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CTAB-mediated, single-step preparation of competent *Escherichia coli*, *Bifidobacterium* sp. and *Kluyveromyces lactis* cells

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ABSTRACT

An efficient and reproducible method for transformation depends on the competency of the organism. We have developed a simple method for the preparation of competent *Escherichia coli*, *Kluyveromyces lactis*, and *Bifidobacterium* sp. by using a buffer containing cetyl trimethyl ammonium bromide (CTAB) and permits efficient uptake of plasmid DNA and ligation-reaction products. Cells are harvested, washed, mixed with 1–10 μg/ml CTAB, incubated, and followed by a buffer wash. For long-term storage of competent cells, bacteria may be frozen in 10% glycerol without the addition of other components. The transformation process is very simple; plasmid DNA and the cells are mixed and incubated for 5–60 min at 4 °C; no heat pulse is required, and the duration of incubation at 4 °C is not crucial.

Introduction

Various methods for permeabilizing cells (chemical- and electrical pulse-mediated) to induce DNA uptake have been devised. For example, Mandel and Higa (1970) first demonstrated that treatment of *Escherichia coli* with $CaCl_2$ renders the cells competent for uptake of bacteriophage DNA. Thereafter, it was shown that the same technique may be used to transform *E. coli* with bacterial chromosomal DNA or plasmid DNA (Oishi and Cosloy, 1972). Since these seminal studies, several important factors have been expounded

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$CaCl_2$ (Cohen et al., 1972), treating bacteria with dimethyl sulfoxide (DMSO), hexamine cobalt, or dithiothreitol in the presence of monovalent and divalent cations by Dargert and Ehrlich (1979). A more recent modification for increasing transformation efficiency involves the use of polyethylene glycol (PEG) (different molecular weights 200–15,000). The efficiency of this process is considered to be, approximately two orders of magnitude lower than that obtainable by other methods (Hanahan, 1983). Chung et al. (1989) introduced the TSS (transformation and storage solution) method and the efficiency was considered to be low. Most of the existing methods emphasize easy (minimized steps, not required a skilled man power, etc.) transformation of plasmid DNA. Serafini et al. (2012) reported a transformation method specifically made for bifidobacteria. The method involves many washing steps and it requires cultivation of bifidobacteria in the presence of high concentrations of complex carbohydrates, requires use of bifidobacteria at the exponential growth phase, osmotic stabilizers are essential, and cells should be maintained at higher temperatures. Therefore, the method is considered to be laborious, and cost effective. However, little attention has been paid for developing a universal method (for prokaryotes and lower eukaryotes) having high-efficiency, reproducible transformation of plasmid DNA or products of ligation-reaction mixtures.

In the present study, we discuss an effective process that uses cetyl trimethyl ammonium bromide (CTAB) for preparation of competent bacterial cells (*E. coli*, and *Bifidobacterium* sp.) and yeast (*Kluyveromyces lactis*). This procedure is convenient, rapid and routinely yields 10^5–10^9 transformants per microgram of plasmid DNA. In addition, the bacteria prepared by this method may be frozen and stored for future use. Thus, this transformation system is universal and advantageous because of its simplicity and multiple uses.

Materials and methods

Chemicals

CTAB, PI (propidium iodide) and PMA (propidium monoazide) were purchased from Sigma-Aldrich (Chemie GmbH Industriestrasse 25 CH-9471, Buchs Sg-Switzerland). *Bifidobacterium* sp. broth, Luria–Bertani (LB) broth, YCB agar, antibiotics (ampicillin, tetracycline, and erythromycin) and DNA staining dyes were purchased from Merck (Darmstadt, Germany).

Bacterial strains and growth conditions

E. coli (BL21DE3) was received from Merck and Co. (Whitehouse Station, NJ). *E. coli* was grown in LB broth at 37 °C, 200 rpm for 12–16 h. *Bifidobacterium* sp. (procured from DSM German culture collection *Bifidobacterium bifidum* DSM20456, *Bifidobacterium breve* 20213, and *Bifidobacterium asteroids* DSM20089) was grown in bifidobacterial broth (15 g/l peptic digest of animal tissue, 10 g/l of casein enzymatic hydrolysate, 20 g/l yeast extract, glucose 20 g/l, tomato juice solids 16.65 g/l, and polysorbate 80.2 g/l pH 6.8, obtained from Merck, Darmstadt, Germany) in an anaerobic condition for 24–36 h and without shaking at 37 °C. The yeast *K. lactis* was obtained from New England Biolabs (Ipswich, MA) along with the *K. lactis* expression kit. They were grown on YCB medium (yeast carbon base containing 5.85 g/l YCB medium powder, 10 g Bacto agar/l and they were autoclaved for 20 min at 121 °C) for 24–36 h without shaking at 24–37 °C. Table 1 illustrates the strains and plasmids used in this study.

Fluorescence activated cell sorting (FACS) and fluorescence microscopy studies

E. coli, *Bifidobacterium* sp. and *K. lactis* were first treated with different concentrations of CTAB, and subsequently they were treated with PMA and PI (50 μM). The resulting cells were used to investigate cell membrane damage and viability. Cells were treated with 10 μg/ml CTAB followed by PI staining. Cells were further washed to remove excessive stain. The same cells were subjected to microscopy and FACS. Un-treated cells were considered as a negative control. Cells were photographed at 1000× with an Olympus I X 71 fluorescent microscope with an inbuilt camera.

