Escherichia coli endA deletion strain for use in two-hybrid shuttle vector selection

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Escherichia coli has been used as the host organism for DNA cloning since its inception (1). As recombinant DNA technology evolves, novel bacterial strains are needed to complement new cloning strategies. In 1989, Fields and Song (2) developed the yeast two-hybrid system. This system uses a pair of yeast-*E. coli* shuttle vectors to detect protein-protein interactions. Plasmids that contain putative interacting proteins are identified in yeast and shuttled back into *E. coli* for further analysis.

Certain yeast genes can complement specific mutations in *E. coli* (3). The *E. coli* strain, KC8, contains *pyrF*, *leuB*, *trpC*, and *hisB* mutations that allow the selection of the *Saccharomyces cerevisiae* *URA3*, *LEU2*, *TRP1*, and *HIS3* genes, respectively (4). However, KC8 does not produce high-quality plasmid DNA preparations due to the lack of an *endA* gene mutation, a consequence-specific endonuclease. To overcome this problem, we have produced a new *E. coli* strain, MG7 α , which contains deletion alleles for each of the *pyrF*, *leuB*, *trpC*, *hisB*, and *endA* genes. MG7 α has some distinct advantages over KC8. In particular, it is sensitive to kanamycin, ampicillin, chloramphenicol, and tetracycline. In addition, it provides α -complementation of the β -galactosidase gene. Finally, MG7 α contains an *endA* deletion mutation. The *endA* deletion allows the production of high-quality plasmid DNA using standard methods. Yeast-*E. coli* shuttle vectors containing any of the *URA3*, *LEU2*, *TRP1*, and *HIS3* genes can be selected and identified in this strain.

The mutations were generated using the one-step inactivation of chromosomal genes method developed by Datsenko and Wanner (5). Bacteria were maintained on LB agar medium, and

the cultures were grown in LB broth (Unless otherwise stated, all chemicals, media, and antibiotics are from Sigma-Aldrich Research Reagents, Oakville, ON, Canada). SOC broth (6) was used for the recovery of bacteria after electroporation. Kanamycin- and ampicillin-resistant transformants were selected on LB plates containing 25 μ g/mL kanamycin and 100 μ g/mL ampicillin, respectively. M9 minimal medium (6) lacking uracil, leucine, tryptophan, or histidine was used to test the auxotrophic phenotype of putative mutants. A 0.2% (w/v) final concentration of arabinose was used for the induction of the red recombinase system (5). PCR primers were produced and desalted by Canadian Life Technologies (Burlington, ON, Canada). The restriction enzyme *DpnI* was purchased from New England Biolabs (Mississauga, ON, Canada), and the *Taq* DNA polymerase was from Canadian Life Technologies. Plasmid DNA was extracted essentially as previously described (7). PCR comprised a 5-min initial denaturation at 94°C, 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 58°C, and 1 min extension at 68°C, followed by a final extension step for 7 min at 68°C. The reaction conditions consisted of 0.2 mM dNTPs, 1 μ M each primer, 0.25 μ L *Taq* DNA polymerase, and approximately 50 ng template DNA per 50 μ L reaction. PCR products were *DpnI*-treated to remove intact template plasmid molecules before being electrophoresed and gel purified, as previously described (8).

The *E. coli* strain DH5 α was used as the parental strain to produce MG7 α . The *E. coli* strain KC8 was used as a benchmark strain for phenotypic testing. Table 1 summarizes the genotype of each strain.

The plasmids pKD46, pKD4, and pCP20 (5) were obtained from the *E. coli* Genetic Stock Center (<http://cgsc.biology.yale.edu/>) and used in the process of gene disruption. Plasmids YEplac112 (*URA3*), YEplac181 (*LEU2*), YEplac195 (*TRP1*) (9), and pRS303 (*HIS3*) (10) were used to test for the complementation of the bacterial auxotrophic mutations.

An overnight culture of bacteria was made electrocompetent and transformed according to the method of Dower et al. (11). The cells were electroporated using a Gene Pulser™ (Bio-Rad Laboratories, Hercules, CA, USA) at 1.25 kV, 25 μ F, with a 400 Ω resistor in parallel with the sample chamber. To prepare the cells for efficient gene disruption, transformants containing the red helper plasmid (pKD46) were grown at 30°C in LB broth containing ampicillin (50 μ g/mL) and arabinose for approximately 3 h. These cells were transformed by electroporation (11) with 50–100 ng PCR-generated gene-disruption cassette. The electroporated cells were immediately suspended in 1 mL SOC broth and incubated for 1 h. Of the 1 mL, 500 μ L were spread onto kanamycin plates immediately after incubation, and the second 500 μ L were spread after standing overnight at room temperature. All colonies selected on kanamycin plates were maintained on LB agar plates without antibiotics. The auxotrophic phenotype of the mutants was tested by replica plating onto the appropriate M9 minimal media plates.

