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Multiple pulse electroporation of lactic acid bacteria *Lactococcus lactis* and *Lactobacillus casei*

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ABSTRACT

Genetic manipulation of lactic acid bacteria is often difficult due to the inability to transform them with high efficiency. Multi-pulse electroporation offers a simple approach to increase transformation efficiencies. Using cells grown with 1% glycine and pretreated with lithium acetate and dithiothreitol, multi-pulse electroporation (five pulses of 12.5 kV cm^{-1}) of *Lactococcus lactis* JB704 cells resulted in a transformation efficiency of up to 1.2×10^6 colony forming units (CFU) μg^{-1} pGK13, an 8-fold increase in the transformation efficiency compared to single pulse electroporation. Other cell growth and pretreatment conditions with JB704 resulted in lower transformation efficiencies but had 4-fold to 27-fold higher transformation efficiencies with the five pulse electroporations. With similarly grown and pretreated *Lactobacillus casei* 32G cells, multi-pulse electroporation (five pulses of 7.5 kV cm^{-1}) resulted in a mean transformation efficiency of 7.3×10^3 CFU μg^{-1} pTRKH2, a 4-fold increase in the transformation efficiency compared to single pulse electroporation.

1. Introduction

Lactic acid bacteria (LAB) have numerous health and industrial uses, particularly in the food industry. Understanding the roles of genes in the physiology and biochemistry of these bacteria is needed to identify strains with beneficial properties. Genetic manipulation of LAB is often stymied by the inability to transform the cells with high efficiency. The typical method for transferring genetic constructs into LAB is by electroporation (Chassy and Flickinger, 1987; Luchansky et al., 1988) and many studies have focused on techniques that can improve transformation efficiency following electroporation. Among other approaches, these methods include modifying the growth medium by addition of glycine or sodium chloride (Holo and Nes, 1989; Palomino et al., 2010) or pretreatment of the cells with a lithium acetate and dithiothreitol mixture prior to electroporation (Papagianni et al., 2007; Welker et al., 2015) which alter the cell membrane or cell wall enhancing the ability of the transforming DNA to enter cells.

Recent reports with other bacteria have shown that multi-pulse electroporation increases the transformation efficiency, compared to that obtained with a single pulse electroporation. Multi-pulse electroporation of *Agrobacterium tumefaciens* strains improved their transformation by 2.5-fold to 5-fold compared to single pulse electroporation (Mahmood et al., 2008). Sato'o and coworkers using an optimized multi-pulse electroporation protocol for *Staphylococcus epidermidis* JMUB20 obtained 1.38×10^3 colony forming units (CFU) μg^{-1} but only 4.15×10^2 CFU μg^{-1} using an optimized single pulse electroporation; a 3.3-fold increase (Sato'o et al., 2018). With *Staphylococcus aureus* multi-pulse electroporation resulted in a 14-fold increase in the transformation efficiency over that seen with a single pulse and brought the transformation efficiency to 3.9×10^5 CFU μg^{-1} (Hisatsune et al., 2016). The latter two studies used the ELEPO21 electroporator (Nepa Gene Co., Ltd) for multi-pulse electroporation with a single poring pulse (16–18 kV cm^{-1}) and five transfer pulses (0.5–1.0 kV cm^{-1}) but the study with *A. tumefaciens* used five pulses at 20 kV cm^{-1} produced with a Cell-Porator® (Life Technologies).

Here we investigate whether multi-pulse electroporation can increase transformation efficiencies of two widely used LAB species, *Lactococcus lactis* and *Lactobacillus casei*.

2. Materials and methods

2.1. Bacterial strains and growth

For preparation of recipient cells of *Lactococcus lactis* strain JB704, M17G medium (100–350 mL) was inoculated using cells stored as

Abbreviations: CFU, colony forming unit; DTT, dithiothreitol; LAB, lactic acid bacteria; lithium acetate, LiAc; MRS, De Man-Rogosa-Sharp medium; PEG, polyethylene glycol 8000

