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Mechanism of artificial transformation of *E. coli* with plasmid DNA – Clues from the influence of ethanol

Suchitra Sarkar, Sujan Chaudhuri and Tarakdas Basu*

Department of Biochemistry and Biophysics, University of Kalyani, Kalyani 741 235, India

The standard method of transformation of *E. coli* with plasmid DNA involves two important steps – binding of DNA to the cell surface, suspended in 100 mM $CaCl_2$ at 0°C, and the subsequent entry of DNA to the cell cytosol by a heat-pulse from 0 to 42°C. When competent *E. coli* cells were transformed with plasmid DNA in the presence of different concentrations (up to 10% v/v) of ethanol, the transformation efficiency $(TR)_E$ decreased gradually with increase in ethanol concentration. This decrease in $(TR)_E$ was directly proportional to ethanol-mediated leaching of lipopolysaccharide (LPS) molecules from the competent cell surface, indicating LPS was the major target site for DNA adsorption to the competent cells. *In vitro* spectrophotometric study showed evidence that there was binding interaction between plasmid DNA and *E. coli* LPS in the presence of a divalent cation, Ca^{2+}. Moreover, plasmid DNA, previously incubated with LPS in $CaCl_2$, had less ability to transform *E. coli* cells. The results suggest that during artificial transformation of *E. coli*, the naked DNA was first bound to the LPS molecules on the competent cell surface and uptake of this LPS-absorbed DNA into the cell cytosol was associated with $CaCl_2$-mediated cell-membrane disintegration.

THE technique of DNA transformation has become important in virtually all aspects of molecular genetics. Transformation is defined as the uptake and expression of foreign DNA by cells. Bacterial transformation occurs naturally in many species such as *Micrococcus*, *Haemophilus* and *Bacillus*[1,2]; all these organisms have proteins on their exterior surface whose function is to bind to DNA in their environment and transport it into the cell. However, it is still a rare event for most bacteria to naturally take up DNA from the environment. But by subjecting bacteria to certain artificial conditions, many of them are able to take up free DNA[3], and the cells in such state are referred to as competent.

In *E. coli* the competence can be developed by suspending the cells in ice-cold $CaCl_2$ and then subjecting to a brief heat-shock at 42°C (refs 4, 5). Although *E. coli* has developed into a universal host organism both for molecular cloning and for a diverse set of assays involving cloned genes, the technique of *E. coli* transformation is highly inefficient even using competent cells. The vast majority of DNA molecules added will not enter any cell, and the vast majority of bacterial cells will receive no DNA. Besides Ca^{2+} ions, other frequently used cations include Mg^{2+}, Mn^{2+}, Rb^+, for competence generation[3]. However, the exact mechanism by which DNA adsorbs to the *E. coli* cell surface and enters the cell cytosol, and why the transformation is stimulated by these treatments, is still largely obscure.

One proposed hypothesis[6] is that DNA crosses through the least-barrier path at zones of adhesion, where the outer and inner cell membranes fuse to pores in the cell wall. The zones of adhesion are rich in negatively-charged lipo polysaccharide (LPS) molecules[7,8] and DNA also being negatively charged, cannot enter the cell easily as the two negative polarities repel each other. A divalent cation such as calcium, is believed to form stable coordination complexes with phosphates, and thus may facilitate the association of the two phosphate-rich structures like DNA and LPS.

The transformation process, being a membrane-bound phenomenon, is most likely to be influenced by the well-

*For correspondence. (e-mail: tbasu@cal3.vsnl.net.in)

known membrane perturbant ethanol. There are reports which show that ethanol leaches LPS from *E. coli* cell surface and this ethanol-mediated leaching of LPS causes reduction in (a) bacteriophage φX174 infectivity in *E. coli*[9], and (b) transformation efficiency of competent *E. coli* cells with plasmid DNA[10]. In this communication we report how the transformation efficiency ($(TR)_E$) of *E. coli* is changed when (i) ethanol remains present during the whole process of transformation, and (ii) ethanol is withdrawn after treating the competent cells for 30 min in ice and then plasmid DNA is added to such cells to carry out the subsequent steps of the transformation process. While searching for the reasons behind the ethanol-mediated changes in $(TR)_E$, some clues have come out regarding the mechanism of DNA adsorption as well as DNA entry into the cells during transformation.