Samples were analyzed using a FACS Caliber flow cytometer (BD FACS caliber flow cytometer, USA) to detect light scattering and fluorescence emission patterns. Cytometer samples were illuminated after passing air-cooled argon ion laser (488 nm, 15 mW) leading to detection of fluorescence using common filter

Table 1
Strains and plasmids used in this study.

Bacterial strain	Source	Reference
B. catenulatum	Fecal isolate 14 month old baby	This study
B. breve	DSM 20213	This study
B. bifidum	DSM20215	This study
B. asteroides	DSM20089	This study
E. coli (DH5-α, JM109)	NEB C2987 H, P9751	This study
XL1blue	Commercial source Stratagene 200268	This study
BL21λDE3	Commercial source Novagen-69387	This study
Enterococcus gallinarum	MTCC7049	This study
K. lactis	Commercial source New England Biolabs-GG799	This study
Plasmids	**Description**	**Reference**
pET23(d)	T7 promoterbased/Novagen	Novagen
pETXFP	xfp gene cloned in pET23(d)	This study
pTraj1[a]	Bifidobacterial expression plasmid Un-published	This study
pTraj2[a]	Bifidobacterial expression plasmid Un-published	This study
p*Klac2*	Commercial source NEB N3742	This study

B. refers to *Bifidobacterium*.
[a] Indigenous bifidobacterial expression plasmids.

configuration (such as the 525-nm band pass filter for FL1, 575-nm band pass filter for FL2, and 620-nm band pass filter for FL3 and FL4). For these experiments, we collected data from more than 10,000 cells and processed. The data acquisition was carried-out by using Cell quest pro software provided by the manufacturer and analysis was done by using DIVA and Cell quest pro software. The standard photomultiplier parameters were set such as linear mode for light scatter data collection and in the logarithmic mode for fluorescence data. Light scattering was set sufficiently large to trigger the flow-cytometer for data collection. Excessive background on stained and un-stained cells was reduced by increasing the threshold limits.

PMA, PI cross-linking experiments

We performed PMA cross-linking experiments for CTAB-treated organisms. *E. coli*, *Bifidobacterium* sp., and *K. lactis* were subjected to 1–10 μg/ml CTAB to differentiate among the healthy cells and cells with faulty membrane. PMA was dissolved to make 20 mM stock in 20% DMSO of which 50 μM was used as working concentration. Bacterial samples (500 μl) were treated with 50 μM PMA, and 10 μg/ml PI was dissolved in double distilled water at 15 mM stock concentration 30 μM working concentration incubated for 5 min in the dark, with occasional gentle mixing followed by light exposure for 5 min using a 500 W halogen light source. Excessive and non-specifically bound PMA was further removed by distilled water wash. *E. coli*, *Bifidobacterium* sp. and *K. lactis* were further used for PCR amplification of whole 16S rRNA (1500 bp), partial 16S rRNA (low molecular weight; 550 bp) and xfp (2475 bp).

Plasmids used/constructed

The plasmid vectors that may survive and replicate in *E. coli* and *Bifidobacterium* sp., (pTraj 1 and 2 both 12.5 kbp having p15A and AMB1 Ori sites. Both of them are *Lactobacillus* plasmids.) were selected for transformation (Table 1). pET23 (d) (Novagen MD, USA) carrying xfp (xylose-6-phosphate phosphoketolase, 2.5 kbp fragment cloned at Nco1 and Xho1 sites, unpublished data) totaling ~7 kbp was used for transformation of *E. coli* alone. p*Klac* (shuttle vector) of 9 kbp was used for transformation of *E. coli*, and *K. lactis* cells. The

ligation mixtures of the xfp gene with pTraj1 and 2 (12.5 kbp), pET-xfp (7 kbp) and p*Klac*-xfp (12 kbp approx. plasmid was received from New England Biolabs, MA, USA. Table 1) vector ligation reactions were used for transformation (xfp gene was cloned in pET23(d), pTraj1 and 2, and p*Klac* plasmids. Un-published data).

PCR amplifications

PCR amplification of f6ppk (fructose 6 phosphate phosphoketolase) from control *E. coli* cells, *Bifidobacterium* sp., and *K. lactis* treated with 1 or 10 μg/ml CTAB was followed, similar to colony screening method of PCR. The following parameters were used for amplification: 98 °C initial denaturation for 30 s, 98 °C denaturation for 5 s, annealing at 64 °C for 5 s, extension at 72 °C for 50 s for 33 cycles and final extension at 72 °C for 1 min with *Phire DNA polymerase* (Finnzymes, Woburn, MA) at standard PCR conditions. The primers were synthesized commercially by Life Technologies Inc., USA and used at a 20 picomole concentration each. Primer sequences are as follows: Forward: $^{5'}$ TCC ATG GTG ACG AGT CCT GTT ATT GGC ACC $^{3'}$ and reverse: $^{5'}$ ATA GCT TTC AGT CTC GTT GTC GCC AGC GGT $^{3'}$. Plasmid vectors such as pET containing the gene of interest and pBluescript (Stratagene Inc.) Vector and other necessary plasmids were used for the study.

Purification of plasmid DNA

A standard alkaline lysis method of Sambrook et al. (2000) was followed.

