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Effect of Inhibitors on the Heat Shock Transformation of DNA

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There is little known about the mechanism of DNA heat shock transformation. Although there are a number of speculations as to how this occurs, no model has been fully analyzed or explained yet. Furthermore, the role of heat shock in the protocol is not identified either. In this report, different types of inhibitors were introduced separately into competent cells *Escherichia coli* DH5α before heat shock. In this manner, we attempted to prove that transformation efficiency is enhanced by protein production during heat shock and also to narrow down the possible regulation mechanism(s). Success rates of DNA transformation were assessed by selective and non-selective plating methods. It was found that the success rates of DNA transformation were improved by protein synthesis during heat shock. Also, there was evidence that the protein(s) involved are likely to be regulated at the translational rather than at the transcriptional level. Our results are in agreement with the molecular model on the regulation of heat shock σ^{32} regulons. However, additional experiments are required to further investigate whether or not the proteins involved are truly σ^{32}-related, and the exact mechanism of heat shock transformation remains to be studied.

The mechanism of DNA transformation is not fully understood even though it is often used in cloning experiments. One of the most popular DNA transformation methods is Hanahan heat-shock transformation (1), where heat shock is defined as the cell growth condition in which there is a sudden temperature increase from 0°C to 42°C in this paper.

If proteins synthesized during heat shock are involved in plasmid DNA uptake, heat shock proteins are the most possible candidates. To further explore a possible involvement of proteins in DNA uptake during heat-shock transformation, studies about heat-shock regulons and their regulations have been carried out. In general, heat shock genes encode groups of proteins, collectively called heat shock proteins (HSPS, 12). HSPS are expressed upon stresses, such as elevated temperature or the presence of organic solvents, and they repair protein misfolding and DNA damage (12). In *E. coli*, there are two sets of heat shock regulons, σ^{32} and σ^{E} regulons, whose promoters are recognized by RNA polymerase holoenzymes with sigma factors σ^{32} and σ^{E}, respectively. Majority of HSPS are encoded in σ^{32} regulons and the rest are encoded in σ^{E} regulons (12). σ^{32} regulon products are usually involved in the folding of cytoplasmic proteins whereas σ^{E} regulon products are engaged in extracytoplasmic and transmembrane proteins (9). Even though both regulons response to heat shock, their regulatory mechanisms are quite different. As shown in Figure 1, σ^{32} (*rpoH*) translation is enhanced upon heat-shock because the secondary structure of *rpoH* mRNA is denatured at an elevated temperature and become accessible for ribosome bindings and thus translation (13). A sudden rise of σ^{32} increases the transcription of σ^{32} regulons (5). On the other hand, σ^{E} activity is negatively regulated by the transmembrane proteins (13). Misfolded proteins caused by heat shock in periplasm act as a stimulus, ensuing signal transduction that leads to an increase in σ^{E} activity (13). As might be expected, σ^{32} is essential for growth at high temperature but it is not required for growth at low temperature (14). However, essentiality of σ^{E} remains controversial (13). Since HSPS from σ^{32} and/or σ^{E} regulons are presumed to be involved in transforming DNA during heat shock, initial approach would be to test if protein synthesis during heat shock is crucial for plasmid DNA uptake. If inhibitors on transcription and translation are added during heat shock, one would expect to see a decrease in the success rates of DNA uptake.

Many biological research projects extensively exploit DNA transformation. However, current heat shock transformation protocol yields low transformation efficiency at about one transformant per 10^4 cells (8). It is very time consuming for isolating transformants that carry plasmids with correct inserts in cloning experiments. Hence, a more efficient protocol is needed. If the mechanism of DNA uptake in transformation is further elucidated, heat shock transformation protocol can be further modified and higher efficiency could be obtained.

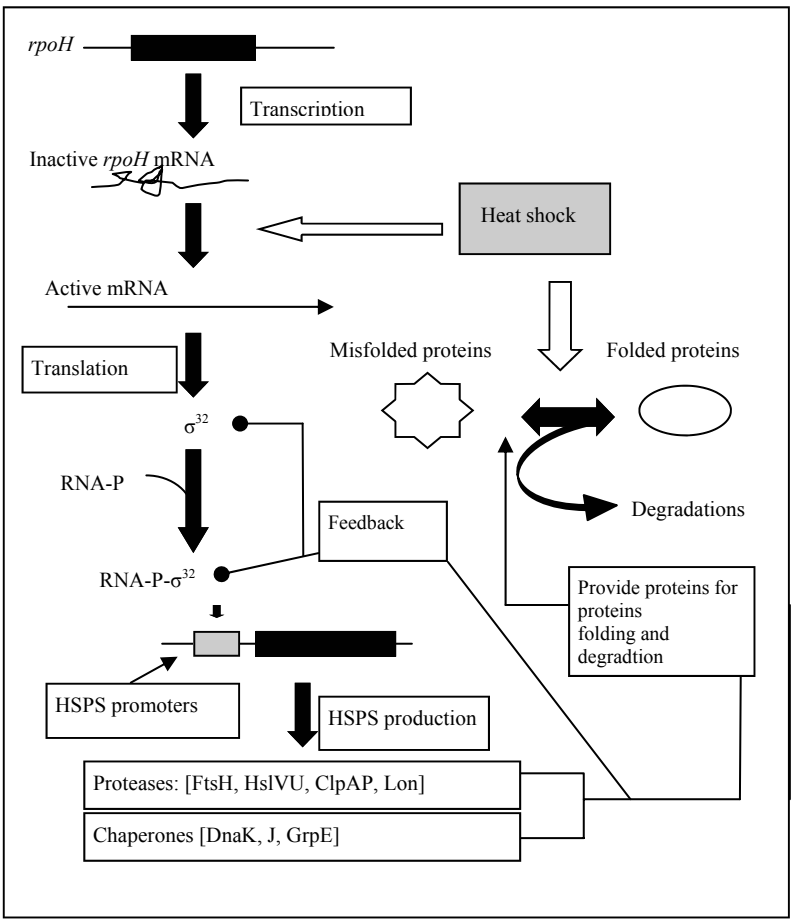

Fig. 1. Representation of regulation systems of heat shock proteins (originated from reference 13).

