Benchmarks

The pKNOCK Series of Broad-Host-Range Mobilizable Suicide Vectors for Gene Knockout and Targeted DNA Insertion into the Chromosome of Gram-Negative Bacteria

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With the completion of the genome sequencing efforts in several Gram-negative (Gram-) bacteria, it becomes important to reveal the function of multiple uncharacterized open reading frames (ORF). The classical way to address this problem involves disrupting an ORF and studying the phenotype of resulting mutants. Two major approaches are currently used for site-specific inactivation of the genes in the chromosome of Gram- bacteria. Allelic exchange allows one to replace a chromosomal copy of the gene with a plasmid-borne mutant one (4,10). The alternative approach is to produce insertions of a suicide vector into a gene of interest (7). While the former approach is in many ways superior to the latter one, it is more time-consuming and is useful only when counter-selection against the donor molecule can be efficiently applied. Heretofore, limiting factors on usefulness of the latter approach include lack of vectors with different antibiotic-resistance markers (other than ampicillin resistance [Ap]) and a versatile polylinker. Pòsfai et al. (8) reported construction of three vectors with either kanamycin, chloramphenicol or ampicillin (Km, Cm or Ap, respectively) resistance genes and nine unique restriction sites. These vectors can be used for insertional mutagenesis in bacterial species for which efficient transformation or electroporation protocols are described. Here, I report on the construction of a series of small plasmids that have more unique restriction sites and that can be introduced to recipient strains by transformation, electroporation or conjugation. Conjugation is advantageous for two reasons: (i) it can occur with up to 100% efficiency per recipient cell (9), which is much higher than efficiency of either transformation or electroporation and (ii) during conjugation, DNA enters the recipient cell in a single-stranded form, which is more conducive to recombination than the double-stranded DNA that enters Gram- cells during transformation or electroporation.

The pKNOCK series of vectors (Figure 1) was constructed by ligating the 700-bp SmaI fragment of pBSL63, containing RP4 oriT and R6K γ-ori (1) to Ecl136II fragments encoding antibiotic-resistance gene cassettes plus the pBluescript® II polylinker from pBSL98, pBSL121, pBSL141, pBSL148 and pBSL190 (2). The suicidal properties of new vectors are determined by the γ-origin of replication of the R6K plasmid, which has activity only in those Escherichia coli strains that can provide in trans the π protein encoded by R6K plasmid (5). These vectors can be propagated in E. coli and introduced into a wide range (9) of Gram- bacteria by means of either conjugation or electroporation.

The general scheme for insertional mutagenesis using newly constructed vectors is the same as described by Miller and Mekalanos (7) and is outlined in Figure 2. Briefly, the internal part (‘gene X’ in Figure 2) of the gene to be mutagenized (gene X in Figure 2) is cloned into the polylinker of the

Figure 1. Structure of pKNOCK vectors. Plasmids are not drawn to scale. Vector sizes are below vector designations. Tc, Gm, Cm and Ap genes confer resistance to tetracyclin, gentamycin, chloramphenicol, kanamycin and ampicillin, respectively. Polylinker: EcoRI-SacI-BsoXI-NotI-EagI-Xbal-Spel-BamHI-Smal-PstI-EcoUI-EcoRV-HindIII-ClaI-SalI-AccI-XhoI-ApaI-DraI-KpnI. The EagI site is not unique in both pKNOCK-Km and pKNOCK-Tc. The EcoRV site is not unique in both pKNOCK-Gm and pKNOCK-Tc. The DraI site is not unique for pKNOCK-Tc and pKNOCK-Ap.
pKNOCK vector. The resulting pKNOCK derivative is mobilized from the appropriate donor (3,6) into the recipient cells (electroporation or transformation can also be used, although with lower efficiencies). As a result of homologous recombination, two mutant copies of the target gene are formed. One copy lacks the 5' region and the other lacks the 3' region, which are separated by insertion of the pKNOCK plasmid. 