Transformation procedure

E. coli (BL21DE3, XL1blue, and DH5-α), *Bifidobacterium* sp., and *K. lactis* were grown in their respective media to (OD_{600} 0.4–0.6) an exponential phase. Cells were harvested by centrifugation at 6000 ×g for 10 min at 4 °C and re-suspended at one tenth of their original volume in ice-cold 1 mM HEPES buffer. The above step was repeated and cells were re-suspended in 1 mM HEPES buffer that contained 1, 5 or 10 μg/ml CTAB. Harvested cells were extensively washed with water and re-suspended in 10% glycerol solution. Cells were grown in 500 ml broth washed twice followed by re-suspension in a 1.5 ml final volume of 10% glycerol. A 0.1-ml aliquot of cells was transferred into a cold polypropylene tube, mixed with 1–2 μl plasmid DNA (10–50 ng) and incubated at 4 °C for 5 min. The transformation mix was transferred to electroporation cuvettes (Bio-Rad, Hercules, and CA), and cells were electroporated in a Bio-Rad Gene Pulsar according to the manufacturer's directions for transformation of bacteria, and yeasts. The following fixed parameters of the Bio-Rad Gene Pulsar were followed, for electroporation of *E. coli* 0.2 cm cuvette, resistance 25 μF, and 200 V, bifidobacteria 0.2 cm cuvette, 25 μF resistance and 200 V, and yeast 0.2 cm cuvette, 25 μF resistance and 200 V. Subsequently, 0.9 ml of fresh medium was added, and the cells were grown at 37 °C. *E. coli* cells with shaking at 200 rpm for 1 h, *Bifidobacterium* sp., and *K. lactis*, without shaking were incubated. This step initiates the phenotypic expression of the antibiotic resistance factor, so that the colonies of the transformants can appear on the antibiotic (ampicillin, 50 mg/ml stock, and 100 μg/ml working concentration) containing plates. For comparison, competent cells were also made by the TSS method of Chung et al. (1989) and by $CaCl_2$.

Transformants were selected by plating cells (in triplicate) on appropriate plates (*Bifidobacterium* sp. agar plates, YCB plates, LB agar plates) with appropriate antibiotic supplementation (according to the resistance gene encoded by the plasmid). Transformation efficiencies (number of transformants per microgram DNA) were determined after incubation of the plates at 30–37 °C for 12–24 h (depending on the organism) (Table 2). To validate the authenticity of the transformants plasmid DNA was purified (by alkaline lyses mini preps, Nco1/Xho1 restriction enzymes were procured from Fermentas/Thermo scientific, PA, USA and were used as per the directions). For long-term storage, the competent cells were immediately frozen and stored in 10% glycerol. Frozen cells were thawed on ice and used immediately in the transformation assay.

Effect of CTAB on the cell viability

Before finalizing the concentrations of CTAB required, experiment was performed to find out the optimal concentration required to permeabilize cells without compromising their cell wall integrity and viability. The cells were incubated in different concentrations of CTAB (Table 3) for 15 min and checked for their viability, after removing non-specifically bound CTAB.

Table 2
Effect of different conc. of CTAB on transformation efficiency.

Serial No.	Conc. of CTAB μg/ml	Transformation efficiency* No. of transformants × 10^{5-9}/per μg DNA	Lysis of cells	Growth of cells
A	10	– 0 –	Yes	Exponential phase
	5	– 0 –	Yes	Exponential phase
	1	$4.1 \pm 0.18 \times 10^9$	Partial	Exponential phase
B	10	$1.10 \pm 0.17 \times 10^5$	10–20%	Exponential phase
	5	$4.10 \pm 0.09 \times 10^4$	No	Exponential phase
	1	$3.8 \pm 0.10 \times 10^5$	No	Exponential phase
C	10	$1.30 \pm 0.08 \times 10^8$	Less than 1%	Exponential phase
	5	$2.40 \pm 0.09 \times 10^8$	No	Exponential phase
	1	$3.1 \pm 0.10 \times 10^8$	No	Exponential phase

A. *E. coli* (DH5α, XL1blue, BL21DE3) JM109, B. *B. catenulatum*, and C. *K. lactis* cells were incubated for 15 min in CTAB for all the above methods.
* Mean ± SEM of triplicate plates.

Cell viability/membrane integrity

In order to understand the adverse effects on nucleic acids (genomic DNA and plasmid DNA) of recombinant cells upon their exposure to different concentrations of CTAB, *E. coli* containing pET with xfp gene cloned, *Bifidobacterium* sp. containing pTraj1 plasmid and *K. lactis* cells containing p*Klac* plasmid were incubated with different concentrations of CTAB. Further, PMA (50 μM) and PI (10 μg/ml) studies were carried out on these recombinant cells to investigate cell membrane damage and viability. The cells were subjected to PCR amplification of the 16S rRNA locus (full length and a shorter, conserved region), fructose-6-phosphate phosphoketolase (f6ppk), and plasmid purification. The cells were also subjected to fluorescence-activated cell sorter (FACS) analysis, and fluorescence microscopy to understand the cell membrane integrity and, status of genomic DNA after exposure to CTAB.

Results

Preparation of competent cells with CTAB

Our rapid, one-step competent cell preparation protocol involves pre-treating cells with the ionic detergent CTAB. It was understood that 1–10 μg of CTAB is suitable and efficiently permeabilizes cell wall without compromising its cell-wall integrity and viability (Table 2). The effect of CTAB on treated and un-treated cells (range of concentrations of CTAB 1–10 μg/ml) was validated by plasmid DNA transformation by electroporation (7–13 kbp) and by transforming a ligation mix of digested plasmid and insert DNA (7–13 kbp). No, or

Table 3
Effect of different concentrations of CTAB on *E. coli* and *B. catenulatum* cell wall integrity and viability.

