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# Study of Transformation Methods for Chemically Competent *E. coli* Cells

**Bachelor's Thesis (12 ECTS)**

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# Study of Transformation Methods for Chemically Competent *E. coli* Cells

**Abstract:**

Bacterial transformation is the ability of bacteria to uptake foreign DNA. High transformation efficiencies (TEs) are important in various molecular cloning processes, DNA library construction and transformation of large plasmids. Therefore, common laboratory strains are made competent by treating their membranes to enhance plasmid uptake.

Transformation and storage solution (TSS) is a buffer for making common laboratory *E. coli* competent cells. (Chung et al., 1989). Two recent studies have reported one to two-log improved TEs (Yang, Liu, et al., 2022; Yang, Yu, et al., 2022), using slightly modified transformation buffers (TSS or TSS-HI) and methods (KCM or Heat shock). Here, we intend to reproduce these two improved methods. We prepared chemically competent *E. coli* cells using TSS or TSS-HI buffers. The competent cells were transformed using KCM, heat shock, or heat shock + KCM methods. Results revealed that *E. coli* strains washed with the modified TSS and TSS-HI buffers and transformed via different methods did not exhibit any significant improvement or difference in the transformation efficiency. However, the modified protocols produce competent cells suitable for quick transformation, eliminating the need for incubation on ice (usually 30 minutes) or recovery (about 1 hour). Our findings refute the reported modifications' effectiveness, reliability, and reproducibility in improving TEs.

**Keywords**: Bacterial transformation, transformation efficiency, competent cells.

**CERCS**: B230 Microbiology, bacteriology, virology, mycology

**Uuring keemiliselt kompetentsete *E. coli* rakkude transformatsioonimeetodite kohta**


**Lühikokkuvõte**

Bakteriaalne transformatsioon on bakterite võime võtta vastu võõrast DNA-d. Kõrged transformatsiooniefektiivsused (TE-d) on olulised mitmesugustes molekulaarse kloonimise protsessides, DNA raamatukogude koostamisel ja suurte plasmiidide transformatsioonil. Seetõttu muudetakse tavapärased laboratoorsed tüved kompetentseks, töödeldes nende membraane plasmiidi omastamise parandamiseks.

Transformatsiooni- ja säilituslahus (TSS) on puhver, mida kasutatakse tavaliste laboratoorsete *E. coli* kompetentsete rakkude valmistamiseks (Chung jt, 1989). Kaks hiljutist uuringut on teatanud ühe kuni kahe suurusjärgu võrra paranenud TE-dest (Yang, Liu jt, 2022; Yang, Yu jt, 2022), kasutades veidi muudetud transformatsioonipuhvreid (TSS või TSS-HI) ja meetodeid (KCM või kuumšokk). Siinkohal kavatseme neid kahte paranenud meetodit reprodutseerida. Valmistasime keemiliselt kompetentseid *E. coli* rakke, kasutades TSS või TSS-HI puhvreid. Kompetentsed rakud transformeeriti KCM, kuumšoki või kuumšokk + KCM meetodite abil. Tulemused näitasid, et *E. coli* tüved, mida oli pestud muudetud TSS ja TSS-HI puhverdustega ja mis olid transformeeritud erinevate meetodite abil, ei näidanud transformatsiooniefektiivsuse osas märkimisväärset paranemist ega erinevust. Kuid muudetud protokollid toodavad kompetentseid rakke, mis sobivad kiireks transformatsiooniks, kõrvaldades vajaduse jää peal inkubeerimise (tavaliselt 30 minutit) või taastumise (umbes 1 tund) järele. Meie tulemused lükkavad ümber teatatud modifikatsioonide tõhususe, usaldusväärsuse ja reprodutseeritavuse TE-de parandamisel.



**Võtmesõnad**: Bakteriaalne transformatsioon, transformatsiooniefektiivsus, kompetentsed rakud.

**CERCS**: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

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# TERMS, ABBREVIATIONS AND NOTATIONS

1xKCM – A salt solution containing potassium chloride (0.1M), calcium chloride (30 mM), and magnesium chloride (50 mM)

cDNA – complementary DNA

CFU – Colony Forming Units

ddH2O – double-distilled water

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

*E. coli – Escherichia coli*

LB – Lysogeny Broth

λ DNA – bacteriophage Lambda DNA

MCS – Multiple Cloning Site

$OD_{600}$ – Optical Density at 600 nanometers

PCR – Polymerase Chain Reaction

PEG – Polyethylene Glycol

rpm – revolutions per minute

SOB – Super Optimal Broth

TE – Transformation Efficiency

TSS – Transformation and storage solution

WT – Wild-type

# INTRODUCTION

DNA cloning is widely used in molecular biology, such as constructing recombinant DNA molecules. Two critical stages are involved in the process: the construction of recombinant DNA and the transformation of the resulting product into a host organism for replication (Sambrook & Russell, 2001). Achieving high efficiencies in these steps is crucial to enhance the success rate of cloning. Bacterial transformation is the ability of bacteria to take up foreign DNA. This process can happen naturally or be induced artificially. Natural transformation occurs when a recipient bacterium absorbs free DNA from its surroundings, which can occur in certain bacteria like *Bacillus subtilis* (Michod et al., 1988). However, many bacteria strains are difficult to transform; hence, there is a need for laboratory strains that are treated to allow DNA entry. Competent cells are those whose cell membranes have been treated to allow DNA uptake. Chemical transformation and electroporation are the two main artificial methods to treat *E. coli* cells for transformation. Mendel and Higa, in 1970, discovered that treating *E. coli* cells with $CaCl_2$ can make the cells take up bacteriophage λ DNA. This method was later used to transform other types of DNA, such as chromosomal and plasmid DNAs (Cohen et al., 1972).

Selecting an appropriate method for preparing and transforming competent cells requires careful consideration of its efficiency, cost-effectiveness, ease of preparation, and relevance to specific experiments. As a result, several techniques have been created to provide a quick and effective way of preparing competent *E. coli* cells with high transformation efficiency. Some of these improved methods involve adding a salt solution containing potassium chloride, calcium chloride, and magnesium chloride (KCM) to the existing Transformation and Storage Solution (TSS) protocol (Yang, Yu, et al., 2022). Another method, TSS-HI, includes an additional 140 mM $Mn^{2+}$ in the TSS buffer, the KCM salt solution, and a heat shock during the transformation process (Yang, Liu, et al., 2022). This study prepares and transforms chemically competent *E. coli* cells using the two improved methods (TSS and TSS-HI) to validate the transformation efficiency of these methods and test a quick transformation using the improved methods.

# 1. LITERATURE REVIEW

## 1.1 DNA Cloning – An Overview

DNA cloning is a method used in molecular biology to manipulate DNA sequences and express them in living cells. The DNA fragment of interest, such as a gene or promoter region, is extracted by cleaving with a restriction enzyme or amplifying with PCR. Then, it is inserted into a plasmid vector and sealed with DNA ligase to produce a recombinant plasmid containing both the original plasmid DNA and the desired DNA fragment. Subsequently, this recombinant plasmid is transferred into competent bacterial cells through an artificial transformation process (Ilves et al., 2020) .

Restriction enzymes recognise and cleave double-stranded DNA at restriction sites and can be divided into two groups based on the type of DNA ends they produce. Most restriction enzymes cut the DNA to produce short, single-stranded overhang sticky ends. e.g. HindIII, PstI, XhoI. If DNA fragments are cut with the same restriction enzyme that creates sticky ends, they create complementary ends. Hence, DNA ligase can seal such ends and restore the restriction site. The other restriction enzymes cleave straight across the DNA, leaving no overhangs and creating a blunt-ended DNA fragment, e.g. EcoRV and SmaI. DNA ligase can join any two DNA molecules that have been cut with any of the restriction enzymes that create blunt ends. However, the ligation efficiency of blunt ends is much lower than that of sticky ends. (Ilves et al., 2020). Lately, DNA assemblies that do not rely on restriction enzymes and instead use short homologous ends have emerged to streamline processes and address experimental challenges (Kosuri & Church, 2014).

The polymerase chain reaction is a method that amplifies a specific DNA fragment using a heat-resistant DNA polymerase (Mullis et al., 1986). To initiate this process, the targeted DNA fragment's nucleotide sequence must be known. Two short single-stranded DNA oligonucleotide primers are created to complement the regions adjacent to the intended amplified DNA section. These primers attach to the single-stranded DNA and prompt initiation of DNA synthesis by the DNA polymerase from the 3’OH-end in a 5’-3’ direction. The key steps in PCR involve denaturing template DNA at around 96 ºC, followed by annealing the primer to complementary sequences on both strands, which typically occurs at temperatures between 50–60ºC depending on the length and nucleotide sequence of the primers. Afterwards, DNA synthesis takes place at approximately 72°C, usually for Taq DNA polymerase. The PCR cycle is usually repeated 20–30 times.

