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Restoration of Gene Function by Homologous Recombination: from PCR to Gene Expression in One Step

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We have developed a simple method for single-step cloning of any PCR product into a plasmid. A novel selection principle has been applied, in which activation of a drug selection marker is achieved following homologous recombination. In this method a DNA fragment is amplified by PCR with standard oligonucleotides that contain flanking tails derived from the host plasmid and the complete λP_R or *rrnA1* promoter regions. The resulting PCR product is then electroporated into an *Escherichia coli* strain harboring both the phage λ Red functions and the host plasmid. Upon homologous recombination of the PCR fragment into the plasmid, expression of a drug selection marker is fully induced due to restoration of its truncated promoter, thus allowing appropriate selection. Recombinant plasmid vectors encoding β-galactosidase and neomycin phosphotransferase were constructed by using this method in two well-known Red systems. This cloning strategy significantly reduces both the time and costs associated with cloning procedures.

Cloning of DNA in *Escherichia coli* plasmids for both bacterial and eukaryotic studies is an invaluable tool. Current molecular biology techniques for cloning genes into plasmids are mainly based on the generation of gene segments by restriction by endonucleases and subsequent joining to a linearized plasmid DNA vector by DNA ligase. These techniques usually require multiple steps such as preparation and restriction of the vector and the gene of interest, purification of the products, ligation, and electroporation. Often, a desired restriction site is either absent or occurs in an unwanted site, and therefore preliminary manipulations are needed as well.

In recent years, a novel approach for molecular cloning based on bacteriophage-encoded recombination functions has been developed (3, 9, 10). This approach, termed "recombineering" (2), involves the use of PCR products or even synthetic oligonucleotides carrying 35 to 70 nucleotides of flanking homology to a target vector as substrates for recombination (3, 9, 10). The use of homologous recombination for in vivo gene cloning overrides the need for restriction and ligation enzymes with as much precision and efficiency (2). Recombineering is based on either the phage λ Red or the RecET recombination functions (6, 8). The λ genes involved in Red recombination are *exo*, *bet*, and *gam*. The *exo* gene product has 5′ to 3′ exonuclease activity, and the *bet* gene product is a single-strand DNA binding protein that promotes annealing. The *gam* gene product inhibits the RecBCD nuclease, thus preventing the degradation of linear DNA fragments.

Zhang and colleagues (10) developed an elegant use of phage homologous recombination functions for cloning any gene of interest into a plasmid. In their method, a plasmid vector containing a backbone of selectable drug marker and an active origin of replication is PCR amplified. The oligonucleotides for this PCR contain in their 5′ ends homology regions that are chosen to define the exact boundaries of the DNA region to be cloned. The chosen DNA region, which is either present in the bacteria or coelectroporated along with the linear plasmid, is inserted into the plasmid backbone by homologous recombination. Gap repair of the electroporated linear plasmid circularizes the plasmid, thus allowing selection for the drug marker. This cloning strategy is straightforward and works very well in subcloning experiments. It excludes the requirement for tedious DNA purification procedures, the required presence of convenient restriction sites, or the mutational risk of PCR. Yet this method requires purification of genomic DNA for cloning genes that are not already cloned on plasmids or bacterial artificial chromosomes or present on the *E. coli* chromosome. In such cases, and especially when large genomes are used for cloning, the efficiency, as well as simplicity, is insufficient. Another drawback of this cloning procedure is the high background resulting from self-circularization that occurs when repeats as short as six bases are present in the vector (10).

In this paper, we describe a reliable and simpler strategy for cloning by recombineering any gene of interest that can be amplified by PCR.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Strain DY378 was described by Yu et al. (9).

The plasmid pTrun-*cat* is a derivative of pACYC184 and was constructed as follows: the pACYC184 plasmid was used as a template in amplification reactions by using Vent polymerase (New England Biolabs, Beverly, Mass.) and the primers Tru-*cat*-for and NcoI-*cat*-rev (Table 2) to amplify the chloramphenicol acetyltransferase (*cat*) gene. The blunt PCR product, encoding the *cat* gene under the control of a truncated λP_R promoter, was cut with NcoI and ligated into an NcoI- and XmnI-linearized pACYC184 plasmid to yield plasmid pTrun-*cat*. The same cloning steps were repeated with the oligonucleotides Full-*cat*-for and NcoI-*cat*-rev to yield the plasmid pFull-*cat* which encodes *cat* under the control of the full λP_R promoter.

