR*lacZ*ins plasmid (Fig. 2). The resulting plasmid, pJR*lacZ*ins(*hel*) was amplified in a MC4100 λ *pir*$^+$ strain and transformed into strain K-12 *E. coli* C600 *hemA* [K-12 strains are unable

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 PII: S1366-2120(08)70076-X

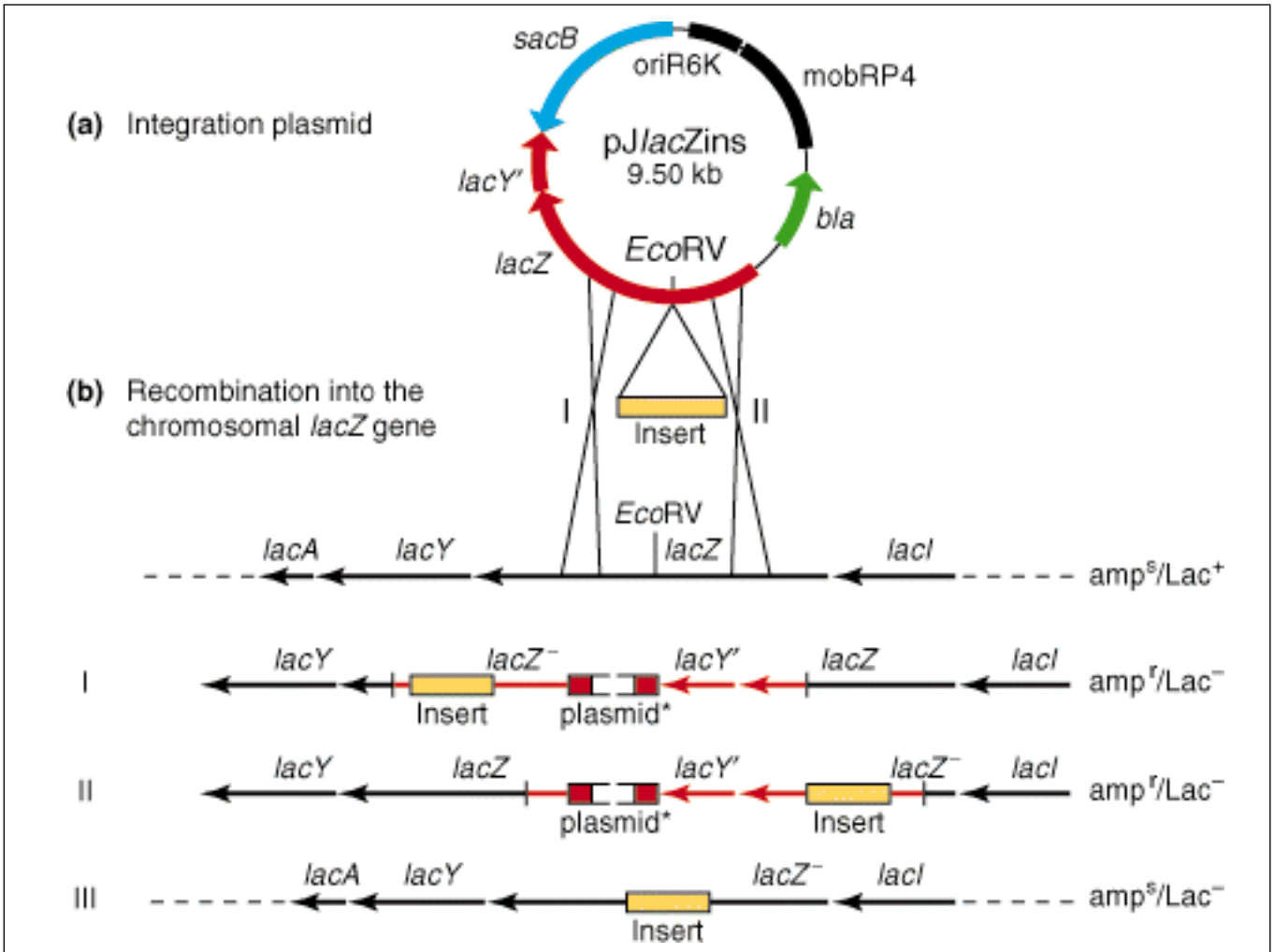

FIGURE 1. Insertion scheme. (a) Plasmid pJR*lacZ*ins, showing the position of the relevant selective markers (*sacB*, *bla*), origin of replication, mob region, the partial *lac* operon, and the unique *Eco*RV cloning site. 'Insert' indicates any blunt-ended DNA fragment subcloned into the unique *Eco*RV site of *lacZ*. (b) Transformation of pJR*lacZ*ins into a *E. coli pir*$^-$ strain and subsequent recombination of the plasmid into the *lac* operon. I and II, homologous single cross-over events (indicated as vertical lines and red colour). After growth-selection in LB (5% sucrose) and subsequent screening for amp^s and *lacZ*$^-$ phenotype, colonies are obtained (III) which contain a defined single copy insert, delivered by targeted integration (for details see text).

to take up hemin, and *hemA* mutants are dependent on supplemented hemin precursor aminolevulinic acid (AlA) to complement hemin auxotrophy, (Ref. 7)]. The transformed cells were tested for phenotypic expression (4 h) in 4 ml Luria Bertani (LB) medium containing 50 μg/ml AlA to allow integration of the plasmid into the *lacZ* loci on the chromosome and were then plated on LB agar plates containing 50 μg/ml AlA, 30 μg/ml ampicillin, 5 mM isopropyl-β-D-thio-galactopyranoside (IPTG), 20 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). After overnight (ON) growth, amp^r *lacZ*$^-$ colonies were screened. Three colonies were chosen and further selected by growth ON in sucrose (5%) to counter-select the *sacB*-containing plasmid marker. Selected colonies were screened for the loss of the ampicillin marker (*bla*) and Southern blot analysis was used to locate the *hel* gene within the *lacZ* gene in the chromosomal *lac* operon (Fig. 2).

Results

Growth analysis showed that the *hel*gene contained in the *lac* operon of strain C600, *hemA hel*$^+$ allowed the *hemA* strains to grow on hemin. However, this integration plasmid also represents a new cloning vehicle that in general allows: (1) the cloning of DNA without need for a multicopy intermediate, resulting in the integration of a single copy of the target gene, and thus preventing lethal or misleading phenotypes due to over-expression and (2) targeted integration of encoding genes (expressed epitopes) at a defined location in the chromosome (e.g. for the construction of vaccine carriers).