## 1.2 The Bacterial Plasmid – A Key Component of DNA Cloning

The bacterial plasmid is a small, circular, double-stranded extrachromosomal DNA molecule. It engages in conjugation, allowing for exchange between bacteria, and can also be taken up by cells from the environment, a process called transformation. Plasmids often carry genes that confer advantageous traits on the bacteria, such as antibiotic-resistance genes. Due to their ability to carry and express genes, bacterial plasmids can be used as vectors to insert genes of interest into bacteria. A simple plasmid vector contains the following elements:

**Origin of replication (ORI):** This is the region where DNA replication begins. Plasmids replicate independently of the chromosome, making several copies within the cell. The copy number depends on the specific ORI and ranges from low (1-10) to high (hundreds) copy number per bacteria cell. During cell division, the copies are segregated between daughter cells.

**Antibiotics resistance gene:** The antibiotic-resistance gene is used as a selection maker to identify the bacteria that took up the plasmid when plated on a solid growth medium that contains the same antibiotics that the plasmid provides resistance against. Thus, only the cells that took up the plasmid can grow and form colonies on the medium.

**Promoter:** The promoter is located upstream of the genes it transcribes. It is used to initiate mRNA synthesis. The promoter can be constitutive, driving continuous gene expression. However, an inducible promoter, such as the tac promoter, is often used in vectors for controlled gene expression. The inductor for the tac promoter is isopropyl β-D-1-thiogalactopyranoside (IPTG), a chemical that mimics lactose but is not metabolised by the bacterium.

**Multiple Cloning Sites (MCS**): This is downstream of the promoter. It is a collection of several unique restriction sites close to one another that allows for the easy insertion of the gene of interest into the plasmid at the restriction site.

**Terminator:** This follows the MCS, which signals the end of transcription.

## 1.3 The Bacterial Transformation

The recombinant plasmid is introduced into the bacteria cell in a process known as transformation. This process can happen naturally or be induced artificially. Natural transformation involves a recipient bacterium taking free DNA from its environment, integrating it into its chromosome or stabilising it outside the chromosome and expressing it to create a new phenotype (Lorenz & Wackernagel, 1994). However, Natural transformation is typically rare in *Escherichia coli* and needs to be induced using artificial techniques.

Chemical transformation and electroporation are the two main artificial methods to prepare *E. coli* cells for transformation. Electroporation involves using short, high-voltage electrical pulses in the range of 12.5 to 15 kV/cm to make cell membranes permeable and facilitate the entry of foreign DNA. While electroporation achieves higher efficiencies $10^9$ to $10^{10}$ transformants/µg DNA than chemical transformation methods (Dower et al., 1988), challenges such as the survival rate of the cells after transformation and plasmid size could result in low cloning efficiency. The chemical method involves the treatment of cells with divalent cations such as $Ca^{2+}$ ($CaCl_2$) or transformation buffers, followed by a brief heat shock (Chung et al., 1989; Cohen et al., 1972; Hanahan, 1983; Inoue et al., 1990).

Transformation efficiency describes how effectively the bacteria cells take up the plasmid and express the genes. This measures the quantity of colony-forming units (CFU) produced by one microgram of plasmid DNA. Achieving high transformation efficiency is essential in various cloning processes; as a result, laboratory-competent cells whose membranes have been chemically treated are used to enhance transformation efficiency **(Fig. 1)**. The plasmid transformed into the bacteria contains an antibiotic resistance gene, which serves as a selection maker to identify the bacteria that have taken up the plasmid when plated on a solid growth medium that contains the same antibiotics against which the plasmid provides resistance. Thus, only the transformed cells can grow and form colonies on the medium **(Fig. 1)**.

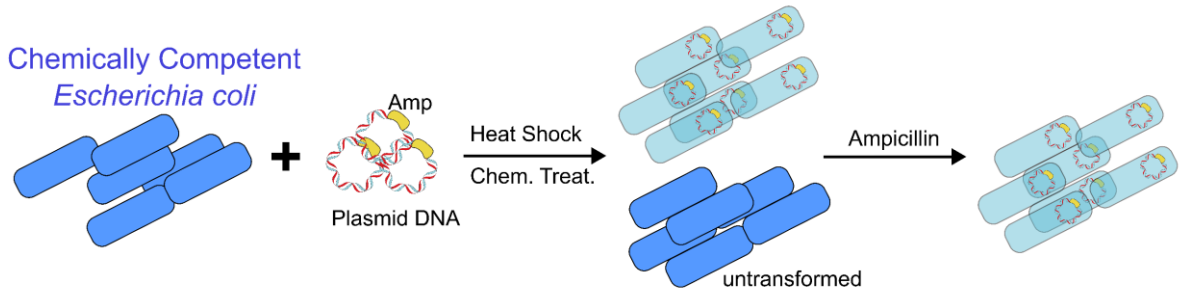


**Fig. 1** | Bacterial transformation using chemically competent *E. coli* cells. Chemically prepared competent cells transformed with plasmid DNA that carry an ampicillin (Amp) antibiotic resistance gene, facilitated by either heat shock or chemical treatment. After transformation, following an overnight incubation period in the presence of ampicillin, only the cells that have successfully taken up the plasmid DNA demonstrate growth and form colonies on the plate.

## 1.4 A Short History of *E. coli* Transformation

In 1970, Mendel and Higa discovered that treating *E. coli* cells with $CaCl_2$ can make the cells take up bacteriophage lambda DNA (Mandel & Higa, 1970). Subsequently, it was discovered that this method can transform other types of DNA, such as DNA from bacterial chromosomes and plasmids, into *E. coli* (Cohen et al., 1972). Since high transformation efficiency is needed in most cloning experiments, several techniques have been created to increase the transformation efficiency. These include incubating the cells in the calcium chloride solution for a longer period, adding a mix of different cations to the transformation mixture, and treating the cells with additional chemicals such as DMSO and PEG (Hanahan, 1983; Sambrook & Russell, 2001).

### 1.4.1 Calcium Chloride Methods for Preparing Chemically Competent *E. coli*

In 1972, Cohen et al. proposed a calcium chloride treatment in *E. coli,* which proved reliable and effective in introducing bacterial plasmid DNAs with antibiotic resistance into the *E. coli* cells (Cohen et al., 1972). Treating the cells with calcium chloride gives a positive charge to the cell membrane, which makes it easier for the DNA to bind to the cell surface, facilitating the entry of DNA into the cells. Cells are grown to the early exponential phase with an $OD_{600}$ of 0.3 to 0.5, harvested by centrifugation, and subjected to multiple rounds of washing and resuspension in $CaCl_2$. During the transformation process, the cells, after being mixed with the DNA, undergo a heat shock at 42°C for 90 seconds with no agitation. The transformation efficiencies achieved through this method range from $10^5$ to $10^6$ transformants/ µg DNA, which is sufficient for most cloning purposes. However, the use of this method is limited due to its lower efficiency and time-consuming preparation process, making it less suitable for experiments that require high transformation efficiencies, such as complex cDNA libraries. The simplified versions of this method, which included magnesium chloride ($MgCl_2$—$CaCl_2$), can produce competent cells capable of transforming up to 5 x $10^6$ to 2 x $10^7$ transformants/µgDNA and can be stored at -70°C (Sambrook & Russell, 2001).

### 1.4.2 Chemical Preparation and Transformation of Competent *E. coli* by Hanahan's Method

In the late 1970s and early 1980s, Doug Hanahan developed a highly efficient bacterial transformation method, achieving transformation efficiencies that reached up to 5x$10^8$ transformants/µg DNA (Hanahan, 1983). This achievement set a new benchmark for bacterial transformation and enabled various developments, such as creating cDNA libraries for functional genomics studies (Sambrook & Russell, 2001).