Induction of recombination functions and preparation of electrocompetent cells. *E. coli* DH5α (Invitrogen) electrocompetent cells were prepared as described by Datsenko and Wanner (3) with minor modifications. Briefly, DH5α

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TABLE 1. Compilation of bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli strains		
DH5α	F^- φ80*lacZ*ΔM15Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *gal-phoA supE44* λ^- *thi-1 gyrA96 relA1*	www.invitrogen.com
DY378	W3110 λcI857 Δ(*cro-bioA*)	9
Plasmids		
pACYC184	p15A origin of replication (Tet^r Chl^r)	1
pFull-*cat*	pACYC184 derived; P_{P_R}*cat* (Tet^r Chl^r)	This study
pTrun-*cat*	pACYC184 derived; $P_{P_R truncated}$*cat* (Tet^r Chl^{low})	This study
pKD46	P_{araB} γ β *exo* (Amp^r)	3

cells harboring both the pKD46 and pTrun-*cat* plasmids (DH5α/pKD46/pTrun-*cat*) were grown overnight at 30°C. Cells were then diluted 20-fold into 1 liter of Luria-Bertani (LB) medium containing 10 mg of tetracycline per liter and 100 mg of ampicillin per liter. When an optical density at 600 nm of ~0.4 was reached, L-arabinose was added to a final concentration of 1 mM in order to induce recombination functions encoded by plasmid pKD46. Following a 1-h induction, cells were washed three times with ice-cold 12% glycerol and resuspended in 2 ml of ice-cold 12% glycerol.

DY378 electrocompetent cells were prepared as described by Yu et al. (9) with minor alterations. Briefly, DY378 cells harboring the pTrun-*cat* plasmid were grown overnight at 30°C, diluted 20-fold into a 2-liter flask containing 1 liter of LB medium with 10 mg of tetracycline per liter and further grown to an optical density at 600 nm of ~0.6. The induction of recombination functions was performed by placing the flask in a water bath at 42°C with shaking for 15 min. Immediately after the induction, the flask was cooled on ice for 10 min. A control culture was cooled on ice without prior heat induction. Cells were then washed three times with ice-cold 12% glycerol and resuspended in 2 ml of ice-cold 12% glycerol. For both DH5α and DY378 electrocompetent cells, aliquots of 50 μl were either immediately used or were frozen and stored at −80°C until use.

PCR preparation of linear cassettes. PCR conditions were as follows: 5 min at 94°C and 3 cycles of 40 s at 94°C, 40 s at 50°C, and 90 s at 72°C, followed by 30 cycles of 40 s at 94°C, 40 s at 65°C, and 90 s at 72°C. The first three cycles were performed under nonstringent conditions (i.e., low annealing temperature) to allow specific and nonspecific initial amplification of the DNA. Following that, 30 cycles of PCR were performed under more stringent conditions. ReadyMix *Taq* PCR (Sigma-Aldrich, Rehovot, Israel) was used for all PCRs unless otherwise stated.

All oligonucleotides are listed in Table 2. Oligonucleotides Km-for-full and Km-rev-790 were used to amplify the Tn*5* neomycin phosphotransferase (*npt*II) gene, yielding λP_R-*npt*II. Oligonucleotides Km-for-rrnA1 and Km-rev-790 were also used to amplify *npt*II, yielding *rrnA1*-*npt*II. The template for the *npt*II gene was a chromosomal DNA to which a pKD4 fragment encoding *npt*II was integrated. Oligonucleotides *lacZ*-for-full and *lacZ*-rev were used to amplify the *lacZ* gene, yielding λP_R-*lacZ*. The plasmid pBluescript II KS(+) (Stratagene) served as a template for λP_R-*lacZ*. PCR mixtures were purified with a PCR nucleospin extract kit (Macherey-Nagel, Düren, Germany) and eluted in double-distilled water.