Cells	0 time (no. of cell)	10 minute incubation (no. of cells)	Reduction in viability after 10 minute incubation (%)
E. coli	11×10^9	12×10^9	0
E. coli with CTAB (10 μg/ml)	11×10^9	9.4×10^9	14.1
E. coli with CTAB (5 μg/ml)	11×10^9	11.004×10^9	8.18
E. coli with CTAB (1 μg/ml)	11×10^9	11×10^9	0
B. catenulatum	11×10^9	11.2×10^9	0
B. catenulatum with 10 μg/ml CTAB	11×10^9	10.24×10^9	6.90
B. catenulatum with 5 μg/ml CTAB	11×10^9	10.86×10^9	1.2
K. lactis	11×10^9	11×10^9	0
K. lactis with 10 μg/ml	11×10^9	10.89×10^9	1
K. lactis with 5 μg/ml	11×10^9	11×10^9	0

negligible number of transformants were observed with bacterial cultures that were un-treated with CTAB or with bacteria that had undergone 6–7 washing steps to achieve permeabilization (Table 4) Competency is increased by increasing the washing step. However, transformants observed were very less when plasmid was transformed in both the cases (with and without CTAB treated). *E. coli*, *Bifidobacterium* sp., and *K. lactis*, pre-treated with CTAB used for competent cell preparation/transformation gave reasonably good transformation efficiency. Hence, we conclude that the use of permissible concentrations of CTAB to permeabilize cell membranes is an effective alternative to the time-consuming traditional procedures and increases the transformation efficiency of (7–13 kbp) plasmid DNA, especially a ligation mixture of plasmid DNA and the insert (Table 5).

We observed that, as the concentration of CTAB in the reaction increased, cell wall integrity decreased, further decreasing the cell viability and subsequent increased cell wall permeability leading to greater efficiency of transformation. A similar process was observed for transformation of *Bifidobacterium* sp., and *K. lactis* for which no cell lysis was seen (Fig. 1A, B, C). The following experiments clearly demonstrated that a particular concentration of CTAB was optimal for cell permeabilization but not for lysis (Table 2). We succeeded in establishing optimal concentrations of CTAB to create competent cells for transformation of plasmid DNA (Tables 2 and 4).

E. coli, *Bifidobacterium* sp., and *K. lactis* at their exponential growth phase are subjected to different concentrations of CTAB (1–10 μg/ml) and incubated for 15 min at 4 °C (Table 2). CTAB at a maximum concentration of 10 μg/μl has shown to lyse all the cells of *E. coli* and (10% of cells only of bifido at 10 μg/ml of CTAB) *Bifidobacterium* sp. and some of the *K. lactis* cells (less than 1% at 10 μg/ml) (Fig. 1A, B, C). The concentration range of 5–10 μg/ml was considered to damage the cell wall of *E. coli*, *Bifidobacterium* sp. and optimal for *K. lactis*, for which the transformation efficiency was considered the highest and cell wall was intact. Hence, we consider that the concentration of 1 μg/ml was suitable for transformation of *E. coli* and *Bifidobacterium* sp. and 10 μg/ml for *K. lactis* (Table 2).

Dependence of transformation efficiency on stage of cell growth at harvesting and cell concentration

We found that the transformation efficiency of *E. coli*, *Bifidobacterium* sp., and *K. lactis* was dependent upon the cell density at the time of harvesting. Experiments were followed with cells harvested at two different stages of their life cycles such as the exponential and late exponential stages. The best possible results among them were selected and presented here. Bacteria subjected to 5 and 10 μg/ml CTAB harvested at the exponential phase of the growth curve were capable of undergoing the transformation but cell wall integrity was not maintained. We found that bacteria harvested in the exponential phase were lysing upon 5–10 μg CTAB treatment, causing the fall of turbidity, which is due to lower scattering of light by the lysed cell debris. However, the bacterial cells treated with a CTAB concentration of 1 μg/ml were resistant and seen to have good transformation efficiency (Table 2). However, the results were much better for *Bifidobacterium* sp. and *K. lactis*, for which the transformation efficiency was high for the exponential phase and they were observed

Table 4
Transformation efficiency of various organisms.

Strain	Transformation efficiency No. of transformants × 10^{5-9}/μg of DNA
A. XL1blue	$5.1 \pm 0.16 \times 10^9$
DH5α	$4.8 \pm 0.18 \times 10^9$
BL21DE3	$5.8 \pm 0.16 \times 10^9$
JM109	$4.5 \pm 0.11 \times 10^9$
B. *B. animalis* subsp. *lactis*	$3.2 \pm 0.19 \times 10^5$
B. indicum	$2.9 \pm 0.17 \times 10^5$
C. *Kluyveromyces lactis*	$3.1 \pm 0.12 \times 10^7$
D. XL1blue	$4.9 \pm 0.12 \times 10^7$
E. XL1blue	$2.5 \pm 0.1 \times 10^9$

All the above cells were subjected to electroporation/Gene pulsing. A represents *E. coli* strains, B corresponds to bifidobacterial strains, C corresponds to yeasts, and D represents XL1blue competent cells made through TSS method of Chung et al. (1989) (−ve control). E represents cells treated with $CaCl_2$ control experiment for comparison. For A and B samples 1 μg, and for sample C 10 μg of CTAB have been used. *B. breve*, *B. bifidum*, and *B. asteroids* were also used but not represented as the efficiency was not encouraging.