In this project, we have attempted to test whether protein production during the period of heat shock enhances the process of Hanahan transformation. Furthermore, to investigate how the proteins are regulated, different types of inhibitors have been introduced separately into competent cells before heat shock. The following inhibitors have been used: rifampicin, which prevents elongation step in transcription (9), tetracycline, which inhibits elongation step in translation (9), and sodium azide, which decreases the overall pool of energy availability inside a cell (http://cancerweb.ncl.ac.uk/cgi-bin/omd?query=aZide). It has been established that DNA uptake is repressed when protein synthesis is blocked by above inhibitors during heat shock. However, further experiments are indispensable to directly prove that the proteins required for DNA uptake during heat shock are indeed HSPS-related.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions: *E. coli* DH5α (http://www.lifetech.com/content.cfm?pageid=102) carrying pUC19 (http://gillnet.lab.nig.ac.jp/cgi-bin/accvec_bro.pl.) were used for plasmid pUC19 isolation. *E. coli* DH5α with no plasmid were used for pUC19 transformation. Cultures were grown aerobically overnight in Luria Bertani (LB) medium at 37 °C with vigorous shaking.

Media and Chemicals: 1x LB medium was prepared by mixing 10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride in 1 L of water and then autoclaving. LB agar plates were prepared following the same recipe with 1.8 % (w/v) agar. LB-ampicillin plates were prepared in the same manner except that filter sterilized ampicillin of concentration at 100 µg/mL was added after the autoclaved LB agar medium was cooled to 42 °C in water bath. Stock solutions of inhibitors for the transformation reactions were prepared according to Miller (6). Rifampicin stock at 4 mg/mL was prepared by dissolving in methanol, sodium azide at 20 mg/mL in sterile distilled water and tetracycline at 1.5 µg/mL in 50 % (v/v) ethanol/distilled water. The inhibitor stock solutions were sterilized by filtration with 0.45 µm pore filters and stored at 4 °C. One µL of each inhibitor was required for each trial of experiment. Ampicillin at 25 mg/mL was prepared by dissolving in sterile distilled water and filtering with 0.45 µm pore; it was prepared fresh every time as ampicillin readily degrades in water. For competent cell preparation, 100 mM calcium chloride was prepared by dissolving $CaCl_2$ salts in sterile distilled water with 15 % (v/v) sterile glycerol.

Competent Cell Preparation: Competent cell preparation was prepared as previously described (4) with few modifications. Plasmid-free DH5α cells were grown in 10 mL 1x LB medium for 10 hours. 100 mL fresh 1x LB was inoculated using the overnight culture and incubated at 37 °C for 3 hours to reach $OD_{600} = 0.38$. Then, cells were transferred into two 50 mL Falcon tubes on ice, following centrifugation at 4000 x g for 10

minutes at 4 °C. Supernatant was removed and cell pellet was resuspended in 10 mL of pre-chilled 100 mM $CaCl_2$ with 15 % (v/v) glycerol. Following the incubation on ice for 30 minutes, the cells were re-centrifuged and re-suspended in 2 mL of 100 mM $CaCl_2$ with 15 % (v/v) glycerol. Then, cells were divided into microfuge tubes so that each tube carried 1 mL and were stored at -80 °C until needed.

Plasmid Isolation: The protocol of pUC19 isolation followed that of Gibco BRL Concert Rapid Miniprep System with few modifications (http://www.lifetech.com/content.cfm?pageid=102). Ten mL of pUC19 carrying *E. coli* DH5α culture was grown for 10 hours in the presence of 100 µg/mL ampicillin. Cells were then washed, lysed and the plasmids were precipitated with propanol and ethanol. The plasmids were eluted with distilled water instead of TE (Tris-EDTA) as indicated, because EDTA molecules would chelate divalent cations such as Ca^{2+} and might affect the results of the subsequent transformation reactions. Qualities of the plasmids were assessed by a preliminary trial transformation and agarose gel electrophoresis. Isolated sample was quantitated by measuring absorbance at 260 and 280 nm (11).

Preliminary Transformation Experiment: Transformation protocol followed the procedure given along with LIBRARY EFFICIENCY® DH5α Competent Cells (http://www.lifetech.com/content.cfm?pageid=102) with some specifications. Two µL of DNA solution carrying 22 ng of plasmid DNA in distilled water was used. Instead of SOC medium, 1x LB medium was used for 1 hour non-selective incubation after heat shock. The cells were spread onto LB and LB-ampicillin plate at a final plated dilution of 10^{-2}. Cells with no plasmid sample introduced were used as a negative control.