The bacterial strain used for transformation was XL1-Blue[11], a genetically engineered, highly transformable strain of *E. coli*. The plasmid DNA used was the cloning vector pUC 19 (ref. 12). LPS of the strain *E. coli* 014 was a gift from B. P. Chatterjee, Department of Biological Chemistry, Indian Association for the Cultivation of Sciences, Kolkata. Ethanol was purchased from Bengal Chemical and Pharmaceutical Works Limited, Kolkata and was used without further purification.

Cells were transformed with the plasmid DNA by the standard method of $CaCl_2$ treatment[10,13]. The steps of ethanol addition and ethanol withdrawal have appropriately been mentioned in figure and table legends. Cells were grown to log phase [up to $(OD)_{600} = 0.10$, i.e. $\sim 5 \times 10^7$ cells/ml] in Luria Broth [LB][13], washed and ultimately concentrated 25 times in ice-cold 100 mM $CaCl_2$. DNA (no more than 50 ng in a volume of 10 μl) was added to 200 μl of competent cell suspension and was allowed to incubate at 0°C for 30 min. The cell–DNA complex was transferred to 42°C for exactly 90 s and was rapidly chilled in ice. Then 800 μl LB was added and the cells were incubated at 37°C for 45 min for expression of antibiotic resistance marker (β-lactamase) encoded by the plasmid. These transformed cells were serially diluted in chilled tryptone broth[10]. Next 100 μl cells from properly diluted samples was spread on agar-LB medium with and without ampicillin (50 μg/ml), to obtain the number of transformants and viable cells respectively. $(TR)_E$ is defined as the ratio of the number of transformants to that of the viable cells (since the presence of 10% v/v ethanol caused 20% cell killing, $(TR)_E$ was not expressed in number of transformants per μg of DNA).

To study *in vitro* DNA–LPS interaction spectrophotometrically, LPS was suspended by sonication in either $CaCl_2$, NaCl or TE buffer, as the case may be, to prepare a homogeneous stock suspension of 2.5 mg/ml. LPS suspension had no significant absorbance in the UV/visible wavelength regions. The pUC19 DNA solution in either $CaCl_2$, NaCl or TE buffer showed characteristic UV

absorption spectra with absorption maxima at 260 nm. To study the effect of addition of LPS on the UV absorbance of DNA, equal amount of LPS was always added in both the sample and reference cuvettes. Spectrophotometric studies were carried out in Shimadzu (UV 160-A) spectrophotometer using 1.0 cm path length cuvettes.

The inducible proteins, alkaline phosphatase and D-serine deaminase, were induced in *E. coli* according to the method of Basu and Podder[14]. For induction of alkaline phosphatase, cells were initially grown to log phase [up to $(OD)_{600} = 0.10$, i.e. $\sim 5 \times 10^7$ cells/ml] at 37°C in tris-glucose medium[14], supplemented with thiamine-HCl (50 μg/ml) and 0.02% casamino acid, washed two times with and finally suspended in the same volume of phosphate-less tris-glucose casamino acid medium supplemented with thiamine-HCL (50 μg/ml). The cells were allowed to grow at 37°C for further 90 min, when high pool of alkaline phosphatase was induced and stored at the periplasmic space of the cell membrane. For induction of D-serine deaminase, cells were initially grown to log phase [up to $(OD)_{600} = 0.10$, i.e. $\sim 5 \times 10^7$ cells/ml] at 37°C in tris-glycerol medium[15], supplemented with thiamine-HCl (50 μg/ml) and 0.02% casamino acid, washed twice and finally suspended in the same volume of the same medium devoid of glycerol. Then 300 μg/ml DL-serine was added to the suspension and the cells were allowed to grow for further 90 min at 37°C to induce and store high pool of D-serine deaminase in the cell cytosol. The induced cultures were then washed with and finally re-suspended in 1/25th volume of 100 mM ice-cold $CaCl_2$ to make them competent. Such competent cells were used to study the release of alkaline phosphatase and D-serine deaminase at the different steps of transformation. Alkaline phosphatase was assayed according to the method of Schlesinger and Levinthal[16], and the enzyme unit was defined according to the definition of Basu and Podder[14]. D-serine deaminase was assayed according to the method of Pollard and Davis[17].

