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Research Article

Evaluation of Parameters for High Efficiency Transformation of *Acinetobacter calcoaceticus* ATCC23055

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Abstract: To optimize parameters influencing transformation efficiency is one of the first steps to develop molecular tools. *Acinetobacter calcoaceticus* is an emerging, potential application bacterium, even a cultivable symbiont that is poorly characterized due to a paucity of genetic tools and methods. Here we reported parameters allowing high efficiency transformation of *A. calcoaceticus*. Using a Orthogonal experimental design we found that growth phase, resistance and the amount of plasmid DNA significantly contribute to transformation efficiency. The highest efficiency (7.51×10^3 transformants/μg DNA) was obtained at the growth phase of the bacterium (OD_{600} = 0.5) using 700 ng of plasmid DNA under 200 Ohms resistance condition. The optimized electroporation parameters might provide a useful tool for genetic manipulation of *A. calcoaceticus*.

Keywords: Evaluation, orthogonal experimental design, parameters, transformation

INTRODUCTION

Acinetobacter calcoaceticus (Pseudomonadales: Moraxellaceae), a Gram-negative bacterium, was extensively studied as which produce the potential application value in agroindustrial wastewater, detergents, pesticides and animal husbandry (De *et al*., 2015; Hošková *et al*., 2015; Pirog *et al*., 2013; Hrenović *et al*., 2003; Srivastava and Srivastava, 2006; Wang *et al*., 2007), Furthermore, since *A. calcoaceticus* has been found under natural conditions involved in plants and even in the insects such as in aphid body and its honeydew(Leroy *et al*., 2011; Uniyal *et al*., 2016), it could be assumed spontaneously to be considered as a cultivable symbiont in its 'habitat' for specific function. Recently it was reported as an abundant cultivable symbionts affecting host's biological characteristic within the Entomophthoralean fungi, *Pandora neoaphidis* which was an obligate fungal pathogens of aphids and has great potential for use in biocontrol (Chen *et al*., 2016).

Commonly, wild-type *A. calcoaceticus* could produce a protective shield, products of industrial fermentation, such as surfactants and emulsifiers (De *et al*., 2015; Hošková *et al*., 2015; Pirog *et al*., 2013). A cell-associated form of emulsan, an extracellular polymeric bioemulsifier produced by *A. calcoaceticus* RAG-1, constitute a minicapsular layer on the surface served as a protective shield (Shabtai and Gutnick, 1985). *A. calcoaceticus* BD4 could possess a heavily capsular polysaccharide, a viscous mixture containing L-rhamnose and D-glucose in the molarratio 4: 1, located external to the cytoplasmic membrane, to the peptodiglycan layer and to the outer membrane (Kaplan *et al*., 1985). Since a protective and clumping bacterium was not particularly suitable for quantitative transformation studies, previous studies on the transformation of *A. calcoaceticus* were carried out frequently by using capsule mutant strains such as transposon mutagenesis or physical and chemical factor mutagenesis (Yildirim *et al*., 2016). In most cases, the preparation of mutant strain was tedious and their original characteristics of the strain were destroyed undoubtedly. Lacking the molecular genetic studies on *A. calcoaceticus* wild-type strains made many interesting works underexplored.

Electroporation is a method commonly used for introducing foreign DNA into cells across many phylogenies. Transformation efficiency in electroporation depends on multiple parameters including: electrical parameters, the amount and purity of DNA used, temperature, cell density, buffer composition and the growth phase of the bacterial cells when made competent (Yildirim *et al*., 2016). Optimal transformation protocols are especially important for organisms with poorly defined or under developed genetic systems such as *A. calcoaceticus*. Therefore, the aim of the present study was to optimize parameters

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influencing transformation efficiency of wild-type *A. calcoaceticus* using an orthogonal experimental design.