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−80 °C stocks with 15% glycerol. The cultures were grown at 30 °C without shaking either without glycine or with 1% glycine to 4–6 × 10^8 CFU mL^{-1} (OD_{600} 0.8–1.0). The cells were recovered from the growth medium by centrifugation (6000*g* for 10 min in a Sorvall GSA rotor), washed once in 100–200 mL sterile water (6000 g for 10 min in a Sorvall GSA rotor), and then washed three times using sterile water in a microcentrifuge by centrifugation at 15000 *g* for 2 min (a 1.7 mL tube with 1 mL water for the cells from 100 mL of the initial culture). The cells were washed once in 30% polyethylene glycol 8000 (PEG) at 15000 g (1 mL per tube), suspended in 30% PEG, and stored frozen at −80 °C in 100–200 μL aliquots. Each 100 μL of frozen cell suspension contained the cells from about 10 mL of the original recipient cell cultures without glycine or with 1% glycine for experiments 1 and 2 and from independently prepared recipient cell cultures without glycine or with 1% glycine for experiment 3.

Recipient cells of *Lactobacillus casei* strain 32G (Broadbent et al., 2012; Welker et al., 2015) were prepared similarly, except that they were grown at 37 °C in De Man-Rogosa-Sharp medium (MRS) with 1% glycine to about 2 × 10^9 CFU mL^{-1} (also OD_{600} 0.8–1.0) and then washed and stored at −80 °C in 30% PEG (Welker et al., 2015). All 32G cells used came from a single recipient cell culture.

2.2. Transformation

A Bio-Rad Gene Pulser (Model 1,652,076) with 0.2 cm cuvettes was used throughout this work with resistance set at 200 Ohms and capacitance set at 25 μF. For JB704 the voltage was set at 2500 Volts corresponding to 12.5 kV cm^{-1}. For 32G, voltage settings were 1500, 2000 or 2500 Volts (7.5, 10 or 12.5 kV cm^{-1}). Multiple pulses were obtained by repeated manual discharge of the machine. Time intervals between pulses were as rapid as could be manually accomplished, about five seconds. Time constants ranged between 4.7 and 3.4, typically 4.6 to 4.4 for both strains. No arcing occurred in this work.

For JB704, recipient cells were thawed, diluted ½ with 30% PEG, and then received: 1, no additional pretreatment (addition of vector DNA, followed by transfer to the cuvettes and electroporation as quickly as possible); 2, a 30 min pretreatment at room temperature using sterile water (150 μL per 100 μL cell suspension); or 3, a 30 min pretreatment at room temperature with lithium acetate and dithiothreitol (LiAc and DTT) (125 μL of a solution containing 200 mM LiAc, 1.2 M sucrose, 20 mM Tris, pH 7.5 and 25 μL of 100 mM DTT per 100 μL cell suspension) (Welker et al., 2015). After the pretreatment with water or LiAc and DTT solution the cells were centrifuged in a microcentrifuge at 15,000*g* for 2 min, the supernatant removed, the cells suspended in 1 mL 30% PEG, centrifuged again at 15,000*g*, the supernatant removed, and the cells suspended in 30% PEG for electroporation. These pretreatments followed the protocol used previously for electroporation of *L. casei* cells (Welker et al., 2015). Each electroporation in experiments 1 and 2 used cells from about 5 mL from the same two recipient cell cultures (one without and one with 1% glycine) suspended in 100 μL of 30% PEG. Each electroporation in experiment 3 used the cells from two additional recipient cell cultures (one without and one with 1% glycine). Within an experiment, cells that received the same pretreatment were prepared in a single tube and then split into smaller aliquots prior to addition of vector DNA. Ten μL of a pGK13 (Kok et al., 1984) DNA solution containing a total of 200 ng vector DNA was added to the cells for each transformation and the mixtures independently transferred to the cuvettes for electroporation. After electroporation the cells were recovered from each of the cuvettes using 0.9 mL M17G Recovery Medium (M17G broth with 0.5 M sucrose), incubated at 30 °C for 4 h, and then inoculated after appropriate dilutions to M17G agar plates with or without 1 μg mL^{-1} erythromycin for incubation at 30 °C. Three plates at each dilution were used per transformation. After colonies appeared, CFUs were tabulated, and the mean of the CFU counts from the three plates calculated to determine the transformation efficiency (CFU μg^{-1} vector DNA) and viable cell numbers for each transformation. Fold differences in transformation efficiencies within each experiment were determined for each growth and pretreatment condition by dividing the mean transformation efficiency obtained after five pulses with that obtained after one pulse. The means of the results for each growth and pretreatment condition from the three experiments were then calculated to determine the fold differences, mean transformation efficiencies, and mean viabilities that we report.