Table 5
Strains and plasmids used in this study.

Bacterial strain	Strain no.	Reference
B. catenulatum	Fecal isolate 14 month old baby	This study
E. coli (DH5-α)	NEB C2987 H	This study
XL1blue	Stratagene	This study
BL21DE3	Novagen	This study
Enterococcus gallinarum	MTCC	This study
K. lactis	NEB	This study
Plasmids		**Reference**
p*ET 23(d)*		Novagen
p*ETXFP*		This study
p*Traj1*		This study
p*KLac*		This study

B. refers to *Bifidobacterium*.

to be partially resistant to higher CTAB concentrations. In *K. lactis* and *Bifidobacterium* sp., the cell wall integrity and transformation efficiency were higher than that of *E. coli* at a concentration of 1–10 μg/ml CTAB. However, partial lysis of *Bifidobacterium* sp. (10–20%) was still observed at a 10 μg/ml CTAB concentration. CTAB at 10, 5 and 1 μg/ml was not detrimental for *K. lactis*, but partial lysis was observed at 10 μg/ml (>1%) and the transformation efficiency was still high. The results pertaining to the CTAB and $CaCl_2$ methods conclude that a 1 μg CTAB concentration is optimal and also does not disrupt the cell membrane and observed to give high transformation efficiency around 10^5–10^9 for *E. coli*, *Bifidobacterium* sp., and *K. lactis*. However, $CaCl_2$ treated cells as well as cells prepared with the transformation and storage solution (TSS) method were able to give lesser transformation efficiency as shown in Table 4 and they are not comparable as both of them are non-CTAB methods and are totally different.

PMA/PI studies of E. coli, Bifidobacterium sp. and K. lactis treated with CTAB

After treating the cells with different concentrations of CTAB, cell wall integrity determination was crucial. The cell viability could be validated through morphological changes and membrane permeability changes or the physiological state inferred from exclusion of certain dyes or uptake and, retention of others. PI, PMA and EMA (Ethidium mono-azide) are known readily to enter the cell whose membrane is damaged. They are DNA-intercalating dyes that penetrate only the cells with compromised/faulty membrane but not viable cells with cell membranes intact (Nocker et al., 2006). PI, PMA and EMA are used for DNA detection from live cells and treatment of samples with PMA is a standard method. The principle behind this is simple, membrane integrity as a general sign of validity.

We exploited this phenomenon to examine the integrity of *E. coli*, *Bifidobacterium* sp. and *K. lactis* treated with different concentrations of CTAB. CTAB-treated cells were subjected to PI labeling to investigate their viability. PI-stained cells were processed via FACS analysis (Fig. 2). The data clearly showed that the granularity of the CTAB-treated cells increased with an increase in the concentration of CTAB during which the cell lysis also increases with CTAB concentration. Fig. 2 clearly shows the effect of CTAB and amount of PI bound to cells. Based on this study, we conclude that as the concentration of CTAB increases, the lysis also increases. Hence, the number of PI stained cells also increases. This phenomenon could be seen in the figure as well as in the inset of the figure with a chromatogram. It is also very well understood from this study that 1 μg CTAB treated cells are not damaged and behave as un-treated cells. Microscopy of CTAB-treated cells followed by PI labeling further validated the above results (Fig. 1A, B and C). As stated earlier PMA binding inhibits PCR amplification, which further determines the cell wall disintegration. PMA free DNA fragments were validated through PCR amplification and purification of plasmid DNA, which further determines no binding of PMA. Therefore, cell wall is intact (Lee and Levin, 2007; Gedalang and Olson, 2009; Feng et al., 2008; Nocker et al., 2006; Nocker and Camper, 2009; Pan and Breidt, 2007).