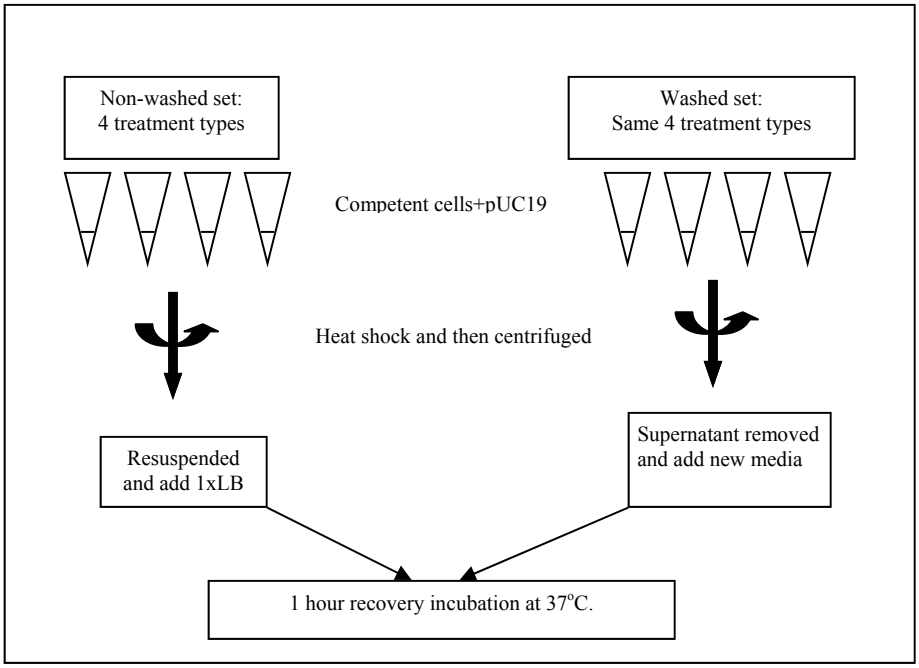

Fig. 2. Schematic of the transformation protocol.

For Transformation 1, the protocol was modified from the protocol given along with LIBRARY EFFICIENCY® DH5α Competent Cells (http://www.lifetech.com/content.cfm?pageid=102). Competent cells were thawed on ice and each 100 µL of cells were transferred into eight pre-chilled sterile 1.5 mL microfuge tubes. 22 ng of pUC19 DNA was added to the cell suspensions in the microfuge tubes, followed by gentle tapping for mixing, and then the tubes were incubated on ice for 30 minutes. The tubes were then divided into four sets, with two tubes a set. Five minutes before heat-shock, 1 µL of each stock inhibitor solution was added into each of the two tubes of competent cells. Each pair of competent cell tubes contained 40 µg/mL rifampicin, 0.2 mg/mL sodium azide, or 15 µg/mL tetracycline. Another two tubes contained 1x LB and served as a positive control. After ice-incubation, the cells were heat-shocked for 45 seconds in a 42 °C non-shaking water bath and then placed on ice for 2 minutes. The cells were then centrifuged at 10000 x g for 1 minute. One of the two tubes for each inhibitor treatment was re-suspended and 0.9 mL of 1 x LB was added; this was called non-washed set. The supernatant of the other tube of the same inhibitor treatment was replaced by 0.9 mL of fresh 1x LB and 0.1 mL of 100 mM $CaCl_2$ in 15 % (v/v) glycerol to re-suspend the cell pellet; this was called washed set. The diluted cells were incubated at 37 °C shaker bath for 1 hour. Figure 2 gives a brief flowchart of the experiment. At 5, 20, and 40 minutes of non-selective incubation, 50 µL of each tube was sampled to perform dilutions, using 4.95 and 4.5 mL 1x LB blanks. 0.1 mL of each diluted samples was plated on LB plates in order to get the desired dilutions. At 60 minutes, the remaining samples were diluted and plated on LB as well as LB-ampicillin plates.

For Transformation 2, the following modifications were made. Firstly, a negative control was included by subjecting 100 µL competent cells to heat shock without the addition of pUC19. These cells were then plated on LB and LB-ampicillin plates at 10^{-1} dilution at 60 minutes. Secondly, since Transformation 1 gave many too-numerous-to-count (TNTC) plates, dilutions in Transformation 2 were increased. For a positive control and inhibitor treated samples, LB dilutions ranged from 10^{-5} to 10^{-7} and LB-ampicillin dilutions ranged from 10^{-1} to 10^{-3}. Lastly, samples at each time point were saved and stored at 4°C refrigeration in case if any anomaly was observed in plate counting.

RESULTS

Prior to any transformation reactions, plasmid purity was examined by gel electrophoresis. In Figure 3, isolated plasmid sample in lane 5 showed two bands. The intense band seems to be a supercoiled conformer whereas the weak band an open circular conformer, given that supercoiled DNA demonstrates higher mobility. The weak band corresponds to about 2.7-2.8 kb in the 1 kb ladder, while the intense band corresponds to about 2.7 kb in the supercoiled DNA ladders. This indicates that the isolated plasmid is pUC19, which is 2.686 kb in size. According to the brightness of these two bands, majority of the pool of pUC19 appeared to be supercoiled. No other band was observed on the lane, suggesting that the isolated pUC19 was reasonably pure. Concentration of plasmid DNA was determined to be 11 ng/μL.