The 32G recipient cells were treated with LiAc and DTT and prepared for electroporation as described above, except that each 100 μL of cell suspension for electroporation contained the cells from 1.7 mL of the recipient cell culture. All cells within each experiment were pretreated for 30 min with LiAc and DTT together in a single tube and then washed and split into the six aliquots needed for the experiment. Ten μL of a pTRKH2 (MG417047; O'Sullivan and Klaenhammer, 1993) DNA solution containing a total of 200 ng vector DNA was added to each of the six aliquots and the mixtures transferred to the cuvettes for electroporation. All recipient cells used in the three experiments came from a single cell culture. After electroporation the cells were recovered from the cuvettes using 0.9 mL MRS Recovery Medium (MRS broth with 0.5 M sucrose), incubated at 37 °C for 4 h, and then inoculated at appropriate dilutions to MRS agar plates with or without 2.5 μg mL^{-1} erythromycin for incubation at 37 °C. Three plates at each dilution were used per transformation. After colonies appeared, CFUs were tabulated, and the mean of the counts from the three plates calculated to determine the transformation efficiency (CFU μg^{-1} vector DNA) and viable cell number for each transformation. Fold differences in transformation efficiencies within each experiment were determined by dividing the result obtained after five pulses with that obtained after one pulse. Mean fold differences, transformation efficiencies, and viable cell numbers were determined from the results obtained from the three experiments. This protocol follows that used previously for transformation of 32G cells (Welker et al., 2015) except that each transformation used 1/6 the number of cells in the earlier work.

3. Results

3.1. Transformation of Lactococcus lactis JB704

Six different treatments of JB704 recipient cells were tested. Cells were grown either without or with 1% glycine, thawed after storage at −80 °C, and then electroporated without further pretreatment, or after pretreatment for 30 min with sterile water, or after pretreatment for 30 min with a LiAc and DTT solution. The transformation efficiencies ranged from about 10^3 to 10^6 among the different treatments. In almost every case the transformation efficiencies obtained were higher after five pulses than after one pulse (Figs. 1 and 2). The sole exception was the second of three trials using cells grown with 1% glycine and which did not have a pretreatment with water or LiAc and DTT solution (Fig. 2.); in this case, the transformation efficiencies were similar for 1 and 5 pulses.

With cells grown without glycine, increases in the transformation efficiencies for the 5 pulse 12.5 kV cm^{-1} electroporations compared to the 1 pulse electroporations were seen with all three pretreatments (Fig. 1). The mean increase for cells that did not receive either a water or LiAc and DTT pretreatment was 27-fold, with water pretreatment it was 25-fold and with LiAc and DTT pretreatment it was 4-fold. The smaller increase seen with the cells pretreated with LiAc and DTT reflects the higher transformation efficiencies obtained with the single pulse electroporations after this pretreatment (Fig. 1). The highest individual transformation efficiency obtained with the cells grown without glycine was 4.8 × 10^5 CFU μg^{-1}, which was seen in the third experiment with five pulses after pretreatment with water. The highest mean transformation efficiency was obtained with the five pulse electroporation using cells pretreated with LiAC and DTT (Fig. 1).

The results with cells grown with 1% glycine were similar to those seen with the cells grown without glycine (Fig. 2). The mean increase in the transformation efficiency after five pulse electroporations compared to single pulse electroporations for cells grown with 1% glycine that did

