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ORIGINAL PAPER

Simple method for transformation of *Ochrobactrum anthropi*

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Abstract A rapid and simple method for preparation of highly efficient *Ochrobactrum anthropi* electrocompetent cells has been developed. The efficiency of transformation increased 200-fold when the cells were prepared from liquid culture compared to agar plates. Effects of different conditions, including cell density, electric field strength, resistance and plasmid size were evaluated to develop an electroporation protocol. The electrocompetent *O. anthropi* prepared by this method were 9-fold more efficient than commercial sources of competent *Escherichia coli*. The method described here will enhance the genetic manipulation of *Ochrobactrum* as a bioremediation tool and a biopesticide agent.

Keywords Biodegradation · Bioremediation · Plasmid

Introduction

Ochrobactrum anthropi is a gram-negative bacterium that constitutes up to 2% of the cultivable bacteria from soil (Lebuhn et al. 2000). In recent decades, *O. anthropi* has become a source of useful enzymes including D-aminopeptidase (Asano et al. 1989b), L-aminopeptidase (DmpA) (Fanuel et al. 1999), D-amino acid amidase (Asano et al. 1989a) and the L-amidase (Sonke et al. 2005). *Ochrobactrum* strains are of particular interest for bioremediation (Lebuhn et al. 2006). As these strains are capable of degrading organophosphorus pesticides such as parathion and methylparathion (Zhang et al. 2005), phenol (El-Sayed et al. 2003), the toxic organic solvent dimethylformamide (DMF) (Veeranagouda et al. 2006), petroleum waste (Katsivela et al. 2005) and soil contaminants like chlorothalonil (Kim et al. 2004). To date, genetic manipulation of *Ochrobactrum* spp. has been limited due to the inefficiency of DNA transformation and the lack of an efficient and stable replicating vector (Seleem et al. 2007). Transformation of *O. anthropi* using the two methods described for related species *Agrobacterium tumefaciens* and *Rhizobium leguminosarum* (Holsters et al. 1978) were not successful (unpublished data). Therefore, the goal of this study was to establish for the first time a rapid and simple method for preparation of highly efficient electrocompetent cells that could be utilized for a variety of genetic procedures.

Materials and methods

Preparation of electrocompetent cells

A plasmid-free, chloramphenicol sensitive *O. anthropi* strain 49237 was used for preparation of the electrocompetent cells. Low speed centrifugation was done at 4°C for 15 min at 3,500 × *g* (Beckman-Allegra 6R). Filter sterilized ice-cold 10% glycerol in de-ionized water was used for all washing processes.

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To prepare *O. anthropi* competent cells, we used two different methods: (A) plate method; 200 µl of *O. anthropi* culture (late log phase) at an $OD_{600} = 2$ were spread on five tryptic soy agar (TSA) plates (Difco), and incubated for 16 h at 30°C. The bacterial cells were collected by scraping from the plates and thoroughly resuspended in 25 ml ice-cold 10% glycerol and kept on ice for 30 min. The cells were harvested by centrifugation and washed twice with 25 ml ice-cold 10% glycerol. The cells were resuspended in 3 ml of 10% ice-cold glycerol (3×10^{11} cells/ml). B) For the second method, liquid culture; *O. anthropi* was grown in 400 ml of filter sterilized YENB salt free medium (0.75% Bacto Yeast extract and 0.8% Nutrient Broth) at 30°C with shaking at 200 rpm. When the cells reached (early log phase) density of $OD_{600} = 0.6$ they were placed on ice for 30 min then collected by centrifugation. The cells were washed twice with 25 ml ice-cold 10% glycerol and resuspended in 2 ml of 10% ice-cold glycerol (2.5×10^{10} cells/ml). The exact CFU of the bacteria were determined retrospectively by plating serial dilutions of the bacterial suspension on TSA plates.

Electrotransformation conditions

To test the efficiency of transformation of the electrocompetent *O. anthropi*, 25 µl from each preparation (plate and liquid broth) were each mixed gently with approximately 100 ng of 2.9 kb broad host range pNSGroE expression vector (Cm^r) (GenBank Accession No. AY576605). The pNSGroE expression vector is a derivative of the pBBR1 plasmid originally isolated from *Bordetella bronchiseptica* (Antoine and Locht 1992) and can be maintained in a wide variety of gram-negative bacteria (Seleem et al. 2004). The DNA was transformed into *O. anthropi* by electroporation using a Gene Pulser ECM-630 (BTX) set at 25 µF capacitance, 200 Ω resistance and 2.4 kV in prechilled 1 mm gap cuvettes (Eppendorf). Competent cells were subjected to the same manipulations using water instead of the plasmid as negative control. Immediately after electroporation, 500 µl of S.O.C medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added to the cuvettes and the cells were transferred into 50 ml tubes and incubated at 30°C, with shaking at 200 rpm, for 2 h to allow expression of the antibiotic resistance gene. The transformation mixture was diluted in Tryptic Soy Broth (TSB) (Difco) and plated on several TSA plates containing 30 µg/ml chloramphenicol. Colonies growing on the plates after 24 h were checked for the presence of the plasmid, by plasmid extraction (QIAprep Spin Miniprep Kit, QIAGEN).