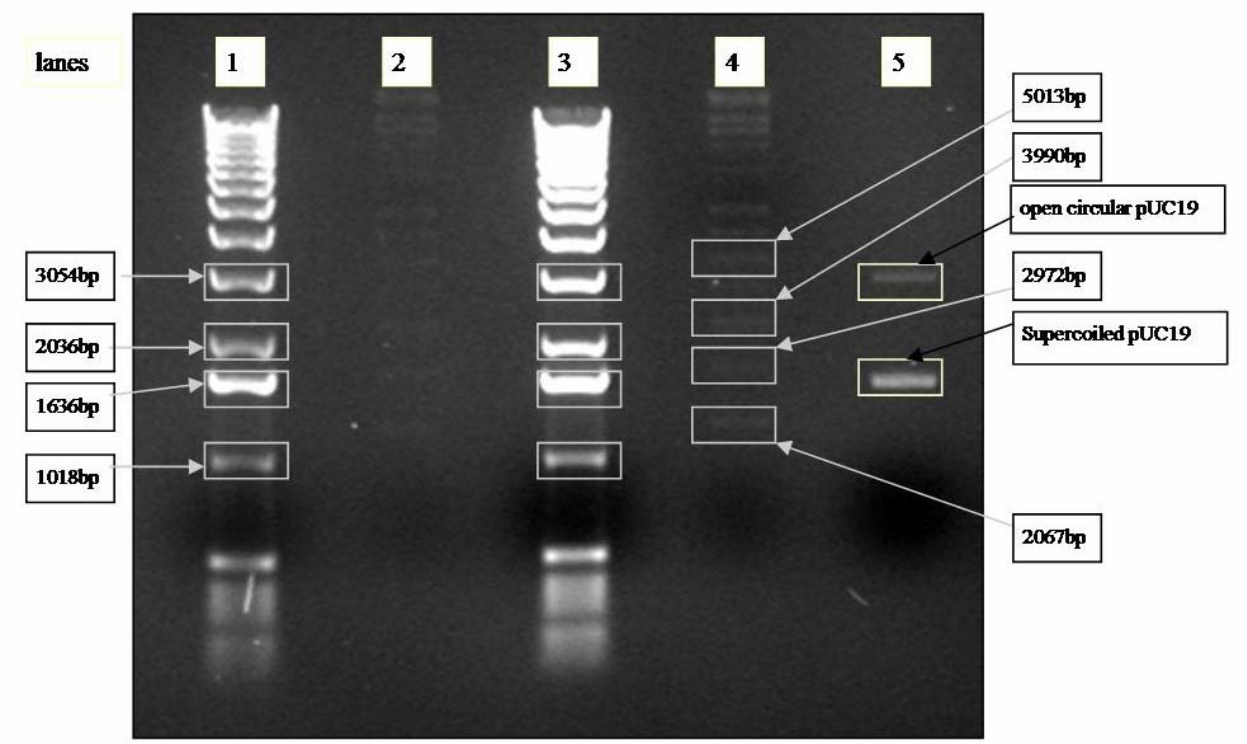

Fig. 3. Photograph of the electrophoresis agarose gel (0.8% w/v) at 80V with isolated plasmid sample from DH5α carrying pUC19. Lanes: 1 and 3 contain 10 and 20 μL of 1 kb ladder respectively, 2 and 4 contain 10 and 20 μL of supercoiled DNA ladder respectively, and 5 contains isolated plasmids.

A preliminary transformation was performed to provide evidence that the isolated plasmids were pUC19 with ampicillin resistance gene. At dilution of 10^{-2}, a confluent lawn of cells was observed on LB plate, whereas approximately 200 colonies were observed on LB-ampicillin plate. A negative control, to which no plasmid was added, had no colony on LB-ampicillin plate, but a confluent lawn of cells on LB plate at dilution of 10^{-1}. This implies that all colonies on LB-ampicillin carried pUC19 and that mutants with endogenous ampicillin resistance gene were not present in empty DH5α cells.

The growth trends of the control and the inhibitor treated cells were assessed by isolating and plating samples taken at various time points during the one-hour recovery period. As shown in Figure 4, great variability in positive controls was observed for both washed and non-washed sets. At 5 minutes, both control sets had the highest concentrations compared to the other time points. For the washed set, the concentration was the lowest at 20 minutes and increased to steady state afterwards. For the non-washed set, the concentrations were lower for 20 and 40 minutes and increased at 60 minutes. In general, all inhibitor treated cells experienced a lag phase for the first 20 minutes, and then the concentrations increased continually. However, there were deviations from this general growth trend for some treatments. Rifampicin non-washed set had a longer lag phase up to 40 minutes and had

lower cell concentrations at all time points than the other treatments. Thus, rifampicin seemed to have the strongest effect on cell viability, followed by tetracycline, and then sodium azide. Sodium azide non-washed set showed no lag phase. A sudden drop at 60 minutes of tetracycline non-washed set was probably due to a plating error.

Samples for each time point were saved at 4 ^{o}C for up to 4 days in case any anomaly was detected in plate counts and further plating was required. However, plating of refrigerated samples resulted in low cfu formations (data not shown). Presumably cells died during the cold incubation.

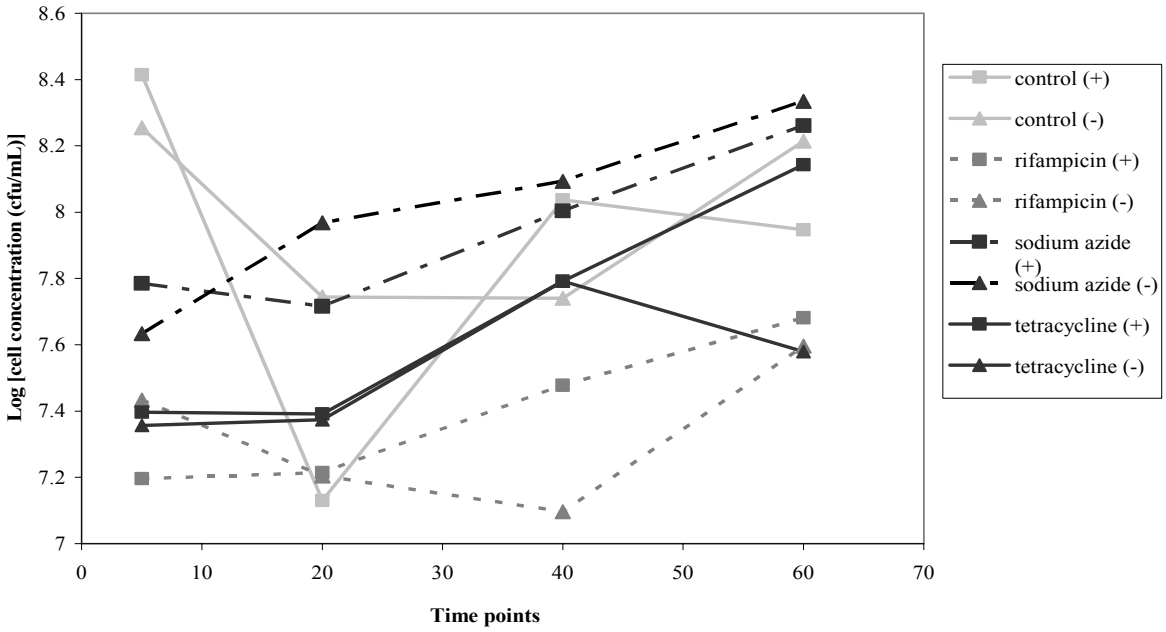

Fig. 4. Growth phase at various time points during the 1 hour recovery period.