Electroporation reactions. Purified PCR products, corresponding to 50 to 200 ng of DNA in 1 to 5 μl, were mixed in the electroporation cuvette with 50 μl of electrocompetent cells. Electroporation was conducted by using a Bio-Rad pulser in 2-mm cuvettes according to the manufacturer's instructions (2,500 kV, 200 Ω, 25 μF). Following electroporation, 1 ml of SOC medium was added to each cuvette. Cells were incubated for 1 h at 30°C (DY378/pTrun-*cat*) or 37°C (DH5α/pKD46/pTrun-*cat*) and then 200 μl from each sample was plated on LB agar plates containing 120 mg of chloramphenicol per liter.

Determination of the percentage of colonies encoding the gene of interest. The frequency of β-galactosidase-expressing colonies obtained following electroporation of the λP_R-*lacZ* fragment was determined as follows: 200 μl of electroporation reaction mixtures with either λP_R-*lacZ* or λP_R-*npt*II (negative control) was plated on LB agar containing 120 mg of chloramphenicol per liter, 1 mM isopropyl-β-D-thiogalactoside (IPTG) (Sigma, Rehovot, Israel), and 60 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (Merck, Darmstadt, Germany) per liter. Plates were incubated overnight at 37°C, and the percentage of blue colonies from total colonies was determined.

The frequency of *npt*II-expressing colonies following electroporation of either λPR-*npt*II or *rrnA1*-*npt*II fragments was determined as follows: colonies selected on chloramphenicol (120 mg/liter) plates were individually picked and streaked on LB agar plates containing 40 mg of kanamycin per liter. Plates were incubated overnight, and the percentage of resistant colonies from total streaked colonies was determined.

Sequencing. Plasmids were isolated by standard procedures from representative colonies (DY378 and DH5α/pKD46) obtained after λPR-*lacZ* electroporation. These plasmids were used as templates for PCRs. PCRs were performed to amplify the upstream region of the λPR-*lacZ* by using the forward primer seq3-pos161-for corresponding to the plasmid and the reverse primer T7 corresponding to the λPR-*lacZ* (Table 2). The PCR fragments obtained were purified from 1% agarose gel and sequenced on an ABI Prism genetic analyzer (Applied Biosystems) by using oligonucleotide seq3-pos161-for as a primer (Table 2).

RESULTS

The cloning strategy. Our aim was to construct a system for rapidly cloning any desired DNA fragment into a plasmid. The system was designed to include an *E. coli* strain harboring the phage λ Red functions and a target plasmid, which encodes *cat* under the control of an upstream truncated promoter. The cloning strategy is detailed in Fig. 1. In essence, the gene of interest is amplified with standard oligonucleotides that contain tails derived from the target plasmid. The 5′ end of the upstream oligonucleotide contains the complementing −35

TABLE 2. List of oligonucleotides used in this study

Oligonucleotide	Sequence[a]
Tru-*cat*-for	5′-TATAGAATTCTTACCTCTGGCGGTGATAATGGTTGCATGTACTAAGGAGGTTGTATGGAGAAAAAAATCACTGG-3′
Full-*cat*-for	5′-TATAGAATTCGTGTTGACTATTTTACCTCTGGCGGTGATAATGGTTGCATGTACTAAGGAGGTTGTATGGAGAAAAAATCAACTGG-3′
NcoI-*cat*-rev	5′-CTTACCATGGTTACGCCCCGCCCTGCC-3′
Km-for-full	5′-*CTCCATACAACCTCCTTAGTACATGCAACCATTATCACCGCCAGAGGTAA***AATAGTCAACAC**TGTAGGCTGGAGCTGCTT-3′
Km-for-*rrnA1*	5′-*TCCAGTGATTTTTTTCTCCATACAACCTCCTTAGTACATGCAACCATTAT***AGGGAGTTATTCCGGCCTGACAAGT**GTAGGCTGGAGCTGCTT-3′
Km-rev-790	5′-*TTGCCGCGGCCCTCTCACTTCCCTGTTAAGTATCTTCCTGGCATCTTCCA*ATGGGAATTAGCCATGGTC-3′
lacZ-for-full	5′-*CTCCATACAACCTCCTTAGTACATGCAACCATTATCACCGCCAGAGGTAA***AATAGTCAACAC**CGTTGGCCGATTCATTA-3′
lacZ-rev	5′-*TTGCCGCGGCCCTCTCACTTCCCTGTTAAGTATCTTCCTGGCATCTTCCA*GAGCTTGACGGGGAAAG-3′
Seq1-pos670-rev	5′-CATATTCTGCTGACGCA-3′
seq2-pos900-rev	5′-GATTTGAGCGTCAGATTTCG-3′
seq3-pos161-for	5′-TCTTTACGATGCCATTG-3′
T7	5′-GTAATACGACTCACTATAGGGC-3′

[a] Italics indicate flanking homology to plasmid. Boldface indicates completion of either λP_R or *rrnA1* promoter.