Effect of cell density on electrotransformation efficiency

To test the effect of cell density on the efficiency of transformation, electrocompetent cells prepared by plate method (3×10^{11} cells/ml) were used as is for transformation or diluted further (1:1) with ice-cold 10% glycerol to obtain 1.5×10^{11} cells/ml, or (1:2) ice-cold 10% glycerol to obtain 1×10^{11} cells/ml or (1:4) ice-cold 10% glycerol to obtain 6×10^{10} cells/ml. The transformation procedures were carried out as mentioned above.

Effect of resistant and voltage on electrotransformation efficiency

The effect of resistance (Ω) and voltage on transformation efficiency was studied in the electrocompetent cells prepared by the plate method. Pulse parameter were set as following: (a) the capacitance and resistance were fixed at 25 µF and 200 Ω, respectively, and voltage was varied (0.5, 1, 1.5, 2 and 2.4 kV); (b) the capacitance and voltage were fixed at 25 µF and 2.4 kV, respectively, and the resistance was varied (100, 200, 400, 600, 800 and 1,000 Ω).

Effect of plasmid size

Different plasmid sizes were used to determine the ability of the electrocompetent cells to be transformed with larger plasmids. A 2.9 kb broad host range pNSGroE, a 6 kb pNSGroE/*lacZ* (pNSGroE expression vector carrying *lacZ* gene) (Cm^r) (Seleem et al. 2004) and 9 kb pBBGroE/lacZ (broad host range pBBGroE expression vector carrying *lacZ* gene) (Cm^r) (Vemulapalli et al. 2000) were tested.

Transformation of ligation reaction

We tested the ability of the electrocompetent *O. anthropi* to be transformed directly with ligation reaction in comparison with commercial sources of competent *E. coli*. Two standard ligation reactions were carried out (Sambrook et al. 1989) using pBBR1MCS broad host range cloning vector (GenBank Accession No. U02374) (Kovach et al. 1994) and either 522 bp *sodC* (Copper/Zinc superoxide dismutase) (BRA0703) or 528 bp *ahpD* (alkylhydroperoxidase-D) (BRA0707).

The same volume of the ligation reactions were used for the transformation of *O. anthropi* as described above and Mach1™ T1 Phage-Resistant (T1R) competent *E. coli* (Invitrogen) according to the manufacturer's instructions. The transformation reactions were spread on several TSA plates containing 30 µg/ml chloramphenicol and colonies were counted and screened for presence of the plasmid and insert by plasmid extraction, restriction digestion and PCR.

Table 1 The effect of cell density on transformation efficiency

Cell density (ml)	Transformation efficiency (CFU/μg of DNA)
3×10^{11}	9.5×10^{6}
1.5×10^{11}	1.2×10^{8}
1×10^{11}	3×10^{7}
6×10^{10}	5×10^{5}

All plasmids were checked for stability by serial passages over on tryptic soy agar plates in the absence of chloramphenicol selection.

Results and discussion

Effect of preparation method

The efficiency of transformation attained its highest level (2.1×10^{9} transformants per microgram of DNA) when the cells were grown on liquid media at the early growth phase; the cells collected from stationary growth phase (plate method) exhibited a lower efficiency of (9.5×10^{6} transformants per microgram of DNA). Although plate method had lower transformation efficiency, it provided enough cells (3×10^{11} cells/ml) to perform at least 300 transformation reactions. No transformants were obtained in the absence of plasmid DNA.

Effect of density, resistant and voltage

To optimize electroporation conditions, first we studied the effect of cell density. Table 1 shows the transformation efficiency of each cell density used. The optimum number of transformants (1.2×10^{8} CFU) was obtained at a concentration of 1.5×10^{11} cells/ml. Higher or lower concentration decreased efficiency of transformation.