The FLP recombinase helper plasmid (pCP20) was used to remove the resistance marker after the successful identification of the target mutation. The plasmid was electroporated into electrocompetent cells prepared from a specific mutant strain. The resulting ampicillin-resistant transformants were selected at 30°C, which facilitated the removal of the antibiotic marker. The colonies were then streaked onto LB plates without antibiotic and grown overnight at 43°C to cure the strain of the pCP20 plasmid. The resulting colonies were tested for antibiotic resistances, and in most cases, the bacteria had become kanamycin- and ampicillin-sensitive.

Lysates from each bacterial strain were prepared from overnight cultures grown in LB broth. The cells were har-

Table 1. Genotype of *Escherichia coli* Bacterial Strains

Strain	Genotype
DH5 α (16)	<i>F</i> ⁻ , <i>endA</i> 1, <i>hsdR</i> 17 (<i>r</i> _K ⁻ <i>m</i> _K ⁺), <i>glnV</i> 44, <i>thi</i> -1, <i>deoR</i> , <i>gyrA</i> 96, <i>recA</i> 1, <i>relA</i> 1, <i>supE</i> 44, Δ (<i>lacZYA-argF</i>)U169, λ -, [ϕ 80 <i>dlac</i> Δ (<i>lacZ</i>) M15].
KC8 (4)	<i>hsdR</i> , <i>leuB</i> 600, <i>trpC</i> 9830, <i>pyrF</i> ::Tn5, <i>hisB</i> 463, <i>lac</i> Δ X74, <i>strA</i> , <i>galU</i> , <i>galk</i> . Note: Tn5 confers <i>kan</i> ^r .
MG7 α	<i>F</i> ⁻ , <i>hsdR</i> 17 (<i>r</i> _K ⁻ <i>m</i> _K ⁺), <i>glnV</i> 44, <i>thi</i> -1, <i>deoR</i> , <i>recA</i> 1, <i>relA</i> 1, <i>supE</i> 44, Δ (<i>lacZYA-argF</i>)U169, λ -, [ϕ 80 <i>dlac</i> Δ (<i>lacZ</i>) M15], <i>pyrF</i> Δ 168, <i>leuB</i> Δ 211, <i>hisB</i> Δ 227, <i>trpC</i> Δ 137, <i>endA</i> Δ 605.

vested and resuspended in Native Binding Buffer (20 mM phosphate, 500 mM NaCl, pH 7.8), part of the ProBond® Purification System (Invitrogen, Carlsbad, CA, USA), containing protease inhibitor cocktail [1 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride (AEBSF), 0.15 mM pepstatin A, 0.15 mM E-64, 0.1 mM bestatin, and 1 mM EDTA]. The cell suspensions were treated with lysozyme (100 μ g/mL) for 15 min and sonicated four times, with flash freezing in liquid nitrogen between each sonication. Finally, the lysate was cleared using a 0.8- μ m syringe filter. Lysates from different strains were normalized for protein content by the Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories) and used for assays of *endA* activity. All

endA assays were conducted in multi-core buffer [25 mM Tris-acetate, pH 7.75, 100 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (DTT)].

For each of the genes, *pyrF*, *leuB*, *hisB*, *trpC*, and *endA*, the location of the gene in the *E. coli* chromosome was determined by a search of the National Center for Biotechnology Information's database (Bethesda, MD, USA) of the *E. coli* K-12 MG1655 complete genome (12). The location and orientation (+ or -) of each target gene was determined, and the sequence was saved for further analysis. These genome sequences were used to design primers that targeted a region of 100–300 bp in size, within 100 bp from the start codon of the gene of interest.

For each target gene, homologous extensions were designed so that a recombination event would occur completely within the target gene and replace part of its sequence with the kanamycin resistance gene-disruption cassette. For each gene, 39–45 bp of homology were used and added onto the 20-bp of priming sequence for the template pKD4 (see Table 2 for all primer sequences).

Bacterial cells containing the plasmid pKD46 were grown in the presence of arabinose to induce the red recombinase system and stimulate homologous recombination (13).

DH5 α or its subsequent derivatives containing pKD46 were electroporated with the purified PCR-generated gene-disruption cassettes. Kanamycin-resistant bacterial colonies were selected and tested for the disruption phenotype by streaking them onto M9 minimal media lacking uracil, leucine, tryptophan, or histidine. A single colony that exhibited the target mutation was then chosen and cured of pKD46. The strain was then used to generate new electrocompetent cells that were electroporated with pCP20 to facilitate the removal of the kanamycin marker as described earlier. Finally, the strain was cured of the second helper plasmid to yield a mutant strain with no residual antibiotic resistance, which allowed additional genes to be targeted for disruption.