MATERIALS AND METHODS

Bacterial strains and plasmids: *A. calcoaceticus* ATCC23055, a wild-type strain, reported that single or combined with other strains can forming biofilm which possess the function of denitrifying and phosphorus removing (Andersson *et al*., 2011), was grown in Luria-Bertani (LB) broth at 30°C with shaking at 180 rpm to the appropriate Optical Density (OD). A shuttle plasmid pWH1266 (ATCC ®77092 ™) for *Escherichiacoli/A. calcoaceticus* was harbored in *E. coli* strain DH5α (Hunger *et al*., 1990). Plasmid DNA from *E. coli*DH5α grown in LB broth was isolated by the TIANprep Mini Plasmid Kit (Takara, Japan) and was digested with restriction enzymes BamHI and SalI (Takara, Japan) and size fractionated by agarose gel electrophoresis. Plasmid pWH1266 DNA was linearized with BamHI and SalI as indicated by manufacturer's protocol. The *Bam H*I-*Sal*I fragment harboring a promoterless *egfp* gene and pWH1266 DNA restriction fragments were purified using the QIAquick PCR purification kit (Qiagen, USA). The ligation products were transformed to *E. coil* DH5α competent cell by heat shock method and the target plasmid was named as pMU 125. Plasmid pWH1266 in *E. coli* and *A. calcoaceticus* was maintained within 100 μg/mL tetracycline (Sigma-Aldrich, USA). Bacterial strains were maintained on LB agar or broth supplemented with the appropriate antibiotics and were incubated overnight at 30°C. Media were supplemented as necessary with antibiotics (final concentrations of Tetracycline and Ampicillin was 100 μg/mL and 100 μg/mL, respectively).

Preparation of Electroporation competence cells: A NaOH-ethanol method for preparation of electrocompetent cells was briefly modified as the method described by Dorsey *et al*. (2002). Appropriate optical density (OD_{600}) bacteria cells from LB broth were harvested by centrifuge (6500 rpm for 10 mins at 4°C). After the pellet was suspended and spinned for three times, 800 μL 0.01 mmol/L cold NaOH was added into 1.5 mL centrifuge tube and washed the protective shield of pellet for 5 mins. Furthermore, 1 mL of 10% cold Ethanol was added to eliminate the shield by washing the pellets twice for 10 mins totally. Subsequently, the pellet was re-suspended by cold distilled water and was harvested in to -80° freezer protected by 200 μL of 10% glycerol.

Electroporation procedure: Electroporation assays were performed as followed. Thaw electroporation competent cells by putting electroporation cuvettes on ice. pMU125 DNA was sampled into thawed electroporation competent cells in 1.5 mL centrifuge tube and kept at 4°C for 30 mins. Subsequently, the mixtures were transferred to 1 mm electroporation cuvettes. Electroporation was performed within a BioRad Gene PulserXcell apparatus (Bio-Rad, USA) which allowed the addition electroporation parameters of a Resistance (ohm), Voltage (V), Capacitance (μF), Cuvettes (mm) and selected Exponential Decay Protocol into the circuit of the electrical discharge. Successive pulse was ran for three times and the cells were immediately recovered in 1mL of LB broth at 180 rpm, 30°C, more than 3 h. Treatment without pMU125 DNA in electroporation mixtures was as blank. To enumerate the response values (the amount of transformants), 200 μL cells was incubated overnight onto LB agar plates supplemented with 100 μg/mL Ampicillin at 30°C. Transformations were confirmed through the specific bands from the target plasmid described above digested by restriction enzymeBamH I and SalI.

Orthogonal experimental design and parameter testing for optimizing transformation: A Orthogonal experimental process was used to optimize electroporation transformation of *A.calcoaceticus*. From all the possible combinations of the four parameters selected, at all levels identified (from low (Andersson *et al*., 2011) to high (Aune and Aachmann, 2010) for each parameter) and in all possible combinations (Table 1) an L_9 (3^4) orthogonal array (Hedayat *et al*., 1999) was used to select a subset of 9 key combinations of parameters over a range of levels for experimental evaluation (Table 1). The response values (transformants/μ g DNA) at all levels of each factor are repeated in twice. The factors, growth phase, voltage, resistance and the amount of plasmid DNA used were denoted by OD_{600}, V (kV/cm), R (Ohms) and DNA (ng), respectively. The response values (transformants/μ g DNA) of the 9 parameter combinations were presented in Table 1.