Leached LPS was assayed by the colorimetric estimation of the unique sugar 2-keto-3-deoxyoctulosonate present in LPS, as described by Sarkar *et al.*[10]. From the standard curve (using known amount of *E. coli* 014 LPS), the amount of leached LPS in the supernatants of the ethanol-treated competent cells was estimated.

The data points in each figure and table correspond to the average of at least four independent experiments.

When transformation of *E. coli* XL1-Blue with plasmid pUC 19 DNA was carried out in the presence of 5 and 10% v/v ethanol, the corresponding $(TR)_E$ reduced by 70 and 95% with reference to that of untreated control cells (see Set I in Table 1). Such reduction in $(TR)_E$ was not caused by loss of cell viability because only 20% cell-killing was observed by 10% v/v ethanol treatment (Table 1). Ethanol, being a well-known membrane perturbant, could affect the DNA adsorption process to the cell surface. Since LPS was hypothesized to be the major

site for adsorption of DNA on the cell surface[6], and ethanol was reported to leach LPS from *E. coli*[9], the supernatant of the ethanol-treated competent cells was checked for the presence of LPS. The results (Table 2) showed that the amount of LPS leaching was proportional to the concentration of ethanol, and approximately 178 and 240 µg of LPS was leached out to the suspending medium by the presence of 5 and 10% v/v ethanol respectively. These amounts were equivalent to 40 and 55% of total cellular LPS [estimated considering the dry weight of an *E. coli* cell to be of the order of 10^{-13} g (ref. 18) and 3.5% of *E. coli* dry weight was due to LPS[19]]. The correlation between the ethanol-induced leaching of LPS and the reduction of $(TR)_E$ was revealed from the following two experimental observations:

(I) From *in vitro* spectrophotometric study it was evident that there was binding interaction between plasmid pUC 19 and *E. coli* LPS. pUC 19 DNA solution taken in 100 mM $CaCl_2$ showed gradual decrease in UV absorbance at 260 nm with the addition of increasing concentrations of LPS (Table 3). The initial absorbance of DNA at 260 nm (in the absence of any LPS) decreased nearly seven-fold upon addition of 140 µg/ml LPS to it. Table 3 also shows that no significant binding between DNA and LPS took place when, instead of $CaCl_2$, the interaction was studied in TE buffer (pH 8.0); and in the presence of monovalent cation Na^+ (in 100 mM NaCl), the interaction was found to be considerably less pronounced compared to that in 100 mM $CaCl_2$.

(II) When pUC 19 DNA was extraneously incubated with different concentrations of LPS in 100 mM $CaCl_2$ at 0°C, it had less ability to transform *E. coli* cells; the $(TR)_E$ gradually reduced with the increase in LPS concentration (Figure 1). The $(TR)_E$ decreased by more than 50% by the pre-incubation of pUC19 DNA with 400 µg/ml LPS with respect to that with LPS-free DNA. In light of the above two observations, it can be stated that in the experiment set I (see Table 1), the binding of a fraction of added DNA to cell-free LPS, leached by ethanol, decreased the effective concentration of free, transforming DNA and thus reduced the $(TR)_E$.

In contrast, the results of the experiment set II shown (see Table 1) indicate that on removing ethanol after treating the competent cells for 30 min at 0°C, when plasmid DNA was added to carry out the subsequent steps of transformation, the $(TR)_E$ was found to increase by 115 and 70% respectively due to pre-treatment of the cells with 5 and 10% v/v ethanol compared to untreated control cells. The two important steps of the artificial transformation process are (i) allowing adsorption of DNA to the cell surface in the presence of 100 mM $CaCl_2$ at 0°C for 30 min, and (ii) shifting the cell–DNA complex from 0 to 42°C to give a heat-shock for 90 s for entry of the adsorbed DNA into the cell cytosol. To observe whether ethanol pre-treatment of cells had any role on any of the above two steps, the $(TR)_E$ of the cells

