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Biotech Method

An efficient transformation method for the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16

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Abbreviations: PHA, polyhydroxyalkanoates; **PHB,** polyhydroxybutyrate

Abstract

Ralstonia eutropha H16 (also known as *Cupriavidus necator* H16) is a Gram-negative lithoautotrophic β-proteobacterium with increasing biotechnological applications, including carbon capture and utilization, biopolymer synthesis and biofuel production. Engineering of this organism is supported by the availability of its genome sequence and suitable plasmid systems. However, the lack of a simple and robust transformation method remains a challenge as it limits both the pace and ease of engineering this organism. To overcome this limitation, a systematic study was performed to evaluate the effects of different parameters on the transformation efficiency of *R. eutropha* H16. The optimized electroporation protocol uses *R. eutropha* H16 cells grown to OD_{600} 0.6. These cells were made competent by a 15-min incubation in 50 mM $CaCl_2$, followed by two cell washes and final resuspension in 0.2 M sucrose prior to electroporation using 2.3 kV. This protocol achieved a transformation efficiency of (3.86 ± 0.29) x 10^5 cfu/µg DNA, a 10^3-fold improvement compared to a previously published value for the same plasmid. This transformation method is a valuable tool for *R. eutropha* H16 research and will further enable the development of other advanced molecular biology methods for this industrially relevant microorganism.

1 Introduction

Ralstonia eutropha H16 (also known as *Cupriavidus necator* H16) is a Gram-negative lithoautotrophic β-proteobacterium found in soil and freshwater habitats. Isolated in 1961 [1], this organism has the ability to accumulate up to 90% per dry cell weight of polyhydroxybutyrate (PHB) under stressed conditions [2] and is often studied as the model organism for polyhydroxyalkanoates (PHA) biopolymer production. *R. eutropha* H16 can metabolize a broad range of substrates as well as grow chemolithoautotrophically using CO_2 and H_2 as its sole carbon and energy source, respectively. These traits have captured the interests of scientists and fueled the research on this non-pathogenic bacterium for carbon capture and utilization, biopolymer synthesis and biofuel production [3-7].

Engineering *R. eutropha* H16 for biotechnological applications requires a set of molecular tools. The availability of its genome sequence [8] and suitable plasmid systems [9, 10] has facilitated the study of this bacterium. However, the lack of a simple and robust transformation method to introduce foreign DNA into *R. eutropha* H16 remains a challenge. Transformation of plasmid DNA into bacteria is a fundamental technique required in recombinant expression of foreign protein(s), genome editing and introduction of foreign metabolic pathways, all of which are key techniques for synthetic biology and strain engineering. With its growing industrial application, an efficient transformation method is urgently needed to support easy investigation and fast engineering of *R. eutropha* H16. The current state-of-the-art for introducing plasmids into *R. eutropha* H16 is via bioconjugation. The basic workflow of bioconjugation involves transforming the plasmid of interest into a donor strain (*e.g.*, *E. coli* S17-1) before transferring it to the final recipient strain (*e.g.*, *R. eutropha* H16) by mating. Despite being effective, bioconjugation is time-consuming as it requires transformation and cultivation

of two strains consecutively compared to a single direct transformation of plasmid DNA into the final recipient strain. Several recent publications included the use of both electroporation and bioconjugation for introducing plasmid DNA into *R. eutropha* H16, but electroporation was never the sole method used in any of these publications. A likely reason is the low electroporation transformation efficiency of *R. eutropha* H16, ranging from 3 cfu/μg to 4 x 10^3 cfu/μg DNA depending on the plasmid used [11-13].

To date, there is only one investigation published for the transformation of *R. eutropha* H16 [13]. Though not well studied for *R. eutropha* H16, transformation of plasmid DNA has been extensively investigated in *Escherichia coli* with a plethora of methods for preparing chemical- or electro-competent cells [14-16]. Between the 2 methods, electroporation frequently gives the highest transformation efficiency, often in the range of 10^9 to 10^{10} cfu/μg of plasmid DNA. Drawing from the available knowledge for *E. coli* transformation, different parameters were investigated for their effects on the electroporation transformation efficiency of *R. eutropha* H16 and reported in this article.