Data on transformants from each parameter combination tested in the orthogonally designed experiment were compared using range analysis to indicate the relative importance of each parameter on transformants and their optimal levels and ANOVA to identify the statistical significance of different levels of each parameter on transformants to confirm indications from range analysis. In range analysis for each parameter, the mean values of the density for each level were calculated (K_1, K_2 and K_3) and the range determined from the difference between the maximum value [K_1, K_2, K_3] and the minimum value [K_1, K_2, K_3] for each parameter. Based on the principle of 'the larger the better' the parameter with the largest range was considered likely to have greatest influence on transformants and the level achieving the greatest transformants, as the optimum. All computations were done using DPS Statistical software (Tang and Feng, 2002)

RESULTS AND DISCUSSION

Experimental designs for optimizing transformation: The response values (transformants/μ

Table 1: Parameters and their levels (1-3 = Low to High) for potential evaluation in the series of experimental treatments following an orthogonal experimental design[1]

Parameter	Designation	Level 1	Level 2	Level 3
Growth phase (OD_{600})	A	0.2	0.5	2
Voltage (kV/cm)	B	1.2	1.6	2
Resistance (Ohms)	C	100	200	400
amount of plasmid DNA (ng)	D	100	300	700

[1]Orthogonal experimental design = L_9 (3^4)

Table 2: Design matrix and the response values (transformants/µ g DNA) based on the orthogonally designed experiment[1]

	Parameters (A-D) and their levels (1-3)				
Treatments	A	B	C	D	Transformants/µ g DNA
1	1	1	1	1	35.0
2	1	2	2	2	3230.0
3	1	3	3	3	80.7
4	2	1	2	3	5891.4
5	2	2	3	1	320.0
6	2	3	1	2	1845.0
7	3	1	3	2	56.7
8	3	2	1	3	1020.0
9	3	3	2	1	185.0

[1]Orthogonal experimental design = L_9 (3^4)

Table 3: Range analysis of the response values (transformants/µ g DNA) achieved in the treatments selected in the orthogonal experimental design[1] and the optimal level of each parameter required to produce the highest transformants

	Transformants/µ g DNA by parameter (A-D)			
K value[2]	A	B	C	D
1	1115.24	1994.37*	966.67	180
2	2685.48*	1523.33	3102.14*	1710.56
3	420.56	703.57	152.46	2330.71*
Range	2039.94	1162.58	2656.68	1937.08

[1]Orthogonal experimental design = L_9 (3^4); [2] K_1, K_2 and K_3 were the average values of the transformants for each level in the orthogonally designed experiment, and the optimal level of each parameter was determined by comparing the value of K_i (i=1, 2, 3); * Optimal level of each parameter to produce the highest transformants; Details of levels are listed in Table 1

Table 4: Results of ANOVA to determine which parameters had a significant effect on the transformants following the orthogonal experimental design[1]

Parameter	DF	SSD	F value	Significance
A (growth phase (OD_{600}))	2	8078097	11.65	0.0043
B (voltage(kV/cm))	2	2560029	3.69	0.0731
C (resistance(Ohms))	2	13923818	20.08	0.0008
D (the amount of plasmid DNA (ng))	2	7352769	10.60	0.0056
Error	8	693419.7		

Details of levels are listed in Table 1. SSD = Sum of Squares of Deviations; [1]Orthogonal experimental design = L_9 (3^4)

g DNA) distribution varied with treatment and ranged from 35.0±7.1 (Treatment 1) to 5891.4±2285.0 (Treatment 4) (Table 2). The highest efficiency (7.51×10^3transformants/µg DNA) was obtained in Treatment 4. The parameters with the largest and smallest ranges in the response values (transformants/µ g DNA) were C, Resistance and B, voltage, respectively. The optimal levels for parameters A, B, C, D were 2 (0.5), 1 (1.2), 2 (200), 3(700) respectively where 1 was low and 3 high (Table 3).

