Heat shock at 37°C with plasmid ligated at 37°C yields more number of *Escherichia coli* transformants than plasmid ligated at 16°C: a possible role of ligated plasmid conformation during heat shock

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The phenomenon of temperature effects on plasmid conformation has been well elucidated. However, the impact of change in plasmid conformation caused by temperature shift (during the heat shock mode) on transformation outcomes has not been reported till now. Here, we analyse transformation efficacy at 37°C of plasmid pBSKS ligated at three different temperatures (4°C, 16°C and 37°C) to *Escherichia coli* DH5*α* strain. Though ligation was effective at all the three temperatures mentioned above, the number of transformants was more with plasmid DNA ligated at 37°C than at 4°C and 16°C. Our study indicates a possible role of plasmid conformation on the effect of heat shock transformation.

Keywords: *Escherichia coli*, heat shock, ligation, plasmid conformation, transformation.

TRANSFORMATION is an important and primordial approach to isolate recombinants using horizontal gene transfer. Bacterium *Escherichia coli* is regarded as naturally incompetent for transformation[1], though there are reports of natural plasmid transformation in stationary phase *E. coli* cells[2] as well as for *E. coli* cells in environments with low concentration of Ca^{++} ions[3]. Artificial competent cells of *E. coli* are developed either for heat shock transformation or for electroporation using specific protocols. Heat shock is a simple and cost-effective method unlike electroporation which requires sophisticated instrument such as an electroporator, specific cuvettes and dialysis membrane. Therefore, heat shock transformation in *E. coli* that was invented in 1970s is still a popular laboratory practice among researchers[1].

During heat shock transformation the steps followed are[4]: (i) setting ligation at 16°C, (ii) exposure to heat shock at 42°C for 90 sec and (iii) growth of the bacterial cells followed by selection of the transformants in medium supplemented with the respective antibiotics. The usual understanding pertaining to heat shock-mediated transformation event at 42°C is that sudden shift in temperature from 0°C (ice temperature) to 42°C causes transient pore formation in the host cell (e.g. *E. coli*) membrane, which in turn allows the ligated plasmid to enter the cell. But whether this temperature shift also affects the conformation of the ligated plasmid entering the host cell is not clearly understood. Generally, after ligation at 16°C, the product is also subjected to a temperature shift when heat shock is given along with the competent host cell at 42°C. This shift of about 26°C (i.e. 42°C – 16°C = 26°C) is also likely to alter the plasmid conformation, as it is known that temperature affects plasmid conformation[5,6]. Hence our hypothesis is that, during transformation by heat shock, temperature shift-induced altered conformation of the ligated plasmid might have a role in the transformation event. A hypothetical model in support of the hypothesis is shown in Figure 1. Inside the bacterium, chromosomal DNA is shown as a relaxed circle, whereas the plasmid is shown in a supercoiled state. This plasmid after isolation is transformation-efficient. Its efficiency can be attributed to the natural conformation of the plasmid, which is negatively supercoiled. After restriction digestion the plasmid gets linearized, which is inefficient for transformation. The linearized plasmid is ligated at 16°C, which attains a relaxed conformation at this temperature. The transformation efficiency of a relaxed plasmid might be low due to

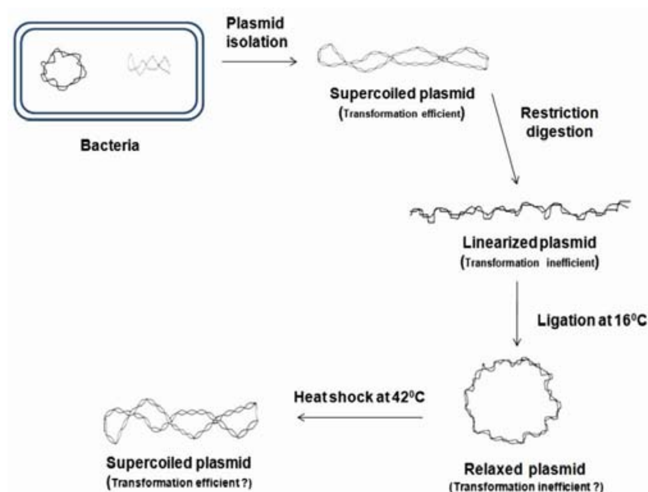

Figure 1. The proposed effect of temperature on plasmid conformation during the heat shock transformation. A hypothetical model describing different conformations of a plasmid starting from isolation to transformation. The plasmid isolated from a cell is naturally negatively supercoiled. After treatment with restriction endonuclease enzymes *in vitro*, the plasmid gets linearized and the torsion strain is removed. By treating with ligase, the plasmid is ligated at 16°C to form a covalently closed circular plasmid, which is in a relaxed state at that temperature. During heat shock, the covalently closed circular plasmid attains positively supercoiled state due to increase in temperature.