Table 1. Transformation efficiency and total viable cell count of *E. coli* cells treated with different concentrations of ethanol. Set I: Competent cells were treated with different concentrations of ethanol for 30 min at 0°C, DNA was then added and the subsequent steps of transformation were performed to study the $(TR)_E$. Set II: After treating the competent cells with different concentrations of ethanol for 30 min at 0°C, ethanol was removed; cells were washed two times by centrifugation and finally suspended in 0.1 M $CaCl_2$. DNA was added to these cells and the steps of transformation were continued to observe the $(TR)_E$

Concentration of ethanol in % v/v	No. of viable cells × 10^8	Transformation efficiency × 10^{-4}	
		Set I	Set II
0.0	3.50 ± 0.3	6.80 ± 0.6	7.0 ± 0.5
5.0	3.35 ± 0.2	2.68 ± 0.2	15 ± 0.9
10.0	2.70 ± 0.4	0.65 ± 0.2	12 ± 0.6

Table 2. Amount of LPS leached from the competent cell surface vs the concentration of ethanol. Competent cells were taken at a concentration of 1.25×10^{11} cells/ml, treated with different concentrations of ethanol for 30 min at 0°C, centrifuged and the supernatants were collected to assay for the presence of LPS

Ethanol concentration in % v/v	LPS leached in µg	Percentage of total LPS leached
0	0	0
5	178.0	40%
10	240.0	55%

Table 3. Change in UV absorbance of DNA at 260 nm with increase in LPS concentration. Both DNA and LPS were individually taken in either 100 mM $CaCl_2$, 100 mM NaCl or TE buffer. A_{260} signifies UV absorbance of DNA at 260 nm

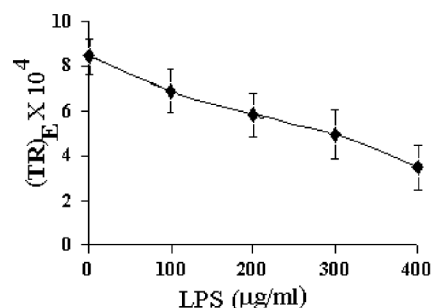|	Absorbance at 260 nm (A_{260})		
LPS (µg/ml)	100 mM $CaCl_2$	100 mM NaCl	TE buffer (pH 8.0)
0	0.641	0.604	0.652
49.0	0.426	0.524	0.652
97.0	0.217	0.506	0.624
142.0	0.088	0.422	0.620
185.0	–	0.400	0.606
227.0	–	0.380	0.584
268.0	–	0.364	0.571

Figure 1. Decrease in transformation efficiency of XL1-Blue cells with increase in LPS concentration in the transforming DNA solution. The amount of DNA required for optimum transformation[13] was allowed to incubate with different concentrations of LPS in 100 mM $CaCl_2$. After incubation for 30 min at 0°C, competent cells were added to each of the incubated complexes and the subsequent steps of the transformation process were then carried out.

was measured after each step. Figure 2 *a* shows that when $(TR)_E$ was measured after the DNA adsorption step, i.e. without applying the heat-pulse $(0 \to 42°C)$ step, 5.0 and 10.0% ethanol pre-treatment enhanced the $(TR)_E$ by 100 and 40% respectively compared to that of the control cells. Figure 2 *a* also shows that by the heat-shock $(0 \to 42°C)$ step, the $(TR)_E$ of both ethanol pretreated and untreated cells was enhanced nearly by the same proportion (approximately twice) compared to the values of $(TR)_E$ obtained without applying heat-pulse. This step-wise study of $(TR)_E$ indicated that $CaCl_2$ helped not only in the adsorption of DNA to the cell surface, but also in the partial uptake of DNA into the cell cytosol, even without the application of the heat-shock step of the transformation process. The heat-shock step nearly doubled this uptake. The following experiment shows that ethanol-pretreatment, perturbing the cell wall of the $CaCl_2$-treated competent cells, increased further the DNA uptake and thus enhanced the $(TR)_E$.