Transformation efficiency was determined by dividing the transformant concentrations after 60 minutes of incubation, over the amount of pUC19 used. As shown in Figure 5, controls had higher efficiencies than inhibitor-treated cells. Except for controls, efficiency in washed set was higher than that in non-washed set for each inhibitor treatment. For washed set, efficiencies decreased in the order of control, sodium azide, rifampicin and tetracycline treatments with 95% confidence intervals (CI) overlapping in the last two. Efficiencies for non-washed sets decreased in the order of control, sodium azide, tetracycline and rifampicin with 95% CI's overlapping in the last two.

The transformation frequency was determined by dividing the concentration of transformants over that of total viable cells after 60 minutes of incubation, as shown in Figure 6. Concentrations of transformants and total cell populations were estimated by counting the number of colonies growing on LB-ampicillin and LB plates, respectively. Frequencies were higher in washed sets than in non-washed sets except for tetracycline samples. In washed set, frequencies decreased in the order of control, rifampicin, sodium azide, and tetracycline treatments. In non-washed set, frequencies decreased in the order of control, tetracycline, rifampicin, and sodium azide. Neither washed nor non-washed sets had 95% CI overlaps. Uncertainty estimations for efficiencies and frequencies were attained using Poisson analysis (10).

DISCUSSION

A careful examination of inhibitor treated cells was essential since the inhibitors might have affected the normal growth of the cells. Samples of different treatments were isolated at different time points during the one-hour recovery period, as shown in Figure 4. For both control washed and non-washed sets, cell concentrations were

expected to show a short lag phase and then increase exponentially as there was no inhibitor added. For inhibitor sets, cell concentrations were expected to exhibit a longer lag phase compared to control as inhibitors distress cell growth. If both washed and non-washed sets had similar growth trends, the effect of the inhibition could be considered to be negligible during the recovery period. This type of observations accounted for sodium azide and tetracycline sets. Comparison between rifampicin washed and non-washed sets showed that washed set had a shorter lag time than non-washed set. Thus, rifampicin seemed to be affecting cells' normal growth if it was not washed away after heat shock. Washed sets were determined to be more reliable than non-washed sets for subsequent interpretations of transformation success rates. This was because all the inhibitor washed sets showed a positive growth trend after 20 minutes during the recovery period whereas some non-washed sets did not. However, deviations were shown on the growth curve in Figure 4. Both washed and non-washed controls displayed large unexpected fluctuations, such as high cell concentrations at 5 minutes and unanticipated drops at 20 minutes. Also, a sudden drop at 60 minutes in tetracycline non-washed set and a lack of lag phase in sodium azide non-washed set were detected. These were probably caused by experimental errors from diluting and/or plating process. Despite all the variations, the cell concentration at 60 minutes of the control was believed to be valid for computation of rate of transformation for the reasons to be discussed later.

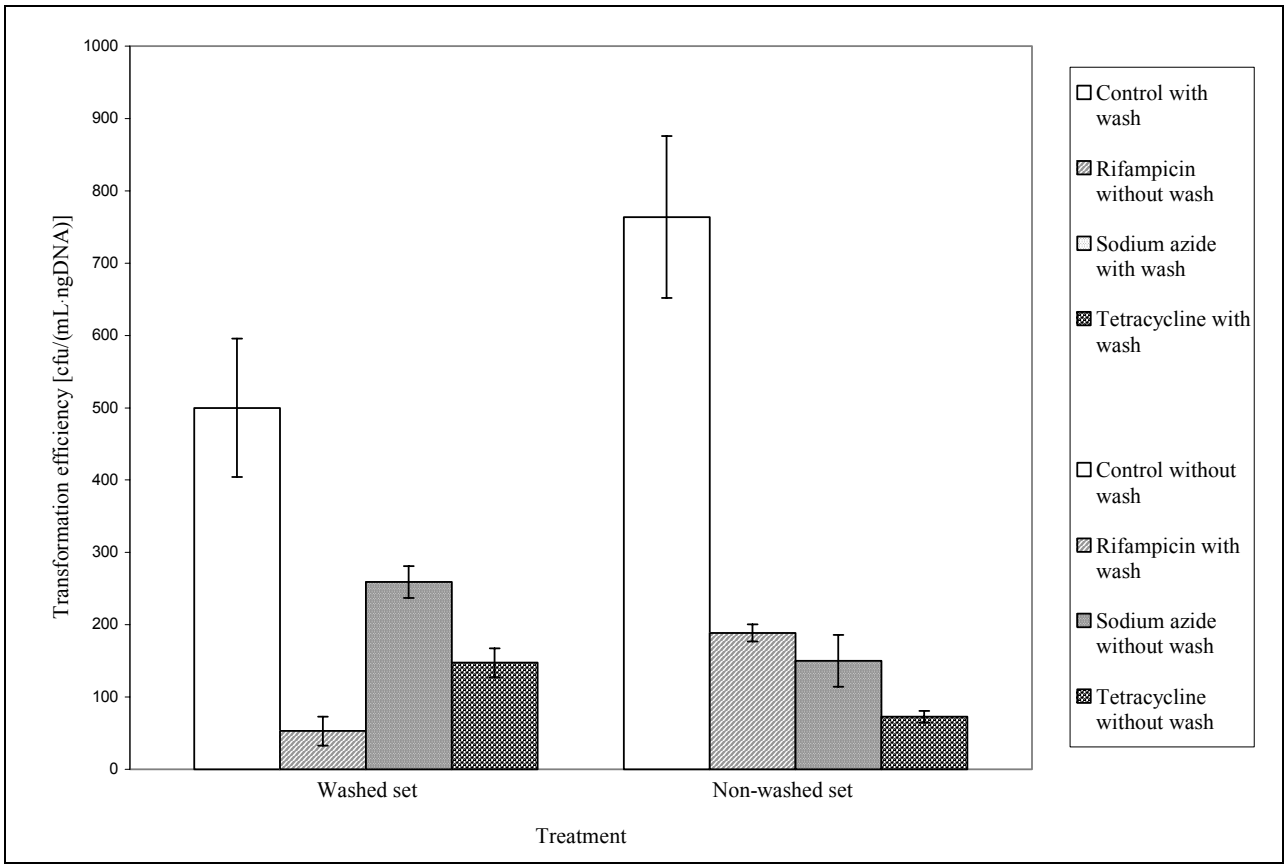

Fig. 5. Transformation efficiencies of different treatments.