For this method, instead of the calcium chloride, cells are washed in TFB buffer solution (10 mM MES at pH 6.3, 45 mM $MnCl_2$, 10 mM $CaCl_2$, 100 mM KCl, 3 mM $HACoCl_3$, water) for transforming fresh competent cells, or FSB buffer (10 mM potassium acetate at pH 7.5, 45 mM $MnCl_2$, 10 mM $CaCl_2$, 10 mM KCl, 100 mM $HACoCl_3$, 10% glycerol, water) for storing competent cells at -70°C (Sambrook & Russell, 2001). To prepare fresh competent cells, additional chemicals such as the DnD solution (1.53g dithiothreitol, 9 ml DMSO, 100 µl 1M potassium acetate at pH 7.5, water to 10 ml) are added to the cells. Meanwhile, DMSO is used to freeze competent cells at 70°C. A heat shock is also applied during the transformation step.
Hanahan's transformation method significantly impacted the development of genetic constructs and libraries with exceptional efficiency and magnitude. However, the method demands top-quality reagents and strict adherence to protocol, which could make it inaccessible for laboratories with limited resources, hence posing challenges in replicating results across researchers.

### 1.4.3 Inoue Chemical Competent *E. coli* Preparation and Transformation Method

The Inoue method in 1990 gave transformation efficiency up to 3 x $10^8$ transformants/µg DNA (Inoue et al., 1990). This method is less precise than the Hanahan approach but is more consistent and easier to reproduce. Also, it is suitable for many *E. coli* strains commonly used in molecular cloning. Unlike the other methods where cultures are cultivated at 37°C, the Inoue method cultivates the cell at 18°C, and temperatures from 20–23°C do not affect the transformation efficiency. However, at these temperatures, cultures grow slowly, which requires preparing cultures overnight and collecting them the next morning (Sambrook & Russell, 2001).

For this method, harvested cells are washed and resuspended in Inoue transformation buffer (10 mM PIPES pH = 6.7, 55 mM $MnCl_2$, 15 mM $CaCl_2$, and 250 mM KCl). DMSO is added to the bacterial suspension before freezing, and a heat shock is applied during the transformation step. A recent study by Liu et al. (2018) has shown that incorporating small concentrations of LFcin-B (0.35 mg/L) with moderately high concentrations of $MnCl_2$ (50 mM) and $CaCl_2$ (30 mM) into the Inoue transformation buffer can increase cell membrane permeability and achieve transformation efficiencies up to 3.7 x $10^9$ transformants /µg DNA (Liu et al., 2018).

### 1.4.4 Preparation and Transformation of *E. coli* by Electroporation

The electroporation protocol marked a significant advancement in *E. coli* transformation. This method uses short, high-voltage electrical pulses to permeabilise the cell membrane (Dower et al., 1988). The process depends on the electric field strength and the pulse length, which

maximise transformation efficiency and minimise cellular damage. The method is more straightforward and yields higher efficiencies up to $10^9$ to $10^{10}$ transformants/µg DNA than the chemical transformation methods. These high efficiencies are due to applying electrical pulses within a range of 12.5-15 kV/cm combined with DNA concentrations between 1 to 10 µg/ml. However, this process results in only 30 to 50% cell survival, but up to 80% of the surviving cells are successfully transformed (Sambrook & Russell, 2001). The concentration of the input DNA has a significant impact on electroporation. While high DNA concentrations boost transformation efficiency, they also increase the likelihood of cotransformation, which might not be desired for specific applications, such as creating cDNA libraries (Dower et al., 1988). Electroporation is highly effective and yields high transformation efficiencies; however, many challenges are associated with the process. Its transformation efficiency can vary across different strains of *E. coli* and plasmid sizes. It generally works well with plasmids smaller than 15 kb, though it can vary depending on the *E. coli* strain (Sambrook & Russell, 2001).

Furthermore, linear plasmid DNAs transform at lower efficiencies than closed circular DNAs. Temperatures also play a role in the success of electroporation; optimal results are achieved between 0 to 4°C (Sambrook & Russell, 2001). DNA is usually purified to avoid arcing problems when large volumes of assembled DNA are used in electroporation. During this process, there could be DNA loss. Cells can also be damaged during the electroporation process, which could result in low cloning efficiency (Tan & Yiap, 2009) .

### 1.4.5 Single-Step Method for Preparation and Transformation of Competent *E. coli*

The development of a single-step method for preparing competent cells using the Transformation and Storage Solution (TSS), as described by Chung et al. (1989), is a simple and straightforward process which yields up to $10^7$ - $10^8$ transformants/µg DNA, marking a substantial improvement in *E. coli* transformation. According to this method, even though cells harvested in the early exponential phase ($OD_{600}$ 0.3-0.4) give optimal results, Cells collected at any growth phase, including the stationary phase, can produce between $10^5$ to $10^7$ transformants/µg DNA, which is sufficient for most cloning experiments. Also, cells can be washed or diluted in the TSS buffer (LB-HCl at pH 6.5, 10% PEG 3350 or 8000, 5% DMSO, 20 or 50 mM $Mg^{2+}$ ($MgSO_4$ or $MgCl_2$)) in a 1:1 ratio, eliminating the need for centrifugation, washing, and long-term incubation of cells. In the transformation step, the DNA is directly mixed with the cells. Heat shock, often employed in other transformation protocols, is unnecessary with the TSS method and may even harm the transformation process. Unlike the other protocols, additional components are not needed to store the cells at -70°C or -80°C.

In a modified version of the TSS method (Yang, Yu, et al., 2022), 10% glycerol was included in the TSS buffer (LB-HCl at pH 6.1, 10% PEG 3350, 5% DMSO, 10% glycerol**,**10 mM $MgSO_4$ and 10 mM $MgCl_2$). During the transformation, 5 × KCM (0.5M KCl, 150 mM $CaCl_2$, and 250 mM $MgCl_2$) was mixed with the DNA and $ddH_2O$ to achieve a final concentration of 1x KCM in the reaction mixture, omitting the heat shock step. Applying the enhanced method to prepare the BW25113 *E. coli* strain gave up to $10^9$ transformants/ µg DNA. In December 2022, yang et al. introduced another improved TSS method named TSS-HI, which included an additional 140 mM $Mn^{2+}$ in the TSS buffer (LB-HCl [pH 6.1], 10% PEG 3350, 5% DMSO, 10% glycerol, 20 mM $MgCl_2$, 140 mM $MnCl_2$). During the transformation step, a 1x final concentration of KCM was also used in the reaction mixture, but it was combined with the heat shock procedure. With the TSS-HI method, cells can be cultured at 18°C or 37°C without any change in transformation efficiency. The BW3KD *E. coli* strain prepared with the TSS-HI method yielded up to 7.21 x$10^9$ transformants/ µg DNA, which is efficient for cloning with large plasmids and multiple fragments.

# 2. THE AIMS OF THE THESIS

The aims and general workflow of the thesis include:

- Preparing chemically competent cells of four bacterial strains: BW25113, *ΔybeX::kan*$^{[BW]}$, MG1655, and *ΔybeX::kan*$^{[MG]}$ using modified versions of TSS protocols : TSS and TSS-H1 (Yang, Liu, et al., 2022; Yang, Yu, et al., 2022).
- Transforming the competent cells with pUC19 plasmid DNA using KCM, heat shock, and combining both methods (KCM + Heat Shock).
- Test the efficiency of quick transformation and compare the results of different transformation methods.

# 3. EXPERIMENTAL PART

## 3.1. Materials and Methods

### 3.1.1 Strains

**Table 1** | All used strains, along with their characteristics and sources.

| Strains | Characteristics | Source |
|---|---|---|
| BW25113 | $F^-$, $\lambda^-$, *Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), *Δ(rhaDrhaB)568, hsdR514, rph-1* | Coli Genetic Stock Center (CGSC) |
| *ΔybeX::kan*$^{[BW]}$ | $F^-$, $\lambda^-$, *Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), *Δ(rhaDrhaB)568*, *ΔybeX*(::kan), *hsdR514, rph-1* | |
| MG1655 | $F^-$, $\lambda^-$, *ilvG*$^-$, *rfb-50, rph-1* | Coli Genetic Stock Center (CGSC) |
| *ΔybeX::kan*$^{[MG]}$ | $F^-$, $\lambda^-$, *ilvG*$^-$, *rfb-50, rph-1, ΔybeX*(::kan) | |

All used strains **(Table 1)** were from the Keio collection. The Keio collection is a single gene knockout library comprising about 4000 *Escherichia coli* strains (Baba et al., 2006). The *ΔybeX::kan* strains are mutant *Escherichia coli* strains in which the *ybeX* gene has been knocked out and replaced with a kanamycin resistance gene. The insertion of the kanamycin resistance gene allows the selection of these mutant strains, as only cells with the resistance gene will grow on a medium containing kanamycin, confirming the successful creation of the mutant strains. The *ybeX* gene is part of the *ybeZYX-Int* operon and is believed to encode a putative Cobalt/Magnesium efflux protein (Gibson et al., 1991). Our lab recently reported that the deletion of *ybeX* is associated with several phenotypic changes, including the accumulation of

17S pre-rRNA, about 1 kb 16S rRNA cleavage product, sensitivity to heat shock and ribosome-targeting antibiotics (Sarıgül et al., 2024).

