

Quick and efficient method for genetic transformation of biopolymer-producing bacteria

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Abstract

In order to genetically modify microorganisms capable of producing polyhydroxyalkanoate (PHA) biopolymers, a simple and rapid method to prepare freshly plated *Pseudomonas* cells for transformation via electroporation was developed. This method can be used to transfer both replicative plasmids and linear DNA to knock out genes into the cells. The transformation efficiencies were in the range of $\geq 10^7$ transformants μg^{-1} DNA for replicative plasmids and $\geq 10^6$ transformants μg^{-1} DNA for linear DNA, which are comparable with commercially available competent cells. Furthermore, this transformation procedure can be performed in less than 10 min, saving a great deal of time compared with traditional methods. Knockout mutants of several *Pseudomonas* species were generated by transformation of linear DNA and these mutations were verified by PCR and analysis of PHA content.

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INTRODUCTION

Pseudomonas putida is a Gram-negative soil bacterium that plays an important role in element cycling in nature, bioremediation, and production of polyhydroxyalkanoates (PHAs), which are environmentally friendly biodegradable plastics.^{1–3} Despite having a fully sequenced genome,³ the functions of many ORFs in this organism remain unknown. A simple and time efficient transformation method is required to functionally analyze newly identified genes. The traditional methods of introducing new genes into *P. putida* include conjugation, chemical transformation, and electroporation, which are all time consuming.^{4–6} An easy, rapid, and inexpensive way to efficiently transfer plasmids and linear DNA into *P. putida* by electroporation of freshly plated cells has been developed. All procedures can be done within 10 min or less. This method can be used for transformation of replicative plasmids or linear DNA for knockout mutant and site-specific gene replacement. The transformation procedure was confirmed by transferring several plasmids that can be replicated in *P. putida* KT2440, and linear DNA into several *Pseudomonas* strains to knock out the *pha* cluster that includes two PHA polymerase genes, *phaC1* and *phaC2*, and one PHA depolymerase gene, *phaZ*. The transformation efficiencies were within the range 10^4 – 10^7 transformants μg^{-1} DNA. The transformation efficiency of cells prepared by this method is comparable with that of commercially available *Escherichia coli* competent cells and this method is adequate for many applications.

MATERIALS AND METHODS

Strains, media, and growth conditions

Table 1 shows the bacterial strains and plasmids used in this study. Plasmids were maintained in *Escherichia coli* JM109. The gene replacement and plasmid transformation experiments were done

using various strains of *P. putida*. Strains were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) with the appropriate antibiotic when necessary. For selection of transformants, kanamycin (Km) and gentamycin (Gm) were added to LB agar plates and liquid media at final concentrations of $50 \mu\text{g mL}^{-1}$ and $20 \mu\text{g mL}^{-1}$, respectively. For determining PHA content, the cells were grown on mineral salts (MS) media⁷.

Construction of *pha* cluster deletion construct

Homologous regions of *phaC1* and *phaC2* from *P. putida* KT2440 were amplified by PCR with two sets of primers harboring engineered restriction enzyme sites, P1 (*EcoRI*): 5'-attgaattc aggtgaacatggatgccttc-3', and P1' (*KpnI*): 5'-atcgggtaccctcgtcgacaaa caaagcaa -3'; P2 (*XbaI*): 5'-atttctagagccacgatctggctcagctt-3' and P2' (*HindIII*): 5'-actaagcttgggtt gatgatgctctggat-3'. Restriction sites are underlined. Resultant fragments were cloned into the same restriction sites of pUC19. A gene encoding gentamycin resistance (*accC1*) was removed from plasmid pGMR5 by restriction digest with *Bam*HI, and inserted into the same restriction site of pUC19, yielding the plasmid, pUC19::phaC1_{Pp}accC1phaC2_{Pp} (Fig. 1). The