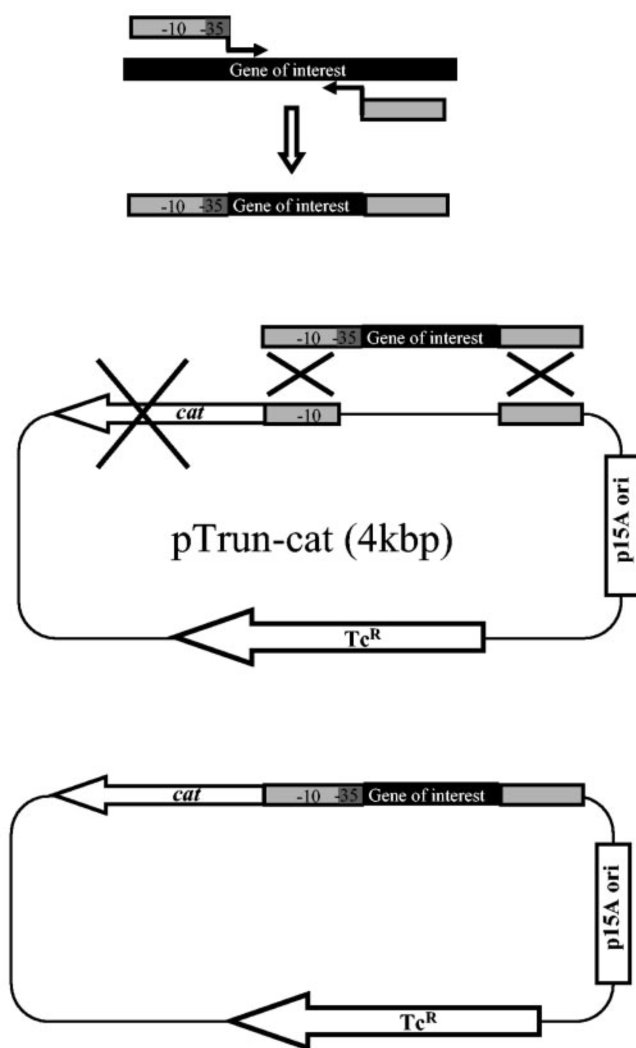

FIG. 1. Diagram of the cloning strategy. (Top) The gene of interest is amplified by PCR by using the indicated upstream (left) and downstream (right) oligonucleotides. The upstream oligonucleotide contains a homology tail to pTrun-*cat* (light gray) followed by complementation of a −35 box of the λPR or *rrnA1* promoter (dark gray) and a PCR primer (right arrow). The downstream oligonucleotide is composed of a pTrun-*cat* homology tail (light gray) followed by a PCR primer (left arrow). (Middle) Following electroporation and targeted homologous recombination of the PCR fragment, the desired DNA is cloned and complements the truncated promoter that drives *cat* expression. (Bottom) Upon homologous recombination of the PCR fragment into the plasmid, the *cat* gene is fully activated and desired colonies are selected on chloramphenicol. The −10 box and the −35 box of the promoter are indicated. Tc^r, tetracycline resistance gene; p15A ori, origin of replication.

box of the truncated promoter. This box is critical for the binding of RNA polymerase via the σ^{70} subunit for appropriate transcription (5). Upon targeted homologous recombination of the PCR fragment into the plasmid and complementation of the truncated promoter, expression of the *cat* gene is greatly enhanced, allowing selection by chloramphenicol of colonies that contain the plasmid with the gene of interest.