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its higher surface area as is evident from the slow migration rate during electrophoresis. The plasmid when subjected to heat shock at 42°C might attain positive supercoiled conformation due to temperature shift which might in turn enhance transformation efficiency.

To prove our hypothesis, we have tried to explore the transformation efficiency with temperature shifts (described in Figure 2). Plasmids were ligated at 4°, 16° and 37°C and transformed at 37°C. This comparative study of heat shock-mediated transformation at 37°C with products ligated at varied temperatures demonstrates that plasmid conformation might play a role during uptake of plasmid by competent cells.

Plasmids were isolated from *E. coli* DH5α cells harbouring pBSKS. The competent *E. coli* DH5α bacterial cells used for the experiment were prepared in the laboratory. *E. coli* DH5α strains harbouring pBSKS were grown in Luria–Bertani (LB) broth (Himedia, Mumbai, 20 g/l) incubated at 37°C and 200 rpm in an orbital shaker incubator (Orbitek, Syngenics Biotech, SN-2007400128) for about 16 h. For plating purposes, 1.5% agar (Himedia, Mumbai) was added to the LB broth and solidified.

pBSKS plasmids were isolated from cultured *E. coli* DH5α strains harbouring pBSKS using commercial plasmid isolation kit (AxyGen, USA). The quality of the isolated plasmid was checked by agarose gel electrophoresis (0.8% agarose; SRL, Mumbai) using ethidium bromide (EtBr; 0.5 μg/ml conc., Biorad, Mumbai) at 70 V, 250 mA, 1 h.

pBSKS plasmids isolated from cultured *E. coli* DH5α cells were digested with *Eco*RI (Fermentas, USA, 5U/μl stock) following the manufacturer's instructions. Restriction digestion of plasmid was checked by agarose gel electrophoresis (0.8% agarose, 70 V, 250 mA, 1 h). Digested product was then purified using plasmid purification kit (Qiagen, Valencia, CA, USA). Gel-extraction procedure to isolate digested DNA was avoided completely to keep away any effect of EtBr on plasmid DNA conformation. Digested pBSKS was then kept for ligation reaction with T4 DNA ligase (Fermentas, USA, 5U/μl stock) at 4°, 16° and 37°C separately for 12 h. Ligation was checked by 0.8% agarose gel electrophoresis in two ways – (a) with EtBr (0.5 μg/ml conc.) incorporated into the gel prior to electrophoresis and (b) staining the gel with EtBr (0.5 μg/ml conc.) post-electrophoresis; other conditions were identical for both the gels.

Competent *E. coli* DH5α cells were prepared following $CaCl_2$ treatment method[4]. Transformation of the competent cells was performed by heat shock at 37°C for 90 sec with the ligated plasmids obtained from all the three ligation set-up events, i.e. 4°, 16° and 37°C separately. Transformants were then checked by plating the cells on LB-agar plates containing antibiotic ampicillin (50 μg/ml conc.) incubated at 37°C for the requisite period of time.

In the present set of experiments, isolated pBSKS plasmids were first linearized by digesting with *Eco*RI. The linearized plasmids were subjected to ligation at three different temperatures, i.e. 4°, 16° and 37°C in order to monitor success of the ligation process at all the three temperatures. During the ligation set-up, ligation mastermix was prepared initially followed by addition of the ligase enzyme at the end. To maintain equal concentration of the linearized plasmid DNA, the ligation-mixture was distributed equally into three tubes. All the reactions were performed in ice to avoid ligase activity prior to their transfer to respective temperature conditions.

The ligation mixture kept at different temperatures was subjected to electrophoresis in two ways. One with EtBr incorporated into the gel prior to electrophoresis and the other with the gel stained after electrophoresis. Multiple bands were observed in the gel after ligation, suggesting that ligation was successful at all the three temperatures (Figure 3). As a band was observed below 3.0 kb (size of pBSKS is 3.0 kb) in both the gels, we predicted that this might be the supercoiled plasmid resulting from intramolecular ligation. The intensity of the bands in the gel in Figure 3 *b* is more than that of the gel in Figure 3 *a* because the gel in Figure 3 *b* was stained later with EtBr. The linearized pBSKS in both the gels (Figure 3) was running as a 3.0 kb band, whereas the supercoiled DNA in gel containing EtBr (Figure 3 *a*) is moving as ~ 1.7 kb band and the supercoiled DNA in the gel without EtBr (Figure 3 *b*) is moving like a ~ 2.2 kb band. In the former case the plasmid DNA has undergone more supercoiling due to intercalation of EtBr. There is a little difference in the migration patterns among the supercoiled DNA ligated at different temperatures (Figure 3 *b*). The supercoiled plasmid obtained during ligation at 37°C is moving faster than that obtained at 16°C and 4°C. In conclusion, in this experiment we demonstrate that ligation of linearized pBSKS had occurred well at 4°C and 37°C apart from at the usual temperatures of 16°C. We had also demonstrated earlier[7] that λDNA *Hin*dIII fragments were ligated efficiently when subjected to ligation at 37°C and 16°C.

