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Effect of Electroporation Versus Hanahan Protocols on the Transformation of *Escherichia coli* HB101 with Chromosomal DNA from *Escherichia coli* HB101, *Escherichia coli* B23, and *Bacillus subtilis* WB746 and the Plasmid p328.5, Including an Analysis of Competent *Escherichia coli* HB101 Cellular Freeze Tolerance

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The plasmid p328.5 was used to transform competent *Escherichia coli* cells, using either the Hanahan or electroporation protocols, and the resulting transformation efficiencies were compared. In each case, competent cells were transformed under four different conditions: (i) plasmid DNA alone, or the plasmid in addition to chromosomal DNA from either: (ii) *E. coli* HB101, (iii) *E. coli* B23 or (iv) *Bacillus subtilis* WB746. Results from the Hanahan protocol showed that, as expected, there was a reduction in transformation efficiency when cells were transformed by both the plasmid and chromosomal DNA, with DNA from *B. subtilis* having the largest net decreasing effect. Upon comparison with the results of electroporation transformation, the opposite was seen; cells transformed with both plasmid and chromosomal DNA from *B. subtilis* showed the highest net rate of transformation efficiency. These results indicated that host cell or foreign chromosomal DNA does reduce transformation efficiencies in the Hanahan protocol. Few conclusions can be drawn from the electroporation results, which appeared erratic and random. These results may have been attributable to differences in the lengths of the chromosomal DNA fragments used. Competent *E. coli* cells, for both the Hanahan and electroporation methods, were frozen in either $CaCl_2$ (chemically competent cells), sterile distilled water (electrically competent cells) or a glycerol cryoprotectant solution and their viability was tested after 48 hours at –20°C. Our results showed that most of the cells died despite the presence of glycerol when frozen at –20°C. Some growth of competent cells frozen with glycerol was seen on nutrient agar plates, but the number of colonies on a final count was still low after more than 48 hours growth. No colonies were seen from samples frozen without any glycerol.

The cellular uptake of DNA and its subsequent addition to the cellular genomic complement is a naturally occurring process that has been studied extensively. The two protocols used in laboratory settings, for the transformation of cells, are the electroporation and Hanahan protocols (also called the calcium chloride method). Electroporation involves subjecting cells to short pulses of electricity in order to facilitate DNA uptake through the cellular membrane. Any extracellular DNA in solution can then move into the cell. Although quite efficient, one disadvantage is the high rate of cellular mortality involved with the procedure. The Hanahan protocol, on the other hand, involves subjecting cells to an ice-cold $CaCl_2$ solution. This treatment facilitates the binding of DNA to the cellular membrane. Following a heat shock, the cells are made more efficient at natural DNA uptake and will take-in any extracellular DNA that is bound to the cellular membrane (2).

In a previous set of experiments (4), *E. coli* HB101 was transformed using only the Hanahan protocol. The cells were transformed with both a plasmid (p328.5) and contaminating chromosomal DNA. The chromosomal DNA was isolated from the *E. coli* strain HB101, another *E. coli* strain (B23) or a *B. subtilis* strain (WB746). The results showed that the foreign, contaminating, chromosomal DNA significantly reduced transformation by the plasmid.

In the following experiment, we have attempted to compare the electroporation and Hanahan methods of transformation under the same conditions, in order to examine further the aforementioned experimental results. Our hypothesis is that electroporation should show a much higher transformation efficiency than the Hanahan protocol, since the DNA is physically dragged into the cell. Binding of DNA to the membrane, as in the Hanahan protocol, may allow the cell to selectively bind certain sequences of DNA. We also hypothesized that the use of the Hanahan method of transformation would show that foreign chromosomal DNA, containing sequences different than those of

the competent cell, would be more effective at preventing transformation by the plasmid, because DNA-binding proteins might preferentially bind these novel sequences.

