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Development of an efficient electroporation method for rhizobacterial *Bacillus mycoides* strains

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ABSTRACT

In order to develop a method for electroporation of environmental *Bacillus mycoides* strains, we optimized several conditions that affect the electroporation efficiency of this bacterium. By combining the optimized conditions, the electroporation efficiency of strain EC18 was improved to $(1.3 \pm 0.6) \times 10^5$ cfu/μg DNA, which is about 10^3-fold increase in comparison with a previously reported value. The method was further validated on various *B. mycoides* strains, yielding reasonable transformation efficiencies. Furthermore, we confirmed that restriction/modification is the main barrier for electroporation of this bacterium. To the best of our knowledge, this is the first systematic investigation of various parameters of electroporation of *B. mycoides*. The electroporation method reported will allow for efficient genetic manipulation of this bacterium.

1. Introduction

Bacillus mycoides is a spore-forming Gram-positive bacterium that is commonly found in soil and the rhizosphere. It belongs to the *B. cereus* sensu lato group, which includes *B. cereus*, *B. thuringiensis*, and *B. anthracis*. *B. mycoides* has received the least attention among this group because it is not a human pathogen as *B. anthracis* and *B. cereus*. Moreover, there are several reports on the insecticidal effect of *B. thuringiensis*, which stimulated further studies using genetic modification (Turchi et al., 2012). The *B. mycoides* species features a unique filamentous growth pattern, either being rotated clockwise or counterclockwise (Di Franco et al., 2002). Nowadays, more and more studies on *B. mycoides* are focusing on their plant-growth promoting activities (Ambrosini et al., 2016; Bargabus et al., 2002; Neher et al., 2009).

In order to study the plant interaction mechanism of *B. mycoides*, it is necessary to be able to perform molecular genetics studies on this bacterial species. A high transformation efficiency is required to establish the genetic manipulation systems, e.g., enabling gene deletion and mutation within the genome of this organism. Several techniques, including phage transduction, protoplast transformation, natural competence, and electroporation have been applied to incorporate exogenous DNA into *Bacillus* cells (Barlass et al., 2002; Lu et al., 2012; Romero et al., 2006). Among these methods, electroporation is usually the quickest and most reproducible method. Di Franco et al. (2002) modified the electrotransformation protocol of Macaluso and Mettus (1991) for *B. thuringiensis*, but the implementation on *B. mycoides* resulted in a very low efficiency (less than 200 cfu/μg plasmid DNA). Our preliminary experiments show that by applying the method reported by Ehling-Schulz et al. (2005), which was originally developed for *B. cereus*, the *B. mycoides* strain EC18 could be transformed, albeit at a low efficiency. Apart from this, no other protocols have been developed on electroporation methods for *B. mycoides*. This work aimed at developing an efficient electroporation method for several environmental *B. mycoides* strains. *B. mycoides* EC18 which was isolated from the endosphere of potato, and displaying potential plant growth-promoting activity (data not shown), was used as a first testing strain. Factors including growth media, growth phase, electroporation buffer, pulse strength, and incubation time that can affect electroporation efficiency were optimized. As a result, a high electroporation efficiency of $(1.3 \pm 0.6) \times 10^5$ cfu/μg DNA) was obtained. Furthermore, we evaluated our optimized protocol on other *B. mycoides* strains as well, which resulted in electroporation efficiencies ranging from $(7.3 \pm 2.1) \times 10^2$ to $(1.3 \pm 0.6) \times 10^5$ cfu/μg DNA.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

Escherichia coli strains were grown in Luria broth at 37 °C, 220 rpm. *B. mycoides* strains were isolated from a potato field in Wijster (the Netherlands) (Table 3) and were grown at 30 °C, 200 rpm in LB. When necessary, 100 μg/ml of ampicillin or 4 μg/ml of chloramphenicol was added to the culture medium. Plasmid DNA from *E. coli* was purified using the NucleoSpin plasmid isolation kit (Macherey-Nagel GmbH & Co. Düren, Germany) according to the manufacturer's instructions. A

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Geobacillus-E. coli shuttle vector pNW33N (Bacillus Genetic Stock Centre) isolated from *E. coli* MC1061 was used as the plasmid for electroporation protocol optimization. pNW33N isolated from *E. coli* JM110 was used to test the plasmid methylation effects on transformation efficiency.

2.2. Screening for growth medium

The *B. mycoides* strain was streaked on LB agar plate and grown at 30 °C overnight. One single colony was inoculated into various media including LB (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L deionized water, pH 7.2), LBS (10 g tryptone, 5 g yeast extract, 10 g NaCl, and 91.1 g sorbitol in 1 L deionized water, pH 7.2), LBSP (10 g tryptone, 5 g yeast extract, 10 g NaCl, 50 mM KH_2PO_4 and K_2HPO_4, and 91.1 g sorbitol in 1 L deionized water, pH 7.2), 2×YT (16 g tryptone, 10 g yeast extract and 5 g NaCl in 1 L deionized water, pH 7.2), and BHIS (34 g BHI, and 91.1 g sorbitol in 1 L deionized water, pH 7.2).

2.3. Preparation of electro-competent cells

B. mycoides cells were grown overnight in an appropriate medium at 30 °C, 200 rpm. The culture was diluted with the same medium to obtain an initial optical density at 600 nm (OD_{600}) of about 0.05 then continually grown. The OD_{600} was measured by a Genesys 20 spectrophotometer (ThermoSpectonic, USA) during bacterial growth. When the OD_{600} reached the appropriate value, the cell culture was transferred into a 50 mL centrifuge tube and cooled on ice for 10 min. Cells were then collected by centrifugation at 4 °C, 3000 ×*g* for 10 min. After being washed four times in corresponding electroporation buffer (pre-chilled), the cell pellets were re-suspended in 1 mL of the electroporation buffer. The resulting electro-competent cells were flash-frozen in liquid nitrogen and stored at −80 °C prior to electroporation.