### 3.1.2 The pUC19 Plasmid

The pUC19 plasmid was used for this experiment **(Fig. 2)**. It is small, approx. 2.7 kb in size, double-stranded, circular DNA with a molecular weight of $1.75\times10^6$ Da (Kulkarni, 2023), facilitating easy manipulation and propagation within the bacteria cell. This vector contains an origin of replication from pMB1 with a high copy number, multiple cloning sites for various restriction enzymes, an Ampicillin resistance gene for selection, and a *lacZ* gene encoding β-galactosidase for blue-white screening. Additionally, it features a 54 bp multiple cloning site poly-linker comprising 13 different hexanucleotide-specific restriction endonuclease sites such as EcoR1, HindIII and BamH1.

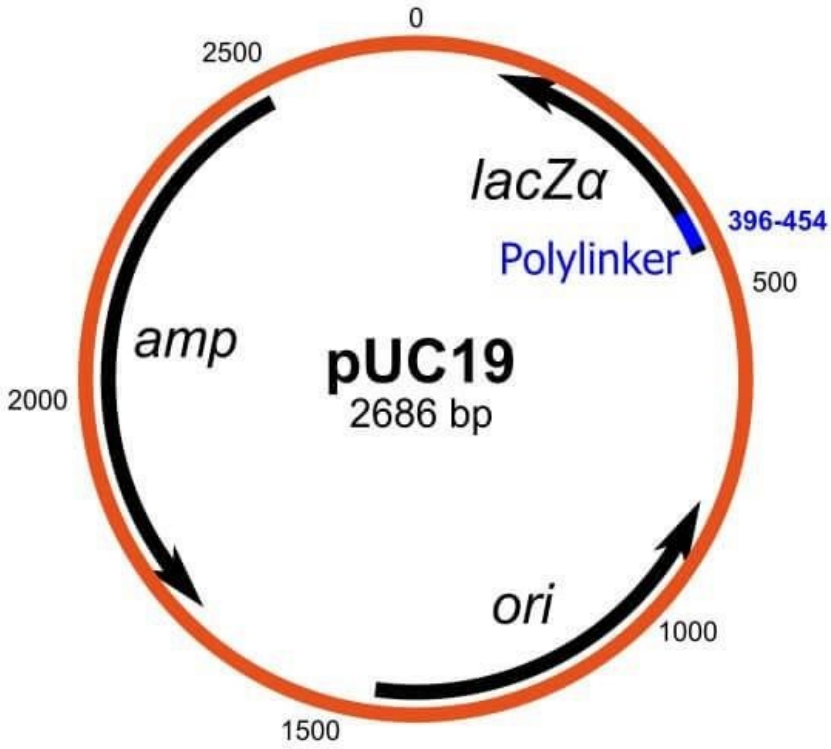


**Fig. 2** | Vector map of pUC19 (Addgene, #50005)

### 3.1.3 Media and Conditions

All wild-type strains were grown on Lysogeny Broth (LB) media (10 g/l Tryptone, 5 g/l Yeast extract, 10 g/l NaCl) and incubated at 37°C, 200rpm overnight. However, the *ΔybeX::kan* mutants were grown on 1 mM $MgCl_2$ (200 µl of $MgCl_2$ into 200 ml of LB) containing 50mg/ml kanamycin and incubated at 37°C, 200rpm overnight. All cells were recovered in SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5mM KCI, 10 mM $MgCI_2$, 10

mM $MgSO_4$, 22.2 mM glucose) at 37 °C, 800rpm for 1 hr. Recovered cultures were plated on selective LB agar plates containing 100 µg/ml carbenicillin and incubated at 37°C overnight.

### 3.1.4 Visualisation and Statistical Analysis

GraphPad Prism V. 10.2.2 was used for all data analysis and visualisation. This includes plotting all the graphs with data points and error bars and performing unpaired t-tests to calculate P-values for statistical comparisons.

## 3.2 Methods

### 3.2.1 Competent Cell Preparation

The competent cells were prepared using the modified TSS and TSS-HI methods (Yang, Liu, et al., 2022; Yang, Yu, et al., 2022). The frozen stock of the wild types (BW25113 and MG1655) at -80°C were streaked on LB agar plates and incubated overnight at 37°C. A single fresh colony was inoculated in 3.5 ml LB and incubated at 37°C, 200rpm overnight. The next day, 1 ml of the overnight cultures were transferred to 500 ml flasks, each containing 100 ml of fresh LB. For the *ΔybeX::kan* mutants, frozen stocks were streaked on 25µg/ml kanamycin-containing LB agar plates and incubated overnight at 37°C. A single fresh colony was inoculated in 3.5 ml of 1 mM $MgCl_2$ (200 µl of $MgCl_2$ into 200 ml of LB) containing 3.5 µl of 50mg/ml kanamycin, and incubated at 37°C, 200rpm overnight.

The next day, 1 ml of the overnight cultures were transferred to 500 ml flasks, each containing 100 ml of the 1 mM $MgCl_2$ and 100 µl of 50mg/ml kanamycin. Then, the fresh LB media were cultured at 37 °C, 200 rpm for about 1.5 hrs. During this period, the optical density at 600nm ($OD_{600nm}$) was monitored by measurements in a spectrophotometer every 30 min. The initial optical density was around 0.04 for BW25113 and *ΔybeX::kan*$^{[BW]}$ and 0.03 for MG1655 and *ΔybeX::kan*$^{[MG]}$. It took about 1 hour for the cells to begin exponential growth. **(Fig. 3)**. When the $OD_{600nm}$ was between 0.25 and 0.3, the flasks were placed on ice for 10 min to stop cell growth. Then, the cells were harvested in ice-cold 50ml falcon tubes by centrifugation at 1000xg for 10 minutes at 4°C using a swing angle rotor. After 10 min, the supernatants were discarded, and the tubes were inverted upside down on a clean paper towel and placed back on ice. The harvested cells were each washed in ice-cold 1ml of the TSS (LB-HCl [pH = 6.1], 10% PEG3350, 5% DMSO, 10% Glycerol, 10 mM $MgCl_2$, and 10 mM $MgSO_4$), and TSS-HI (LB-HCl [pH 6.1], 10% PEG 3350, 5% DMSO, 10% Glycerol, 20 mM $MgCl_2$, 140 mM $MnCl_2$) by gently swirling the mixture in the cold room (-4°C) until the cells were all in the medium. Then the cells were incubated on ice for 10 minutes, after which 100 µl of the cells were aliquoted

into 20 labelled ice-cold Eppendorf tubes (10 tubes for each strain), snap-frozen in liquid nitrogen, and stored at -80 °C freezer.

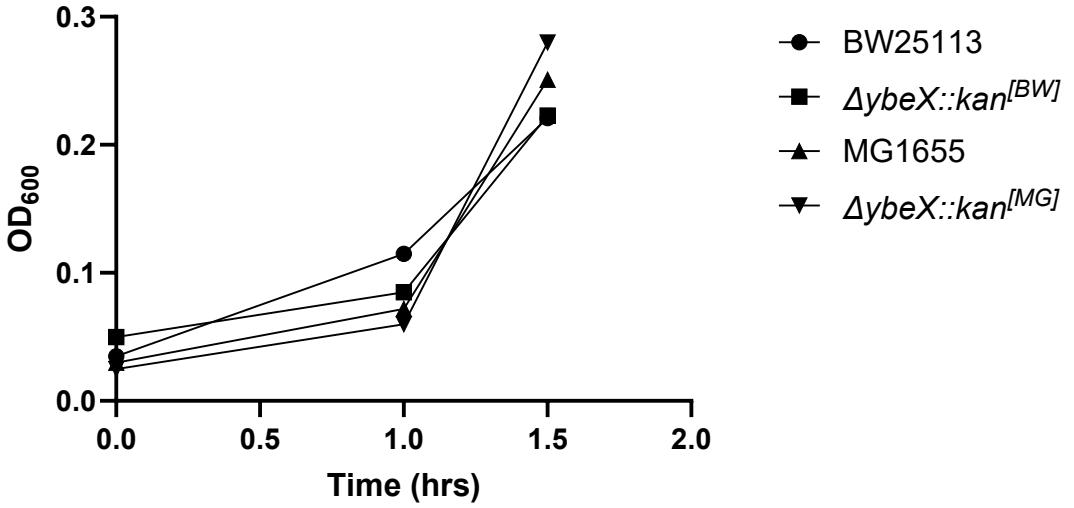


**Fig. 3** | Monitored growth of *E. coli* strains used in this study in LB medium at 37°C. A single colony of indicated strains was picked and grown overnight at 37°C with good aeration. The following morning, the cells were diluted to an $OD_{600}$ of 0.05, and the growth was monitored every 30-60 minutes. After about an hour, the cells were collected at an $OD_{600}$ between 0.25 and 0.3 at the exponential phase.

