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Influences of growth temperature and preparation of competent cells on efficiency of chemically-induced transformation in *Escherichia coli* DH5α.

JOANNA CHAN, COLETTE DAVIS, AND IVA JOKIC
Department of Microbiology and Immunology, UBC

It is well known that cellular cultures grown at different temperatures will display altered membrane lipid composition to maintain a relatively constant membrane fluidity across a range of temperatures. One of the proposed theories on the mechanism of chemically-induced transformation is that a higher temperature of the heat-shock step increases the fluidity of the host cell membrane, allowing plasmid DNA to insert into the cell through transient gaps in the membrane. We sought evidence for this model by growing *Escherichia coli* DH5α at two different temperatures (37 ºC and 42 ºC) and transforming cells from each culture with pUC19 plasmid DNA. At a constant heat-shock temperature of 42 ºC, the cells grown at 42 ºC were expected to have a higher survival rate, but a lower degree of membrane fluidity and thus lower transformation efficiency than those cells grown at 37 ºC. Our results showed no evidence that the differences in fluidity between cells grown at these two experimental temperatures were sufficient to affect any kind of change in transformation efficiency. However, our results did show a benefit to using cells grown at 42 ºC for chemically-induced transformations as a result of their higher survival of heat-shock.

There are currently at least two competing models to explain the mechanism of induced transformation of bacterial cells. The first postulates that exogenous plasmid DNA is bound by receptor complexes in the cell membrane and transported through transmembrane channels in either a compact-condensed or open-circular conformation. This model has received growing support with evidence for an important role of poly-beta-hydroxybutyrate(PHB)/calcium polyphosphate channel in uptake of exogenous DNA (6).

A second possibility is that the heat-shock step of transformation destabilizes the membrane, allowing plasmid to insert loops of DNA through transient gaps in the membrane, rather than through lipid or protein channels. Receptor complexes in the cytoplasm would then anchor the loop ends inside the cell and facilitate uptake of the remainder of the plasmid (1). This model suggests that increased membrane fluidity would increase efficiency of transformation by more frequently creating gaps in the membrane through which loops of the plasmid could insert.

This study sought evidence for the latter model by growing cells at two different temperatures, 37 ºC and 42 ºC, before treating them with $CaCl_2$ and heat-shock. Previous studies (4, 11) have shown that when *E. coli* cells were grown at different temperatures, the molecular ratio of saturated to unsaturated fatty acids increases as a function of growth temperature. If DNA uptake during the $CaCl_2$ cell transformation protocol occurs via DNA crossing through transient holes in the membrane rather than through PHB-based ion channels, and the degree of membrane fluidity at the constant heat-shock temperature of 42 ºC is higher in the cells grown at 37 ºC than in those grown at 42 ºC, an associated rise in transformation efficiency in the 37 ºC culture over the 42 ºC culture should result.

METHODS AND MATERIALS

Media. Luria-Bertani (LB) broth: 10 g tryptone (BDH (Merck)), 5 g yeast extract (Becton Dickinson), 5 g NaCl, pH adjusted to 7.0 with NaOH in 1 L dH_2O. Solid LB-agar for plates was made by adding 15 g/L Select agar (Invitrogen). The concentration of ampicillin (AMP) for LB-AMP selective medium was 100µg/mL.

Bacterial strain. Overnight cultures of *E. coli* DH5α (*deoR endA1 gyrA96 hsdR17(r_k^- m_k^+) recA1 relA1 supE44 thi-1 D(lacZYA-argFV169) f80lacZ DM15 F^-*) were grown in Luria Broth at experimental temperatures of 37 ºC and 42 ºC in a shaking water bath.

Plasmid. pUC19 (2.7 kb) was selected for its traditionally high degree of transformation efficiency and the ease of selection of transformants on ampicillin.

Plasmid isolation. Plasmids were isolated from *E. coli* DH5α host cells using phenol-chloroform extraction (13). Acceptable levels of plasmid purity were confirmed both by spectrophotometric methods and visualization on 0.8% agarose gel.

Preparation of competent cells. Two batches of log-phase cells from 37 ºC and 42 ºC cultures were treated with $CaCl_2$ in preparation for transformation. One set of cells was made competent according to the protocol in Sambrook, Fritsch and Maniatis (15). Cells were treated with 100 mM $CaCl_2$ solution and then frozen at -85 ºC until they were needed. A second set of cells was made competent by treatment with Tris-$CaCl_2$ solution (10 mM Tris, 50 mM $CaCl_2$ buffered at pH 8.0) (12) and were kept chilled on ice for same-day use in transformation.