We have also examined the effects of freezing on competent *E. coli* cells that were not stored in a cryoprotectant (glycerol) solution prior to transformation. This study arose from a previous round of experimentation (data not shown). Experimental and technical difficulties resulted in freezing competent cells for several days in a –20°C freezer. The experimental results were inconclusive because most plates had very few colonies, regardless of dilution. We therefore decided to investigate whether the poor results were a consequence of freezing the competent cells without glycerol.

MATERIALS AND METHODS

Cultures of *E. coli* HB101, *E. coli* B23 and *B. subtilis* WB746 were grown overnight in 20 ml of nutrient broth in a water bath at 37°C, shaking at 180 rpm. The following day the culture was centrifuged at 9000 rpm for 9 minutes. Following centrifugation and decanting of the supernatant, the cells were resuspended in 1 ml of TE buffer (pH 7.6). 1 ml of 5 mg/ml lysozyme was added to the mixture which was then incubated at 37°C for 30 minutes. Following incubation, 100 µl of 10 mg/ml proteinase K was added. The mixture was then incubated at 37°C for 15 minutes. After this second incubation, 500 µl of 10% SDS was added and the mixture was allowed to reach room temperature. 200 µl of 3M sodium acetate was added, followed by gentle shaking and the addition of 2 ml of phenol that had been equilibrated with TE buffer. The mixture was shaken for 10 minutes and then spun at 7000 rpm for 10 minutes. After centrifugation, the aqueous layer was removed and placed in a new corex tube to which 2 ml of 1:1 phenol chloroform was added. Care was taken not to get any of the interface mixed with the aqueous phase. The aqueous phase was then mixed for 10 minutes and centrifuged for 10 minutes at 7000 rpm. The process of removing the aqueous phase, adding phenol-chloroform, shaking and spinning was repeated once more. Once the centrifugation step was complete, the aqueous layer was removed and placed into a new tube. The DNA was slowly spun out using a pasteur pipette. The DNA was then washed twice using 2 ml 70% ethanol and 2 ml 0.1M sodium acetate. The DNA on the pasteur pipette was then placed into a 1.5 ml Eppendorf tube to which 200 µl TE buffer was added to allow the DNA to dissolve.

The plasmid p328.5 was isolated from *E. coli* HB101 as previously described (4). This culture was grown overnight in nutrient broth with both ampicillin and chloramphenicol to enhance plasmid production (4). For the first round of experimentation, *E. coli* HB101 cells were made competent one week prior to transformation and stored at –20°C without any cryoprotectant. For the second round of experimentation the cells were made competent the same day as transformation. To assess the impact of chromosomal DNA on the uptake of plasmid DNA, various combinations of plasmid and chromosomal DNA were used to transform the competent cells. Each DNA mixture was used to transform cells in either the Hanahan protocol or electroporation as previously described (2). Four vials of electro-competent and Hanahan-competent cells from the second round were saved and stored for one week at –20°C. Two of each type of competent cells were stored in water and the remaining pair were stored in glycerol at a final concentration of 20%. The following week, these cells were streaked onto nutrient agar plates to assess viability. They were also transformed: one set with plasmid and another without to determine the effects on the two storage methods on the two transformation protocols (Appendix A).

RESULTS

Table 1 indicates the actual number of observed colonies at different amounts of plasmid for the Hanahan method. The transformation efficiencies are represented by the number of transformants per microgram of supplied plasmid (Table 2). For cells that received 5 µg of plasmid, the transformation efficiency of the pure plasmid is only about twice that of the cells that had added HB101 or B23 chromosomal DNA as well. When the cells received 10 µg of plasmid, the pure plasmid condition tripled in its efficiency while the number of transformants per microgram of plasmid increased to a smaller extent in the conditions where chromosomal DNA was present. Overall, however, transformation efficiencies did not change dramatically between the different amounts of plasmid, possibly due to the fact that the values were initially rather low.

TABLE 1. Effects of Nucleic Acid Concentration on Transformation for Hanahan method in the presence and absence of chromosomal DNA from E. coli HB101, E. coli B23, B. subtilis WB746.

