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Transformation of the rhizospheric *Bacillus cereus sensu lato B*25 strain using a room-temperature electrocompetent cells preparation protocol

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ABSTRACT

Bacterial transformation is a crucial step in the genetic manipulation of a bacterium. However, Gram-positive bacteria are difficult to transform and consequently many different methodologies have been developed. Here, we examined the transformation efficiencies of an electroporation protocol by varying three main factors: the composition of the electroporation buffer, the strength of the electric pulse, and the composition of the recovery media. Overall, transformation efficiency was enhanced when we prepared the electrocompetent cells at room temperature instead of an ice-cold temperature. The protocol detailed in this work was demonstrated to be applicable to another *B. cereus* strain and two other *Bacillus* species, and has the potential to be applied to other undomesticated Gram-positive and/or rhizospheric bacterial strains that are difficult to transform using current methodologies.

1. Introduction

The *Bacillus cereus* group comprises several species of Gram-positive, spore-forming bacteria. Due to their highly conserved genome sequence (70–75%), many species such as *B. anthracis*, *B. mycoides*, *B. thuringiensis*, *B. weihenstephanensis* and *B. cereus* belong to the *B. cereus sensu lato* group (Bazinet, 2017). The bacteria in this group are ubiquitous and display a wide variety of phenotypes, such as the human pathogen *B. anthracis*, and some function as plant-growth promoters, such as *B. cereus* (Rasko et al., 2005).

Bacillus cereus sensu lato strain *B*25 has been isolated from the maize rhizosphere and studied as a biocontrol agent for use against the maize-infecting phytopathogenic fungus *Fusarium verticillioides* (Figueroa-López et al., 2016). Its effectiveness has been demonstrated in greenhouse experiments as well as in field trials (Lizárraga-Sánchez et al., 2015). To understand deeper the plant-growth promoting traits of *B. cereus*, it is crucial to be able to genetically manipulate its genome. However, in contrast to type strains or lab-cultivated strains such as *B. subtilis*, many rhizospheric *Bacillus* strains have proven difficult to manipulate, particularly when routine protocols are not always applicable for introducing plasmids (Schurter et al., 1989; Turgeon et al., 2006). In addition, different studies have shown that transformation of some *Bacillus* species requires non-methylated plasmid DNA (Macaluso and Mettus, 1991), while other species require methylated plasmid DNA (Groot et al., 2008).

Various *Bacillus* transformation protocols have been reported, including some that improve the natural competence of strains or transform protoplasts, and others that modify the electroporation buffers with hyperosmotic agents, vary the voltage pulses, or even use wall-weakening techniques (Masson et al., 1989; Turgeon et al., 2006; Xue et al., 1999; Zhang et al., 2011). Likewise, Jirásková et al. (2005) described an electroporation protocol for *Clostridium perfringens* that yielded better transformation efficiencies when the cells were prepared at room temperature rather than at 4 °C. Tu et al. (2016) reported that a Gram-negative electroporation protocol could be improved by raising the temperature at which the competent-cells are made (which typically requires ice-cold temperatures). Here, we describe a novel way to improve an existing protocol for room-temperature electro-transformation of *Bacillus cereus* that could be applied to other Gram-positive bacteria.

2. Materials and methods

2.1. Bacterial strains and plasmids

*Bacillus cereus B*25, *B. cereus B*14, *B. subtilis B*6, *B. thuringiensis B*29 and the *Escherichia coli* strains Top10, BL21 (cytosine methylation deficient: *dcm*$^-$), GM33 (adenine methylation deficient: *dam*$^-$) and

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GM2163 (adenine and cytosine methylation deficient: *dam*$^-$, *dcm*$^-$) were routinely grown on LB agar and LB broth at 37 °C with orbital shaking at 225 rpm. The following antibiotics concentrations were used: 150 μg mL^{-1} ampicillin for *E. coli* and 5 μg mL^{-1} chloramphenicol for *B. cereus*. The plasmid pAD4325, which harbors a gene coding for a green fluorescent protein (GFP) and confers resistance to ampicillin and chloramphenicol, was propagated into each of the four *E. coli* strains mentioned above. Plasmid DNA was purified using the Plasmid Miniprep Kit (QIAGEN, Cat No. 27106; Hilden, Germany) and was quantified with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA).