It was earlier reported[20] that *E. coli* cells, when subjected to a competence regimen involving a heat-shock

Figure 2. *a*, Effect of ethanol on transformation efficiency before and after heat-pulse step of transformation $CaCl_2$-mediated competent cells were treated with different concentrations (0, 5 and 10% v/v) of ethanol for 30 min at $0°C$. Ethanol was then removed by centrifugation and the cell pellets were re-suspended in the same volume of 100 mM $CaCl_2$ to incubate with DNA at $0°C$ for a further 30 min. Before and after giving heat-shock $(0 \to 42°C)$, aliquots from the cell–DNA complex were withdrawn to study the $(TR)_E$; *b*, Effect of ethanol on release of AP from the $CaCl_2$-treated competent cells before and after heat-pulse step of transformation. After treatment of competent cells containing heavy pool of induced alkaline phosphatase, with different concentrations of ethanol for 30 min at $0°C$, ethanol was then removed by centrifugation and cell pellets were re-suspended in the same volume of 100 mM $CaCl_2$ to incubate in ice for a further 30 min (this incubation was equivalent to the time of DNA adsorption to the competent cells). Just before and after the heat-shock $(0 \to 42°C)$, aliquots of cells were collected, centrifuged and supernatants were assayed for the presence of AP.

from $0 \to 42°C$, released an enzyme β-lactamase from the periplasm to the extracellular environment, indicating membrane disintegration. If membrane disintegration was a consequence of the transformation procedure, release of other membrane proteins was also expected to occur. Therefore, release, if any, of an inducible periplasmic protein alkaline phosphatase (AP) of *E. coli* was studied, and it was found to be positive. To investigate the reason behind the enhancement of $(TR)_E$ in ethanol-pretreated cells, release of AP was studied after each of the two important stages of the transformation procedure. Figure 2 *b* shows that normal *E. coli* cells, kept in $CaCl_2$ at $0°C$ for 30 min, released AP from the periplasmic space of the cell membrane to the cell-suspending medium, suggesting loss of membrane integrity by the $CaCl_2$ treatment only. The release remained unaltered by the subsequent heat-shock $(0 \to 42°C)$ step. When ethanol-treated competent cells, after removal of ethanol, were subjected to the two different steps of transformation, similar feature of AP release was observed (Figure 2 *b*); the amount of release was much higher, e.g. more than three times in case of 5% ethanol-pretreated cells than that from the control cells. Figure 2 *b* also shows that more the concentration of ethanol, more was the magnitude of AP release. This happened because leaching of LPS molecules from the cell surface during 30 min of ethanol treatment obviously disordered the cell wall, which perhaps facilitated further release of AP from the periplasmic space (between the inner and outer membrane) of ethanol-pretreated *E. coli* cells. Thus the results on AP release signified that the ethanol pre-treatment of the competent cells for 30 min caused more disruption of the cell membrane than that produced by the $CaCl_2$ treatment only. No such release of the inducible cytoplasmic protein D-serine deaminase from both ethanol-pretreated and untreated cells was observed at any of the two important stages of the transformation process, indicating that the integrity of the inner or cytoplasmic membrane of the cell remained unaltered, i.e. disruption occurred at the level of the cell wall. From the experimental results, the following important conclusions can be drawn: (a) $CaCl_2$-mediated generation of cell competence was associated with disintegration of cell wall, as was evident from the release of membrane protein AP. (b) In each of the steps of transformation [i.e. the steps of $CaCl_2$ treatment and the heat-shock] DNA uptake took place in the *E. coli* cell. In the former step, besides DNA binding to the cell surface, the $CaCl_2$-mediated cell-wall disruption caused partial uptake of bound DNA. In the latter step, the heat-shock through the disintegrated cell wall facilitated further entry of DNA into the cell cytosol. The cumulative DNA uptake through these steps gave the ultimate $(TR)_E$. (c) As there was enhanced release of membrane protein AP from the ethanol-pretreated cells (Figure 2 *b*), the consequent enhanced cell-wall perturbation was responsible for the corresponding increase in $(TR)_E$ of such cells (Figure 2 *a*).
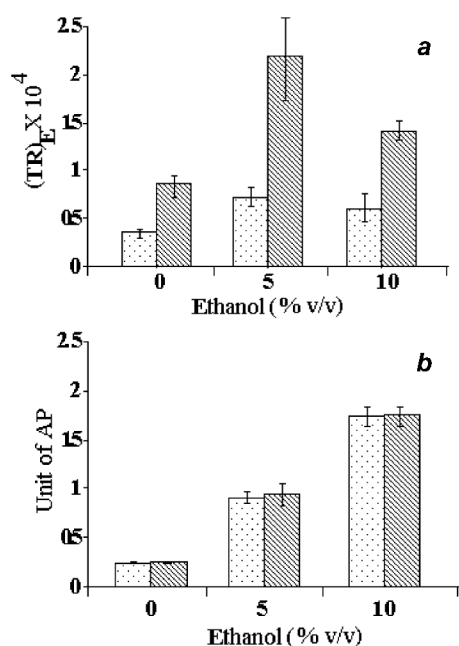
(d) In spite of much larger release of AP from the 10% ethanol-treated cells (Figure 2 b), its $(TR)_E$ reduced nearly by 20% from that of the 5% ethanol-treated cells (Figure 2 a); this was perhaps because 10% v/v ethanol treatment caused approximately 20% cell killing (Table 1). Hence, during transformation, the more the membrane was disintegrated (without causing cell death by ethanol treatment), the more was the uptake of plasmid DNA. (e) After the heat-shock step, where no noticeable increase in the release of AP took place (Figure 2 b), $(TR)_E$ of both ethanol-pretreated and untreated cells was nearly doubled compared to the values obtained without applying the heat-pulse step (Figure 2 a). Thus it can be suggested that no further cell-wall disruption occurred by the heat-pulse step; on the other hand, this step helped DNA entry into the cell cytosol through the disintegrated membrane of competent cells. (f) This ethanol-mediated enhancement of $(TR)_E$ can be considered as an improved technique of artificial transformation of $E.$ $coli$ with plasmid DNA.