To examine the transformation efficacy of ligated pBSKS at 4°, 16° and 37°C, the ligated plasmids were transformed to competent *E. coli* DH5α cells by heat shock at 37°C separately. From earlier perceptions, higher temperature (as 37°C is used for heat shock in our case) is thought to introduce positive supercoils in plasmid ligated at lower temperatures (as 4° and 16°C have been used in our experiment). Whereas heat shock at higher temperatures like 37°C will not introduce positive supercoils in plasmid molecules ligated at 37°C. So, during transformation, heat shock at 37°C with ligation products at lower temperatures such as 4°C and 16°C is expected to yield better transformation outcomes than the ligated product at higher temperatures like 37°C (Figure 1).

Heat shock-mediated transformation (at 37°C) of *E. coli* DH5α, set separately with intact pBSKS, digested

pBSKS, and ligated pBSKS from ligation tubes kept at 4°, 16° and 37°C respectively, manifested interesting outcomes. Figure 4 depicts a comparison of the transformation events set at the respective experimental conditions.

Transformation with intact pBSKS yielded maximum average transformants per plate. This might be due to the integral conformation of plasmid, as these were not treated with *Eco*RI. Digested or linearized pBSKS showed negligible transformant yield. Among the ligated products from three separate ligation events, transformation with pBSKS ligated at 37°C resulted in highest average transformants per plate. The next best transformation yield obtained was with pBSKS ligated at 4°C followed by 16°C. The average transformants per plate resulting with pBSKS ligated at three temperatures is in descending order as $107.75 > 82.75 > 58$, i.e. $37° > 4° > 16°$C. The transformation event was executed at least three times to obtain the best result and all the events showed similar outcomes. Figure 4 is a graphical representation of the average transformants per plate with respect to ligation conditions. The possible explanation for this is discussed below.

In this study we have shown that linearized pBSKS plasmid ligated at 37°C yields more number of transformants by heat shock at 37°C than when ligation was

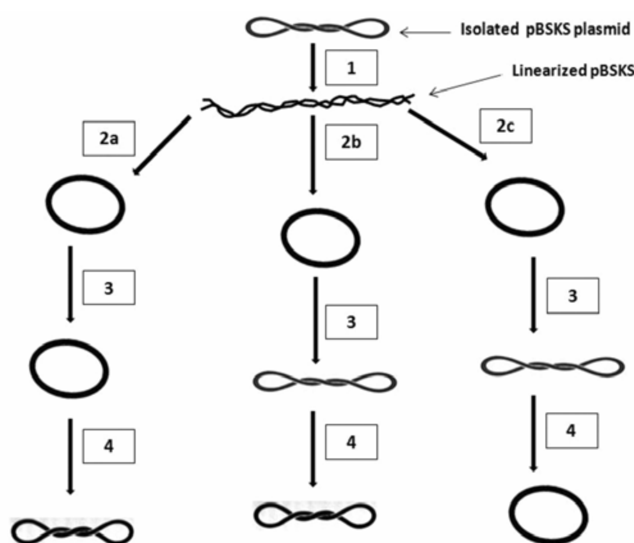

Figure 2. Schematic representation of the plasmid conformations proposed during the course of experiments designed in this study. 1, The isolated pBSKS (negatively supercoiled) was treated with *Eco*RI to obtain linearized pBSKS. 2, The linearized pBSKS was treated with T4 DNA ligase for ligation followed by incubation at three different temperatures separately at 4°C (2a), 16°C (2b) and 37°C (2c). Ligation results in covalently closed circular plasmids that are in relaxed conformation at the respective temperatures. 3, Before heat shock, the ligated product is mixed with competent cell and incubated in ice (~ 4°C). The lowering in temperature results 16°C (2b) and 37°C (2c) ligated plasmid attaining negative supercoiled state, whereas the 4°C ligated plasmid remains relaxed. 4, During heat shock at 37°C, the 4°C (2a) and 16°C (2b) ligated plasmids attain positive supercoiled state owing to increase in temperature, whereas the 37°C (2c) ligated product attains relaxed conformation.