2.2. Electroporation buffers

Electrocompetent cells were prepared using six different reported buffers: PEB (272 mM sucrose, 1 mM $MgCl_2{\cdot}6H_2O$, 7 mM phosphate buffer, pH 7.4), HEB (272 mM sucrose, 1 mM $MgCl_2{\cdot}6H_2O$, 7 mM HEPES, pH 7.3) (Luchansky et al., 1988), ETM (0.5 M sorbitol, 0.5 M mannitol, 10% glycerol, pH 7.0) (Zhang et al., 2011); SHMG (250 mM sucrose, 1 mM HEPES, 1 mM $MgCl_2{\cdot}6H_2O$, 10% glycerol, pH 7.0) (Turgeon et al., 2006), KS (1 mM HEPES, glycerol 10%, pH 7.0) (Shatalin and Neyfakh, 2005), and SA (10% sucrose) (Aukrust et al., 1995).

2.3. Preparation of electrocompetent cells

For each electroporation buffer (EB), a 5-mL overnight LB culture of *Bacillus cereus B*25 was diluted 100-fold in 50 mL fresh LB and incubated (37 °C, 225 rpm) until the culture reached an OD_{600} of 0.4. Then, the cell culture was centrifuged (10 min at 4500 rpm) at room temperature. The cell pellet was then resuspended with 5 mL of EB (1/10 of the original culture volume) by gently pipetting. Next, the cell suspension was immediately divided into five 1.6 mL microcentrifuge tubes (1 mL each) and centrifuged at room temperature for 1 min at 10,000 rpm. In total, three washes with 1 mL of EB were performed. For each wash, the cell pellet was gently resuspended with the micropipette and centrifuged at room temperature for 1 min at 10,000 rpm. Finally, the cells in each microcentrifuge tube were resuspended in 100 μL EB. Each step in the preparation of ice-cold electrocompetent cells was the same except that once the culture reached an OD_{600} of 0.4, the cells were cooled on ice for 10 min and the washing steps were performed at 4 °C.

2.4. Electroporation

Electroporation was carried out in an Eppendorf® Electroporator 2510. 90 μL of electrocompetent cells were mixed with 400 ng of plasmid and loaded into a previously cooled 1 mm electroporation cuvette. The cells were given an electric pulse (either 620, 1250 or 1800 kV/cm) for 5 ms. After the electric pulse, 900 μL of LB or SOC (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2{\cdot}6H_2O$, 10 mM $MgSO_4{\cdot}7H_2O$, 20 mM glucose) media were added to the cells. The suspension was incubated for 2 h at 37 °C without agitation, after which it was plated onto LB agar supplemented with chloramphenicol and incubated for 16 h at 37 °C. Electroporation controls were performed by electroporating cells lacking any added plasmid DNA.

2.5. Data analysis

All transformation experiments were conducted three times independently. The transformation efficiency (number of transformants (CFU)/μg of plasmid DNA) was calculated for each experiment and the statistical significance of transformation efficiency differences among treatments was determined using a one-way ANOVA test.

2.6. Fluorescence microscope observation

*Bacillus cereus B*25 transformation was confirmed by epifluorescence microscopy, based on the GFP expression of plasmid pAD4325. For this, strain *B*25 as well as *B*25 transformed with pAD4325 were grown overnight in 5 mL LB broth. The next day, 5 μL of the culture were spotted onto a 1-cm^2 agarose pad (UltraPure™ Low Melting Point Agarose, Thermo Fisher Scientific, Cat No. 16520050; Waltham, MA, USA) placed previously on a glass slide. Immediately after pipetting, the agarose pad was covered with a coverslip and bacteria were imaged using a Leica DM6000 fluorescent microscope with the I3 filter cube (BP 450-490 excitation filter).

3. Results

3.1. Determination of the DNA methylation requirements for transformation

Our initial attempts to electroporate *B. cereus B*25 with plasmid pAD4325 isolated from *E. coli* Top 10 were unsuccessful when using each of the six electroporation buffers (EB) with a 620 kV/cm electric pulse. As a minimum, we had expected a poor transformation efficiency in which at least one EB worked. Since there were no transformants, we hypothesized that strain *B*25 might require non-methylated DNA, which is necessary for *B. anthracis* (Marrero and Welkos, 1995) but contrary to what has been reported for other *B. cereus* strains (Groot et al., 2008).