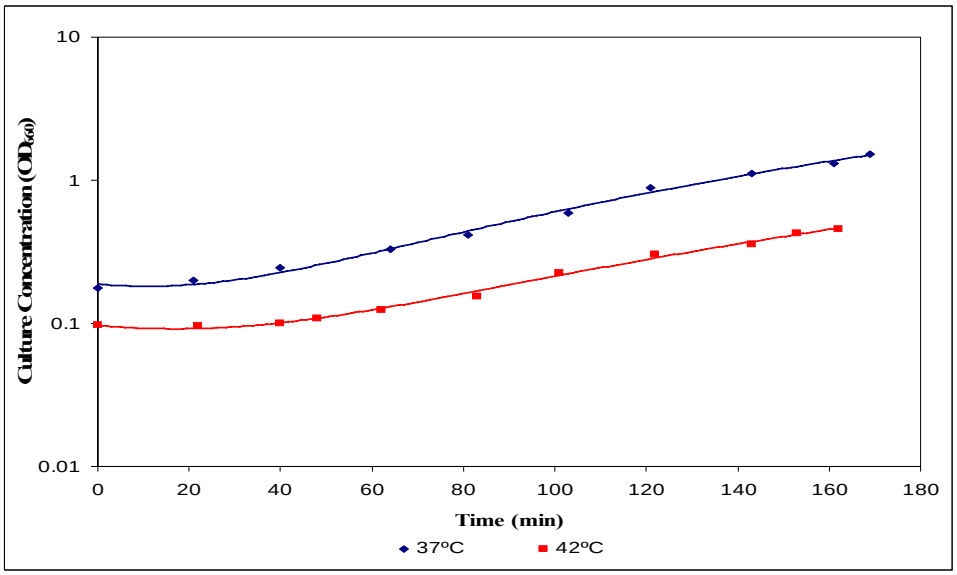

Fig. 1 Growth curves of *E. coli* DH5α grown at 37 ºC and 42 ºC in Luria broth with vigorous agitation.

Transformation and plating. Cells were transformed with pUC19 plasmid DNA (5). Briefly, 4.6 µL (2 µg) of plasmid DNA was added to 50 µL competent cells and left to incubate for 10 minutes on ice. Cells were then subjected to a 30 second heat-shock in a 42 ºC water bath. Cells were left to recover on ice for two minutes before the addition of 0.95 mL LB. After one hour recovery in LB in a 37 ºC water bath, cells were plated on LB for calculation of heat-shock survival, and on LB-AMP selection of transformants and calculation of transformation efficiency. Transformations were performed twice. The first set of transformations made use of the frozen competent cells and the heat-shock was carried out in 5 mL glass test tubes. The second set used the freshly-prepared competent cells and subjected them to heat-shock in plastic microfuge tubes.

Controls. Three controls were run. The first was a negative control to which no plasmid was added and in which the cells were not heat-shocked. These cells were plated directly, without a one hour recuperation step. The second was a positive control to which an equivalent volume of dH_2O was added instead of plasmid and the cells were heat-shocked at 42 ºC and plated after a one hour recovery. One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen; Cat. No. C4040-10) acted as a control on the procedure and were used in the second set of transformations only.

Calculation of Transformation Efficiency. Transformation efficiency was calculated using three different methods. In the first method, the concentration of transformants was divided by the concentration of non-heat-shocked cells. In the second method, the concentration of transformants was divided by the concentration of cells surviving heat-shock at 42 ºC. The third method divided the number of transformants by the mass (µg) of plasmid used.

RESULTS

E. coli DH5α growth rate at the experimental temperatures yielded a calculated doubling time of 45 min for the 37 ºC culture and 55 min for the 42 ºC culture (Fig. 1). The cells grown at 37 ºC showed an approximately 30% shorter lag phase (25 min) than those grown at 42 ºC (35 min). Both of the cultures remained in log phase after 2.5 hours growth. pUC19 plasmid concentration was calculated at 435 µg/mL assuming 1 A_{260} unit to be equivalent to 50 µg/mL. The purity was assessed by A_{260}:A_{280} ratio which yielded a value of 2.00, indicating a reasonable level of purity.

The first set of transformations using frozen stock of competent cells yielded minimal numbers of colonies on LB and no growth on LB-AMP (Table 1). Viable cell concentration before heat-shock for these cells was considerably lower than anticipated, and survival of heat-shock was also low. The second transformations yielded higher numbers of both viable cells and transformants. Furthermore, death resulting from heat-shock was found to be considerably reduced (Table 2).