Determining the optimal chloramphenicol concentration for selection. To achieve our goal, we chose to use plasmid pACYC184 which encodes the tetracycline resistance gene and the *cat* gene under constitutive promoters (1). The endogenous *cat* promoter was replaced with a truncated λP_R promoter or with the full λP_R promoter. The constructed plasmids were designated pTrun-*cat* and pFull-*cat*, respectively. In order to determine the chloramphenicol concentration for use in selection, we compared the resistance of a DH5α strain harboring pFull-*cat* versus pTrun-*cat* to elevated concentrations of chloramphenicol. We observed that DH5α/pFull-*cat* could still grow at a concentration of 400 mg of chloramphenicol per liter in liquid and on solid LB medium after overnight incubation, whereas DH5α/pTrun-*cat* was already inhibited at 50 mg of chloramphenicol per liter under the same conditions. We chose to use a selective concentration range of 100 to 150 mg of chloramphenicol per liter, which was later found to be optimal for selecting colonies that harbor the target insertion while maintaining a null background of pTrun-*cat*-harboring bacteria (data not shown). This was probably due to the following reason: a targeted homologous recombination event, prior to chloramphenicol selection, forms a mixture of plasmids containing mostly pTrun-*cat* and only a minority of plasmids with a fully active *cat* promoter in the desired bacteria. Therefore, these bacteria are more resistant than the parental DH5α/pTrun-*cat* bacteria but more sensitive than DH5α bacteria harboring pFull-*cat*.

In vivo cloning of PCR fragments into the plasmid by using DH5α/pKD46/pTrun-*cat*. To enable efficient homologous recombination, we first used the previously described Red helper plasmid pKD46 (3). The Red enzymes encoded by the pKD46 plasmid are under an arabinose-inducible promoter. Transformants carrying both pKD46 and pTrun-*cat* plasmids were made electrocompetent as described in Materials and Methods. In order to test the cloning system, the Tn*5* neomycin phosphotransferase (*npt*II), which confers kanamycin resistance, and the *lacZ* reporter gene were amplified with their endogenous promoters. These genes were chosen in order to allow an easy verification of targeted homologous recombination events. Oligonucleotides were designed as depicted in Fig. 1. The 5′ end of the upstream oligonucleotides used for amplifying the gene of interest contained 50 nucleotides (nt) of homologous sequence of both the *cat* gene and the truncated promoter followed by the −35 box of the λP_R promoter or *rrnA1* promoter sequence. A 17- to 20-nt primer of the gene of interest was designed at the 3′ end (Table 2, oligonucleotides Km-for-full, Km-for-*rrnA1*, and *lacZ*-for-full). The downstream oligonucleotides contained a 50-nt sequence homologous to the pTru-*cat* plasmid followed by a 17- to 20-nt primer for amplifying the gene of interest (Table 2, oligonucleotides Km-rev-790 and *lacZ*-rev). The amplified DNA of the *npt*II gene with the full upstream λP_R promoter or an alternative promoter, *rrnA1*, were named λP_R-*npt*II and *rrnA1*-*npt*II, respectively. The *lacZ* PCR fragment with the full λP_R promoter was designated λP_R-*lacZ*. We electroporated 50 to 200 ng of the products amplified by PCR into DH5α/pKD46/pTrun-*cat*. Following electroporation, cells were spread on LB agar plates containing 120 mg of chloramphenicol per liter and were incubated overnight at 37°C. Incubation at 37°C greatly reduces pKD46 presence as this plasmid has a temperature-sensitive origin of replication (3). The recombination functions are thus eliminated, and construct stability is maintained. Table 3 sum-

TABLE 3. Summary of results

PCR insert[a]	Red source	Results of electroporation			
		Fresh aliquots		Frozen aliquots	
		No. of colonies obtained[b]	% Correct insertion[c]	No. of colonies obtained[b]	% Correct insertion[c]
λP_R-*npt*II	DY378	550	82	430	86
λP_R-*npt*II	DH5α/pKD46	350	88	315	90
*rrnA1-npt*II	DY378	146	92	172	84
*rrnA1-npt*II	DH5α/pKD46	140	88	133	90
λP_R-*lacZ*	DY378	224	ND	196	ND
λP_R-*lacZ*	DH5α/pKD46	230	91	220	88

[a] λP_R-*npt*II encodes *npt*II flanked by pTrun-*cat* homologies containing complementation of the λP_R promoter. *rrnA1-npt*II encodes *npt*II flanked by pTrun-*cat* homologies containing complementation of the *rrnA1* promoter. λP_R-*lacZ* encodes β-galactosidase flanked by pTrun-*cat* homologies containing complementation of the λP_R promoter.