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>Escherichia coli</i> JM109	<i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 relA1 λ⁻ lac</i> [F' proAB lacIq ZΔM15]	Takara
<i>Pseudomonas putida</i> KT2440	<i>P. putida</i> mt-2 <i>hsdR1 hsdM</i> ⁺	8
<i>Pseudomonas putida</i> KT2442	spontaneous <i>rif^r</i> mutant of KT2440	9
<i>Pseudomonas putida</i> IFO14164	Wild type	10
<i>Pseudomonas putida</i> rpoN	Km ^r , RpoN ⁻ derivative from KT2440	11
<i>Pseudomonas putida</i> GPp104	PHA synthase negative mutant of <i>P. putida</i> KT2442	12
<i>Pseudomonas</i> sp. ATCC 29 347	OCT plasmid, PHA production	13
pUC19	Amp ^r , Car ^r , the <i>lac</i> promoter (<i>Plac</i>)	Invitrogen
pUC19::phaC1' _{pp} accC1phaC2' _{pp}	pUC19 derivative, phaC1' _{pp} , accC1(Gen ^R), phaC2' _{pp}	This study
pGMR5	pUC19 derivative, Gm ^r , Amp ^r , <i>accC1</i>	Dr Niels-Ulrich Frigaard
pBBR1-MCS2	Broad-host-range vector, lacPOZ', Km ^R	14
pJRD215	Km ^r , Sm ^r , <i>mob</i> ⁺	15
pBBRSTQKAB	pBBR1-MCS2 derivative, <i>phaC1</i> (STQK) _{ps} , phaA _{Re} , phaB _{Re}	16

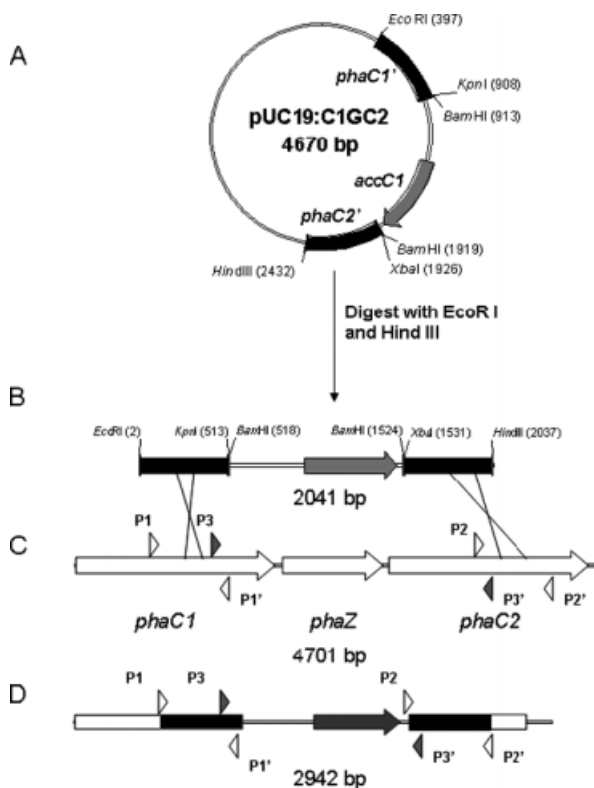


Figure 1. Proposed process for knocking out the *pha* cluster of *P. putida* KT2440. A. Insertion of *phaC1'*_{pp}, *accC1* (Gen^R), and *phaC2'*_{pp} into pUC19 to construct pUC19:C1GC2. B. The linear DNA digested from pUC19:C1GC2. C. The *pha* Cluster including *phaC1*, *phaZ*, and *phaC2* in genome of wild-type *P. putida*. P1 and P1' were the primers to amplify *phaC1'*, P2 and p2' were the primers to amplify *phaC2'*, and P3 and P3' were the primers for verifying the knockout mutant. D. Knockout mutant of *P. putida* after double crossover.

plasmid was then digested with *EcoRI* and *HindIII* to yield the linear DNA for transformation.

Preparation of competent cells and transformation by electroporation

P. putida cells were streaked on LB agar plates and incubated at 30 °C or 37 °C overnight. Cells were transferred to 1.5 mL

microcentrifuge tubes from the plates, and resuspended in 1 mL sterile 15% glycerol (OD₆₀₀ ~ 0.4). The cell suspensions were centrifuged at 13 000 rpm for 1 min, and the supernatants were removed. The cells were washed three times with 15% sterile glycerol, and then resuspended again with 100 μL 15% sterile glycerol for electroporation. Approximately 10 ng of plasmid DNA or linear DNA was added to the prepared cells, and the DNA–cell suspensions were transferred to 2 mm gap electroporation cuvettes (BTX, USA.) on ice. The cuvettes were pulsed at 36 μF; 150 Ω; with an ECM 399 electroporation system (BTX, MA, USA) at various voltages. A volume of 1 mL SOC media (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mmol L⁻¹ KCl, 20 mmol L⁻¹ glucose) was added to each cuvette immediately after pulsing. The cell mixtures were then transferred to sterile microcentrifuge tubes and incubated for 0 h or 1 h at 30 °C or 37 °C, 250 rpm. After incubation, 10 μL or 100 μL of the transformation mixtures were spread on to selective plates and incubated at 30 °C overnight.