Transformation condition	Observed colony numbers at different amounts of plasmid (µg)		
	0	5	10
Plasmid Control	0	110	610
Plasmid and HB101	0	50	240
Plasmid and B23	0	50	140
Plasmid and WB746	0	0	190

TABLE 2. Effects of Chromosomal DNA on Transformation by Plasmid for Hanahan method in the presence and absence of chromosomal DNA from E. coli HB101, E. coli B23, B. subtilis WB746.

Transformation condition	10^{-6} Transformants per viable cell at the following microgram amounts of supplied plasmid			Transformants per microgram of supplied plasmid at the following microgram amounts of supplied plasmid		
	0	**5**	**10**	**0**	**5**	**10**
Plasmid Control	0	3.33	18.5	0	22	61
Plasmid and HB101	0	1.52	7.27	0	10	24
Plasmid and B23	0	1.52	4.24	0	10	14
Plasmid and WB746	0	0	5.76	0	0	19

Table 3 illustrates the observed colony numbers after transformation by electroporation. In the category of cells that received 5 μg of plasmid, the transformation efficiency of the pure plasmid was higher than that of the pure 10 μg of plasmid in the Hanahan protocol (Table 4). For the condition that had B23 chromosomal DNA in addition to 5 μg of plasmid, efficiency was only slightly below the pure plasmid condition. The efficiencies of samples given 5 μg plasmid plus HB101 or WB746 chromosomal DNA were respectively almost 2-fold and 10-fold greater than that of the pure plasmid. For the cells that received 10 μg of plasmid, the pure plasmid condition's efficiency increased almost 7-fold. The transformation efficiencies in the 10 μg plasmid plus chromosomal DNA decreased from their values at 5 μg of plasmid.

TABLE 3. Effects of Nucleic Acid Concentration on Transformation for Electroporation method in the presence and absence of chromosomal DNA from E. coli HB101, E. coli B23, B. subtilis WB746.

Transformation condition	Observed colony numbers at different amounts of plasmid (μg)		
	0	**5**	**10**
Plasmid Control	0	360	4700
Plasmid and HB101	0	700	0
Plasmid and B23	0	330	480
Plasmid and WB746	0	3220	1810

TABLE 4. Effects of Chromosomal DNA on Transformation for Electroporation method in the presence and absence of chromosomal DNA from E. coli HB101, E. coli B23, B. subtilis WB746.

Transformation condition	10^{-6} Transformants per viable cell at the following microgram amounts of supplied plasmid			Transformants per microgram of supplied plasmid at the following microgram amounts of supplied plasmid		
	0	**5**	**10**	**0**	**5**	**10**
Plasmid Control	0	468	1660	0	72	470
Plasmid and HB101	0	34.5	0	0	140	0
Plasmid and B23	0	75.0	133	0	66	48
Plasmid and WB746	0	460	369	0	644	181

The viability tests were performed to see if cells could survive freezing either with or without glycerol. Transformation by either the Hanahan method or electroporation was performed on aliquots of competent cells that had been frozen. The results were that cells that had not been treated with glycerol were not viable prior to the transformation, and cells that were treated with glycerol prior to freezing were viable, but produced few colonies that took more than 48 hours to grow (Tables 5 and 6). Since very few viable cells were put through transformation, no cells were obtained after transformation by either electroporation or the Hanahan protocol.