A BLAST search of the *B*25 strain genome (Douriet-Gámez et al., 2017) allowed us to find an McrA homologue identical to that of *B. cereus* ATCC 14579, and a protein with 80% identity to *B. anthracis* McrB3P. These proteins belong to the type IV restriction-modification system, which are defense mechanisms used by bacteria to restrict foreign DNA. The restriction enzymes of this system are methyl-dependent, specifically, both McrA and McrB3P cleave the 5-methylcytosine methylated form of DNA. The presence of this MDRE (Methyl-Dependent Restriction Enzyme) in *B. cereus B*25 means that this strain restricts methylated DNA (as when plasmid DNA is isolated from *E. coli* Top10). Consequently, its own DNA remains non-methylated, which can explain why we were unable to transform it.

To verify whether strain *B*25 DNA is non-methylated, we performed genomic DNA digestions with the restriction enzymes *Mbo*I, *Dpn*II (which are both blocked by *dam* methylation, *i.e.* adenine methylation) and *Eco*RI (which is not sensitive to methylation) and used methylated plasmid DNA digestion as a control. With this experiment we suggested that the *B*25 DNA was not methylated, since it was restricted by *Mbo*I and *Dpn*II, whereas the plasmid DNA was not. Furthermore, *Eco*RI was able to restrict both genomic and plasmid DNA, as expected (Fig. 1).

Since these results indicate that transformation of the *B*25 strain requires non-methylated plasmid DNA, we then isolated plasmid DNA from the *E. coli* strains BL21 (*dcm*$^-$), GM33 (*dam*$^-$) and GM2163 (*dam*$^-$, *dcm*$^-$) to determine if any transformants could be obtained under these circumstances. We expected to obtain transformants when using plasmid isolated from strains GM33 (*dam*$^-$) and GM2163 (*dam*$^-$, *dcm*$^-$) being that in both strains, adenines are not methylated and consistently, the *B*25 MDRE should not restrict the plasmid DNA. Nonetheless, transformants were only obtained with the plasmid from strain GM2163.

We then verified the methylation status of plasmids from all four *E. coli* strains tested. We found that plasmid DNA from strain Top10 was only cleaved by *Eco*R1 and both *Mbo*I and *Dpn*II were blocked as expected. Plasmid DNA from strain GM2163 (that is no adenine or cytosine methylated) was cleaved by the three restriction enzymes also as expected. Plasmid DNA from BL21 strain which is adenine-methylated was cleaved with *Eco*R1 but partially cleaved with *Mbo*I and *Dpn*II. Finally, plasmid DNA from strain GM33 was cleaved with *Eco*R1 but only partially cleaved with *Mbo*I and *Dpn*II (Supplemental Fig. 1). This

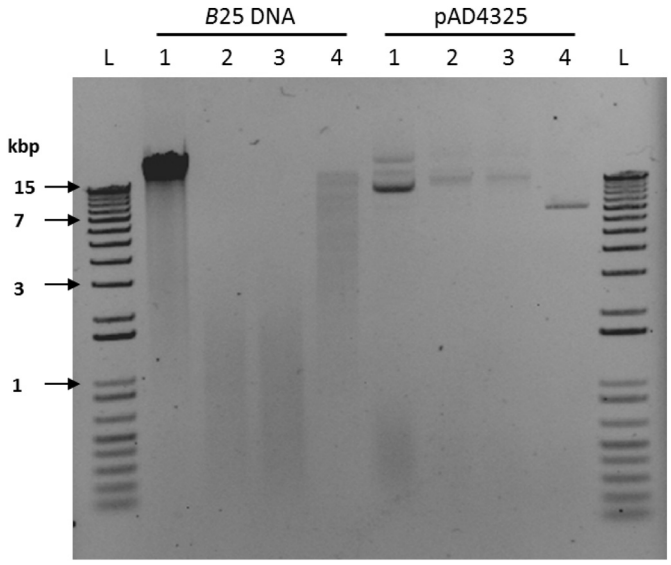

Fig. 1. DNA digestion of genomic DNA of strain *B*25 and pAD4325 plasmid isolated from *E. coli* Top10. Lanes: **L**, DNA Ladder 1 Kb plus (Invitrogen); **1**, control without enzyme; **2**, restriction with *Mbo*I; **3**, restriction with *Dpn*II; **4**, restriction with *Eco*RI. Restriction enzymes *Mbo*I and *Dpn*II are blocked by Dam methylation while *Eco*RI is not sensitive to methylation. This experiment shows that the *B*25 genomic DNA is not methylated since it is digested by *Mbo*I and *Dpn*II whereas the plasmid pAD4325 is not. pAD4325 length: 7.262 kbp. *Eco*RI sites in pAD4325: one. Kbp: kilobase pair. Lanes 1 correspond to 300 ng gDNA and 192 ng plasmid. Lanes 2, 3 and 4 correspond to 5 μL of each restriction reaction where plasmid and gDNA are diluted 10 times.

experiment shows that DNA of strain GM2163 is not methylated while DNA of strain Top10 is. Interestingly, DNA of strains BL21 and GM33 are somehow hemi-methylated since they were partially cleaved by *Mbo*I and *Dpn*II. Thus, we continued using this plasmid DNA to search for the best conditions for our electroporation method.