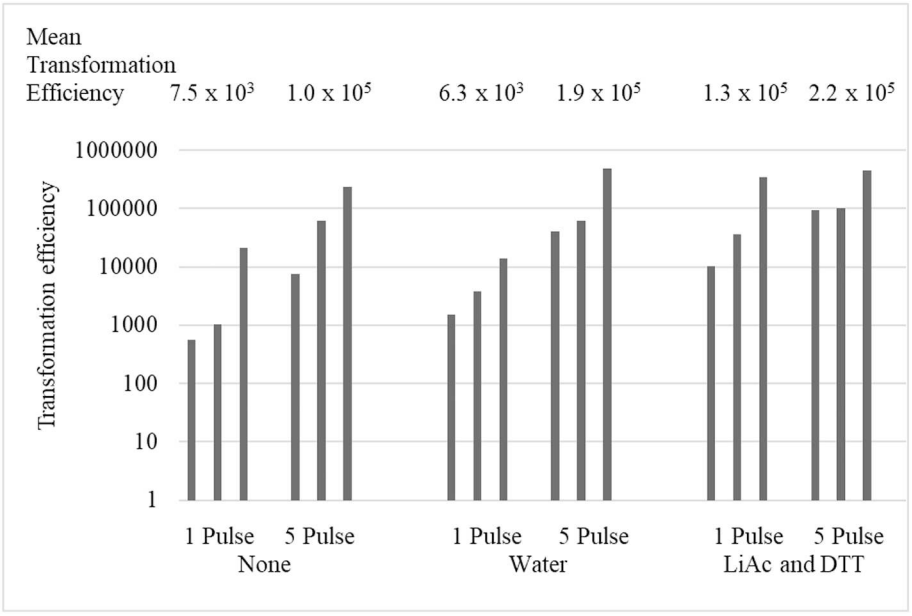

Fig. 1. Transformation efficiencies for *L. lactis* JB704 after 1 or 5 pulses given as CFU μg^{-1} pGK13. Each bar represents the data from a single electroporation. Data from three experiments are shown in order, left to right. Cells were grown in M17G without glycine and received either no pretreatment with water or LiAc and DTT (None), a 30 min pretreatment with water (Water), or a 30 min pretreatment with a LiAc and DTT solution (LiAc and DTT). Mean transformation efficiencies are given as CFU μg^{-1} pGK13.

not receive either a water or a LiAc and DTT pretreatment was 7-fold, after water pretreatment it was 14-fold, and after LiAc and DTT pretreatment it was 8-fold. The highest transformation efficiency seen with these cells was 1.2×10^6 CFU μg^{-1} with cells pretreated with LiAc and DTT and given five pulses in the second of the three experiments. The highest mean transformation efficiency was obtained with the five pulse electroporation using cells pretreated with LiAC and DTT (Fig. 2).

One concern with multiple pulse electroporations is whether the additional electroporations decrease cell viability. With JB704, the mean viability remained high and was similar within treatments between single pulse and five pulse electroporations (Fig. 3). The lowest mean viability was seen with cells grown with 1% glycine and then exposed to five electroporation pulses after a LiAC and DTT pretreatment.

3.2. Transformation of Lactobacillus casei 32G

Three different treatments of 32G recipient cells were tested in which cells were electroporated with pTRKH2 DNA at 1500, 2000 or 2500 Volts (7.5, 10.0, or 12.5 kV cm^{-1}) (Fig. 4). All cells were grown with 1% glycine and then pretreated for 30 min with a LiAc and DTT solution prior to electroporation. The growth condition and pretreatment were chosen since it yielded a high transformation efficiency for 32G in earlier work (Welker et al., 2015). At 1500 V, five pulse electroporation resulted in a 4-fold increase in the transformation efficiency over that seen with single pulse electroporation. At 1500 V the mean transformation efficiency obtained after five pulses (7.3×10^3 CFU μg^{-1}) was also 4-fold higher than after one pulse (1.8×10^3 CFU μg^{-1}). However, the mean transformation efficiencies at 2000 V were similar with five pulses or one pulse (6.8×10^3 CFU μg^{-1} and 6.7×10^3 CFU μg^{-1}, respectively) and at 2500 V the mean transformation efficiency after five pulses was lower than that with one pulse (2.6×10^3 CFU μg^{-1} and 7.0×10^3 CFU μg^{-1}, respectively).