TABLE 5. The Effects of Glycerol on the Viability of Frozen Cells (chemically competent)

Cell Storage condition	Viability Pre Transformation	Viability Post Transformation
With Glycerol	Very little growth*	No growth
Without Glycerol	No growth	No growth

**After 48 hours, very few colonies (<20) were observed on these plates*

TABLE 6. The Effects of Glycerol on the Viability of Frozen Cells (electrocompetent)

Cell Storage condition	Viability Pre Transformation	Viability Post Transformation
With Glycerol	Very little growth*	No growth
Without Glycerol	No growth	No growth

**After 48 hours, very few colonies (<20) were observed on these plates*

DISCUSSION

When the results from the Hanahan protocol in this study are compared to those of the previous study (4), several differences can be seen. First, the transformation efficiencies with both the 5 and 10 μg samples of plasmid were lower than expected. The previous study showed a substantial increase in the number of transformants with pure plasmid compared to plasmid with B23 or WB746 DNA. In some unreported experiments, the foreign DNA eliminated transformation by p328.5 altogether. Our results showed that the foreign DNA had a negative effect that was not as pronounced as those seen in the earlier set of experiments (4). The B23 DNA lowered transformation efficiency by about 50% when 5 μg of plasmid was present; no transformation by the plasmid was observed in the presence of WB746 at 5 μg of plasmid. At 10 μg of plasmid, the effect of both B23 and WB746 on the transformation by p328.5 was diminished, while the transformation efficiency nearly tripled with pure plasmid. These observed differences most likely resulted from the increased amount of p328.5. At 10 μg, the amount of plasmid would have been equal to the amount of added chromosomal DNA.

The effect of HB101 chromosomal DNA seemed to be larger in our experiment than expected. Unlike the previous study (4), transformation by plasmid did not completely out-compete the addition of HB101 chromosomal DNA when the concentration of plasmid was increased. One reason for this result may be that the amount of plasmid DNA in our experiment was doubled rather than quadrupled as in the first study; therefore, there may not have been enough plasmid DNA present to eliminate the effect of added chromosomal DNA even at 10 μg. However, since the number of transformants with pure plasmid at both amounts of plasmid was much lower than expected, it is difficult to draw this conclusion. In contrast to the previous experiment, the ratio of plasmid to foreign DNA used was 1:1 at 10 μg of plasmid. The cells may have taken up host cell-type chromosomal DNA and plasmid DNA equally.

Although transformation efficiency in the Hanahan protocol almost tripled from 5 μg to 10 μg of plasmid, it should be noted that the actual number of transformants per microgram of supplied plasmid (Table 1) was far lower in our study than in the first study (4). Part of the reason for this decrease may be the isolation method used in obtaining pure plasmid, and the storage of plasmid DNA. The plasmid DNA was isolated from a culture containing p328.5 about three weeks before being used in our final experiment. It was stored in the –20°C freezer, but was thawed several times for use in prior studies before the final experiment from which our results are drawn. These freeze-thaw cycles may have caused some of the DNA to become nicked or sheared (Nucleic Acid Isolation and Purification Manual, p. 154, Roche Diagnostic Corporation). Nicked or fragmented plasmid DNA would have led to a lower transformation efficiency. Our initial hypothesis was that the electroporation method would generally produce more transformants per microgram of supplied plasmid than the Hanahan method. The results from Tables 2 and 4 indicate that this hypothesis was supported in all test conditions, except 10 μg plasmid plus HB101 chromosomal DNA. This particular result appears to be anomalous, and may be due to a technical error rather than an experimental variable. In the other conditions, electroporation appears to be a more effective method of transformation of HB101 cells by p328.5, even in the presence of foreign DNA.

The first study led to the conclusion that both types of foreign DNA affected the transformation more strongly than host cell type DNA (4). B23 DNA, from a strain of *E. coli*, and *B. subtilis* WB746 DNA, had a larger negative effect on transformation than host *E. coli* HB101 DNA. This result was seen to some extent in the Hanahan method. Electroporation, however, appears to randomize the effects of foreign and host cell chromosomal DNA. There were more transformants with plasmid and WB746 DNA than with plasmid DNA and HB101 DNA, or B23 DNA. In the 5 μg plasmid condition, transformation efficiency was nine-fold greater with plasmid plus WB746 DNA than pure plasmid. These results concur with the hypothesis that electroporation allows cells to pick up any DNA that surrounds the cells rather than to specifically bind plasmid DNA. If the WB746 DNA was fragmented or sheared during the process of isolation or further experimentation, there may have been less physical hindrance of the plasmid DNA that surrounded the cells during electroporation; as a result, transformation efficiency would have increased. However, it is not known why the transformation efficiency for the 5 μg of plasmid plus WB746 DNA was so much higher than that of the control condition (Table 4). We suspect this anomaly to be a result of experimental error. The viscosity of both plasmid and chromosomal DNA preparations may have led to pipetting errors; it is possible that more than 5 μg of plasmid DNA was added to the tube. Replicating the procedure would provide a clearer indication of whether error caused this result.