3.2. Transformation efficiency and the varying of electroporation buffers, voltage pulse and recovery medium

The aim of electroporation is to generate pores on the bacterial membrane so that exogenous molecules such as plasmid DNA can enter the cell (Prasanna and Panda, 1997). After reviewing the extensive literature, we selected six different reported electroporation buffers (EB) that vary in electrolyte composition and pH (*see Materials and Methods*), with three different voltage pulses (620, 1250 and 1800 kV/cm) and two recovery media (LB broth or SOC). *B. cereus B*25 was then electroporated with plasmid pAD4325, a low-copy number Gram-positive – *E. coli* shuttle vector that carries a constitutively expressed GFP. The transformation efficiencies for each condition are listed in Table 1. No transformants were recovered using the ice-cold protocol.

Table 1 shows that the best transformation efficiency ($4.50 \pm 0.25 \times 10^3$ CFU μg^{-1}) was obtained with the electroporation buffer PEB, a voltage pulse of 620 kV/cm, and the SOC bacterial culture recovery medium. This value was significantly different ($P \leq .05$) from the values for cells recovered in LB broth ($3.45 \pm 0.12 \times 10^3$ μg^{-1}), or cells that received a voltage pulse of 1250 kV/cm in either LB or SOC ($3.29 \pm 0.17 \times 10^3$ UFC μg^{-1}). In contrast, the sucrose and KS buffers yielded the lowest efficiencies. No transformants were recovered at 1800 kV/cm, except for when the PEB buffer was used with SOC. Overall, the transformation efficiencies were the highest when the voltage pulse was 620 kV/cm for all the EB except KS ($P \leq .05$).

After electroporation, several transformants were verified by plasmid extraction (data not shown) followed by fluorescent microscopy, in order to observe the GFP expression (Fig. 2, panels **a** and **b**). To verify plasmid maintenance, transformed *B. cereus B*25 were re-

Table 1
Transformation efficiencies of *Bacillus cereus* (as number of CFU μg^{-1}) with the non-methylated plasmid pAD4325. Each experiment was performed three times and SD is presented. NT: no transformants were obtained.

Buffer	Voltage (kV/cm)	Transformation efficiency	
		SOC	LB
HEB	620	$2.45 \pm 0.24 \times 10^3$	$2.15 \pm 0.22 \times 10^3$
	1250	$1.34 \pm 0.09 \times 10^3$	$1.20 \pm 0.11 \times 10^3$
	1800	NT	NT
SHMG	620	$2.13 \pm 0.15 \times 10^3$	$2.42 \pm 0.11 \times 10^3$
	1250	$1.85 \pm 0.17 \times 10^3$	$1.55 \pm 0.90 \times 10^3$
	1800	NT	NT
PEB	620	$4.50 \pm 0.25 \times 10^3$	$3.45 \pm 0.12 \times 10^3$
	1250	$3.29 \pm 0.17 \times 10^3$	$2.98 \pm 0.09 \times 10^3$
	1800	$1.09 \pm 0.17 \times 10^1$	NT
SA	620	$2.23 \pm 0.26 \times 10^2$	$2.25 \pm 0.01 \times 10^2$
	1250	$1.03 \pm 0.22 \times 10^2$	$1.30 \pm 0.01 \times 10^1$
	1800	NT	NT
ETM	620	$1.53 \pm 0.22 \times 10^3$	$1.24 \pm 0.11 \times 10^3$
	1250	$0.91 \pm 0.08 \times 10^3$	$0.97 \pm 0.06 \times 10^3$
	1800	NT	NT
KS	620	$1.30 \pm 0.10 \times 10^1$	$1.08 \pm 0.05 \times 10^1$
	1250	$1.15 \pm 0.08 \times 10^1$	$0.90 \pm 0.01 \times 10^1$
	1800	NT	NT

plated consecutively for five days, and again observed under fluorescence microscopy (Fig. 2, panels **c** and **d**). These observations indicate that strain *B*25 was indeed transformed with plasmid pAD4325 and was able to maintain the plasmid in subsequent generations.