Resistance had the most significant impact on the response values (transformants/µ g DNA), followed by growth phase, the amount of plasmid DNA and finally voltage (Table 2). In contrast, the voltage had no significant effect on transformants (Table 4). Accordingly, the optimal electroporation conditions to achieve the highest efficient transformation of *A. calcoaceticus* were, an intermediate growth phase (OD_{600} = 0.5) of strain, an intermediate Resistance of electroporation (200 Ohms) and a high amount of plasmid DNA of 700 ng. *A. calcoaceticus* was isolated from the mycelium of *P. neoaphidis* (Entomophthoromycotina, Entomophthorales) and might be of an important symbiont for the pathogenicity of fungi (Chen *et al*., 2016). So the molecular genetic studies are of forward to explore the biological function of bacterial symbionts. The GFP-labeled *A. calcoaceticus* is to explore the interaction mechanism between symbionts and *P. neoaphidis*.

Large capsules characteristic of wild-type *A. calcoaceticus*s train was able to form chains which also easily form clumps of cell (Juni and Janik, 1969). This physiological phenomenon may be causes the electroporation transformation with foreign DNA to be difficult to succeed. In this study, an improved protocol for making electroporation competence cell using Sodium hydroxide (NaOH) and ethanol to explore transformation were described. The biological function of microorganisms cell membrane was able to maintain osmotic pressure balance and to regulate the exchange

of solutes between the living cell and the external circumstance (Weber and de Bont, 1996; Sajbidor, 1997). Therefore, a non-lethal NaOH concentration could be employed to wash the *A. calcoaceticus* and strip the CPS which might prevent the foreign DNA into recipient in transformation. Besides, 10% ethanol was able to increased transformation efficiency by improving the membranes (Chu-Ky *et al*., 2005; Assad-García *et al*., 2008; Aune and Aachmann, 2010). According to the NaOH-ethanol method treatment in advance, we successfully obtained the transformation and explored optimizing transformation conditions (Table 2).

Actually, in order to strip the polysaccharide capsule and change cell membrane structure of *A. calcoaceticus*, some cell-wall-weakening agents, such as bismuth subsalicylate, amino acids and enzymes have been shown to increase transformation efficiencies in some species and strain (Domenico *et al*., 1991). However, the toxic drawbacks and species- and strain-dependent manner made such additives restricted (Aune and Aachmann, 2010). In addition, Capsular Polysaccharide (CPS), a polyanionic heteropolysaccharide produced by *A. calcoaceticus*, could be precipitated by the cationic detergent amphipathic cationic surfactants such as cetyltrimethylammonium bromide (CTAB) which sheds the CPS from cell surface (Shabtai and Gutnick, 1985). The most effective, both increases cell permeability and specifically alters the outer membrane by releasing Lipopolysaccharide (LPS) and without caused enzyme inhibition, ribosomal breakdown and death, performed by EDTA combined with Tris buffer at alkaline PH (Leive, 1974). Besides 0.01 mmol/L NaOH combined with 10% ethanol, we also explored the effectiveness of cetyltrimethylammonium bromide (CTAB), Ethylene diaminetetraacetic acid (EDTA) and hydrochloric acid (HCl).

Different concentration including 0.1%, 0.01% and 0.0001% CTAB were evaluated the transformation efficiency by similar with the above NaOH-ethanol method. These concentration CTAB obtained few colonies which did not meet the requirement of the cell quantity of electroporation transformation. As for 1mmol/L EDTA-treated, the transparent gelatinous material was observed on the wall of the centrifuge tube after centrifugation. This phenomenon is the same with 0.01 mol/L NaOH-treated. However, it failed to harvest the electroporation competent cell by either 1 mmol/L EDTA or 1 mmol/L EDTA combined with 10% ethanol. Additionally, Hydrochloric acid (HCl)-treated was also investigated. Unfortunately, milky white flocculation was observed after HCl-treated and it was hard to proceed the following electroporation transformation. The reason might be that acid shock lead to the membrane was more rigid than its initial state (Chu-Ky *et al*., 2005).

CONCLUSION

To conclude, results indicated that high efficiency transformation of *A. calcoaceticus* wild-type strains ATCC23055 were obtained at the growth phase of the bacterium (OD_{600} = 0.5) using 700 ng of plasmid DNA under 200 Ohms resistance. The electroporation protocol conditions presented here should facilitate the genetic manipulation of *A. calcoaceticus* strains, including the generation of knockout mutants, side-directed mutagenesis, heterologous gene expression and random insertion mutagenesis.

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