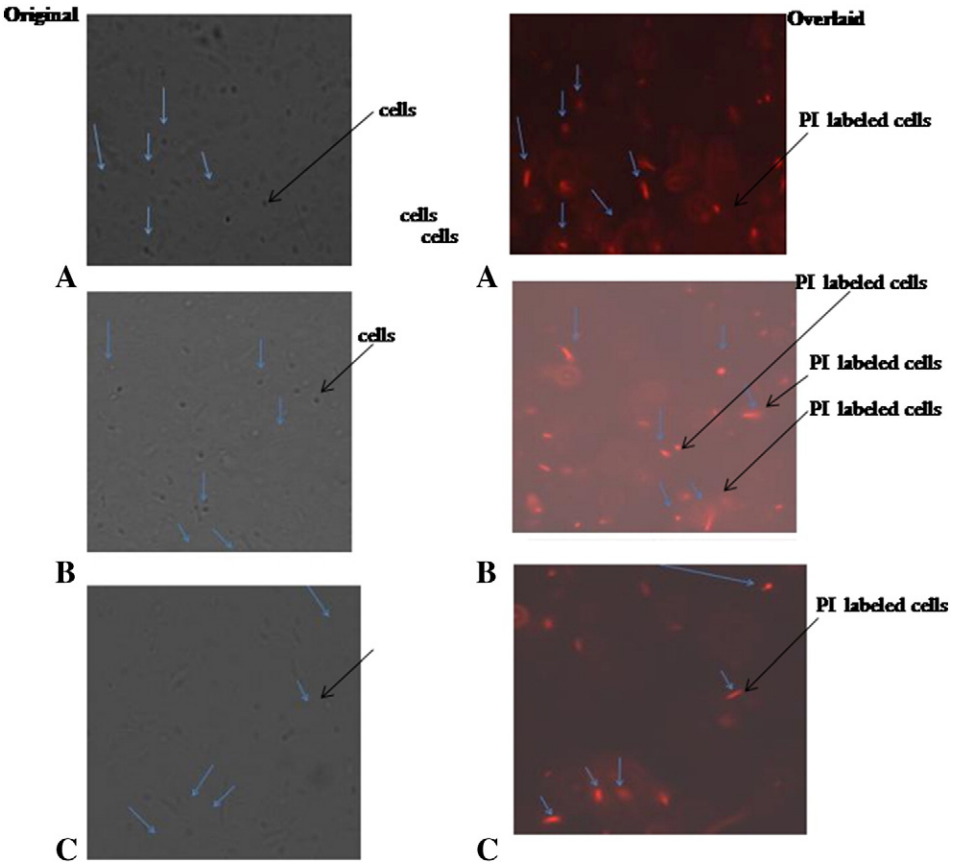

Fig. 1. A, B, and C. Fluorescence micrographs of CTAB treated recombinant *E. coli* subjected to PI staining. Microscopy was used to investigate cell membrane damage and viability. Phase contrast (1000×) and fluorescence microscopy photos of *E. coli*. These images illustrate the cells and some granules take up nuclear stain. A: Cells treated with 10 μg/ml CTAB followed by PI staining (20 μl/ml), they were further washed to remove the excessive stain. Same cells were subjected to fluorescence microscopy. B: Cells treated with 5 μg/ml CTAB followed by PI staining, they were further washed to remove excess stain. C: Cells treated with 1 μg/ml CTAB followed by PI similar procedure as above was followed. Note: left row shows original (cells in black and white), and right row shows overlaid. The arrows indicate PI labeled cells.

Plasmid DNA isolation understood genetic integrity of the cells. Full-length 16S rRNA amplification confirmed that *E. coli* cells were viable after treatment with 1 μg/ml, but they were lysed when they were treated with higher concentrations of CTAB (Fig. 3A and B). The cells treated with 1 μg/ml successfully amplified complete and partial 16S rRNA (Fig. 3A Lanes 1, 2, 3 full length 16S rRNA, Lanes 7–10 partial 16S rRNA) and full length f6ppk gene (Fig. 3B: Lanes 8, 9, yeast and *E. coli*). Similarly, plasmid DNA (7 kbp) was observed to be intact when it was purified (Fig. 3B: Lanes 1, 2, 3) successfully. Further, changes were not observed in the restriction fragment sizes of the plasmid DNA, which suggests that CTAB treated cells are viable without compromising their cell wall integrity. The cells treated with 10 μg/ml of CTAB were subjected to PMA studies, Fig. 3A Lanes 4 and 6 clearly show that there was no amplification. Hence, PMA treatment completely suppressed PCR amplification, which confirms that cell lysis/death was induced by treatment with 10 μg/ml of CTAB.

Bifidobacterium sp. treated with 5–10 μg/ml CTAB were subjected to PMA studies. Here, the results clearly showed the successful amplification of complete and partial 16S rRNA (Fig. 3A: Lanes 3 and 10). Plasmid DNA was also successfully purified (Fig. 3A: Lane 3). There was successful amplification of whole f6ppk (Fig. 3B: Lane 8) in *K. lactis* cells that were subjected to 5–10 μg/ml CTAB (data not shown), demonstrating their ability to resist CTAB effectively.

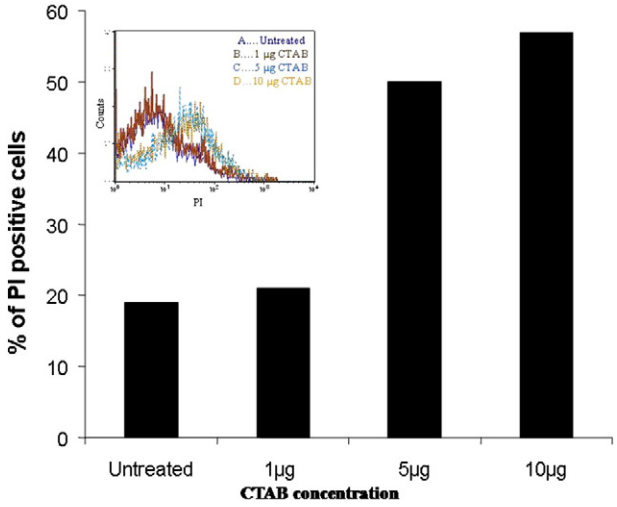

Fig. 2. FACS analysis of the cells treated with different concentrations of CTAB (1 to 10 μg) subsequently they were subjected to PI staining. The PI stained cells were subjected to FACS to capture only the PI stained cells. (A) Control cells not treated with CTAB but treated with PI only, (B) cells treated with 1 μg of CTAB followed by PI staining, (C) cells treated with 5 μg of CTAB followed by PI staining, and (D) cells treated with 10 μg of CTAB followed by PI staining. Fluorescence data: FL1, 525 emissions: FL3, 620 nm emission. Inlet picture signifies extent of fluorescence.