In the first round of experimentation (data not shown), competent cells were made one week prior to transformation. The cells were stored in distilled water at –20°C. After electroporation or chemical transformation, very few or no colonies were seen on the plates. Because no cryoprotectant was used, the freezing likely caused cellular damage. This damage was so severe that the cells were unable to survive once they were returned to more favourable conditions. For our second round of experimentation, the effect of freezing competent cells was examined. As expected, the cells stored only in water failed to survive freezing. Low levels of cells stored using 20% glycerol as a cryoprotectant grew on agar at 37°C. Still, none survived transformation, thus indicating that storing cells at –20°C reduces their capability to survive transformation regardless of protocol. The freezer in the lab may also have been a factor; it may not have kept the temperature constant at –20°C. As well, it may have been kept open at times, causing the internal temperature to rise. A better procedure for long-term storage would be to suspend cells in aliquots of TSS (transformation and storage solution), treat the aliquots with liquid nitrogen, and freeze tubes of cells at –70°C (1).

Future experiments may include investigation of the effect of digested (fragmented) foreign chromosomal DNA on transformation by plasmid. Results from this study have led to the hypothesis that chromosomal DNA physically inhibits plasmid uptake by *E. coli* cells. An experiment could be designed to test this hypothesis by using restriction enzymes to digest the DNA, and using electrophoresis to examine and isolate various sizes of DNA fragments. If transformation efficiency with pure plasmid is not significantly different from transformation with plasmid and added chromosomal DNA fragments, the hypothesis will be supported.

Optimizing long-term storage of competent cells may also prove to be a worthwhile investigation. Other methods that could be tested include stab agar storage and lyophilization. Our results, however, seem to indicate that cells should be used immediately after being made competent. Even if competent cells can be stored for a long period of time, they may lose viability; this effect could be limited if new cells are made competent for every round of experimentation.

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Appendix
A. Competent Cell Survival in –20°C Environment

1. Media & Reagents

All media & reagents required for Electroporation and Hanahan Protocols

4 vials containing 40 µl of electrocompetent cells

4 vials containing 50 µl of Hanahan competent cells

50% Sterile glycerol

2. Equipment

All equipment required for Electroporation and Hanahan Protocols

1 –20°C Freezer

1 Ice bath

1 Stab inoculator

1 Bunsen burner

4 Nutrient Agar plates

3. Protocol

1) Prepare four vials of each electrocompetent and Hanahan competent cells using the specific protocol as previously described (2, 4)
2) Store four vials of competent cells, two from each protocol in the –20°C freezer without adding any cryoprotectant.
3) For the remaining four vials of competent cells, two from each protocol, add enough 50% glycerol to the vial to achieve a final concentration of 20%. Then store these vials in the -20°C freezer.

4) After one week, remove the cells from the freezer and thaw them on ice.

5) Using a sterile stab, streak culture from each vial on to a nutrient agar plate.

6) For transformation use the following scheme

    a) *No DNA*: For one electrocompetent vial and one Hanahan competent vial that was stored in glycerol and one vial of each that was not stored in glycerol

    b) *5 μg p328.5 DNA*: For one electrocompetent vial and one Hanahan competent vial that was stored in glycerol and one vial of each that was not stored in glycerol

7) Complete the rest of the experiment following the Electroporation and Hanahan Transformation protocols as previously described (2, 4).