2 Materials and methods

2.1 Chemicals, bacterial strains, cultivation conditions and plasmids

All chemicals used were from Sigma-Aldrich Ltd (Dorset, UK), Fisher Scientific (Loughborough, UK) or Formedium (Norfolk, UK). *R. eutropha* H16 (ATCC 17699) strain used was obtained from ATCC (Middlesex, UK). *R. eutropha* H16 was cultivated either in nutrient broth (NB) or mineral salts medium (MSM) [1] and transformants were selected on NB agar plates at 30°C. When necessary the medium used for *R. eutropha* H16 cultivation was supplemented with 250 μg/mL kanamycin, 25 μg/mL chloramphenicol or 10 μg/mL gentamicin, while 50 μg/mL kanamycin or 25 μg/mL chloramphenicol was used for *E. coli*. DH5α strain used for plasmid replication was cultivated in 2×TY medium at

37°C. Plasmid used in this work is the broad-host-range mobilizable vector pBHR1 (5.3 kb; MoBiTec GmbH, Germany). Plasmid DNA was prepared using the E.Z.N.A.® Plasmid Mini Kit I (Omega Bio-tek, USA) and eluted in double-distilled water. The plasmid DNA concentration was measured using VersaWave (Expedeon Inc, USA). Two-tailed t-test was performed using Graphpad Prism (La Jolla California, USA).

2.2 Electroporation of *R. eutropha* H16

2.2.1 Electroporation procedure

Unless stated otherwise, the following transformation procedure was used. A preculture of *R. eutropha* H16 was cultivated in 5 mL of NB supplemented with gentamicin for 38 h to 42 h at 30°C. For electrocompetent cell preparation, fresh NB supplemented with gentamicin was inoculated with the preculture at a 1:50 dilution and cultivated at 30°C. When the cells reached an optical density at 600 nm (OD_{600}) of 0.4–0.6, they were transferred to ice and chilled for 5–10 min. Two mL of the chilled cells were transferred to a pre-chilled 2-mL microcentrifuge tube. Cells were centrifuged at 17000 g for 30 s and the supernatant was removed by pipetting. The cell pellet was washed thrice by resuspension in 1 mL of ice-cold transformation buffer, followed by centrifuging at 17000 g for 30 s and removal of the supernatant by pipetting. For experiments with chemical treatment, the first cell wash was replaced by resuspension of the cell pellet using different chemicals instead of the transformation buffer and incubated for 0–60 min. After the final wash, the cell pellet was resuspended in 100 µL of ice-cold transformation buffer. Plasmid DNA pBHR1 (0.25 µg) was added to the cells and gently mixed before transferring the cells and DNA into a chilled 2-mm electroporation cuvette (Bio-Rad, USA) and electroporated at 2.5 kV (Eppendorf Eporator, Eppendorf, Germany). In all experiments, the volume of DNA is between 1–2% of cell volume. One mL of NB was immediately added after electroporation and the cells were then transferred to a 2-mL centrifuge tube for outgrowth at 30°C for 2

h. Following outgrowth, cells were plated on NB agar plates with 250 µg/mL kanamycin and incubated for 40–48 h. Typically, 9% (v/v) of the cells were plated after transformation. Gentamicin was not used in the agar plates. The above procedure was used for the parameters investigated in section 3.1 to 3.4 unless otherwise stated.