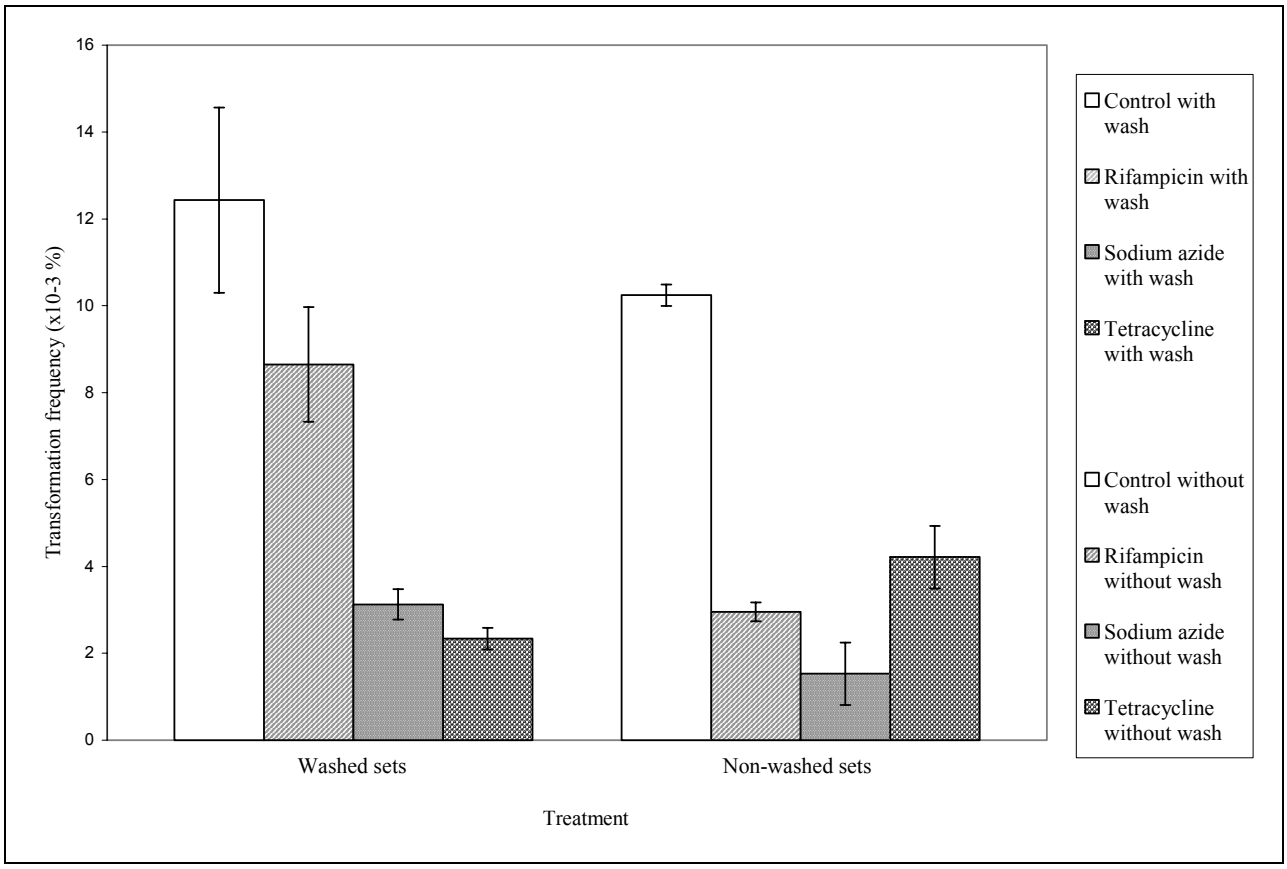

Fig. 6. Transformation frequencies of different treatments.

The purpose of introducing washed and non-washed sets was to test whether the inhibitors were affecting cells' growth during the one-hour non-selective incubation. Even though the concentrations of inhibitors were diluted in non-washed set, the remaining amount of inhibitors still might have had exerted effects on plasmid replication in the transformants. If inhibitors affected transformants during the incubation, plasmid replication might have decreased, resulting in an insufficient copy number to pass onto daughter cells; this would lead to a lower number of transformants than expected. The replication of plasmid is controlled by a complex system of enzyme interactions and RNAs priming at the origin of replication and it is reported to be more susceptible to inhibition than chromosomal DNA replication (3). Therefore, plasmid instability could have been another factor contributing to low transformation efficiencies and frequencies. In Figure 5, all inhibitor treated cells had higher efficiencies in washed sets than in non-washed sets. This indicates that if the inhibitors were not washed away, they would decrease transformability. Also, inhibitors might have affected plasmid stability, plasmid replication frequency and the expression of ampicillin resistance gene. The same explanations can be accounted for higher frequency values in washed than non-washed set for each treatment in Figure 6, except tetracycline samples. The opposite trend of frequencies in tetracycline samples was probably due to low cfu's on LB plate in non-washed set as pointed out earlier. Another factor that may contribute to low efficiencies and frequencies was that the cells could have been killed by the presence of inhibitors. On the other hand, as mentioned above, cell concentrations were not significantly affected in the inhibitors washed sets.

Consequently, based on the growth curve during recovery period and the plasmid instability, efficiencies and frequencies of the washed sets are more reliable than those of the non-washed sets for comparing the rates of transformation among treatment types.