### 3.2.2 Transformation of Competent Cells Using KCM

The pUC19 plasmid was used for all transformations, and a 5 × KCM salt solution (0.5M KCl, 150 mM $CaCl_2$, and 250 mM $MgCl_2$) was used in the KCM transformation step. Competent cells from -80°C were placed on ice for about 5 minutes to thaw. A total volume of 100 µl of the KCM reaction mixture (20 µl 5xKCM, 2 µl 2ng/µl pUC19, and 78 µl MQ; final concentration of KCM = 1x) was gently mixed with 100 µl of the cells. The mixtures were incubated on ice for 20 minutes and then at room temperature for 10 minutes. The cells were then recovered in 800 µl SOC medium (2 ml/100 ml 20% glucose, 98 ml/100 ml SOB) at 37°C and 800 rpm for 1 hour. Serial dilutions ($10^{-1}$,$10^{-2}$, and $10^{-3}$) of the recovered cells were made, and 80 µl of each dilution was spread onto an LB plate containing carbenicillin and incubated at 37°C overnight. The transformation procedure was repeated using different concentrations of pUC19: 0.1, 0.2, 4, 6, and 8 ng/µl. The next morning, the colonies were counted, and the transformation efficiency was calculated.

### 3.2.3 Transformation of Competent Cells with Heat Shock

Competent cells from -80°C were placed on ice for about 5 minutes to thaw. 4 ng/µl pUC19 was gently mixed with 100 µl of the cells and incubated on ice for 20 minutes. Heat shock was applied at 42°C, 0 rpm, for 90 seconds, and then the mixture was cooled on ice for 2 minutes to prevent intracellular nucleases from degrading the plasmid. 900 µl of SOC medium was used to recover the cells at 37°C, 800 rpm, for 1 hour. Serial dilutions ($10^{-1}$,$10^{-2}$, and $10^{-3}$) of the recovered cells were made, and 80 µl of each dilution was spread onto an LB plate containing carbenicillin and incubated at 37°C overnight. The procedure was repeated using different concentrations of pUC19: 0.1, 0.2, 4, 6, and 8 ng/µl. The next morning, the colonies were counted, and the transformation efficiency was calculated.

### 3.2.4 Transformation of Competent Cells Using Heat Shock + KCM

Competent cells from -80°C were placed on ice for about 5 minutes to thaw. A total volume of 50 µl of the KCM reaction mixture (10 µl 5× KCM, 2 µl 2 ng/µl pUC19, and 38 µl MQ; final concentration of KCM = 1×) was gently mixed with 50 µl of the cells and incubated on ice for 20 minutes. Heat shock was applied at 42°C, 0 rpm, for 90 seconds, and then the mixture was cooled on ice for 2 minutes. 900 µl of SOC medium was used to recover the cells at 37°C, 800 rpm, for 1 hour. Serial dilutions ($10^{-1}$,$10^{-2}$, and $10^{-3}$) of the recovered cells were made, and 80 µl of each dilution was spread onto an LB plate containing carbenicillin and incubated at 37°C overnight. The procedure was repeated using different concentrations of pUC19: 0.1, 0.2, 4, 6, and 8 ng/µl. The next morning, the colonies were counted, and the transformation efficiency was calculated.

### 3.2.5 Calculation of Transformation Efficiency

Transformation efficiency (TE) is defined as the number of colony-forming units (cfu) produced by 1µg of plasmid DNA. It is calculated as follows:

$$\text{TC (cfu)} = \frac{\text{The number of bacterial colonies} \times \text{dilution ratio} \times \text{original transformation volume}}{\text{Plated volume}}$$

$$\text{TE (cfu/µg)} = \frac{\text{TC}}{\text{Plasmid DNA (µg)}}$$

# 4 RESULTS

## 4.1 Comparison of Transformation Methods for BW25113 *E. coli* Cells Washed with TSS

Transformation and storage solution (TSS) is a buffer used to prepare competent *E. coli* cells (Chung et al., 1989). A recent study by Yang et al. (2022) reported improved transformation efficiencies using a slightly modified TSS buffer, including a salt solution containing potassium chloride, calcium chloride, and magnesium chloride (KCM) in the transformation step. When the BW25113 *E. coli* strain was transformed using this improved method, the transformation efficiency exceeded $10^9$ transformants/µg of DNA.

Following the improved TSS protocol by Yang et al., we aimed to test whether the reported transformation efficiencies for the BW25113 *E. coli* strain could be replicated in our experiments. The BW25113 *E. coli* strain was washed with TSS buffer and transformed using the KCM mixture as detailed by Yang et al. (see Materials and Methods, KCM transformation method). Our results indicated a transformation efficiency of 3.14 x$10^6$ **(Fig. 4)**, which was lower than expected. Additionally, alternative transformation methods involving heat shock and heat shock combined with KCM were explored, resulting in transformation efficiencies of 1.07 x$10^7$ and 9.86 x$10^6$, respectively **(Fig. 4)**. These findings suggest that the BW25113 *E. coli* strain, prepared using the improved TSS protocol and transformed via heat shock and heat shock with the KCM mixture, exhibited higher transformation efficiency than when only the KCM mixture was used. However, the observed differences between the transformation methods were not statistically significant, and the results did not align with those proposed by Yang et al.

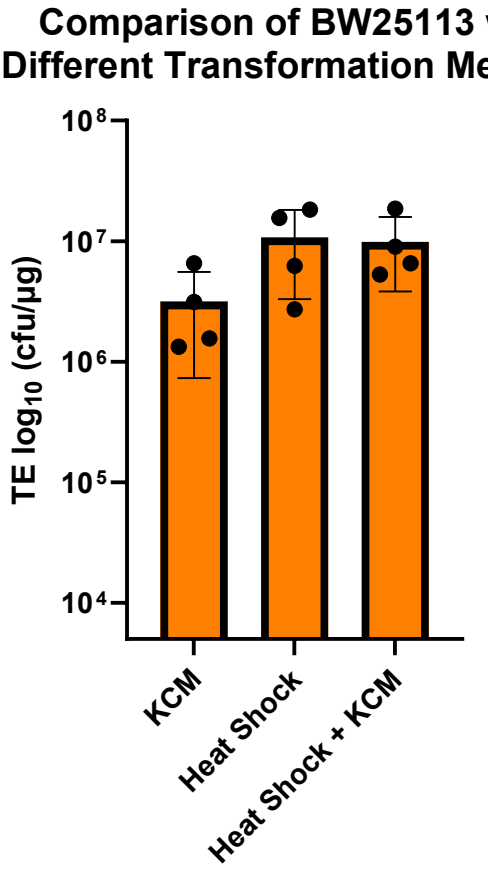


**Fig. 4** | BW25113 *E. coli* strain washed with TSS and transformed using KCM mixture, heat shock, and heat shock + KCM. Transformation efficiency (TE) of the different methods is measured by estimating $\log_{10}$ scale of colonies obtained for each 1 µg of plasmid (Y-axis). The shown data represent average mean values from four independent experiments with standard deviation (error bars) (n = 4). The BW25113 exhibited higher transformation efficiency with heat shock and heat shock + KCM transformation methods; however, the differences were not statistically significant, and the results did not align with those reported by the improved TSS.

## 4.2 Comparison of Transformation Methods for *ΔybeX::kan*$^{[BW]}$ *E. coli* Cells Washed with TSS

We also employed the improved TSS and KCM protocol by Yang et al. to compare its effect on *ΔybeX::kan* mutant strain. As seen in Fig. 4, the transformation efficiency of the BW25113 was 3.14 x $10^{6,}$ whereas that of *ΔybeX::kan*$^{[BW]}$ was 1.43 x $10^7$ **(Fig. 5)**. Under this protocol, the *ΔybeX::kan*$^{[BW]}$ showed an increased transformation efficiency with the KCM method. However, the difference between the transformation methods is insignificant, and the

transformation efficiency did not match that reported in the improved TSS protocol but fell within the range reported in the original protocol by Chung et al. (1989).