Discussion

The CTAB-mediated competent cell preparation method described here shares no similarities with any of the existing methods for producing competent cells. Our method requires only one permeabilizing reagent, the cationic detergent CTAB, for efficient and reproducible transformation. It does not need any other reagents such as Mg^{2+} ions, PEG and DMSO, which are essential elements of other methods. We identified two important factors to achieve highest transformation efficiencies: 1) CTAB concentration, which varies according to the organism being studied and 2) the growth phase of the harvested cells where, harvesting cells in the exponential phase worked best for all different kinds of cells namely *E. coli*, *Bifidobacterium* sp. and *K. lactis* (at OD_{600} 0.4–0.6).

A unique feature of this new method is that, the treated cells can be efficiently transformed with both plasmid DNA and ligation-reaction mixtures. Several factors could contribute to the greater transformation efficiencies of plasmid as well as ligation reactions. Based on the organism to be transformed, we use different stages of the cell cycle to enhance its efficiency (exponential stage cells were best suitable and presented in the manuscript). However, the protocol used for all the organisms was the same. Hence, the prepared buffers that contained CTAB could be used to make any competent cells such as *E. coli*, *Bifidobacterium* sp. and *K. lactis*. Thus, the main advantage of our protocol is the significantly higher transformation efficiencies and the universal use of the same transformation buffers for many different organisms.

Although the efficiency and reproducibility of our transformation assay system are comparable and even superior to those of more widely used methods (Dargert and Ehrlich, 1979; Ishikawa et al., 2002; Sambrook et al., 2000; Smith et al., 1975), we understand that, there are several advantages to prepare competent bacteria with CTAB. First, our method is relatively simple as a single buffer that contains CTAB can be used for transformation of plasmid DNA in different organisms. It is a highly efficient, reliable and reproducible method of making competent cells. The existing methods for making competent cells are lagging behind in validating the transformation efficiency with plasmid DNA. It is also understood that, the efficiency of transformation is much less and occasionally, no transformants were observed when the molecular weight of the plasmid, especially the ligation mix of molecular weight 7–13 kbp or higher is transformed. The prepared, competent cells can be used for transformation after 10–12 months of storage at −70 °C without losing their efficiency. The CTAB-mediated competent cell preparation method yields at least 10^5–10^9 transformants per microgram of DNA (Tables 2 & 4), a level that is satisfactory for routine sub-cloning experiments. Second,