2.2.2 Electroporation procedure for large volume cell preparation

In the investigation of cell concentration, cell numbers and phase of cell growth (section 3.5 and 3.6), large volume of cells was required and was thus prepared using Erlenmeyer flasks. The cells were cultivated and chilled as before and then transferred to a 50-mL falcon tube and centrifuged at 6000 g, 4°C for 1.5 min. The supernatant was decanted and cells were resuspended in 25 mL of 50 mM $CaCl_2$ and incubated for 15 min on ice. The cells were then centrifuged at 6500 g, 4°C for 2 min and the supernatant was decanted. Cells were washed twice using 25-mL and 15-mL of ice-cold 0.2 M sucrose respectively. At the end of each wash, cells were centrifuged at 6500 g, 4°C for 2–3 min and the supernatant was decanted. The cell pellet was finally resuspended in the desired volume of transformation buffer (*e.g.*, 50 mL initial cell culture to 500 µL final resuspension volume for a 100-time cell concentration). Aliquots of competent cells were transferred into 1.5-mL centrifuge tubes and pBHR1 plasmid DNA was added. Each electroporation sample was then transferred into a chilled 2-mm electroporation cuvette and electroporated. One mL of NB was immediately added after electroporation and the cells were then transferred to a 2-mL centrifuge tube for outgrowth at 30°C for 2 h. After the outgrowth, cells were diluted and depending on the initial cell concentration 1–9% (v/v) of the transformed cells was plated. For transformation frequency calculation, the same transformed cells after outgrowth were diluted 10^6 times before plating on NB agar plates with no antibiotics to obtain the corresponding number of survivor cells.

3 Results and discussion

Common factors that influence transformation efficiency were investigated, including the choice of electroporation buffer, the use of chemical agents to increase cell wall permeability, amount of DNA, electroporation voltage, supplementation of preferred carbon source, cell number and cell concentration. It is important to mention that transformation efficiency varies across different batches of competent cells prepared (see Table S1 in Supporting Information). As such, it necessitates the use of relative transformation efficiencies for comparison in this work. This was achieved by including in each cell batch a triplicate of the reference sample; 100 μL of 20-time concentrated competent cells prepared using 0.2 M or 0.3 M sucrose and electroporated with 0.25 μg of pBHR1. The transformation efficiency of this reference sample was arbitrarily assigned 100% and the efficiencies of other samples were calculated as relative transformation efficiency against this reference sample.

3.1 Sucrose - the most efficient transformation buffer

For efficient electroporation of most microorganisms, a high resistance medium (*i.e.*, low conductivity) is used. This is generally achieved by removal of ions in the growth media through cell wash during electrocompetent cell preparation [14]. Five common transformation buffers used for cell wash were first investigated, including double-distilled water, 10% (v/v) glycerol, 0.3 M sucrose, 0.3 M glucose and 10% (w/v) fructose (0.56 M). A comparison of the relative transformation efficiencies obtained is shown in Figure 1A. Using 0.3 M sucrose as transformation buffer yielded the best transformation efficiency. Interestingly, two of the most commonly used transformation buffers for *E. coli*, double-distilled water and 10% (v/v) glycerol performed poorly with very few transformants. Further optimization of the sucrose concentration from 0.1–0.6 M identified the optimum concentration for high transformation efficiency to be 0.2 M

sucrose (Figure 1A). Combinations of sucrose with glycerol or fructose were also investigated but both resulted in poorer transformation efficiency. The optimum transformation buffer was thus set as 0.2 M sucrose.