Finally, we tested the best conditions found (buffer PEB, voltage pulse of 620 kV/cm, and SOC as recovery medium) with three other strains of rhizospheric *Bacillus: B. cereus B*14, *B. subtilis B*6 and *B. thuringiensis B*29 with transformation efficiencies of $1.35 \pm 0.10 \times 10^3$ CFU μg^{-1}, $1.79 \pm 0.12 \times 10^3$ CFU μg^{-1} and $1.04 \pm 0.08 \times 10^3$ CFU μg^{-1} respectively.

4. Discussion

The genetic manipulation of a bacterium requires the ability to transform it. Electroporation is an easy and efficient transformation technique that can facilitate the entry of exogenous molecules such as plasmid DNA by creating pores on the bacterial membrane *via* electrical shock (Prasanna and Panda, 1997). This methodology has been widely and successfully used in Gram-negative bacteria, however its application with Gram-positive microorganisms requires some adaptations, due to differences in membrane composition (Moran et al., 2017; Piggot et al., 2011).

*Bacillus cereus B*25, a Gram-positive maize rhizospheric bacterium, has been shown to act as a biocontrol agent against fungal infections (Lizárraga-Sánchez et al., 2015). One recent report proposes that it exerts its action against phytopathogenic fungi through the production of chitinases, although this remains to be confirmed (Figueroa-López et al., 2017). Therefore, the ability to transform the *B*25 bacterium by electroporation is a prerequisite for manipulating its genome. However, as various reports in the literature point out, environmental strains such as this one, are not as easy to transform as type- or lab-cultivated strains.

We did an extensive search for the best electroporation conditions for this strain that exposed a wide variety of possible electroporation techniques. Therefore, we decided to examine the most representative approaches. In addition, we improved the reported technique by raising the temperature at which the electrocompetent cells are made (usually an ice-cold temperature). Importantly, Tu et al. (2016) demonstrated that the ice-cold temperatures affect the fluidity and permeability of the bacterial membrane, which makes it more difficult for them to survive, thus decreasing the transformation efficiency.