[b] One-fifth of each electroporation reaction mixture was plated and counted. Results represent the number of chloramphenicol-resistant colonies obtained per reaction mixture. Standard deviations of multiple platings from the same sample were less than 10% in all cases.

[c] At least 50 obtained colonies electroporated with either λP_R-*npt*II or *rrnA1-npt*II were tested for kanamycin resistance as described in Materials and Methods. The percentage of kanamycin-resistant colonies was determined accordingly. DH5α/pKD46/pTrun-*cat* colonies electroporated with λP_R-*lacZ* were plated on LB agar containing chloramphenicol, X-Gal, and IPTG, and the percentage of blue colonies was determined from total colonies (Fig. 2). The percentage of DY378/pTrun-*cat* blue colonies was not determined (ND) due to endogenous *lacZ* background activity.

marizes the results obtained by using this simple protocol. We obtained 350 chloramphenicol-resistant colonies per λP_R-*npt*II electroporation reaction. Of these colonies, 82% were also kanamycin resistant, thus containing the correct insertion of the *npt*II gene (Table 3). Similar results were obtained when electroporation of λP_R-*lacZ* was performed. Of 230 colonies that were resistant to chloramphenicol, 91% turned blue on LB plates containing X-Gal, IPTG, and 120 mg of chloramphenicol per ml, as shown in Fig. 2, indicating a very high cloning efficiency. We further tested this cloning system by amplifying the same *npt*II gene by using a different upstream oligonucleotide, which converted the truncated promoter into a full *rrnA1* promoter (*rrnA1-npt*II). In this case, too, sufficient cloning efficiency was observed, as shown in Table 3. All the above procedures were repeated at least once more with comparable results.

In vivo cloning of PCR fragments into the plasmid by using DY378/pTrun-*cat*. The cloning system was also tested by using another well-characterized Red function-containing bacterium, strain DY378 (9). The DY378 strain harbors a prophage encoding the λ Red genes *gam*, *bet*, and *exo*. These genes in the prophage are under the control of a temperature-sensitive repressor and are expressed at 42°C but remain repressed at 32°C. Electroporation was performed as described in Materials and Methods. Results obtained with this strain were similar to those obtained with the DH5α/pKD46 strain (Table 3). Uninduced bacteria did not yield resistant colonies, confirming the involvement of the Red recombination functions in this cloning system. Most importantly, in both DY378 and DH5α strains, no chloramphenicol-resistant colonies were detected in reactions where the PCR fragment was replaced by the corresponding amplifying oligonucleotides or by water.

Sequence analysis of the cloned products. A primer corresponding to the plasmid backbone and a primer corresponding to the gene of interest were used to amplify the insertion regions. These PCRs that were performed on representative colonies from λP_R-*npt*II, *rrnA1-npt*II, and λP_R-*lacZ* electroporations of both the DY378 and DH5α strains confirmed the presence of the insert in the plasmid (data not shown). In addition, all (five of five) PCR products that were subjected to sequence analysis confirmed that the intended target DNA was fully inserted into the plasmid without any mutational errors.

Testing efficiency of frozen aliquots. Frozen electrocompetent cells in glycerol lose some potency compared to fresh cells (7). We wanted to examine whether such stocks harboring recombination proteins can still be used efficiently for our procedures. Therefore, electrocompetent cells harboring pTrun-*cat* were prepared, and fresh versus frozen (−80°C) stocks were tested for efficiency. The frozen stocks demonstrated only a slight decrease in electrotransformation efficiency and a sim-