3.2 Calcium chloride treatment improves transformation efficiency

Various chemicals are known to facilitate DNA transfer into cells. Though the exact mechanisms by which they function were sometimes unclear, the general consensus is they increase cell wall permeability [17, 18]. This strategy is frequently combined with heat-shock [15] for transformation of Gram-negative bacteria and with electroporation for Gram-positive bacteria [19]. To explore the effects of these chemicals in combination with electroporation, a 30-min chemical treatment step was introduced to replace the first cell wash. The 5 chemicals tested were 50 mM $CaCl_2$, 20 mM $MgCl_2$, 100 mM EDTA, 1% (w/v) glycine and TSS buffer [20]. Results in Figure 1B show both the TSS and 50 mM $CaCl_2$ to improve transformation efficiency upon a 30-min incubation while all other chemicals decreased the transformation efficiency with 1% (w/v) glycine giving no transformants. As 50 mM $CaCl_2$ showed the greatest improvement, the time dependency of 50 mM $CaCl_2$ treatment was further investigated. Treatment time was varied from 0−60 min and results showed the 15-min incubation to be optimal, with a 5-fold increase in transformation efficiency compared to the sample with no chemical treatment. A 50 mM $CaCl_2$ treatment for 15 min was thus included in the final optimized protocol. Calcium chloride is commonly used to generate chemical competent bacterial cells [15] but not electro-competent cells. It was suggested that calcium ions bring the DNA molecule closer to the cell by forming a coordination complex between the negatively charged DNA and lipopolysaccharide for chemical competent cells [21]. Since the $CaCl_2$ and DNA were added at different stages in this investigation, its mechanism in the electroporation protocol remains obscure.

3.3 Optimum electroporation voltage and DNA amount for high transformation efficiency

At the beginning of this study, 0.25 µg of pBHR1 was electroporated in a 2-mm electroporation cuvette using 2.5 kV electroporation voltage. Figure 1C and 1D illustrate the effect of changing electroporation voltage and DNA quantity respectively. Varying the electroporation voltage between 2.1 to 2.5 kV demonstrated that the initial set voltage of 2.5 kV had the lowest efficiency within the tested range (Figure 1C). Using 2.3 kV for electroporation increased the efficiency 3.7 folds. The final optimal voltage for a 2-mm electroporation cuvette was thus set as 2.3 kV (11.5 kV/cm). The electroporation voltage used varies significantly in literature [11-13]. However, Park *et al.* previously investigated this specific property and also obtained 11.5 kV/cm as the optimum electroporation voltage [13].

Increasing DNA amount from 0.25 µg to 0.5 µg to 1.0 µg resulted in very similar transformation efficiencies (Figure 1D), where quadrupling the DNA quantity from 0.25 µg to 1.0 µg generated a 1.4-fold increase. Since transformant number is the multiplication of transformation efficiency and DNA amount, the number of transformants did increase proportionally with the increase in DNA quantity. Similar observation was made by Park *et al.* where the transformant number increased proportionally with increased DNA amount of up to 1 µg DNA [13]. This same trend was also reported for *E. coli* where transformation efficiency varied only 4.5 folds when DNA quantity increased 10^6 folds with transformant number increasing linearly with DNA quantity [14]. Thus increasing DNA quantity is especially advantageous when high number of transformants are desired (*e.g.*, generating mutant libraries) or for difficult-to-transform plasmids (*e.g.*, very large plasmids).

3.4 Fructose improves transformation efficiency

Fructose is one of the three carbohydrates assimilated by *R. eutropha* H16 [22]. When 1% (w/v) fructose was added to the growth and outgrowth medium, the transformant number increased (167 ± 47)% compared to that without fructose. It is postulated that the addition of a preferred carbon source may have facilitated the recovery of cells during outgrowth.

R. eutropha H16 is known to have natural resistance against low concentrations of kanamycin. To determine if transformant number was affected by the choice of kanamycin or chloramphenicol as selection marker, transformed cells (pBHR1 plasmid has both kanamycin and chloramphenicol resistant genes) were plated on agar plates with either kanamycin or chloramphenicol. No significant difference was observed; the chloramphenicol plates had transformant number (112 ± 24)% relative to the kanamycin plates.