DH5 α cells electroporated with a purified PCR product containing homologous regions for the *pyrF* gene yielded several kanamycin-resistant colonies. When streaked onto M9 minimal medium that lacked uracil, some of these colonies did not produce any growth. One of these auxotrophic mutants was chosen for subsequent disruption. This new *pyrF* Δ 168 deletion strain was prepared for the deletion of the *leuB* gene as described earlier. The process of gene disruption was repeated, and the *leuB*, *hisB*, and *trpC* genes were each disrupted and verified by testing on the appropriate minimal medium.

The *pyrF* Δ 168, *leuB* Δ 211, *hisB* Δ 227, *trpC* Δ 137 deletion strain was then prepared for a disruption of the *endA* gene. The *endA*-PCR gene-disruption cassette was electroporated into these cells, yielding several hundred *kan*^R colonies. To test for the correct disruption

Figure 1. Assays of *endA* activity. (A) Qualitative assay of *endA* activity. The pUC9 plasmid DNA was treated with lysates from the strains KC8 (K), MG7 α (M), and DH5 α (D). Supercoiled plasmid DNA was added to *E. coli* lysates and incubated at 37°C. The samples were electrophoresed on 0.7% agarose gels, and the degradation of the plasmid DNA was observed by ethidium bromide staining after gel electrophoresis. (1) Open circular plasmid. (2) Linear plasmid. (3) Supercoiled plasmid. (B) Electrophoresis of QIAprep Spin Plasmid DNA. Plasmid preparations from KC8 (K) and MG7 α (M) cells were electrophoresed on a 0.7% agarose gel and observed by ethidium bromide staining.

Table 2. Sequences of Amplification Primers

Primer Name	Sequence
<i>pyrF</i> -pKD4 Fwd.	5'-CGCTGTTACGAATTCTCCTGTGGTTGTTGCCCTTGA gtgtaggctggagctgcttc -3'
<i>pyrF</i> -pKD4 Rev.	5'-GCAGCAGCGACAGCGTGCGCTGCAGTGTTGGGGATA catatgaatatcctccttag -3'
<i>leuB</i> -pKD4 Fwd.	5'-AACCGCTTTGCGATGCGCATCACCACCAGCCATTACGAT gtgtaggctggagctgcttc -3'
<i>leuB</i> -pKD4 Rev.	5'-GCAATGTCTGCACGCAGCGGACAGAATGCTTCCAGCCCC catatgaatatcctccttag -3'
<i>hisB</i> -pKD4 Fwd.	5'-ATTTTCAGGTGGACCGTTTTGATAAACTCGCCTTTGAAC gtgtaggctggagctgcttc -3'
<i>hisB</i> -pKD4 Rev.	5'-GCGATCCATCGCTTGCTCAGCCAGATAACGTTCCACCAG catatgaatatcctccttag -3'
<i>trpC</i> -pKD4 Fwd.	5'-TTTTAGCGAAAATCGTCGCAGACAAGGCGATTTGGGTAG gtgtaggctggagctgcttc -3'
<i>trpC</i> -pKD4 Rev.	5'-GGCGGCAATGCGTGCTGGATCGAAATCATCACGGATCAC catatgaatatcctccttag -3'
<i>endA</i> -pKD4 Fwd.	5'-ATGTACCGTTATTTGTCTATTGCTGCGGTGGTACTGAGCGCAGCA gtgtaggctggagctgcttc -3'
<i>endA</i> -pKD4 Rev.	5'-GCCTGGCAAGCGCGTTGCACATACGGGTTATGATTGCCCTGCACC catatgaatatcctccttag -3'
<i>endA</i> -Test Fwd.	5'- tcgcctg cgggaaaaactcgcccaggccaa -3'
<i>endA</i> -Test Rev.	5'- caccaggcttc cagatccatcgctggacgg -3'

Homologous extensions: sequences that target the gene of interest are uppercase.
Sequences used for amplification of the knock-out cassette from the template plasmid, pKD4, or verification of the *endA* disruption, are bold and lowercase.

tion of the *endA* gene, we designed test primers to amplify the region flanking the *endA* gene, and the disruption was confirmed by the size of the PCR products that were amplified (Table 2). Genomic DNA from several kanamycin-resistant colonies was used to test for *endA* gene disruption using this PCR assay. The *endA* gene disruption was verified in several *kan^R* colonies. With the *kan^R* gene inserted into the *endA* gene, the PCR product was found to be the expected 2683 bp. After the *kan^R* marker was evicted, the product was found to be the expected 1296 bp in size. These products were easily distinguished from the 1811-bp product expected for the parent strain DH5 α . The resulting strain containing each of the deletion mutations described earlier was designated MG7 α (see Table 1 for genotype).