In addition to inhibitors, there were some other factors contributing to variations of growth among different treatments. First, the starting concentrations of competent cells could have been slightly different. This could lead to large variations by the end of the incubation. In order to start with reasonably similar number of competent cells, cells were aliquoted from one tube of well-suspended cells. Secondly, small amount of cells could have been removed during the washing step. Rifampicin treated cells had the lowest cell concentration among all types of

treatments. It is unlikely that rifampicin inhibited growth of cells during the recovery period, especially for the washed-set, because an obvious log phase was observed after 20 minutes. Instead, it was more likely that the cells started at a lower concentration. This would not affect the subsequent calculation of transformation frequency, but would affect transformation efficiency as would be discussed below.

Transformation efficiency was determined by two factors, the amount of pUC19 and the number of transformants, where the former remained constant while the latter varied. As mentioned above, the number of transformants could vary due to the differences in the starting competent cell concentrations. Although an attempt to minimize any discrepancy on the starting number of competent cells was made as stated above, slight variations might still have influenced the number of transformants to a large extent. This will result in an unreliable efficiency, as the number of transformants on LB-ampicillin is the only determinant for efficiencies. To avoid this problem, emphasis was placed on transformation frequency in evaluating the success rate of transformation. Transformation frequency is more reliable because it normalizes the number of tranformants to each sample's own total viable population. However, it should be noted that frequency is more prone to sampling errors since it takes cell counts on both LB and LB-ampicillin plates. In order to minimize errors, dilutions were performed with thorough mixing, careful pipetting, and three different dilutions were plated at 60 mintues.

Transformation efficiencies and frequencies had a similar pattern in that control had the highest values among all treatments as illustrated in Figures 5 and 6. This means that the inhibitors did affect efficiencies and frequencies. Because frequency values are more dependable for interpretation as previously discussed, the majority of our results analysis would be focused on frequencies. In control, frequency is approximately 0.01%, which is close to the literature values (8); thus this provides presumptive evidence that plating at 60 minutes had little or no experimental error. The lowest frequency appeared in the tetracycline washed set. Tetracycline is a translation inhibitor that binds to ribosome A site and prevents peptide elongation (9). Thus, it is very likely that protein synthesis during heat shock enhances plasmid DNA uptake. Rifampicin washed set had the second highest frequency. Rifampicin is a transcription inhibitor that binds to RNA polymerase (9). The fact that tetracycline resulted in lower frequency than rifampicin suggests that the protein(s), which enhance frequency, is regulated at translational rather than transcriptional level. In other words, mRNAs of these proteins are always present at a basal level under normal condition yet remain untranslated, but they start to be translated at elevated temperature. However, frequency of rifampicin was still relatively low compared to that of control, which denotes that RNA production may be induced by heat shock and somehow further enhance plasmid DNA uptake. Frequency of sodium azide was slightly higher than tetracycline with overlapping 95% CI's, but was significantly lower than that of rifampicin. Sodium azide inhibits cell growth by decoupling the respiratory chains in cells (http://cancerweb.ncl.ac.uk/cgi-bin/omd?query=aZide). This uncoupling decreases the pool of ATP, thus dNTP and NTP pools. It also inhibits protein translocation (2). Therefore, any process that requires energy such as transcription and translation would be prohibited in the presence of sodium azide. Frequency of sodium azide was close to tetracycline probably because sodium azide inhibits protein production indirectly. Since frequency was much smaller in sodium azide than in control, plasmid transformation seems to be energy-dependent and/or requires specific protein production and translocation.

Although we previously suggested that frequency values are more dependable for interpretation, transformation efficiencies gave a similar pattern as frequency. As shown in Figure 5, the control had the highest efficiency among all of the treatment types; rifampicin had higher efficiency than tetracycline washed samples. These two observations further support that the protein production during heat shock enhances transformation and it is regulated at translational level. Note that the difference in efficiencies between rifampicin and tetracycline was not as significant as it was for frequencies. A probable explanation could be that the starting cell concentration in rifampicin sets was lower than that in tetracycline sets. Sodium azide washed set had the second highest efficieny. Again, this could be a consequence of sodium azide having higher starting cell concentrations than the ones in rifampicin.

Even though it is evident that the inhibitor treatments caused a decrease in the rate of transformation, none of the efficiencies and frequencies was zero. In other words, transformants were obtained regardless of the treatment type. Both Clark and Hanahan reported that transformation could be very unsuccessful in the absence of favourable substances (1, 5). For example, without divalent cations, frequency decreased by 10-fold compared to control (1, 5). In our experiment, the concentrations of inhibitors introduced were inhibitory to cells (6), so it was unlikely that non-zero efficiency and frequency values in the inhibitor sets were due to insufficient working concentration of the inhibitors. Frequency decreased by two- to six- fold in inhibitor treated cells compared to control. A more plausible explanation is that the presence of the proteins engaged in heat shock improved the success rates of transformation but is not essential. It is also probable for these proteins to be essential but be expressed at a low level under normal condition and be increased upon heat shock.

In conclusion, this report showed that protein synthesis during heat shock enhances the rate of transformation. Using different inhibitor treatments, the proteins involved in enhancing transformation were found to be regulated at the translational rather than at the transcriptional level. Furthermore, sodium azide affects all energy-dependent processes in cell; thus, transformation could be affected due to translation inhibition, or the actual process of transformation has a direct need for energy.

FUTURE EXPERIMENTS

The observations made in this report are in agreement with what is known about the regulations of σ^{32} regulons. Heat shock proteins are regulated by σ^{32}, which is, in turn, autogenously regulated at translational level (13). Since HSPS can be induced by the presence of organic solvents (12), it is plausible to test if organic-solvent shock can replace heat shock in plasmid DNA transformation. Competent cells with plasmid DNA would be subject to different types of organic solvents at different concentrations. Examples of organic solvents that could be used are ethanol, propanol and benzoate (12). If organic-solvent shock can replace the function of heat shock, it provides extra presumptive evidence that DNA transformation requires HSPS production.