3.5 High cell number and concentration improve transformation efficiency

Figure 2A shows the result of varying cell concentration and number. Using a constant number of cells prepared from a 2 mL-cell culture, transformation efficiency increased as cell concentration increased from 5× to 20×. The transformation efficiency also increased with competent cell number, as evident when the competent cell volume increased from 0.1 mL to 0.4 mL. Interestingly, the sample with 0.2 mL competent cell showed an unusual spike in transformation efficiency. Since the sample resistance within the electroporation cuvette is inversely proportional to the sample volume, it can only be postulated that a 0.2 mL sample generated the optimum resistance for high transformation efficiency. As increasing competent cell concentration and number both proved to increase

transformation efficiency, these parameters were increased further by maintaining a final competent cell volume of 0.1 mL and increasing competent cell concentration from 20× to 40× and 80×. The 80× cell concentration generated a 7.8-fold improvement in transformation efficiency relative to the 20× concentration. Among all the factors investigated thus far, increasing cell number and concentration showed the most pronounced effect on increasing the transformation efficiency which aligns to the trend observed by Park *et al.* [13].

3.6 Optimum cell growth phase for high transformation efficiency

Using the optimized parameter obtained thus far, the effect of cell growth phase was investigated. The phase of cell growth is known to affect transformation efficiency with most transformation methods using competent cells in the early to mid-logarithmic phase of growth. Different optima had also been suggested [23, 24]. Different phase of cell growth affects both cell number and the ease of transporting DNA across the cell membrane. In this study, the stage of cell harvest was optimized between the early to mid log-phase (OD_{600} of 0.2 to 0.8).

Figure 2B shows the transformation efficiency and transformation frequency obtained at various OD_{600}. Within this tested range, transformation efficiency increased 2.4 folds from (1.58 ± 0.21) x 10^5 cfu/μg DNA to (3.86 ± 0.29) x 10^5 cfu/μg DNA as OD_{600} increased, likely contributed by the increase in competent cell number. In this set of experiments, the transformation frequencies were also determined. Transformation frequency is defined as the number of successful transformants per survivor cell and it increased 10 folds as OD_{600} increased from 0.2 to 0.6 before it decreased 2.6 folds at OD_{600} of 0.8. This final fall in transformation frequency was due to a 3-fold increase in survivor cells but a relatively smaller increase in the number of successful transformants compared to OD_{600} of 0.6. This

fall in transformation frequency suggests that increasing cell number by increasing OD_{600} beyond 0.6 is counterproductive as the transport of DNA across cell membrane becomes less efficient. The optimal OD_{600} for competent cell preparation was thus set at 0.6.

3.7 High transformation efficiency for *R. eutropha* H16 variant and different plasmids

To demonstrate the versatility of the optimized protocol, it was applied to *R. eutropha* H16 cells cultivated in mineral salts medium (MSM) [1] with 1% (w/v) sodium gluconate as carbon source instead of NB. Due to the different growth rate of *R. eutropha* H16 in NB and MSM, cells were cultivated to early-logarithmic phase of OD_{600} of 1.0 in MSM before competent cell preparation. A transformation efficiency of (3.07 ± 0.44) x 10^5 cfu/µg DNA was achieved for the MSM cultivated cells, which is similar to the (3.86 ± 0.29) x 10^5 cfu/µg DNA obtained in NB under the same conditions. Cell growth in MSM however has a longer lag phase compared to NB; consequently 11−12 h is required to complete the whole procedure compared to the 7−8 h required when cells were cultivated in NB.

In most circumstances, the plasmids and *R. eutropha* H16 strains used would likely differ from that used during this method development; pBHR1 (5.3 kb) and wild type *R. eutropha* H16. The electroporation protocol was thus tested on the *R. eutropha* H16 ΔPhaC1 variant and an efficiency of (7.70 ± 2.14) x 10^5 cfu/µg DNA was achieved; almost twice as efficient compared to the wild type strain. This result suggests that transformation efficiency may vary slightly across different variants. The protocol was also used to transform pBBR1MCS1-based [25] L-arabinose-inducible broad host range plasmids; pBBR1c-RFP (6.7 kb) and pBBR1c-DD (9.7 kb). The former carries the red fluorescence protein open reading frame and showed a transformation efficiency of (4.56±0.99) x 10^4 while the latter has a 5-gene operon and a transformation efficiency of

(4.35±1.46) x 10^4 (see Table S2 for individual transformation efficiency values). The pBBR1c-RFP transformants were further tested for protein expression; 96 individual transformants were transferred to a microtiter plate and cultivated at 30°C in the presence of 0.1% (w/v) arabinose as inducer and 94 transformants showed clear expression of RFP (see Figure S1).