DNA degradation was observed qualitatively by treating samples of YEplac112 plasmid DNA (4.99 kb) with lysates from each *E. coli* strain (at 37°C) for various time intervals and analyzing (approximately 500 ng) by gel electrophoresis. Figure 1A shows that the plasmid DNA that was treated with lysate from KC8 was dramatically degraded by 25 h and continued to degrade with further incubation (indicat-

ed by the smear of low molecular weight DNA). The samples treated with DH5 α and MG7 α lysates showed some nicking after 25 h. This is observed as a conversion of supercoiled plasmid to the open circular form. Further incubation converts the open circular form of the plasmid to the linear form and produces more degradation products in both the MG7 α and DH5 α samples. The MG7 α lysate appears to have less degradation potential when the 125-h time point is inspected.

The quality of QIAprep[®] spin plasmid DNA preparations (Qiagen, Mississauga, ON, Canada) that were produced by each strain was also tested. The YEplac112 plasmid DNA was prepared from KC8 and MG7 α cultures, and approximately 300 ng were analyzed on a gel (Figure 1B). The KC8 lane shows a mixture of supercoiled and open circular plasmid, compared to the MG7 α sample, which is composed of supercoiled plasmid.

The quality of DNA produced by the strains MG7 α and KC8 was compared quantitatively by using the plasmid DNA for electroporation. Plasmid samples (YEplac112) extracted from MG7 α produced significantly more transformants than plasmid samples ex-

tracted from KC8 ($P < 0.001$). Twelve samples were prepared from each strain, and the MG7 α plasmid preparations produced on average three times as many transformants. This test was also conducted using cosmid DNA, and the effect was more pronounced. The MG7 α cosmid preparations produced 4.5 times more transformants than KC8 ($P < 0.001$).

MG7 α cells were transformed by electroporation (11) with comparable efficiencies to that of DH5 α . MG7 α showed no growth on M9 drop-out media lacking uracil, leucine, histidine, or tryptophan. However, each of these mutations was successfully complemented by the *URA3*, *LEU2*, *TRP1*, and *HIS3* yeast genes carried on the plasmids YEplac195, YEplac181, YEplac112, and pRS303, respectively. In addition, MG7 α has been successfully used to rescue numerous LEU2 plasmids from a two-hybrid screen conducted in our laboratory (R.D. Gietz, unpublished results).

We have produced a new *E. coli* strain, MG7 α , with genetic markers equivalent to KC8 but superior plasmid DNA production characteristics. The strain MG7 α contains four auxotrophic mutations that make it ideally suited for

the selection of the vectors that are used in two-hybrid strategies. MG7 α contains a deletion of the *endA* gene, compared to the point mutation in DH5 α , which still allows for a residual level of endonuclease activity (14,15). The *endA* knock-out removes any residual *endA* activity and thus allows MG7 α to produce higher quality plasmid DNA preparations than strains carrying *endA* point mutations. In addition to its use in the yeast two-hybrid system, the qualities that it inherits from its parent strain, DH5 α , and the *endA* deletion we have introduced, make it a good all-purpose laboratory strain. This strain will be made available through ATCC (Reference no. MBA-78; Manassas, VA, USA).

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Rapid isolation of *Arabidopsis thaliana* developing embryos

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Arabidopsis thaliana is a model plant for which many genetic and molecular tools are available. Another advantage of *A. thaliana* is its small size, allowing many plants to be grown in limited space. However, the diminutive size makes some experiments difficult. In particular, it is laborious to obtain enough isolated embryos for molecular or biochemical studies. Experiments involving developing embryos are further complicated because tissues containing *A. thaliana* embryos, such as siliques and seeds, can be difficult to work with because of secondary metabolites and polysaccharides. For example, we have found that RNA cannot be obtained from siliques or seeds using commercially available RNA isolation kits, such as RNeasy® (Qiagen, Valencia, CA, USA) or TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), methods that are widely used because they are fast and easy. Protocols to obtain RNA

from these tissues are considerably more involved (1,2).

Here we describe a simple method, based on density gradient centrifugation, to isolate relatively large quantities of *A. thaliana* embryos. Our method is faster and requires less handling of individual seeds and embryos than does manual dissection. Approximately 100 mg torpedo to bent cotyledon stage embryos can be isolated in an afternoon. It is possible to isolate even younger embryos, as early as late heart stage. The embryos are suitable for a variety of experiments, including RNA isolation and protein extraction and analysis.

Developing siliques were opened and seeds removed into a few drops of MC buffer for protein work (3) (MC buffer is 10 mM potassium phosphate, pH 7.0, 50 mM NaCl, 0.1 M sucrose), or RNeasy™ (Qiagen) for RNA isolation, on a glass microscope slide.