With the 32G cells, viability was affected by increasing voltage (Fig. 5). Even with a single pulse the viability of the cells decreased as voltage increased; the mean viability after a single pulse at 2500 V was 2/3 of that at 1500 V. The mean viabilities at all three voltages with five pulses were lower than the mean viabilities at those voltages with a

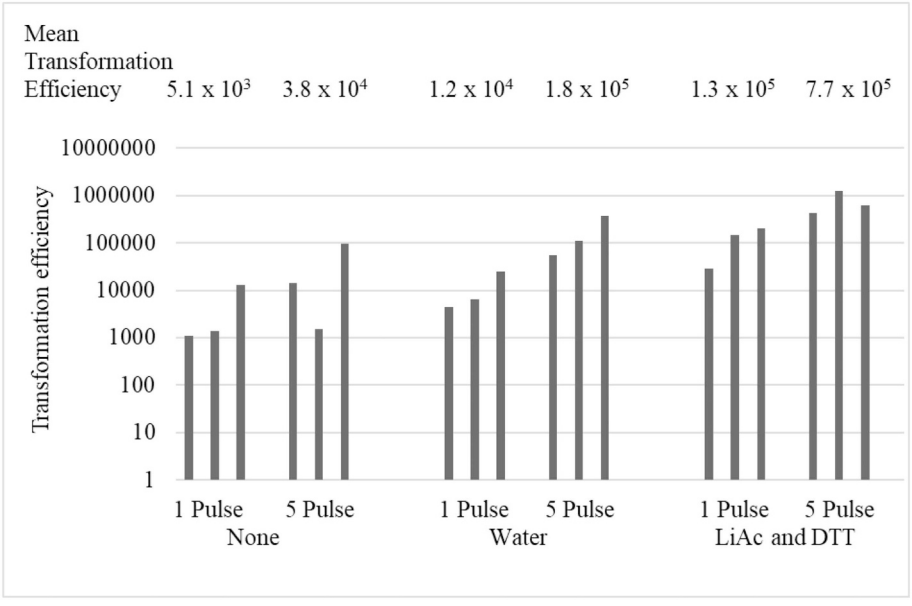

Fig. 2. Transformation efficiencies for *L. lactis* JB704 after 1 or 5 pulses given as CFU μg^{-1} pGK13. Each bar represents the data from a single electroporation. Data from three experiments are shown in order, left to right. Cells were grown in M17G with 1% glycine and received either no pretreatment with water or LiAc and DTT (None), a 30 min pretreatment with water (Water), or a 30 min pretreatment with a LiAc and DTT solution (LiAc and DTT). Mean transformation efficiencies are given as CFU μg^{-1} pGK13.

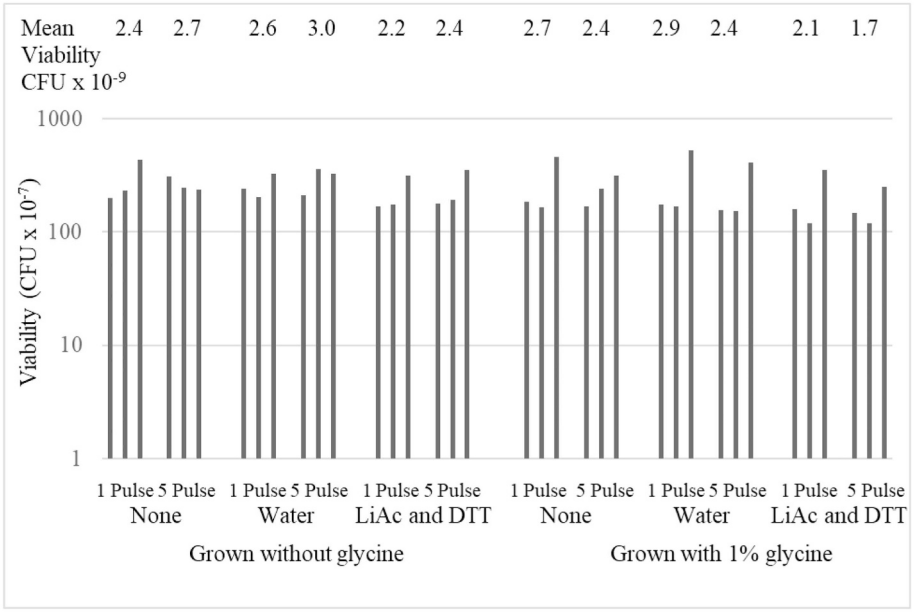

Fig. 3. Viability for *L. lactis* JB704 after 1 or 5 pulses given as CFU x 10^{-7}. Each bar represents the data from a single electroporation. Data from three experiments are shown in order, left to right. Cells were grown in M17G without or with 1% glycine and received either no pretreatment with water or LiAc and DTT (None), a 30 min pretreatment with water (Water), or a 30 min pretreatment with a LiAc and DTT solution (LiAc and DTT). Mean viabilities are given as CFU x 10^{-9}.