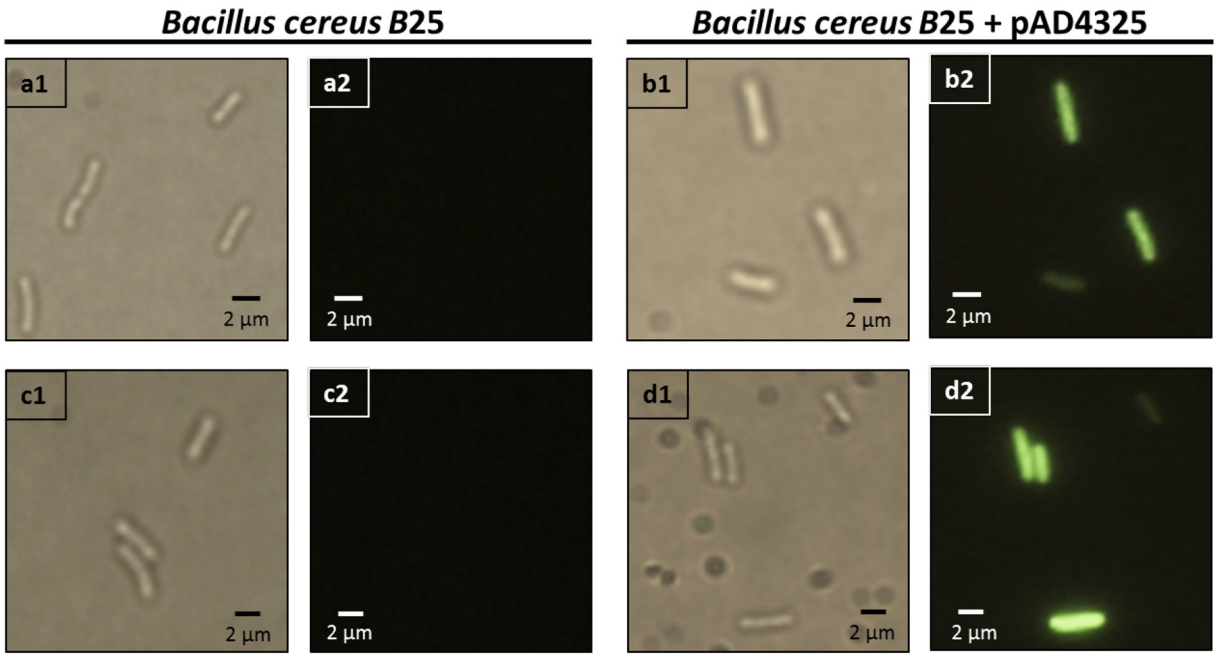

Fig. 2. Brightfield (panels numbered with a **1**) and fluorescence (panels numbered with a **2**) imaging of *B. cereus B*25 and *B. cereus B*25 transformed with plasmid pAD4325. Cells were observed one day after electroporation (**upper** row) and after five consecutive re-plates (**lower** row). The imaging reveals that plasmid pAD4325 constitutively expresses GFP.

Since our initial attempts to transform *B. cereus B*25 strain were unsuccessful no matter the electroporation buffer used, we hypothesized that this strain might require a specific methylation status of DNA for an efficient transformation. One way to investigate this is through restriction-modification (RM) systems, which are defense mechanisms used by bacteria (and archaea) against viral infections. These systems consist of two proteins: a restriction endonuclease (RE) that recognizes and cleaves specific DNA sequences, and a methyltransferase (MT) that transfers methyl groups to the bacterial DNA (specifically, to adenines by Dam MT and to cytosines by Dcm MT) in order to distinguish it from foreign (*i.e.* viral) DNA. RM systems are classified into four groups based on their mechanism of action, DNA sequence recognition and substrate specificity. Types I, II and III RM systems cleave non-methylated DNA, whereas type IV restriction enzymes are methyl-dependent (MDRE) (Marinus and Lobner-Olesen, 2014; Vasu and Nagaraja, 2013).

The MDRE system has been primarily studied in *E. coli*, where four enzymes have been found: McrA (which cleaves cytosine-methylated DNA), Mrr (which cleaves adenine and cytosine-methylated DNA), and McrB/McrC (which cleaves cytosine-methylated DNA) (Tock and Dryden, 2005). Interestingly, *B. anthracis* encodes for type IV MDRE: Mrr, McrBP, McrB2P and McrB3P. It should be noted here that the effective transformation of *B. anthracis* requires non-methylated plasmid DNA (Sitaraman and Leppla, 2012).

We found a McrA homologue identical to that of *B. cereus* ATCC 14579 and a protein with 80% identity to *B. anthracis* McrB3P whose presence means that this strain required not methylated DNA for transformation. This hypothesis was corroborated with genomic DNA digestions that showed that the *B*25 DNA was not methylated (Fig. 1) and thus, requires not methylated plasmid DNA to be transformed.

We expected to be able to transform the *B*25 strain with plasmid DNA isolated from the *E. coli* strain GM33 (dam^-) but our attempts were unsuccessful. We digested this plasmid DNA with *Mbo*I and *Dpn*II (Dam sensitive) restriction enzymes and we observed that the DNA was partially digested (Supplemental Fig. 1), meaning that this DNA is somehow still partially methylated. Previous work has demonstrated that *dam* deficient mutants retain residual DNA adenine-methylation (Marinus and Lobner-Olesen, 2014), and this phenomena could explain our results. We obtained transformants when we used plasmid DNA isolated from strain GM2163 (dam^-, dcm^-) confirming our results obtained with the restriction enzymes.

In our experiment, the PEB buffer resulted in the best performance. No transformants were recovered from the conditions using 1800 kV/cm electric pulses, which could be due to the formation of a non-viable number of pores leading to membrane disruption (Kotnik et al., 2015; Tieleman, 2004).

The pore formation depends on the electric strength applied to the membrane and is affected by the electric conductivity, osmolarity and pH of the solution in which the bacteria are electroporated, as well as the bacterial membrane properties (Moran et al., 2017). In addition to pore formation, transformation efficiency also depends on DNA concentration, rapid pore resealing, and the fitness recovery of the cells (Kotnik et al., 2015). While most of these parameters can be empirically determined, several reports that have studied the optimal conditions listed above provide an approximate idea of the best conditions needed for electroporation.

Gram-positive bacteria are difficult to transform due to their membrane traits, which could explain the variety in available electroporation methodologies (Moran et al., 2017; Rauch and Leigh, 2015).