PCR amplification for verification of *pha* knockout mutant

PCR reactions were done using genomic DNA isolated from wild-type *P. putida* KT2440 and knockout mutant strains with a set of test primers, P3: 5'-caccacatggacaaccaggttgcttggctg-3' and P3': 5'-gctttcgaaacttgctgtccagcaagctgaccagat-3' that amplify a region of (2612 bp for WT and 1106 bp for the knockout). The reaction conditions were one cycle for 4 min at 94 °C, 30 × (40 s at 94 °C, 40 s at 60 °C, 4 min at 72 °C), 8 min at 72 °C.

GC analysis of PHA composition

Liquid MS cultures (100 mL) were centrifuged at 5000 g for 10 min at 4 °C and lyophilized for a minimum of 24 h. The cell dry weight was measured. The dry cells (~15 mg) were treated with 2 mL of methanol-sulfuric acid (85 : 15) solution and 2 mL chloroform at 100 °C for 140 min. A total of 0.5 mL chloroform mixture containing the soluble methyl ester and 0.5 mL 0.1% caprylic acid in chloroform were mixed well, and these samples were assayed using a GC 2010 Gas Chromatograph (Shimadzu, Japan).⁷ All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Transformation with replicative plasmids and linear DNA

The broad host plasmid pBBR1-MCS2 was used to evaluate the transformation efficiencies of *P. putida* KT2440 at different

Table 2. Effect of various parameters on transformation efficiencies of *P. putida* KT2440

DNA	Size	Selection	Voltage	Transformation efficiencies	
				30 °C	37 °C
pBBR1-MCS2	5.1 kb	Km ^r	1200 V	1.0×10^4	4.0×10^4
pBBR1-MCS2	5.1 kb	Km ^r	2000 V	2.4×10^7	1.0×10^6
pBBR1-MCS2	5.1 kb	Km ^r	2500 V	5.3×10^7	6.7×10^6
pBBRSTQKAB	8.8 kb	Km ^r	2500 V	1.0×10^3	–
pJRD215	10.2 kb	Km ^r	2500 V	1.2×10^6	–

Table 3. Transformation efficiencies of linear DNA of several *Pseudomonas* species

Species	Size	Selection	Transformation efficiencies	
			0 h	1 h
<i>P. putida</i> KT2440	2029 bp	Gm ^r	5×10^4	5×10^5
<i>P. putida</i> IFO14164	2029 bp	Gm ^r	8.4×10^5	3.1×10^6
<i>P. sp.</i> ATCC 29347	2029 bp	Gm ^r	6.6×10^5	1.0×10^6
<i>P. putida</i> rpoN	2029 bp	Gm ^r	1.3×10^6	2.5×10^6
<i>P. putida</i> GPp104	2029 bp	Gm ^r	2.4×10^5	3.0×10^5
<i>P. putida</i> KT2442	2029 bp	Gm ^r	1.3×10^5	1.6×10^5

voltages, when the cells were incubated at different temperatures (Table 2). The transformation efficiencies ranged from 1.0×10^4 to 5.3×10^7 transformants μg^{-1} DNA. Cells transformed at 2500 V and incubated at 30 °C had the highest transformation efficiency. The plasmid pJRD215 (10.2 kb) is another broad host range plasmid but is much larger than pBBR1-MCS2, had a transformation efficiency of 1.2×10^6 transformants μg^{-1} DNA with 1 h recovery time after transformation and 8.6×10^5 transformants μg^{-1} DNA without recovery time. The transformation efficiency of pBBRSTQKAB was only 1×10^4 transformants μg^{-1} DNA, which might be due to the overexpression of PhaC1 (STQK), PhaA, and PhaB proteins affecting the growth of the cells.