The whole study can be summarized as follows: during artificial transformation of $E.$ $coli$, the DNA was first bound to the LPS molecules on the competent cell surface and uptake of this LPS-adsorbed DNA into the cell cytosol was associated with cell-wall disintegration.

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Carbon sequestration estimates for forestry options under different land-use scenarios in India

Suruchi Bhadwal* and Roma Singh
Tata Energy Research Institute, Darbari Seth Block, Habitat Place, Lodhi Road, New Delhi 110 003, India

Land resources have been under tremendous pressure since the very beginning of civilization for food and forest products. For better land-use management, these resources must be used sustainably and in accordance with the demands of the masses. To manage the land resources effectively and for future food security we need to find out the demands and potential of our land. In this study, a comparative estimate of land-use and carbon sequestration potential of different forestry options has been done for India. This has been done using the Land Use and Carbon Sequestration (LUCS) model. This model systematically incorporates Indian agricultural and forest statistics with geographical and demographic data. Besides this, various pressures exerted on land-use system due to the existing growth rates of population, fuelwood requirements, export of agricultural commodities and probable transfer of lands from one category to the other, are also considered here.

Three scenarios (LUCS-I, LUCS-II and LUCS-III) are generated with different land-use options following the demands and present land-use pattern prevailing in the country. The scenario LUCS-I puts maximum amount of land into the forestry sector and is an economically feasible scenario. The scenario LUCS-II is a 'business as usual' scenario, as it is projected according to the current five-year plan. The scenario LUCS-III puts maximum amount of land in the plantations category and is a potential scenario. These scenarios were generated for a time period of fifty years starting from the year 2000. The LUCS model estimates the amount of carbon sequestered by approximating land-use and relative biomass changes in the landscape over time. The amount of carbon sequestered in scenario LUCS-III is estimated to be 6.937 billion tonnes, which is the highest among those sequestered in all the three scenarios. According to this scenario, the carbon sequestered in aboveground vegetation of India will be more than double by the year 2050.

ANTHROPOGENIC activities have known to affect the biosphere through changes in land-use and forest management activities, thus altering the natural balance of greenhouse gases in the atmosphere. Over the last three centuries, forests have decreased by 1.2 billion hectares (bha), i.e. 19% and grasslands by 560 million hectares (mha)[1]. This has resulted due to an increase in croplands