As for the buffers, the sole difference between PEB and HEB was the buffering agent (phosphate and HEPES, respectively). HEPES is a zwitterionic buffer and does not contribute to the ionic strength of the solution, whereas phosphate does contribute to the ionic strength and could help the current flow into the cell and promote cell viability (Kotnik et al., 2015). In conclusion, this report demonstrates that the efficient transformation of *B. cereus B*25 requires non-methylated DNA, and that the protocol reported here can be applied to obtain transformants from other *Bacillus* species. Furthermore, room-temperature preparation of competent cells could improve the transformation of other undomesticated strains, thereby enabling their molecular analysis and genetic manipulation.

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References

Aukrust, T.W., Brurberg, M.B., Nes, I.F., 1995. Transformation of *Lactobacillus* by electroporation. Methods Mol. Biol. 47, 201–208. https://doi.org/10.1385/0-89603-310-4:201.

Bazinet, A.L., 2017. Pan-genome and phylogeny of *Bacillus cereus sensu lato*. BMC Evol. Biol. 17, 1–16. https://doi.org/10.1186/s12862-017-1020-1.

Douriet-Gámez, N.R., Maldonado-Mendoza, I.E., Ibarra-Laclette, E., Blom, J., Calderón-Vázquez, C.L., 2017. Genomic analysis of *Bacillus sp*. strain *B*25, a biocontrol agent of maize pathogen *Fusarium verticillioides*. Curr. Microbiol. 0, 1–9. https://doi.org/10.1007/s00284-017-1372-1.

Figueroa-López, A.M., Cordero-Ramírez, J.D., Martínez-Álvarez, J.C., López-Meyer, M., Lizárraga-Sánchez, G.J., Félix-Gastélum, R., Castro-Martínez, C., Maldonado-Mendoza, I.E., 2016. Rhizospheric bacteria of maize with potential for biocontrol of *Fusarium verticillioides*. Springerplus 5, 330. https://doi.org/10.1186/s40064-016-1780-x.

Figueroa-López, A.M., Leyva-Madrigal, K.Y., Cervantes-Gámez, R.G., Beltrán-Arredondo, L.I., Douriet-Gámez, N.R., Castro-Martínez, C., Maldonado-Mendoza, I.E., 2017. Induction of *Bacillus cereus* chitinases as a response to lysates of *Fusarium verticillioides*. Rom. Biotechnol. Lett. 22, 12722–12731.

Groot, M.N., Nieboer, F., Abee, T., 2008. Enhanced transformation efficiency of recalcitrant *Bacillus cereus* and *Bacillus weihenstephanensis* isolates upon in vitro methylation of plasmid DNA. Appl. Environ. Microbiol. 74, 7817–7820. https://doi.org/10.1128/AEM.01932-08.

Jirásková, A., Vítek, L., Fevery, J., Ruml, T., Branny, P., 2005. Rapid protocol for electroporation of *Clostridium perfringens*. J. Microbiol. Methods 62, 125–127. https://doi.org/10.1016/j.mimet.2005.01.012.

Kotnik, T., Frey, W., Sack, M., Haberl Meglič, S., Peterka, M., Miklavčič, D., 2015. Electroporation-based applications in biotechnology. Trends Biotechnol. 33, 480–488. https://doi.org/10.1016/j.tibtech.2015.06.002.

Lizárraga-Sánchez, G.J., Leyva-Madrigal, K.Y., Sánchez-Peña, P., Quiroz-Figueroa, F.R., Maldonado-Mendoza, I.E., 2015. Bacillus cereus sensu lato strain B25 controls maize stalk and ear rot in Sinaloa, Mexico. F. Crop. Res. 176, 11–21. https://doi.org/10.1016/j.fcr.2015.02.015.

Luchansky, J.B., Muriana, P.M., Klaenhammer, T.R., 1988. Application of electroporation for transfer of plasmid DNA to *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Listeria*, *Pediococcus*, *Bacillus*, *Staphylococcus*, *Enterococcus* and *Propionibacterium*. Mol. Microbiol. 2, 637–646. https://doi.org/10.1111/j.1365-2958.1988.tb00072.x.

Macaluso, A., Mettus, A., 1991. Efficient transformation of *Bacillus thuringiensis* requires nonmethylated plasmid DNA. J. Bacteriol. 173, 1353–1356.

Marinus, M.G., Lobner-Olesen, A., 2014. DNA Methylation. EcoSal Plus 6, 1–62. https://doi.org/10.1128/ecosalplus.ESP-0003-2013.DNA.