4 Concluding remarks

An efficient transformation protocol for *R. eutropha* H16 is developed in this work; cells cultivated in NB to OD_{600} of 0.6 were treated for 15 minutes in 50 mM $CaCl_2$, followed by 2 cell washes and final resuspension using 0.2 M sucrose before the sample was electroporated with 2.3 kV in a 2-mm cuvette. The maximum transformation efficiency obtained for wild type *R. eutropha* H16 was (3.86 ± 0.29) x 10^5 cfu/µg DNA when 200 µL of 100× concentrated cells were used. Table 1 summarizes the electroporation conditions and efficiencies previously reported for *R. eutropha* H16. Despite the overlap of some parameters investigated in this work with previous publications (Table 1), the combination of all the optimized electroporation conditions has not been previously reported. Based on the transformation efficiency, this work achieved at least a 100 times more efficient method compared to any of the earlier reported values. Solaiman *et al.* previously reported a transformation efficiency of 450 cfu/µg for electroporation of pBHR1 [12], the same plasmid used in this study. This suggests a 10^3-fold improvement was attained through a systematic investigation as reported here.

Bacterial conjugation is currently the most widely used method to transfer plasmids into *R. eutropha* H16 and has a reported conjugation frequency of 8 x 10^{-6} transconjugants per donor cfu [26]. The transformation efficiency of (3.86 ± 0.29) x 10^5 cfu/µg DNA translates to 2 x 10^{-6} cfu per molecule of pBHR1. As electroporation and bacterial conjugation are

vastly different techniques, direct comparison of their efficiency or frequency is difficult. The key advantage of electroporation is its shorter time requirement. *R. eutropha* H16 transformation with a target plasmid can be accomplished within 2 days, while bacterial conjugation typically requires 4–5 days.

Although the transformation efficiency achieved here remains lower than the 10^8 to 10^9 cfu per µg DNA commonly reported for *E. coli* [14], it is a significant improvement for *R. eutropha* H16. Not only is the procedure faster compared to conjugation, the transformation efficiency achieved here will enable other biotechnological applications, for example the generation of mutant libraries during directed evolution experiments. We foresee that this transformation method will further stimulate the development of more molecular biology tools to facilitate the investigation of *R. eutropha* H16 and development of biotechnological applications for this bacterial strain.

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Conceived and designed the experiments: KLT and TSW. Performed the experiments: KLT JG, AMO, MGV and AOJ. Wrote the paper: KLT and TSW.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

5 References

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Table 1. Transformation efficiency of *R. eutropha* H16 reported in the literature.

Plasmid		Cells	Electroporation condition		Transformation efficiency (cfu/µg DNA) [a]	Ref.
Name	Size (kb)	OD_{600} [b]	Buffer	Voltage (kV/cm)		
pBS29-P2-gfp	8.7	n.r.	0.3 M sucrose	25	$(4.04 \pm 0.07) \times 10^3$	[12]
pBHR1	5.3	n.r.	0.3 M sucrose	25	$(4.70 \pm 0.02) \times 10^2$	[12]
pKK-II 89-6	7.8	n.r.	0.3 M sucrose	25	$(1.20 \pm 0.04) \times 10^2$	[12]
pCUP3	7.6	0.5	ddH_20	7.5	$(1.11 \pm 0.08) \times 10^3$	[11]
pJRD215	10.2	0.5	ddH_20	7.5	3.4 ± 0.6	[11]
pKT230	11.9	0.8	10% glycerol	11.5	8.0×10^1 [c]	[13]
pBHR1	5.3	0.6	0.2M sucrose	11.5	$(3.86 \pm 0.29) \times 10^5$	This work