Furthermore, a series of cloning experiment can be used to provide strong evidence that σ^{32} is obligate for transformation. First, *rpoH* can be cloned into a marked plasmid under inducible control, such as *lacI/O* regulation. Then, the cloned plasmid will be transformed into a strain of *E. coli*, whose chromosomal *rpoH* gene has been knocked out (14). This mutant cell will then be separated into two growth conditions: one that induces *rpoH* transcription and the other that does not. These two types of cells will be subjected to heat-shock transformations. By comparing the success rates of DNA uptake under these two conditions, it is possible to determine the importance of σ^{32} in heat shock transformation. This method can be used to monitor any other genes, such as σ^{E} regulons, that may be involved in heat shock transformation.

In addition to σ^{32}, there could be other genes that are responsible for plasmid DNA uptake. To identify other possible gene product(s) that might be involved in heat shock transformation, a sequence of experiments are required. First, subtractive hybridization can be used to select for genes that are expressed in increased levels upon heat shock. Secondly, the putative gene products would be subjected to testing on their essentiality in plasmid DNA uptake via the method as suggested above. Since our finding of protein regulation at translational level was performed in an indirect manner, the degree of difference in efficiencies and frequencies between rifampicin and tetracycline treatments may not be substantial. Thus, after identifying a protein that is upregulated upon heat shock and essential for DNA transformation, the next step would be to test if it is regulated at transcriptional or translational level, in a direct fashion. C terminal of the test protein would be fused with a reporter gene that encodes green fluorescence protein (GFP). One can test the difference in mRNA level and protein expression, by employing RT-PCR and FACS scan, respectively, before and after heat shock for transformation. Upon heat shock, regulation at transcriptional level would lead to increases in both mRNA and protein expression, whereas translational level regulation would result in an increase in only protein expression.

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APPENDIX I : Raw Data

Table A1. The number of viable cells counted as cfu's on the LB plate at each time points during the recovery period.

Types of treatment	Time points	With wash, Final plated dilution			Without wash, Final plated dilution			
		10^{-5}	10^{-6}	10^{-7}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
A= Control	5	TNTC	260	\[a]	TNTC	180	\	\
	20	\	8, 19[b]	\	\	48, 63	\	\
	40	\	109	(9)[c]	\	55	(6)	\
	60	\	80, 97	(12,20)	\	126, 202	(12,5)	\
B= Rifampicin	5	157	(17)	\	252	29	\	\
	20	78	25	\	160	(19)	\	\
	40	312	29	\	125	(18)	\	\
	60	TNTC	48	(3)	299	49	(9)	\
C= Sodium azide	5	TNTC	61	\	TNTC	43	\	\
	20	TNTC	52	\	(353)	93	\	\
	40	TNTC	101	\	TNTC	124	\	\
	60	\	175	19	TNTC	216	68	\
D= Tetracyclin	5	258	24	\	215	24	\	\
	20	313	18	\	284	19	\	\
	40	(677)	62	\	264	32	\	\
	60	(1034)	139	(14)	(418)	38	(4)	\

[a] \ means no data.
[b] Numbers separated by a comma indicate duplicate counts.
[c] Numbers in parentheses were ignored in analysis due to their unacceptable ranges.

Table A2 – The number of transformants counted as cfu's after one hour on the LB-ampicillin plates.

Types of Treatment	With wash, Final plated dilution			Without wash, Final plated dilution		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
A=Control	\[a]	110	\	\	168	\
B=Rifampicin	309	48, 35[b]	\	136, 97	(20, 18)	\
C=Sodium azide	\	27, 87	27, (4)[c]	\	32, 34	(1, 8)
D=Tetracyclin	179	47, (26)	\	114, 206	(15, 28)	\

[a, b and c] The same rules are applied as in the Table 1.

APPENDIX II : Calculations using the raw data

Concentration of the isolated pUC plasmid

A_{260} of the 1-in 25 diluted plasmid = 0.0087

A_{280} of the 1-in 25 diluted plasmid = 0.0030

Apply to the $C_{DNA}=63A_{260} - 36A_{280}$

[pUC] = 63(0.0087) – 36(0.0030)= 11□/□ = 11ng/□

To obtain error in plate counting, Poisson analysis was employed:

For cfu's on the LB plate for the washed control treated sample

$$\% \text{ standard deviation} = \frac{\sqrt{(80+97)}}{(80+97)} \times 100\% = 7.5\ \%$$

Thus, 95% confidence interval (CI) of controls' cfu on LB-ampicillin=2 x 7.5 %=15 %
The 95% CI's of other samples are calculated in similar fashion.

To obtain transformation efficiency (Figure 5)

$$\text{Tranformation efficiency} = \frac{\text{Transformants}}{\text{Amount of plasmid DNA used}}$$

For the washed control treated sample

The amount of DNA used = 11ng/□ × 2□ = 22ng

$$\text{Tranformation efficiency} = \frac{11000 \pm 19\%}{22\text{ng}}$$

$$= 500 \pm \sqrt{(19\%^2)}$$
$$= 500 \pm 19\%$$
$$= 500 \pm 95 \text{ transformants/(ngDNA)}$$

To obtain the transformation frequency and its error (Figure 6)

$$\text{Tranformation frequency} = \frac{\text{Transformants}}{\text{Total viable cells}} \times 100\%$$

For the washed control treated sample

$$\text{Tranformation frequency} = \frac{11000\ (\pm 19\%)}{8.05 \times 10^7\ (\pm 14.5\%)} \times 100\%$$

$$= 1.3 \times 10^{-1} \pm \sqrt{(14.5\%^2) + (19\%^2)}$$
$$= 1.3 \times 10^{-1} \pm 23.9\ \%$$
$$= (1.3 \pm 0.3) \times 10^{-1}\ \%$$