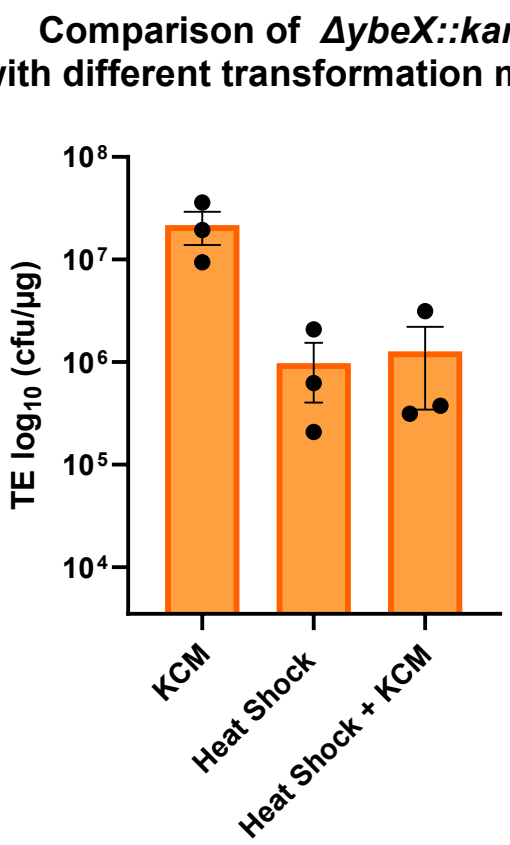


**Fig. 5** | Transformation efficiency (TE) of *ΔybeX::kan*$^{[BW]}$ *E. coli* strain, washed with TSS and transformed using KCM, heat shock, and heat shock + KCM. TE is measured as the $\log_{10}$ scale of colonies obtained per 1 µg of plasmid (Y-axis). The data represents mean values from three independent experiments, with standard deviations shown as error bars (n = 3). An unpaired t-test was performed to calculate P-values between the different methods, and the results were not statistically significant ($P > 0.05$).

## 4.3 Comparison of Transformation Methods for MG1655 and *ΔybeX::kan*$^{[MG]}$ *E. coli* Cells Washed with TSS

Following the improved TSS protocol, we also transformed MG1655 and *ΔybeX::kan*$^{[MG]}$ *E. coli* strains using KCM, heat shock, and heat shock + KCM. Across all transformation methods, the MG1655 maintained a transformation efficiency above $10^7$ **(Fig. 6)**. Meanwhile, the transformation efficiency of the *ΔybeX::kan*$^{[MG]}$ was below $10^7$ transformants/µg DNA. The heat shock and heat shock + KCM significantly decrease the transformation efficiency of the *ΔybeX::kan*$^{[MG]}$ mutant.

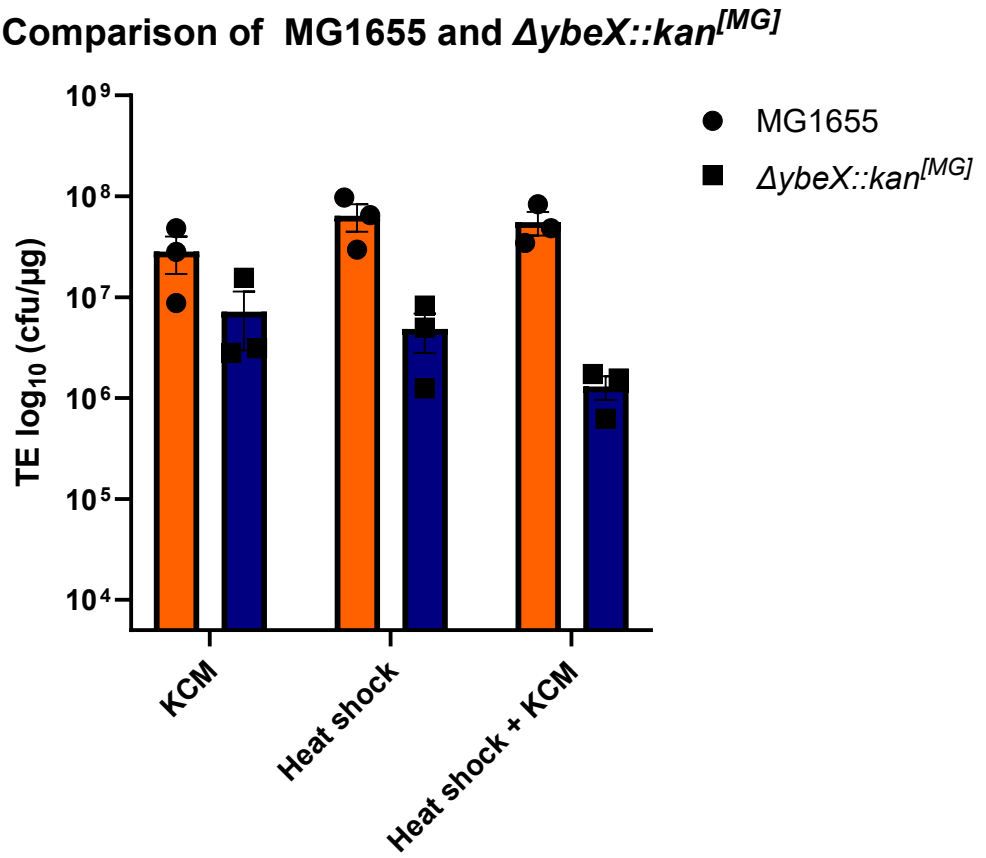


**Fig. 6** | Transformation efficiency (TE) of MG1655 and *ΔybeX::kan*$^{[MG]}$ *E. coli* strains, washed with TSS and transformed using KCM, heat shock, and heat shock + KCM methods. TE is measured as the $\log_{10}$ scale of colonies obtained per 1 µg of plasmid (Y-axis). The data represents mean values from three independent experiments, with standard deviations shown as error bars (n = 3). The MG1655 strain maintained a transformation efficiency above $10^7$ transformants/µgDNA across all transformation methods, while the *ΔybeX::kan*$^{[MG]}$ strain had a transformation efficiency below $10^7$ transformants/µgDNA. An unpaired t-test was performed to calculate the P-values for the differences between the two strains when transformed using heat shock and heat shock + KCM methods. The P-values were 0.0395 (*, $P < 0.05$) and 0.0203 (*, $P < 0.05$), respectively.

## 4.4 Comparison of BW25113 and *ΔybeX::kan*$^{[BW]}$ with TSS-HI

The improved TSS protocol by Yang et al. was further enhanced to TSS-HI by including an additional 140 mM $Mn^{2+}$ in the TSS buffer, along with the combination of the KCM salt solution and a heat shock during the transformation process (Yang, Liu, et al., 2022).

According to this protocol, the BW3KD *E. coli* cells washed with TSS-HI and transformed using KCM + heat shock showed high transformation efficiency, reaching 7.21 x $10^9$ transformants/µg DNA, compared to those transformed using only KCM. In an earlier

publication, Yang et al. reported that BW25113 cells prepared using the TSS method and transformed using only KCM had a transformation efficiency of up to $10^9$ transformants/ µg DNA, equivalent to that of BW3KD.

Following this, we used different transformation methods to compare BW25113 and *ΔybeX::kan*$^{[BW]}$ washed with the TSS-HI (**Fig. 7**). Our result shows that the BW25113 cells achieved a transformation efficiency of around 2.6 x $10^7$, both when only KCM was used in the transformation method and when heat shock was added to the KCM **(Fig. 7)**. In contrast, under the TSS protocol, BW25113 cells reached an efficiency of 1.07 x $10^7$ transformants /µg DNA with only heat shock **(Fig. 4)**. The highest transformation efficiency for *ΔybeX::kan*$^{[BW]}$ was 1.43 x $10^7$ with the TSS and 2.77 x $10^7$ with the TSS-HI, both achieved using only the KCM transformation method **(Fig. 5, 7)**. This suggests that the BW25113 cells washed with TSS-HI exhibit increased transformation efficiency both when heat shock is added to the KCM, as described by Yang et al., and when only KCM is used in the transformation. However, the differences between the transformation efficiencies achieved with the TSS and TSS-HI protocols were not significant, and none of the efficiencies matched the 7.21 x $10^9$ transformants/µg DNA reported by Yang et al.