The linear DNA containing *phaC1'* (a fragment of *phaC1*_{pp}), *accC1* (Gen^R), and *phaC2'* (a fragment of *phaC2*_{pp}) was transferred into several *Pseudomonas* strains to knock out the *pha* cluster. The transformation efficiencies of different *Pseudomonas* species ranged from 9×10^4 to 3.1×10^6 transformants μg^{-1} DNA (Table 3). This study also showed that transformation efficiencies can still reach 10^4 – 10^6 transformants μg^{-1} DNA without a recovery incubation time ($T = 0$ h), which reduced the overall transformation time to less than 10 min.

Confirmation of knockout mutant with PCR and PHA production

After linear DNA was transferred into *P. putida* KT2440, gentamycin resistance colonies were selected and checked by PCR to confirm the replacement of the wild type *pha* gene cluster with the knockout construct (Fig. 2). The results showed the DNA isolated from individually isolated colonies in lane 5, 6, 7 and 8 contained two copies of chromosomal DNA, one containing the wild-type *pha* cluster, the other containing the knockout *pha* cluster. DNA isolated from individually isolated colonies in lane 4 and 9 had only the knockout *pha* cluster amplicon. The transformed strain

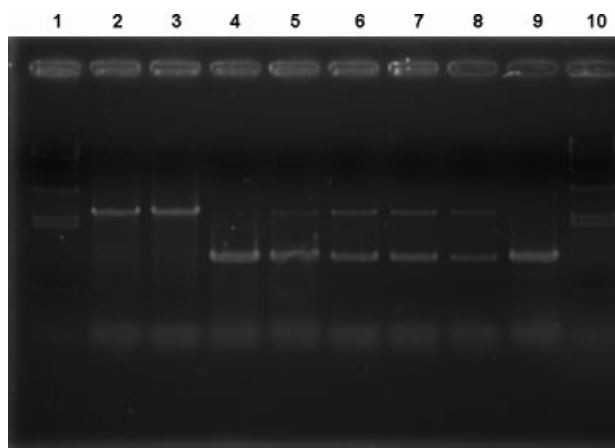


Figure 2. Verification of knockout mutations by PCR. Lanes 1 and 10 are markers. Lanes 2 and 3 have PCR products from chromosomal DNA isolated from wild-type *P. putida* colonies, and the size of the PCR product is 2.6 kb. Lanes 4 to 9 are PCR products for chromosomal DNA isolated from gentamycin resistant transformants of *P. putida*. The band at 2.6 kb is the wild type copy of the *pha* cluster and the lower band at 1.1 kb represents the amplified knockout mutation. Lanes 4 and 9 appeared to be products for a double-crossover event to knockout the *pha* cluster.

from which the DNA had been isolated in lane 4 was grown on MS medium to determine PHA content. The knockout cells still produced about 4% CDW of PHA (Table 4). Although PHA production was significantly lower than that of the wild-type *P. putida* KT2440 (14%), it was not entirely eliminated, indicating that the strain may still be merodiploid at the *pha* cluster and that the wild type PCR amplicon was beyond the detection limit of PCR (Fig. 2).

Table 4. PHA accumulation in wild-type *P. putida* KT 2440 and its knockout mutant strain

Strains	CDW ^a (g L ⁻¹)	PHA%	Composition (mol %) ^b			
			3HHx	3HO	3HD	3HDD
<i>P. putida</i> KT2440	0.2 ± 0.02	13.0 ± 0.1	2.6 ± 0.1	28.1 ± 0.4	62.9 ± 0.4	6.4 ± 0.1
<i>P. putida</i> Gen ^r	0.2 ± 0.01	4.0 ± 0.4	2.6 ± 0.2	24.5 ± 0.4	63.8 ± 0.4	9.1 ± 0.2

All the cells were grown on MS medium with the NH₄Cl concentration of 1.35×10^{-3} mol L⁻¹.

^a CDW, cell dry weight.

^b 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate.

CONCLUSIONS

This paper describes a simple and rapid method to transfer replicative plasmids and linear DNA into several *Pseudomonas* species. All procedures can be done within 10 min. The transformation efficiencies could reach $\geq 10^7$ transformants μg^{-1} DNA for replicative plasmids and $\geq 10^6$ transformants μg^{-1} DNA for linear DNA. This method can be used for transferring entire plasmids for cloning and expression and linear DNA for knockout mutations and gene replacements.

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