Marrero, R., Welkos, S.L., 1995. The transformation frequency of plasmids into *Bacillus anthracis* is affected by adenine methylation. Gene 152, 75–78. https://doi.org/10.1016/0378-1119(94)00647-B.

Masson, L., Prefontaine, G., Brousseau, R., 1989. Transformation of *Bacillus thuringiensis* vegetative cells by electroporation. FEMS Microbiol. Lett. 60, 273–277. https://doi.org/10.1016/0378-1097(89)90409-6.

Moran, J.L., Dingari, N.N., Garcia, P.A., Buie, C.R., 2017. Numerical study of the effect of soft layer properties on bacterial electroporation. Bioelectrochemistry. https://doi.org/10.1016/j.bioelechem.2017.09.004.

Piggot, T.J., Holdbrook, D.A., Khalid, S., 2011. Electroporation of the *E. coli* and *S. aureus* membranes: molecular dynamics simulations of complex bacterial membranes. J. Phys. Chem. B 115, 13381–13388. https://doi.org/10.1021/jp207013v.

Prasanna, G.L., Panda, T., 1997. Electroporation: basic principles, practical considerations and applications in molecular biology. Bioprocess Eng. 16, 261–264. https://doi.org/10.1007/s004490050319.

Rasko, D.A., Altherr, M.R., Han, C.S., Ravel, J., 2005. Genomics of the *Bacillus cereus* group of organisms. FEMS Microbiol. Rev. 29, 303–329. https://doi.org/10.1016/j.femsre.2004.12.005.

Rauch, C., Leigh, J., 2015. Theoretical evaluation of wall teichoic acids in the cavitation-mediated pores formation in gram-positive bacteria subjected to an electric field. Biochim. Biophys. Acta, Gen. Subj. 1850, 595–601. https://doi.org/10.1016/j.bbagen.2014.12.004.

Schurter, W., Geiser, M., Mathé, D., 1989. Efficient transformation of *Bacillus thuringiensis* and *B. cereus* via electroporation: transformation of acrystalliferous strains with a cloned delta-endotoxin gene. MGG Mol. Gen. Genet. 218, 177–181. https://doi.org/10.1007/BF00330581.

Shatalin, K.Y., Neyfakh, A.A., 2005. Efficient gene inactivation in *Bacillus anthracis*. FEMS Microbiol. Lett. 245, 315–319. https://doi.org/10.1016/j.femsle.2005.03.029.

Sitaraman, R., Leppla, S.H., 2012. Methylation-dependent DNA restriction in *Bacillus anthracis*. Gene 494, 44–50. https://doi.org/10.1016/j.gene.2011.11.061.

Tieleman, D.P., 2004. The molecular basis of electroporation. BMC Biochem. 5, 1–12. https://doi.org/10.1186/1471-2091-5-10.

Tock, M.R., Dryden, D.T.F., 2005. The biology of restriction and anti-restriction. Curr. Opin. Microbiol. 8, 466–472. https://doi.org/10.1016/j.mib.2005.06.003.

Tu, Q., Yin, J., Fu, J., Herrmann, J., Li, Y., Yin, Y., Stewart, A.F., Müller, R., Zhang, Y., 2016. Room temperature electrocompetent bacterial cells improve DNA transformation and recombineering efficiency. Sci. Rep. 6, 1–8. https://doi.org/10.1038/srep24648.

Turgeon, N., Laflamme, C., Ho, J., Duchaine, C., 2006. Elaboration of an electroporation protocol for *Bacillus cereus* ATCC 14579. J. Microbiol. Methods 67, 543–548. https://doi.org/10.1016/j.mimet.2006.05.005.

Vasu, K., Nagaraja, V., 2013. Diverse functions of restriction-modification Systems in Addition to cellular Defense. Microbiol. Mol. Biol. Rev. 77, 53–72. https://doi.org/10.1128/MMBR.00044-12.

Xue, G.P., Johnson, J.S., Dalrymple, B.P., 1999. High osmolarity improves the electrotransformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus licheniformis*. J. Microbiol. Methods 34, 183–191. https://doi.org/10.1016/S0167-7012(98)00087-6.

Zhang, G.Q., Bao, P., Zhang, Y., Deng, A.H., Chen, N., Wen, T.Y., 2011. Enhancing electrotransformation competency of recalcitrant *Bacillus amyloliquefaciens* by combining cell-wall weakening and cell-membrane fluidity disturbing. Anal. Biochem. 409, 130–137. https://doi.org/10.1016/j.ab.2010.10.013.