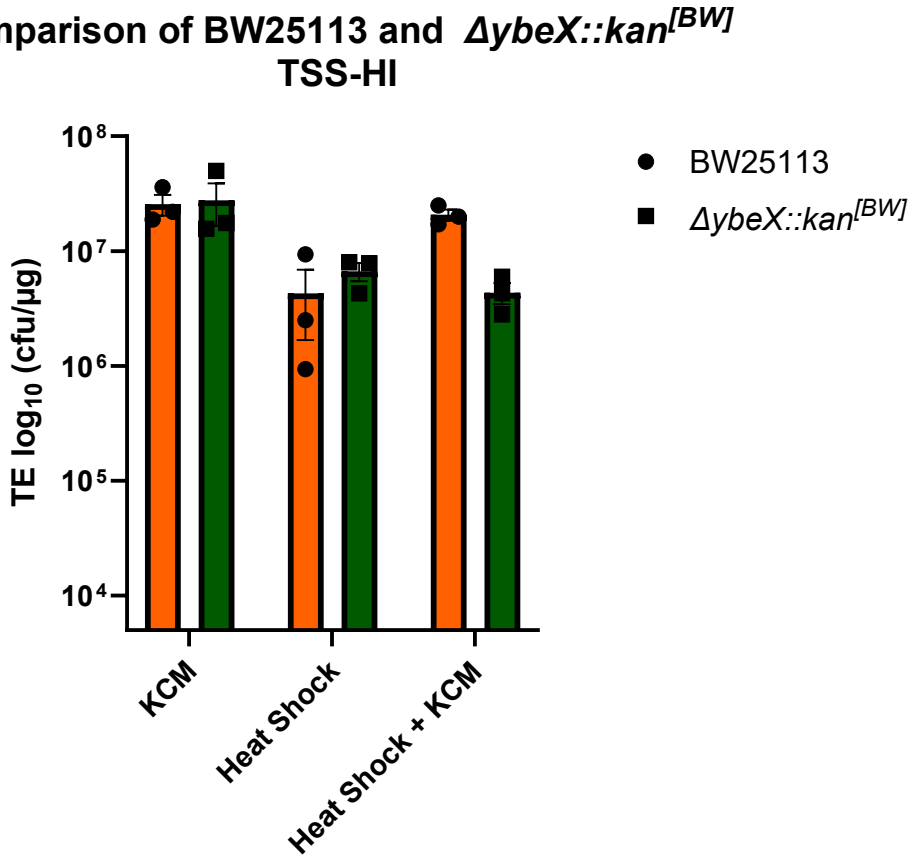

**Fig. 7** | Transformation efficiency (TE) of BW25113 and the *ΔybeX::kan*$^{[BW]}$ *E. coli* strain, washed with TSS-HI and transformed using KCM, heat shock, and heat shock + KCM. TE is measured as the $\log_{10}$ scale of colonies obtained per 1 µg of plasmid (Y-axis). The data represents mean values from three independent experiments, with standard deviations shown as error bars (n = 3).

## 4.5 Comparison of TSS and TSS-HI Quick Transformations

We further performed a quick transformation using the BW25113 and *ΔybeX::kan*$^{[BW]}$ washed with TSS and TSS-HI to determine if there would be transformants. The quick transformation was done by thawing frozen cells on ice for 10 minutes and gently resuspending them to achieve an even mixture. Then, the DNA was gently mixed with the cells and incubated on ice for 2 minutes. This was followed by a heat shock at 42°C with no agitation for 90 seconds. After this, SOC medium was added, and the cells were immediately plated **(Fig. 8)**. Surprisingly, all samples showed colonies with efficiencies of at least $10^5$ transformants/ µg DNA **(Fig. 9)**. The BW25113 prepared with the TSS-HI showed up to $10^6$ transformants/ µg DNA.

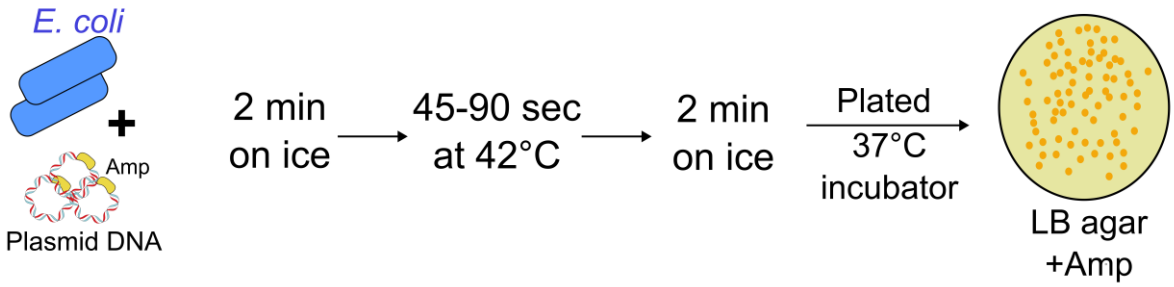


**Fig. 8** | Quick transformation process using chemically competent cells washed with TSS and TSS-HI. Frozen cells were thawed on ice. DNA was gently mixed with the cells and incubated on ice for 2 minutes, followed by a heat shock at 42°C for 45 to 90 seconds. The cells were then placed back on ice for 2 minutes before being plated on selective LB agar containing 100 µg/ml carbenicillin and incubated at 37°C overnight.

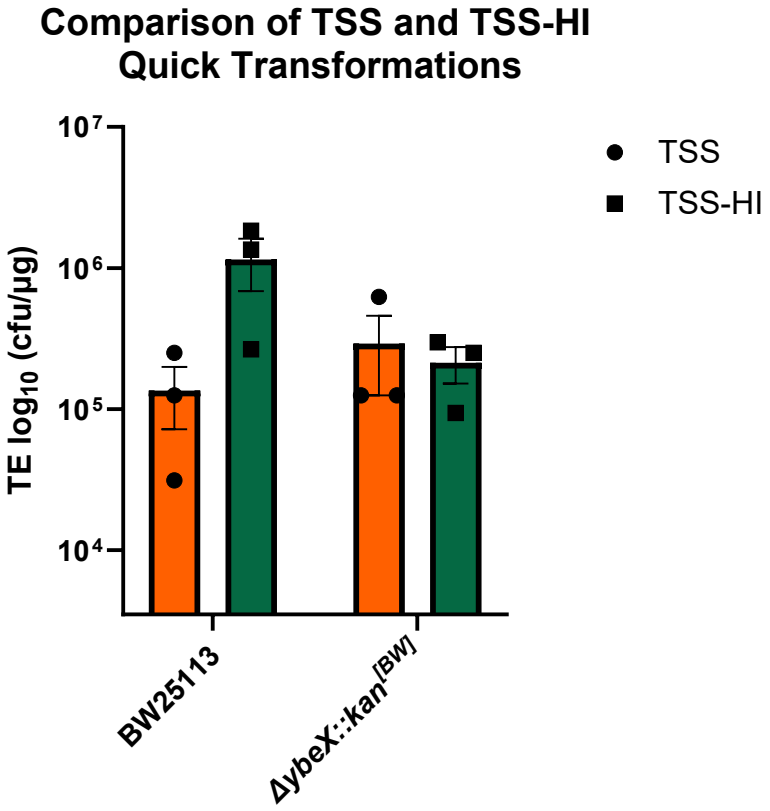


**Fig. 9** | Transformation efficiency (TE) of BW25113 and the *ΔybeX::kan*$^{[BW]}$ *E. coli* strain, washed with TSS and TSS-HI and transformed using KCM, heat shock, and heat shock + KCM. TE is measured as the $\log_{10}$ scale of colonies obtained per 1 µg of plasmid (Y-axis). The data represents mean values from three independent experiments, with standard deviations shown as error bars (n = 3). All samples showed colonies of at least $10^5$ transformants/µg DNA. BW25113 prepared with the TSS-HI showed up to $10^6$ transformants/µg DNA.

## 4.6 Discussion

In 1989, Chung et al. developed a single-step method for preparing competent *E. coli* cells using the Transformation and Storage Solution (TSS), which yielded between 5.35 x $10^7$ and 2.38 x $10^8$ transformants/µg DNA and eliminates the need for heat shock during the transformation process. In 2022, Yang et al. modified this method by including a salt solution containing potassium chloride, calcium chloride, and magnesium chloride (KCM) in the transformation step. The BW25113 *E. coli* strain washed with this improved method achieved unmatched transformation efficiencies of up to $10^9$ transformants/ µg DNA compared to the original method by Chung et al.

The two protocols share several similarities, including pH values, concentration and molecular weight of PEG, concentration of DMSO and divalent cations, incubation time, and cell growth phase. According to the original protocol, no transformants were found at pH below 4 or above 8. Transformants were observed within pH 4 to 8, with the highest transformation efficiencies between pH 6.4 and 6.8. Hence, a slightly acidic pH of 6.5 was used in the TSS buffer. Similarly, the improved protocol employed a final pH of 6.1.