2.4. Cell wall-weakening treatment

Serial concentrations of Glycine or DL-threonine were added when bacterial cultures reached an $OD_{600} = 0.85$ for the cell-wall weakening treatment. These treated bacterial cultures were continued to shake for 1 h. After that, electro-competent cells were prepared with the methods mentioned above.

2.5. Electroporation

The electroporation was performed as previously reported method (Peng et al., 2009; Turgeon et al., 2006) with slight modification. 100 μL of frozen electro-competent cells was thawed on ice and mixed with 2–3 μL of plasmid DNA (1 μg). Cells mixed with the same amount of deionized water served as negative control. The mixture was loaded into an electroporation cuvette (2 mm electrode gap, pre-chilled) and exposed to a single pulse in a Gene Pulser System (Bio-Rad, USA) with the settings of 25 μF capacitance, 100, 200, or 400 Ω resistance, and voltage ranges between 5 and 12.5 kV cm^{-1}. After pulse-shock, cells were immediately added with 1 ml of pre-warmed corresponding growth medium and transferred to a 2 mL Eppendorf tube. After 2 h incubation at 30 °C, 180 rpm, the dilutions of the recovered cell culture were plated on LB agar with 4 μg/ml of chloramphenicol. The plates were incubated at 30 °C overnight and transformation efficiencies (cfu/μg DNA) were calculated by counting the colonies on plates.

3. Results and discussion

3.1. Optimization of growth conditions

For determining the optimal growth medium, we transformed *B. mycoides* according to the protocol published by Ehling-Schulz et al. (2005). Strain EC18 was grown in different media before preparing the electro-competent cells. Media including LB, LBS, LBSP, 2×YT and BHIS were chosen according to reported electroporation methods for *Bacillus* species (Zhang et al., 2011a, 2015). When the OD_{600} reached a value of 0.6, the competent cells were prepared by washing the cell pellet with increasing concentrations of ice-cold glycerol (2.5, 5, and 10%). Electroporation was performed with 1 μg pNW33N plasmid DNA and recovered for 2 h with the corresponding growth media. The efficiencies were calculated after 1 day (Table 1) on 4 μg/mL chloramphenicol selection media. Among the five tested media, the super rich medium BHIS showed the highest efficiency of $(6.2 \pm 1.4) \times 10^3$ cfu/μg plasmid DNA. Zhang et al. (2011a) reported that the transformation efficiency of *B. amyloliquefaciens* was positively correlated to concentrations of salts, whereas negatively related to the nutritional ingredient concentration. Surprisingly, we observed an opposite effect of the nutritional and salt concentrations on transformation efficiencies in our study. The transformation efficiency by culturing in the LBSP medium is lower than culturing in LBS medium. Moreover, *B. mycoides* EC18 could not grow in the hypertonic media NCM and M9YE (data not shown). We hypothesized that *B. mycoides* EC18 is sensitive to salt concentrations.

According to previous reports, cells of *B. cereus* and *B. thuringiensis* collected at early growth-stage show better electroporation efficiency than late growth-stage cultures (Peng et al., 2009; Turgeon et al., 2006), while *B. subtilis* WB800 has high electroporation efficiency of 1.88×10^5 cfu/μg DNA at late growth-stage ($OD_{600} = 2.2–2.3$) (Lu et al., 2012). To investigate the effects of the growth phase of *B. mycoides* on the electroporation efficiency, *B. mycoides* cells were cultured in BHIS medium to OD_{600} from 0.3 to 1.8 for competent cell preparations. Our results indicated that when OD_{600} is between 0.9 and 1, the highest electroporation efficiency was obtained (Fig. 1A). According to the growth curve (Fig. 1B), cells are in the early stage of exponential growth. A similar phenomenon was also reported for *B. subtilis* ZK by Zhang et al. (2015). This indicates that cells from the early exponential phase result in higher electroporation efficiency of *B. mycoides*.

3.2. Optimization of cell wall-weakening agents

The cell wall-weakening agents, glycine and DL-threonine, are widely used to improve the electroporation efficiency of environmental strains. These amino acids can reduce the peptidoglycan bonds and loosen up the cell wall by replacing the L- and D-alanine bridges (Hammes et al., 1973). In this study, EC18 cells were first grown in BHIS medium, and when the OD_{600} reached about 0.85, glycine and threonine were added at different concentrations (0%, 1%, 2%, 3%, 4% and 5%). After 1 h of additional incubation, the cells were collected and concentrated to obtain competent cells. Electroporation treatments were performed and the greatest transformation efficiency was obtained in the 2% glycine treatment group. The same concentration of threonine also improved the transformation efficiency, which was however lower than that of glycine (Fig. 2). Cell growth rate was slightly reduced in the presence of both glycine and DL-threonine, and high concentrations of glycine treatments resulted in cellular lysis in the samples

Table 1
Effect of growth media on the electroporation efficiency of *B. mycoides* EC18.

Medium	Transformation efficiency (cfu/μg DNA)
LB	$(3.4 \pm 1.0) \times 10^2$
LBS	$(8.3 \pm 2.5) \times 10^2$
LBSP	$(2.0 \pm 1.1) \times 10^2$
2×YT	$(5.4 \pm 1.3) \times 10^2$
BHIS	$(6.2 \pm 1.4) \times 10^3$

Cells were grown in different media to OD600 ~0.6 to prepare the electro-competent cells using series concentration of glycerol solution as electroporation buffer. 1 μg of pNW33N plasmid was used for electroporation with the settings 25 μF, 10 kV cm^{-1}, 200 Ω. Data are shown as mean ± standard deviation based on 3 replications.