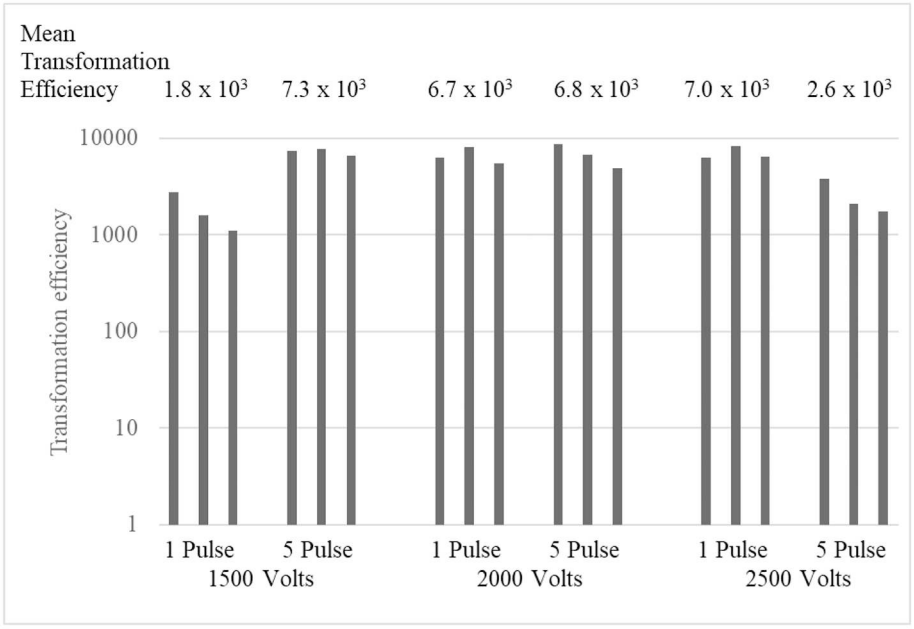

Fig. 4. Transformation efficiencies for *L. casei* 32G given as CFU μg^{-1} pTRKH2. Each bar represents the data from a single electroporation. Data from three experiments are shown in order, left to right. Cells were grown in MRS with 1% glycine and received a 30 min pretreatment with a LiAc and DTT solution and then electroporated with 1 or 5 pulses at 1500 V, 2000 V or 2500 V (7.5, 10.0 or 12.5 kV cm^{-1}, respectively). Mean transformation efficiencies are given as CFU μg^{-1} pTRKH2.

single pulse. The viability at 2500 V with five pulses was only 0.25 of that at 1500 V with a single pulse. The decrease in viability explains the loss of transformation efficiency observed with five pulses at 2500 V. At 2500 V, the number of transformants per viable cell were similar for the single pulse and five pulse electroporations, but the lower number of viable cells after five pulses resulted in a lower transformation efficiency.

4. Discussion

In this work, we showed that multi-pulse electroporations enhanced the transformation efficiencies obtained with two different lactic acid bacteria *L. lactis* JB704 and *L. casei* 32G. These experiments made use of equipment that is widely available and therefore can be tried at low cost in other laboratories with other species and strains of bacteria.