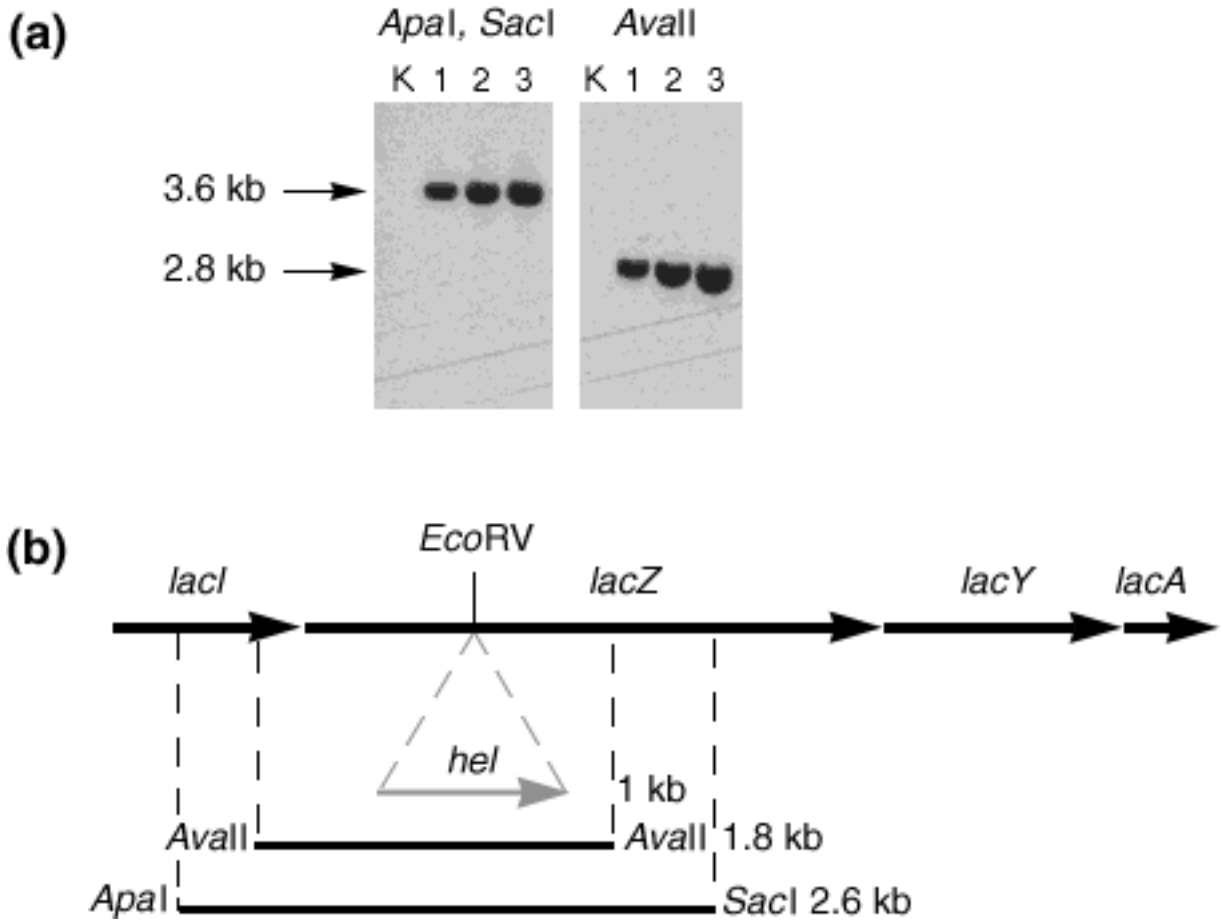

FIGURE 2. Southern-blot analysis of *hel*gene inserted into chromosomal *lacZ* locus. (a) Three amp^s and *lacZ*$^-$ colonies were isolated and chromosomal DNA was prepared. Restriction enzymes ApaI (Life Technologies GIBCO-BRL), ScaI (Life Technologies GIBCO-BRL) and AvaII (Life Technologies GIBCO-BRL), which cut in *lacIZ*, were chosen to obtain specific patterns of DNA fragments from the chromosomal *lacIZ* region (*lacI*lacks pJR*lacZ*ins). Lanes 1, 2 and 3, independent isolated *hel* insertions; K, receptor strain C600 *hemA* control DNA. DNA fragments were separated by 0.7% agarose gel electrophoresis and blotted onto a nylon membrane (Amersham). A *hel*-gene-specific DNA probe (1 kb) was generated with specific *hel* oligonucleotide in a PCR reaction (data not shown) and hybridized with the blotted DNA fragments. Specific DNA bands can be recognized with a photo-sensitive peroxidase-labeling reaction [ECL (Amersham)]. Bands indicated show the specific location of the *hel* insert in the *lac* operon, as shown in (b).

Acknowledgements

We thank M. Dziejman and J.J. Mekalanos for providing plasmid pMD35, and M.S. Donnenberg and J.B. Kaper for plasmid pCVD422. This work was funded by the BMBF grant 01KI8906.

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Products Used

Klenow: Klenow from Life Technologies (Gibco BRL)

nylon membrane: nylon membrane from Amersham Pharmacia Biotech

ECL: ECL from Amersham Pharmacia Biotech