Regarding PEG, the initial protocol observed that concentrations greater than 20% or less than 5% yielded no transformants. The most efficient transformations were achieved with 10% PEG. PEG with a molecular weight of 200 was ineffective, while PEG 1000 was modestly effective in inducing DNA transformation. PEG 3350 and 8000 in the TSS buffer provided equivalent results and yielded the highest transformation efficiencies. Chemical derivatives of PEG were ineffective in inducing transformation. Similarly, the improved protocol utilised 10% PEG 3350. Also, both protocols identified 5% DMSO in the TSS buffer as the optimal concentration for achieving high transformation efficiency.

In the original TSS protocol, $Mg^{2+}$ as $MgSO_4$ or $MgCl_2$ at a concentration ranging from 10 to 100 mM provided stable and high transformation efficiency, leading to the use of 20 or 50 mM $Mg^{2+}$ in the experiments. This was also true for the optimised protocol as $MgSO_4$ and $MgCl_2$ at 10 mM each were used. The old protocol noted that incubation on ice for 30 minutes resulted in the highest transformation efficiency; the new protocol also adopted this. Regarding the growth phase, the protocol of Chung et al. found that cells in the early exponential phase provided the highest transformation efficiencies of up to $10^8$. Other growth stages, including the stationary phase, yielded transformation efficiencies ranging from $10^5$ to $10^7$. Similarly, cells at the early growth phase yielded optimal results in the Yang et al. experiment.

Despite their similarities, the protocols showed differences in their approaches. The improved protocol included 10% glycerol in the TSS buffer, which was not present in the original protocol. In the transformation step by Chung's original method, the plasmid is added directly and mixed with the cells; meanwhile, In the enhanced protocol, 5x KCM was mixed with the plasmid and $ddH_2O$ so that the final concentration of KCM in the reaction mixture was 1x and then mixed with the cells in a 1:1 ratio.

In Summary, Chung et al.'s 1989 TSS buffer consists of LB-HCl at pH 6.5, 10% PEG 3350 or 8000, 5% DMSO, and 20 or 50 mM $Mg^{2+}$ ($MgSO_4$ or $MgCl_2$). In the transformation step, DNA is directly mixed with the cells. Meanwhile, the TSS buffer by Yang et al. (2022) contains LB-HCl at pH 6.1, 10% PEG 3350, 5% DMSO, 10% glycerol,10 mM $MgSO_4$ and 10 mM $MgCl_2$.

In the transformation step, 5x KCM is mixed with DNA and dd$H_2O$ to a final concentration of 1x KCM in the reaction mixture.

In December 2022, Yang et al. introduced another improved TSS method named TSS-HI. This method combines the advantages of the TSS, Hannahan, and Inoue protocols to simplify the preparation of competent cells and achieve high transformation efficiency. The BW3KD *E. coli* strain prepared with this method yielded up to 7.21 x$10^9$ transformants/ ug DNA.

Several similarities exist between the TSS-HI method by Yang et al. and the previous TSS protocol. As in the earlier TSS protocol, 1x KCM was also introduced in the transformation step. The cells were grown to an $OD_{600nm}$ of 0.55 and concentrated 50x. Also, cells can be snap-frozen in liquid nitrogen and stored at -80°C. All components in the previous TSS buffer, which include LBHCl [pH 6.1], 10% PEG 3350, 5% DMSO, 10% glycerol and 20 mM MgCl2, were all present in the TSS-HI.

The differences in the TSS-HI method include an additional 140 mM $Mn^{2+}$ in the TSS buffer. Also, after adding the KCM mix to the cells in the transformation step, a heat shock at 42°C for 90 seconds (a method derived from the Hanahan protocol) was introduced. Cells can be cultured at 18°C or 37°C (Inoue, Hanahan, TSS method) without any change in transformation efficiency. Furthermore, cells can be recovered in LB, LB+$Mg^{2+}$, or SOC without affecting the transformation efficiency.

In summary, the TSS-HI transformation buffer contains LB-HCl [pH 6.1], 10% PEG 3350, 5% DMSO, 10% glycerol, 20 mM $MgCl_2$, 140 mM $MnCl_2$. In the transformation step, 1x final concentration of KCM in a reaction mixture and a heat shock at 42°C for 90 seconds was introduced.

Achieving high transformation efficiency is crucial in various molecular cloning processes. However, it's equally important to develop a protocol that is cost-effective, reliable, and easy to reproduce. Despite reports of achieving $10^9$ transformants/µgDNA using the two improved TSS and TSS-HI protocols (Yang, Liu, et al., 2022; Yang, Yu, et al., 2022), we were unable to replicate these results. Both *E. coli* wild-type strains (BW25113 and MG1655) and their *ΔybeX::kan* mutant strains were washed with the improved TSS and TSS-HI and transformed via KCM, heat shock, and heat shock + KCM as detailed. Our results did not match the reported values. Although TSS-HI increased the transformation efficiency of BW25113, the difference between the two methods (TSS and TSS-HI) was insignificant. Clearly, there is no substantial difference in transformation efficiency when using either method. However, the transformation efficiencies observed in our experiment closely align with the original protocol by Chung et al.

(1989), indicating that this original protocol can be deemed reliable and easily reproducible. Therefore, conducting cross-laboratory studies to compare the reproducibility and efficiency of different transformation protocols could ensure that the findings are not specific to just one laboratory condition and can be trusted more broadly.

An interesting discovery is that a quick transformation with competent cells prepared using these two improved protocols produces a transformation efficiency of up to $10^6$ transformants/ µg DNA, which is suitable for most cloning purposes. The quick transformation eliminates the need for incubation on ice and cell recovery, which typically takes 30 minutes and 1 hour, respectively.

## Conclusion

In conclusion, we could not reproduce $10^9$ transformants/µgDNA as reported for the improved TSS and TSS-HI protocols, questioning the reliability and reproducibility of these protocols in improving transformation efficiency. Competent cells prepared with these protocols achieve transformation efficiencies of up to $10^7$ transformants/µgDNA aligning with the transformation efficiencies reported in the original protocol (Chung et al., 1989). A quick transformation using competent cells prepared with the two improved TSS and TSS-HI can produce up to $10^6$ transformants/ µg DNA.

# SUMMARY

Bacterial transformation is the ability of bacteria to uptake foreign DNA. Achieving high transformation efficiency is needed for various molecular cloning processes, such as constructing DNA libraries. However, high transformation efficiency can be challenging with many bacterial strains. Therefore, competent cells treated to enhance plasmid uptake are often used. Several techniques have been developed to enhance plasmid uptake in *E. coli* cells. Among these is an improved transformation and storage solution (TSS) protocol (Yang, Yu, et al., 2022), which involves using a TSS buffer to wash *E. coli* cells to make them competent. The transformation method employs a salt solution containing potassium chloride, calcium chloride, and magnesium chloride (KCM) to facilitate DNA uptake. Another technique, the improved TSS-HI protocol, adds 140 mM $Mn^{2+}$ to the TSS buffer. This method also uses the KCM salt solution combined with a heat shock for transformation (Yang, Liu, et al., 2022).

This thesis utilised these two improved protocols to prepare and transform chemically competent *E. coli* cells. Single colonies of BW25113 and MG1655 *E. coli* wild types and their *ΔybeX::kan* mutant strains were grown overnight in LB medium. The following morning, the cells were diluted and cultured until the optical density at 600 nm reached between 0.25 and 0.3. The cells were then harvested by centrifugation and washed in the TSS and TSS-HI buffers. For transformation, three methods were tested. In the first method, we tested the KCM method, where a mixture of 1x KCM, DNA, and dd$H_2O$ was gently mixed with the competent cells, followed by incubation on ice and at room temperature. The cells were then recovered in SOC medium before being plated. Secondly, we performed a heat shock transformation where the DNA was directly mixed with the cells, incubated on ice, and then subjected to a heat shock. Then, the cells were recovered in SOC medium and plated. Thirdly, we combined the KCM and heat shock by mixing the cells and the KCM mixture in a 1:1 ratio, followed by a heat shock. The cells were then recovered and plated. Lastly, a quick transformation method was tested using competent cells prepared with the TSS and TSS-HI protocols. This involved directly mixing the DNA with the cells, applying a heat shock, and plating the cells without recovery.

Our results indicated that the reported values for the improved protocols were not reproducible. However, competent cells prepared with these protocols achieved transformation efficiencies consistent with the original protocol (Chung et al., 1989). Interestingly, the quick transformation method using cells prepared with the TSS and TSS-HI protocols yielded transformation efficiencies sufficient for common molecular cloning experiments.

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