Table 1 Effect of treatment on viability and transformation of competent *E. coli* DH5α cells.

Growth temp. (ºC)	Concentration (cfu/mL)			
	Non-heat-shocked cells	Heat-shocked cells	Heat-shocked with pUC19	Transformants
1st Set				
37	3.3×10^4	1.0×10^2	6.0×10^1	0
42	1.7×10^5	2.4×10^2	1.3×10^2	0
2nd Set				
37	2.8×10^8	2.1×10^8	2.7×10^8	7.8×10^3
42	1.3×10^7	3.8×10^7	6.1×10^7	1.8×10^3
TOP10	n/a	n/a	1.5×10^8	6.3×10^4

n/a = data not collected

Table 2 Comparison of heat-shock survival in the first and second sets of competent *E. coli* DH5α cells.

Growth temp. (ºC)	Survival of heat-shock with pUC19 (%)
1st Set	
37	0.18
42	0.076
2nd Set	
37	96
42	470

Results from the attempted transformations of the first set of competent cells indicated both low cell concentration and low competence levels, so a second set of cells was prepared. Trends in transformation efficiency for this second set are shown in Table 3. Overall transformation efficiency was low in all growth conditions investigated. No significant difference in transformability was found between the 37 ºC and 42 ºC cultures, although the commercially prepared One Shot® TOP10 cells displayed a success rate nearly 20 times higher than those we prepared.

Table 3 Effect of growth temperature on transformation efficiency of *E. coli* DH5α cells.

Growth temp.	Calculated Transformation Efficiency			Expected transformation efficiency
	Transformants (heat-shocked cells) (%)	Transformants (non-heat-shocked cells) (%)	Transformants /µg plasmid DNA	Transformants /µg plasmid DNA
37ºC	0.0029	0.0027	3900	2.3×10^6 [a]
42ºC	0.0029	0.014	880	n/a
TOP10	0.043	n/a	32000	1.0×10^9 [b]

[a] Tu et al (18)
[b] Invitrogen manual, Invitrogen Life Technologies, Carlsbad, Ca.
n/a = data not collected

DISCUSSION

Growth curves determined from the cultures of DH5α *E. coli* cells grown at 37 ºC and 42 ºC displayed similar growth kinetics. The overall similarity in growth kinetic revealed no compelling reason why cells grown at one temperature should be favoured over another with regards to practicality of culture preparation.

Assessment of transformation efficiency in cells grown at the two temperatures tested revealed no indication of any difference in the mechanism of plasmid uptake. In both cases, only 0.0029% of cells heat-shocked in solution with pUC19 plasmid DNA were successfully transformed (Table 3). When survival of the heat-shock step is accounted for, cells grown at 42 ºC showed a marked advantage over the 37 ºC cells. The cells prepared from the 37 ºC culture yielded approximately 1 transformant for every 37,000 viable cells, whereas the cells from the 42 ºC culture produced 1 transformant for every 7,100 viable cells. We believe this significant difference can be explained by the fact that the cells grown at 42 ºC had a membrane composition that was better adapted to the heat-shock conditions. These cells would therefore have been more resilient to heat-stress than the 37 ºC cells. Thus, while there is no evidence to support the proposed model of plasmid DNA uptake, there is an indication from our data that cells grown at 42 ºC may be favourable for use in heat-shock transformation over the traditional 37 ºC cultures.

Initial difficulties during transformation with competent cells revealed several intricacies in the process of preparing and storing competent cells that should be considered in any protocol. The original preparation of cells yielded unexpectedly low numbers of viable cells both before and after heat-shock and no successful transformants were observed. It is believed that the former problem was attributable to the storage conditions at -85 ºC, namely the lack of glycerol. Results from a previous study show that when glycerol is included in the storage medium, viability is preserved (10).

Pre-storage conditions at -85 ºC are also likely to have been a causative factor in the low number of cells surviving heat-shock. Competent cells frozen before use have been shown to be more sensitive to transformation procedures than freshly prepared cells (9). It has also been demonstrated that the recovery period has no effect on and may even be detrimental to previously stored cells (7). The use of freshly prepared competent cells in the second set of transformations improved heat-shock survival considerably (Table 2).

The low numbers of viable cells alone do not account for the lack of successful transformants. A transformation rate as low as 0.01% should have yielded some transformants. Therefore, it is believed that the prepared cells had a reduced level of competence.

