**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 required to complement the current cloning systems. 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 nonsequence-specific endonuclease. To overcome this problem, we have produced a new *E. coli* strain, MG7α, which contains deletion alleles for each of the *pyrF*, *leuB*, *trpC*, *hisB*, and *endA* genes. MG7α has some distinct advantages over KC8. In particular, it is sensitive to kanamycin, ampicillin, chloramphenicol, and tetracyclin. In addition, it provides α-complementation of the β-galactosidase gene. Finally, MG7α 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 µg/mL kanamycin and 100 µg/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 recombination 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 µM each primer, 0.25 µL *Taq* DNA polymerase, and approximately 50 ng template DNA per 50 µL 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 DH5α was used as the parental strain to produce MG7α. The *E. coli* strain KC8 was used as a benchmark strain for phenotypic testing. Table 1 summarizes the genotype of each strain.
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 µg/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-µm 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).

DH5α 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 competent 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.

DH5α 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 pyrFΔ168 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 pyrFΔ168, leuBΔ211, hisBΔ-227, trpCΔ137 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 kanR colonies. To test for the correct disrup-

Table 1. Genotype of Escherichia coli Bacterial Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>DH5α (16)</td>
<td>F-, endA1, hsdR17 (rK mK), glnV44, thi-1, deoR, gyrA96, recA1, relA1, supE44, Δ(lacZYA-argF)U169, λ−, [φ80lacΔ(lacZ) M15].</td>
</tr>
<tr>
<td>KC8 (4)</td>
<td>hsdR, leuB600, trpC8380, pyrF−:Tn5, hisB463, lacΔX74, strA, galU, galK.</td>
</tr>
<tr>
<td>MG7α</td>
<td>F−, hsdR17 (rK mK), glnV44, thi-1, deoR, recA1, relA1, supE44, Δ(lacZYA-argF)U169, λ−, [φ80lacΔ(lacZ) M15], pyrFA168, leuBΔ211, hisBΔ227, trpCΔ137, endA Δ605.</td>
</tr>
</tbody>
</table>

Note: Tn5 confers kanR.

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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), MG7α (M), and DH5α (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 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 MG7α (M) cells were electrophoresed on a 0.7% agarose gel and observed by ethidium bromide staining.
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 kanR colonies. With the kanR gene inserted into the endA gene, the PCR product was found to be the expected 2683 bp. After the kanR 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 endA disruption, are bold and lowercase.

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 each strain, and the MG7α 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 MG7α cosmid preparations produced 4.5 times more transformants than KC8 (P < 0.001).

MG7α cells were transformed by electroporation (11) with comparable efficiencies to that of DH5α. MG7α 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, MG7α 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, MG7α, with genetic markers equivalent to KC8 but superior plasmid DNA production characteristics. The strain MG7α contains four auxotrophic mutations that make it ideally suited for

### Table 2. Sequences of Amplification Primers

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>pyrF-pKD4 Fwd</td>
<td>5′-CGCTGTTACGAATTCTCTGTTGTTGCTTTGAAGTACGAGGATTCgtgtagctgagctgcttc-3′</td>
</tr>
<tr>
<td>pyrF-pKD4 Rev</td>
<td>5′-GGAGCAAGAGCACTGTGCGATGTTGGGATAcataaadatatctctccttag-3′</td>
</tr>
<tr>
<td>leuB-pKD4 Fwd</td>
<td>5′-ACCCGCTTTGCCATCACCCACCCACCCATTACGATgtgtagctgagctgcttc-3′</td>
</tr>
<tr>
<td>leuB-pKD4 Rev</td>
<td>5′-GGAAATTCTGACGACGCTGATACACATACGGTCCACCCCTATCAGGACAccatagatataactcctctctc-3′</td>
</tr>
<tr>
<td>hisB-pKD4 Fwd</td>
<td>5′-ATTCGGGCTTTATCTGAATACGTTTCCTGCTCCAGACATACCATACCCACCCGACCAgtgtagctgagctgcttc-3′</td>
</tr>
<tr>
<td>hisB-pKD4 Rev</td>
<td>5′-GGAAATTCTGACGACGCTGATACACATACGGTCCACCCCTATCAGGACAccatagatataactcctctctc-3′</td>
</tr>
<tr>
<td>trpC-pKD4 Fwd</td>
<td>5′-CTTATTGCAGGAAATTCTGACGACGCTGATACACATACCCACCCGACCAgtgtagctgagctgcttc-3′</td>
</tr>
<tr>
<td>trpC-pKD4 Rev</td>
<td>5′-GGAAATTCTGACGACGCTGATACACATACGGTCCACCCCTATCAGGACAccatagatataactcctctctc-3′</td>
</tr>
<tr>
<td>endA-pKD4 Fwd</td>
<td>5′-GACGACGCTGATACACATACCCACCCGACCAgtgtagctgagctgcttc-3′</td>
</tr>
<tr>
<td>endA-pKD4 Rev</td>
<td>5′-GACGACGCTGATACACATACCCACCCGACCAgtgtagctgagctgcttc-3′</td>
</tr>
<tr>
<td>endA-Test Fwd</td>
<td>5′-GACGACGCTGATACACATACCCACCCGACCAgtgtagctgagctgcttc-3′</td>
</tr>
<tr>
<td>endA-Test Rev</td>
<td>5′-GACGACGCTGATACACATACCCACCCGACCAgtgtagctgagctgcttc-3′</td>
</tr>
</tbody>
</table>

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.
the selection of the vectors that are used in two-hybrid strategies. MG7α contains a deletion of the endA gene, compared to the point mutation in DH5α, which still allows for a residual level of endonuclease activity (14,15). The endA knock-out removes any residual endA activity and thus allows MG7α 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, DH5α, 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).

REFERENCES


Received 10 March 2003; accepted 16 May 2003.

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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 commercial 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 RNAlater® (Qiagen) for RNA isolation, on a glass microscope slide.