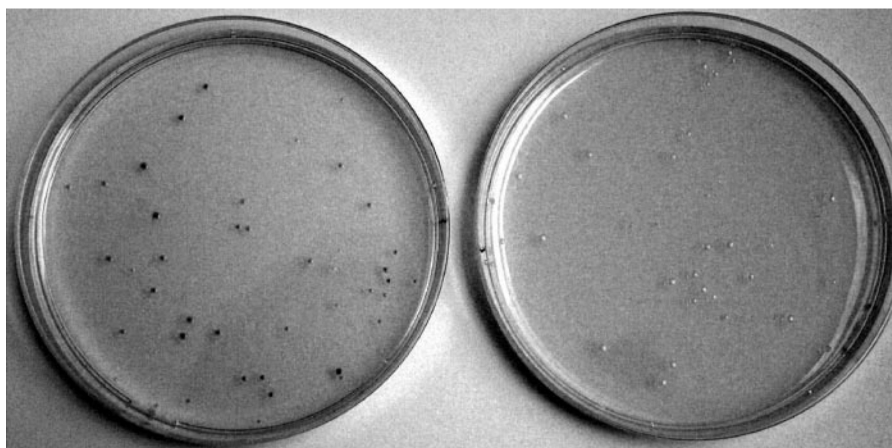

FIG. 2. About 90% of the colonies selected on chloramphenicol contain the gene of interest. The λP_R-*lacZ* fragment encoding *lacZ* (left) or λP_R-*npt*II fragment encoding Tn*5* neomycin phosphotransferase (right) were electroporated into DH5α/pKD46/pTrun-*cat* electrocompetent cells. SOC medium was added for 1 h, and bacteria were then spread on LB plates containing chloramphenicol, X-Gal, and IPTG. Representative plates were pictured after overnight incubation at 37°C, and the percentage of blue colonies was determined as described in Material and Methods.

ilar insertion percentage compared to fresh stocks (Table 3). We thus conclude that electrocompetent cells harboring recombination functions can be prepared in advance for routine use with the described cloning procedures.

DISCUSSION

The cloning system that we have developed is based on general principles of recombineering (2). However, we have demonstrated for the first time restoration of gene function by using homologous recombination. We introduced this novel principle for driving the expression of a drug marker gene that is required for efficient selection following targeted homologous recombination. Our cloning system has many advantages over currently used molecular cloning techniques for protein expression. The only components of the system are electrocompetent cells harboring recombination functions and a target plasmid. These cells can be prepared and stored frozen for long periods of time. Designing the oligonucleotides for use in this system is extremely easy as they are based on the presence of fixed sequences at their 5′ ends, joined with the primers used to amplify the gene of interest.

We demonstrated efficient expression of the β-galactosidase gene product and of the *npt*II gene that were cloned with their endogenous promoters (Fig. 2 and Table 3). Our cloning strategy could be further applied to any parallel expression system simply by transferring the *cat* gene with its truncated promoter to any expression plasmid (e.g., prokaryotic and eukaryotic expression vectors). Alternatively, a prokaryotic or eukaryotic promoter can be cloned into the pTrun-*cat* plasmid, converting it to an expression vector. In addition, the principle of using a truncated promoter, which confers very low chloramphenicol resistance (50 mg of chloramphenicol per liter) versus a full promoter, which confers high chloramphenicol resistance (400 mg of chloramphenicol per liter), can be further utilized. For example, constructing a promoterless *cat* gene (completely sensitive to chloramphenicol) and using the selection principle with a truncated promoter on a low chloramphenicol concentration may offer the advantage of using the obtained construct for a second cloning step on a higher chloramphenicol concentration. This way one can engineer fusion genes, site-directed mutagenesis, and similar constructs by using only two consecutive steps.

The Red recombination functions are known to cause multimerization in colE1-type plasmids in the presence of Gam (2, 4). In addition, when this cloning system with pTrun-*cat* already established in the bacteria is used, a mixture containing parental and recombinant plasmids is formed despite selection for bacteria highly resistant to chloramphenicol. The parental pTrun-*cat* plasmids are not eliminated because a negative selection force against them is absent. Both phenomena are acceptable when the cloning system is used for protein expression and for related purposes. Our strategy could also be applied for obtaining pure recombinant plasmids simply by using low-copy-number vectors, like pSC101 derivatives, and/or performing coelectroporation of the target plasmid along with the PCR insert, thus avoiding multimerization (2).

Recombineering is still not accepted as the method of choice for plasmid construction, despite its enormous potential. In this paper we present new principles for utilizing recombineering as a simple and straightforward gene expression methodology.

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