Another factor that could have had an effect on transformation efficiency was the handling of competent cells during preparation. It has been noted that maintenance of electro-competent cells at low temperatures (below 4 ºC) is important for optimal transformation efficiency (16) and would likely be applicable to chemically competent cells (W. D. Ramey, personal communication). Therefore, the use of non-chilled pipettes to transfer $CaCl_2$ solution to the *E. coli* DH5α cell pellet may have warmed up the solution sufficiently to render the cells non-competent despite all other steps being performed in chilled centrifuge or microfuge tubes. This potential source of

error was corrected in the second preparation of competent cells.

A second possible factor for the low transformation efficiency is the use of glass test tubes instead of plastic microfuge tubes during heat-shock. At least one study has shown that the use of glass tubes during heat shock reduces competency 10 fold over plastic tubes (8). Again, speculation that this may have played a role in reducing competency lead us to switch to the use of plastic tubes the second time competent cells were prepared.

The prolonged incubation in $CaCl_2$ could also have improved competency of the cells. The first set of cells was incubated in $CaCl_2$ for only one hour before they were flash-frozen, and the second set of competent cells incubated in $CaCl_2$ on ice for 1.5 hours before transformation. By contrast, incubation of cells in $CaCl_2$ for a longer period (24 hours) improves competency 20-30 times over cells used immediately (2).

Another potential source of error in preparing the first set of competent cells was the use of an unbuffered $CaCl_2$ solution, as opposed to the second set, in which the $CaCl_2$ was buffered at pH 8.0. The pH of the $CaCl_2$ solution was not specified in the protocol (15), likely because it was expected to be within the pH range of distilled water in which the $CaCl_2$ salt was dissolved in. However, it was later found that the pH of the $CaCl_2$ solution used was actually acidic, at a pH of 5. Since the heat-shocked cells were diluted in and neutralized by LB broth before the recovery step, the acidity is not likely to be a factor in diminished growth, and hence, the number of transformed cells recovered. However, decreasing pH has been linearly correlated to decreasing conductance of PHB channels to bivalent cations such as Ca^{2+} (3). This is significant because DNA translocation *in vitro* across a lipid bilayer appears to be an electrophoretic process (17). The potential difference required for this process is generated by the activity of high conductance channels in the membrane, thus a decreased conductance of the PHB channels due to low pH would likely result in defective translocation of DNA. This notion of the role of PHB in transformability of cells is supported by the high amounts of PHB found in the membranes of competent cells in correlation to their transformability (14).

Despite the noted compensations for low competence in the first set of cells, the second set of cells also yielded unusually low numbers of transformants per µg DNA (Table 3). Another point to be considered in this analysis is that the starting concentration of cells was different in each case (Table 1), and the concentration of transformants per µg DNA is intrinsically dependent on this parameter. Nevertheless, comparison of calculated and expected rates of transformation in the One Shot® TOP10 cells indicated unidentified problems with the heat-shock protocol and post-transformation recuperation. Here, for example, the use of SOC medium instead of LB is recommended for better recovery (Invitrogen manual, Invitrogen Life Technologies, Carlsbad, Ca.). Post-heat shock concentration of total numbers of One Shot® TOP10 cells surviving the heat-shock step (1.5×10^8 cfu/mL, Table 1) would indicate that the choice of medium indeed played a role, as this concentration is already lower than the expected value of 1.0×10^9 cfu/mL transformants using SOC (Invitrogen manual, Invitrogen Life Technologies, Carlsbad, Ca.).

In conclusion, this study found no appreciable difference in transformation efficiency between cells grown at 37 ºC and 42 ºC that would support a model of plasmid uptake involving DNA entering the cell via transient gaps in the membrane. This may be due to the fact that the differences in membrane fluidity between 37 ºC and 42 ºC were not sufficient to elicit significant improvement of the transformation process. There was, however, convincing evidence to show that cells grown at 42 ºC are preferable for use in chemically-induced transformation, owing to their higher survival rates of heat-shock.

FUTURE EXPERIMENTS

It is possible that the experimental growth temperatures selected for this study did not effect sufficient differences in membrane composition to bring about the necessary changes in fluidity. A wider difference in growth temperature could provide the support for this model. Preliminary testing with cells grown at 27 ºC indicated higher transformation efficiency in these cells (data not shown). Therefore, we recommend a follow-up study with growth temperatures of 27 ºC and 17 ºC, for example, to test the same parameters at wider temperature extremes. We further recommend that any such follow-up be conducted with the aforementioned 24 hour incubation of competent cells in $CaCl_2$ as well as plating on SOC medium as opposed to LB.

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