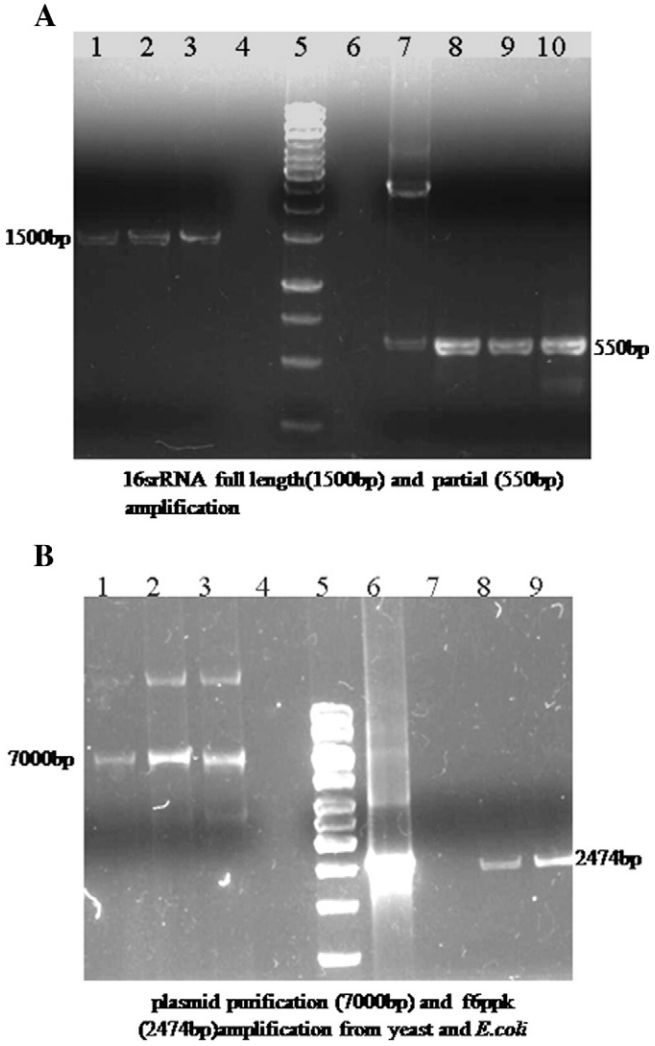

Fig. 3. A. Cells were subjected to CTAB treatment followed by PMA and were screened for plasmid DNA. The same cells were further subjected to heat treatment and centrifugation. The resultant supernatant was used as a template for amplification of full-length and partial 16S rRNA and f6ppk. Isolated plasmid DNA and complete, partial and full-length f6ppk were amplified and subjected to 1% Agarose gel electrophoresis. Lane 1: Amplification of full-length 16S rRNA from control cells; not treated with CTAB and PMA. Lane 2: Amplification of full-length 16S rRNA from *E. coli* treated with CTAB and PMA (1 μg). Lane 3: Amplification of 16S rRNA from *Bifidobacterium* treated with CTAB and PMA (5 μg). Lane 4: Amplification of 16S rRNA from *E. coli* treated with 10 μg/ml CTAB and PMA. Lane 5: Molecular weight markers for 100 (not seen in the gel picture), 300, 500, 750, 1000, 1500, 2000, 3000 and 5000 bp. Lane 6: Amplification of 16S rRNA from *Bifidobacterium* treated with 10 μg/ml CTAB and PMA. Lane 7: Amplification of full-length f6ppk from *Bifidobacterium* treated with 5 μg/ml CTAB and PMA. Lane 8: Amplification of partial 16S rRNA from control *E. coli* (un-treated with CTAB and PMA). Lane 9: Amplification of partial 16S rRNA from *E. coli* treated with 5 μg/ml CTAB and PMA. Lane 10: Amplification of partial 16S rRNA from *Bifidobacterium* treated with 5 μg/ml CTAB and PMA. B. Cells were subjected to CTAB treatment followed by PMA and were screened for plasmid DNA and PCR amplified. The same cells were further subjected to heat treatment and centrifugation. The resultant supernatant was used as a template for amplification of full-length and partial f6ppk and purification of plasmid DNA. Isolated plasmid DNA and complete, partial and full-length f6ppk were amplified and subjected to 1% Agarose gel electrophoresis. Lane 1: Plasmid DNA isolated from control *E. coli*. Lanes 2 and 3: Plasmid DNA isolated from *E. coli* and yeasts treated with 1 and 10 μg/ml CTAB. Lane 4: Plasmid DNA isolated from *E. coli* treated with 10 μg/ml CTAB. Lane 5: Molecular weight markers for 1, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 kbp. Lane 6: PCR amplification of f6ppk from control *E. coli* cells. Lane 7: PCR amplification of f6ppk treated with 10 μg/ml CTAB (*E. coli*). Lanes 8 and 9: PCR amplification of f6ppk from yeasts and *E. coli* treated with 1 or 10 μg/ml CTAB.

the bacteria and yeasts in the CTAB method do not require repetitive washing (as followed in conventional methods), which not only restricts cell rupture, but also increases DNA uptake. Third, the bacteria can be maintained on ice for periods as short as 2 min or as long as 1 h, without significant loss in transformation efficiency. The competent cells have the capacity to uptake plasmid DNA. Hence, they can also be used for making cDNA libraries with plasmid DNA fragments. The same procedure can also be universally used for transformation of *E. coli*, *Bifidobacterium* sp. and *K. lactis*. Therefore, it may be possible that this protocol could be generally applicable and used as an inexpensive and reliable alternative to existing methods.

Based on the FACS data, an increase in the concentration of CTAB led to the cell wall permeability and affected cell viability. Wherever there was permeability, PI binding to DNA was observed. However, when the cells were treated with 5 and 10 μg/ml CTAB, PI binding was not observed. Such findings demonstrate that these concentrations of CTAB permeabilize the cell membrane and lead to cell death and lysis (Fig. 1A–C). The cell walls of organisms that were treated with a lower concentration (1–5 μg) of CTAB were permeabilized where PI could bind lesser number of cells i.e. 10–20% of *E. coli* and less than 1% of *K. lactis*. When the cells were treated with the highest concentration of CTAB (10 μg/ml), no PI-stained cells were noted, which suggests a lack of viable cells and that cell lysis has occurred (Fig. 1). The primary mechanism of the bactericidal action of cationic detergents such as CTAB appears to consist of general membrane damage and destruction of semi-permeability.

It is also understood that CTAB has a completely different effect on growing cultures. The culture turbidity did not decrease as with equivalent concentrations of cetrimide. Microscopically, we observed that large clumps of cells formed after the addition of CTAB, and after 4 h, a few single cells remained. With higher concentrations of CTAB (5–10 μg), there was a marked increase in turbidity immediately following the addition of the drug. This increase could have been brought about by changes in the density of the cells or the light scattering properties of the bacteria. A study by Smith et al. (1975) has clearly shown that CTAB does affect cell viability, but it leads to increased cell wall permeability. Hence, the higher the permeability of the cell wall, the greater the chances for DNA uptake by the cell.

Flow cytometry and microscopy studies further reveal that treatment of cells with higher concentrations of CTAB affected their viability, which led to cell lysis and death (Table 3), as confirmed by colony forming unit (cfu) counting. However, higher levels of PI staining were observed in cells treated with 1 μg/ml CTAB and PI, which infers that FACS has the ability to detect PI in faulty cell membranes only, but not PI-stained cell debris and inclusion bodies. However, microscopy detects PI-stained cells as well as debris. Hence, we observed less PI staining by FACS for cells treated with 5–10 μg/ml CTAB, which infers an increase in the number of non-viable cells. 5–10 μg CTAB treated cells after the PI treatment exhibited the expected large shift in fluorescence intensity (Fig. 2) relative to unstained and 1 μg CTAB treated cells. After further treatment with CTAB and PI, these cells were observed by microscopy (Fig. 1A, B, C). This suggested that the number of PI-stained cells increased with the concentration of CTAB. These images verified that granules as well as cells take up nuclear stain. It is also possible that the large levels of fluorescence observed in the cell debris may, in theory, be because of nucleic acid contamination and adhesion to cell components, especially those with inclusion bodies, following cell disruption. To validate this, we treated cell debris with a DNase and did not observe a significant decrease in fluorescence (data not shown). Therefore, binding of the dye to the cell debris is non-specific and dye becomes trapped without binding to nucleic acids.

Author contributions

Conceived and designed the plan of action: Analysis, writing and initiated the idea, Dr K. Rajagopal. Experimental setup and follow-up by Praveen Kumar Singh, and Rajesh Kumar. Kaneez Fateeema Siddiqui carried-out microscopy and FACS.

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