The functional utility of the vectors was tested in the experiment on inactivation of the E. coli lacZ gene. Briefly, the 827-bp EcoRV-EcoRI36I fragment of the E. coli lacZ gene was cloned in the unique Smal site of the pKNOCK-Ap, pKNOCK-Km, pKNOCK-Cm, pKNOCK-Gm and pKNOCK-Tc plasmids (Nos. PC-V3404, PC-V3405, PC-V3406 and PC-V3408, respectively; Phabagen Collection, Utrecht University, Utrecht, The Netherlands) yielding pBSL286, pBSL287, pBSL288, pBSL289 and pBSL290, respectively. The resulting plasmids were introduced into E. coli BW17975 [RP4-2tet::Mu-1 kan::Tn7 integrant/ΔudA::pir+ hsdR17 srlC300 creB510 endA1 zbf-5 thi, (6)] and conjugally transferred into spontaneous rifampicin resistant (RifR) mutant of the E. coli strain LE392. Putative exconjugants were selected for resistance to 50 µg/mL of rifampicin (Rif) and to a corresponding plasmid marker of either 25 µg/mL of kanamycin, 20 µg/mL of chloramphenicol, 5 µg/mL of tetracyclin, 150 µg/mL of ampicillin or 5 µg/mL of gentamycin. The frequency of exconjugants varied between 10^{-5} and 10^{-6} per recipient cell. After replica plating, fifty colonies from each experiment on plates supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 58% to 96% of colonies were selected on plates supplemented with IPTG and X-Gal. Of 50 colonies, 58% to 96% of exconjugants appeared to be lac\(^{-}\) (results are average of two experiments). Miniprep DNAs from sixteen lac\(^{+}\) colonies were analyzed. All 16 clones appeared to contain plasmid DNA of the same molecular weight as the introduced plasmid, suggesting that the suicidal system of the plasmids had been impaired in these clones. Impairing the suicidal system in our experiments could not have happened due to induction of the λ prophage bearing the pir gene in the donor cells and subsequent lysogenization of the recipient cells, as it has been explained earlier (3), because strain BW17975 has no such prophage (6). Therefore, it could have resulted from Hfr-like behavior of the RP4 derivative integrated into the E. coli BW17975 chromosome (9,11). The rate of spontaneous mutation of donor strain to Rif\(^{R}\) was 5 × 10\(^{-8}\), which is too low to explain the phenomenon. The number of putative exconjugants that acquired donor plasmid as an autonomously replicating unit should be significantly lower either when other bacteria are used as recipients (which will reduce the rate of Hfr-like transfer of the pir gene into recipient strain) or when markers other than that for rifampicin resistance are used for counter-selection of donors. Alternatively, use of electroporation, rather than conjugation, for introduction of the plasmid should remedy the problem.

To further demonstrate the ability of pKNOCK vectors to deliver genes into predetermined sites in a bacterial chromosome, insertion of the Ap\(^{R}\) gene into the lacZ locus in E. coli LE392 was performed. pBSL298, which is a pBSL287 derivative bearing a 1.3-kb BamHI fragment of pBSL167 encoding the Ap\(^{R}\) gene (2) in its unique BamHI site, was mobilized from E. coli S17-1[pir into E. coli LE392 Rif\(^{R}\) and Km\(^{R}\), and Rif\(^{R}\) colonies were selected on plates supplemented with IPTG and X-Gal. Of 50 white colonies that were replica-plated on plates containing Ap, all were Ap\(^{R}\). Therefore, pKNOCK vectors can be used for site-specific insertion of genes into the chromosome of Gram\(^{-}\) bacteria. It seems obvious that pKNOCK vectors can also be used for the construction of the conditional mutants and reporter gene fusions. To accomplish the first goal, the 5' portion of the gene, with its native promoter replaced by an inducible one, should be cloned in the polylinker of the pKNOCK plasmid. To create a reporter gene fusion in the chromosome, the reporter construct should be first created in the polylinker of the pKNOCK plasmid and then introduced into the strain of interest. Importantly, in this case, artifacts sometimes associated with complementation of chromosomal mutations with genes cloned on multicopy plasmids (e.g., repressor/activator titration) will be virtually eliminated, since the number of operators per cell remains unchanged. Although not explicitly demonstrated,
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Amplification of FADD mRNA Using RT-PCR Results in an Artifact

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Reverse transcription-polymerase chain reaction (RT-PCR) is one of the methods of choice for analyzing gene expression. It has been proven to be fast, sensitive and semi-quantitative. While studying apoptosis in human leukemia Jurkat cells, we investigated the regulation of the transcription of the Fas-associating protein with death domain (FADD) gene. FADD/MORT1 is a mediator molecule that bridges the external apoptotic signal and the amplification of caspases that leads to programmed cell death (9). Upon stimulation of the Fas receptor, FADD and the cytosolic domain of Fas receptor interact through a domain known as death domain (DD), located in the C terminus of both proteins. Simultaneously, the FADD N-terminal region, termed death effector domain (DED), interacts with at least one member of the caspase family, caspase-8 (FLICE, MACH, Mch5), linking it to Fas receptor (2,3,5,8). The FADD gene comprises 2 exons, 1 and 2, which code for DED and DD domains, respectively (7).

Analysis of FADD mRNA by RT-PCR followed by Southern blot after the induction of Fas/FasL-mediated apoptosis in Jurkat and normal human lymphocytes showed the over-expression of a form of FADD with lower molecular weight. Using the RNeasy® Total RNA System (Qiagen, Valencia, CA, USA) RNAs were extracted from untreated or treated Jurkat cells with phytohemagglutinin (PHA) (20 µg/mL) and from human lymphocytes that had been induced to proliferate with IL-2 for 5 days and were subsequently treated with phorbol 12-myristate 13-acetate (PMA) (10 ng/mL) and ionomycin (400 ng/mL). RNA concentrations were determined spectrophotometrically, and RNA integrity was verified by electrophoretic analysis. The RT reaction was performed according to the manufacturer’s specifications (Life Technologies, Gaithersburg, MD, USA). After cDNA synthesis using Super-