The increases in transformation efficiency after multi-pulse electroporation are similar in magnitude to those seen with other bacteria. With other bacteria, transformation efficiencies were increased by 2.5-fold to 14-fold using multi-pulse electroporations (Mahmood et al., 2008; Hisatsune et al., 2016; Sato'o et al., 2018). The mean transformation efficiency of *L. casei* 32G cells using five 7.5 kV cm^{-1} pulses (7.3×10^3 CFU μg^{-1}) was 4-fold higher than with a single 7.5 kV cm^{-1} pulse. Transformation efficiencies up to 1.2×10^6 CFU μg^{-1} were obtained with *L. lactis* JB704 cells grown with 1% glycine, pretreated prior to electroporation with a LiAc and DTT solution, and then given five 12.5 kV cm^{-1} pulses. JB704 cells treated this way showed an 8-fold increase in transformation efficiency compared to similarly prepared cells given a single pulse electroporation. Increases in the transformation efficiency after multi-pulse electroporation were also seen with the other five treatments of JB704, but they resulted in lower overall transformation efficiencies. These increases were 27-fold (no glycine, no pretreatment), 25-fold (no glycine, water pretreatment), 4-fold (no glycine, LiAc and DTT pretreatment), 7-fold (1% glycine, no pretreatment) and 14-fold (1% glycine, water pretreatment) compared to similarly treated cells that were exposed to a single pulse electroporation.

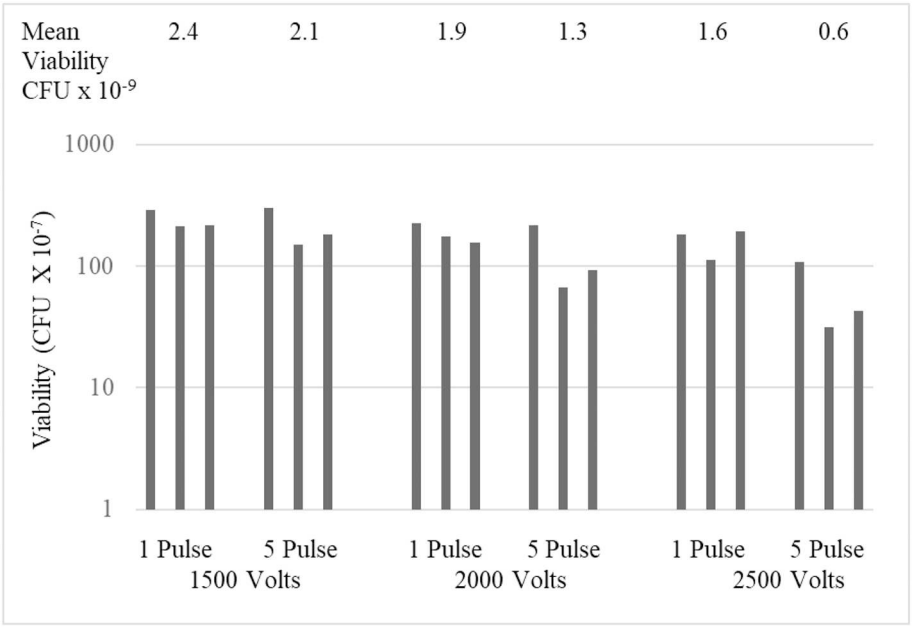

Fig. 5. Viability for *L. casei* 32G after 1 or 5 pulses given as CFU x 10^{-7}. Each bar represents the data from a single electroporation. Data from three experiments are shown in order, left to right. Cells were grown in MRS with 1% glycine and received a 30 min pretreatment with a LiAc and DTT solution and then electroporated with 1 or 5 pulses at 1500 V, 2000 V or 2500 V (7.5, 10.0 or 12.5 kV cm^{-1}, respectively). Mean viabilities are given as CFU x 10^{-9}.

Previous work showed that pretreatment of *L. lactis* spp. *lactis* and *L. casei* strains with a LiAc and DTT solution increased transformation efficiencies after single pulse electroporations (Papagianni et al., 2007; Welker et al., 2015). The results obtained with JB704 consistently showed higher mean transformation efficiencies following the LiAc and DTT pretreatment than following either of the other two pretreatments. This was seen with both the single and the multi-pulse electroporations.

Even though the mean transformation efficiency obtained with JB704 cells grown with 1% glycine, pretreated with LiAc and DTT, and given a multi-pulse electroporation was the highest, it may have been even higher except that cell viability for this treatment was the lowest observed in these experiments. The results with 32G also show that cell viability is an important factor contributing to the transformation efficiencies obtained with single and multi-pulse electroporations.

Author contributions

DLW designed this project, performed experiments and wrote the manuscript. BMC and JHM assisted with the experiments and data analysis. JRB managed the project and helped write the paper.

Declaration of Competing Interest

None.

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