[a] The standard error of mean is provided whenever the information is available or can be calculated from the referenced article

[b] n.r.: value not reported

[c] The competent cells were frozen and thawed before transformation

Figure legends

Figure 1. Relative transformation efficiency of *R. eutropha* H16. **A.** Effect of 10 different transformation buffers. **B**. Effect of chemical treatment during competent cell preparation. **C**. Effect of different electroporation voltage ranging from 2.1–2.5 kV. **D.** Effect of DNA quantity. Bar charts show the mean and standard error of mean for each sample after at least 3 replicates. The reference sample (red bar; 100%) was 100 µL of competent cells prepared using 0.3 M sucrose as transformation buffer to a 20-time cell concentration and transformed with 0.25 µg of pBHR1 and 2.5 kV electroporation voltage. Two-tailed t-test was used to compare the means of the varied conditions against the reference sample; * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$ and **** denotes $P < 0.0001$.

Figure 2. A. Effect of varying cell concentration and cell number on the relative transformation efficiency of *R. eutropha* H16. The reference sample (red bar; 100%) was 100 µL of competent cells prepared using a 15-min $CaCl_2$ incubation step, 0.2 M sucrose as transformation buffer and transformed with 0.25 µg of pBHR1 using 2.5 kV electroporation voltage. Cell concentration and number were controlled by varying the initial cell culture volume and/or final resuspension volume of the competent cells. **B**. Effect of cell growth phase on the transformation efficiency (black bar) and frequency (grey bar) of *R. eutropha* H16. Competent cells (200 µL) were prepared using a 15-min $CaCl_2$ incubation step, 0.2 M sucrose as transformation buffer to a 100-time cell concentration and transformed with 0.25 µg of pBHR1 using 2.3 kV electroporation voltage. Both bar charts show the mean and standard error of mean for each sample after at least 3 replicates. Two-tailed t-test was used to compare the means of the varied conditions in **2A** against the reference sample; * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$ and **** denotes $P < 0.0001$.

Figure 1:

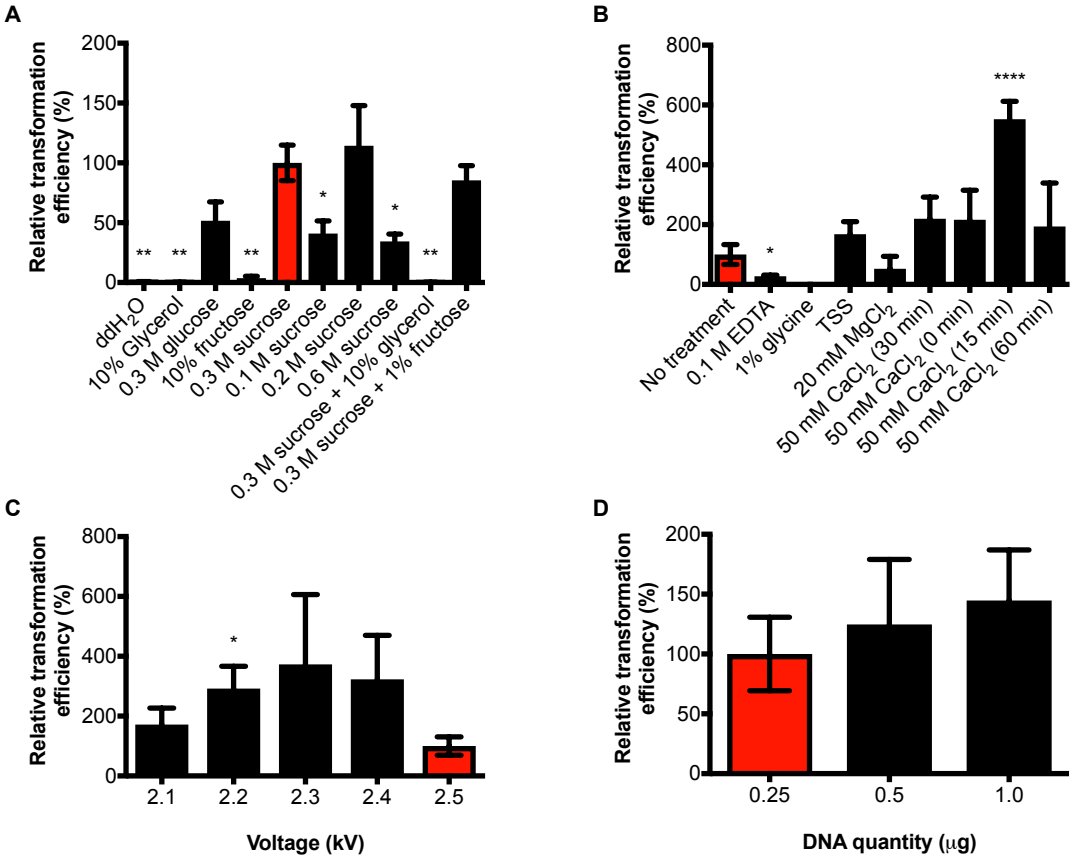
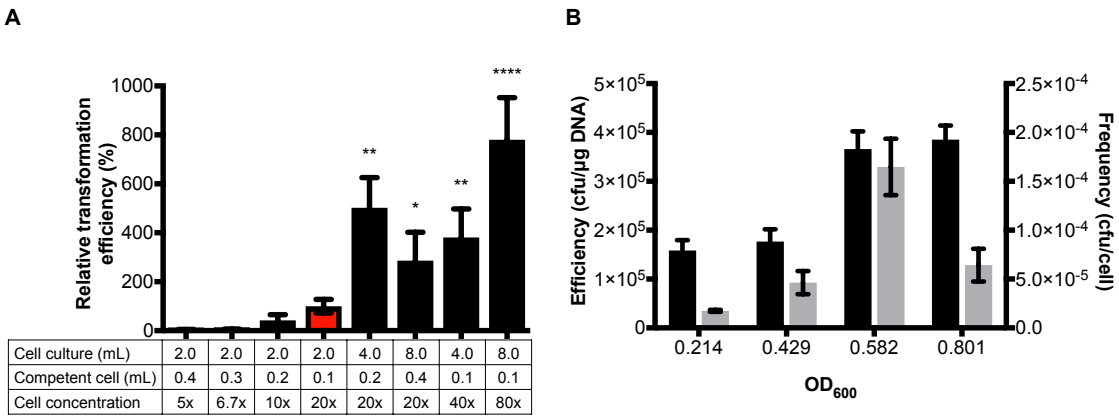

A
Relative transformation efficiency (%)
200
150
100
50
0
**
**
**
*
*
**
ddH2O
10% Glycerol
0.3 M glucose
10% fructose
0.3 M sucrose
0.1 M sucrose
0.2 M sucrose
0.6 M sucrose
0.3 M sucrose + 10% glycerol
0.3 M sucrose + 1% fructose
B
Relative transformation efficiency (%)
800
600
400
200
0
*
****
No treatment
0.1 M EDTA
1% glycine
TSS
20 mM MgCl2
50 mM CaCl2 (30 min)
50 mM CaCl2 (0 min)
50 mM CaCl2 (15 min)
50 mM CaCl2 (60 min)
C
Relative transformation efficiency (%)
800
600
400
200
0
*
2.1
2.2
2.3
2.4
2.5
Voltage (kV)
D
Relative transformation efficiency (%)
200
150
100
50
0
0.25
0.5
1.0
DNA quantity (μg)

Figure 2:



Cell culture (mL)	2.0	2.0	2.0	2.0	4.0	8.0	4.0	8.0
Competent cell (mL)	0.4	0.3	0.2	0.1	0.2	0.4	0.1	0.1
Cell concentration	5x	6.7x	10x	20x	20x	20x	40x	80x