The electric field strength was the most important factor in *Ochrobactrum* electroporation. As seen in Fig. 1a the transformation efficiency increased proportionally to the electric field strength. A 2×10^{5}-fold higher efficiency was obtained at 2.4 kV than that at 0.5 kV with no arcing. No sign of saturation was observed at 2.4 kV suggesting that higher efficiency could be achieved with higher voltage (Chen et al. 2006).

There was no significant change observed in the transformation efficiency due to increasing resistance, with the maximum number of transformants (2.2×10^{8}) obtained at 600 Ω (Fig. 1b).

Effect of plasmid size

The transformation efficiency as expected was inversely proportional to plasmid size. A 2.9 kb pNSGroE plasmid had a transformation efficiency of 9.5×10^{6} transformants per microgram of DNA, a 6 kb pNSGroE/*lacZ* had a transformation efficiency of 4.7×10^{4} transformants per microgram of DNA, while a 9 kb pBBGroE/*lacZ* had a transformation efficiency of 2.5×10^{3} transformants per microgram of DNA.

Plasmids DNA recovered from the transformants were indistinguishable from that introduced on the basis of restriction enzyme digestion and agarose gel electrophoresis.

Transformation of a ligation reaction mixture

The transformation efficiency of ligation reaction directly into *O. anthropi* was 9-fold more efficient than commercial sources of competent *E. coli*. (Mach1). This feature facilitated the use of *Ochrobactrum* as a cloning host with very high efficiency without the need of the expensive commercial cloning competent cells (*E. coli*).

Although there was no sign of homologous recombination or alternation of the cloned genes in *Ochrobactrum*

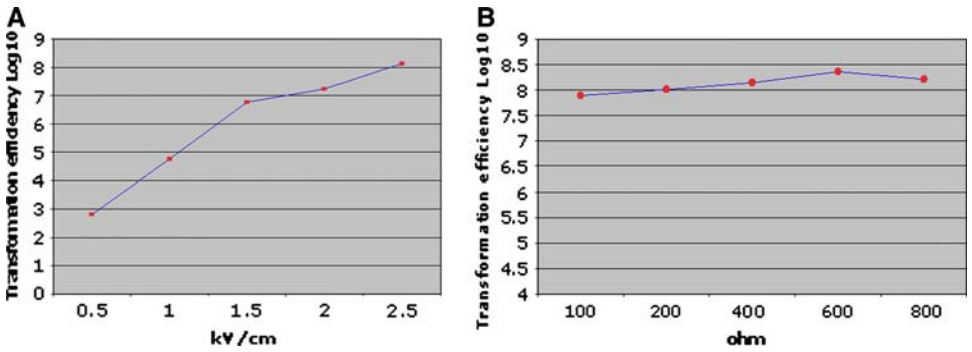

Fig. 1 Effect of electric field strength (**a**) and resistance (**b**) on transformation efficiency of *O. anthropi*. (**a**) The capacitance and resistance were fixed at 25 μmF and 200 Ω, respectively, and 0.5, 1, 1.5, 2 or 2.4 kV were used. (**b**) The capacitance and voltage were fixed at 25 μmF and 2.4 kV, respectively, and 100, 200, 400, 600, 800 or 1,000 Ω were used. Values shown are the means of three independent experiments

and the plasmid yield was similar to *E. coli*, further genetic modification might be needed to facilitate the use of *Ochrobactrum* as cheaper cloning and/or expression host and also to obtain a cleaner plasmid extract.

Recently the ability of *O. anthropi* to express recombinant fusion proteins was revealed in one-step detection and purification of recombinant green fluorescence protein (*gfp*) without using *E. coli* as an expression host (Seleem et al. 2007). We were able to obtain enough electrocompetent cells (2.5×10^{10} cells/ml) and (3×10^{11} cells/ml) from liquid culture and plate method, respectively, to perform 80 (2 ml of 25 µl reaction) and 120 (3 ml of 25 µl reaction) transformation reactions with efficiency 2.1×10^9 and 9.5×10^6 transformants per microgram of DNA, respectively. All plasmids were stably maintained after eight generations over 16 days on TSA plates in the absence of chloramphenicol selection.

The protocol applied here simplified the procedures for the preparation and transformation of *Ochrobactrum* and does not require any expensive chemicals or enzymes to be added to the procedure. Among the several factors that were studied that could affect the efficiency of the transformation, preparation method and the voltage were the most critical factors. No significant differences were observed due to increased resistance. Coupled with efficient expression system, this simple and rapid technique will facilitate the genetic manipulation and the use of *O. anthropi* as a potential bioremediation tool or as a biopesticide agent.

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