performed at 4°C and 16°C. In fact, ligation performed at 4°C resulted in more number of transformants than at 16°C. The consequence is surprising because ligation is usually performed at 16°C and our finding shows that the number of transformants with products ligated at 37°C and 4°C was more than that at 16°C. The higher number of transformants of plasmids ligated at 37°C and 4°C might be due to the different levels of supercoiled state of the ligated plasmid during heat shock at 37°C. However, transformation by electroporation with the same ligated samples might reveal a different story.

The observation of slow migration of the DNA bands at lower front without EtBr suggests that the presence of EtBr in the gel introduces supercoils in the plasmid. Even without EtBr the ligated plasmid is able to attain some amount of supercoiling. The incidence of supercoiling here is likely to be due to the electrophoresis conditions as well as temperature of buffers used for treatments. This implies that the temperature has a role in alteration of conformation in covalently closed circular DNA.

The surprising inquiry in this study is pertaining to the low transformant yield with 16°C ligation product as against the other two. Then, what might be the reason behind the high transformant yield with plasmid DNA ligated at 37°C? There is least possibility of increment in the intramolecular ligation happening during ligation at 37°C as manifested by the similar intensity of the supercoiled plasmid bands in the gel. In fact the 37°C ligated product showed lower intensity compared to the other two. Hence, increased number of intra-molecular ligations leading to more number of transformant yields during transformation would not hold. A critical observation of the fact is necessary to arrive at a conclusion. Earlier, transformation of *E. coli* at 37°C has been shown in foodstuffs[8]. Recently, transformation viability in *E. coli* with plasmid pUC19 by heat shock at 37°C and three other temperatures has also been reported[9]. Adding to this, a group reported about a finding pertaining to transformation feasibility of pre-incubated plate at 37°C that resulted in double time the transformation efficiency in comparison with the routine 42°C heat shock treatment[10]. Considering their report, we chose 37°C temperature to be feasible for transformation by heat shock as well. However, in none of the previous studies involving transformation at 37°C has any comparison been made for transformation efficiency of products ligated at different temperatures, which we have illustrated here.

One of the rationalizations relating to transformation by heat shock can be that DNA remains naturally in negatively supercoiled state inside the cell. So, negative supercoiled state of a plasmid can be attained easily from a relaxed state of the plasmid than that from a positive supercoiled state. In addition, the transient pore formed due to heat shock at 37°C is good enough for the uptake of both relaxed and less supercoiled (ligated at 37°C) as well as the positive supercoiled form (ligated at 16°C and

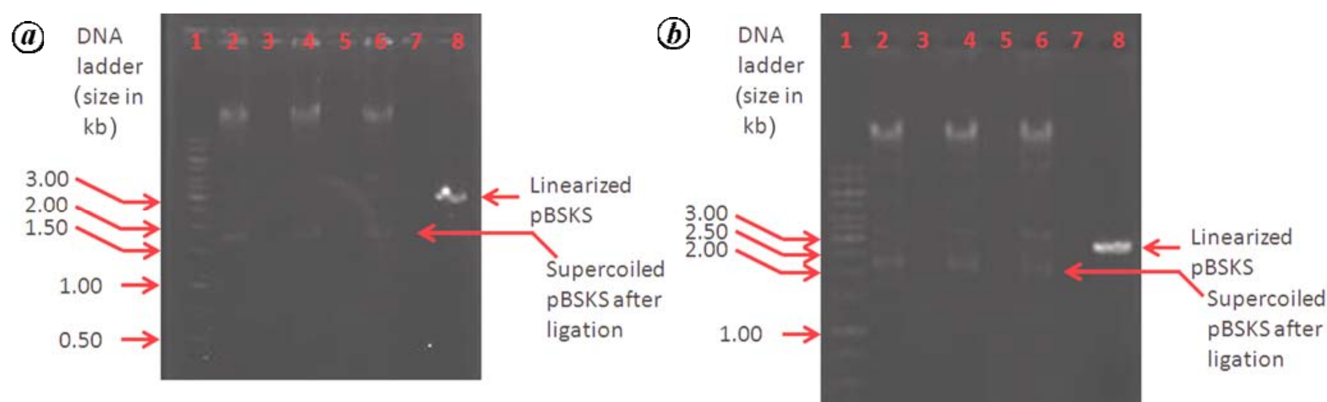

Figure 3. Agarose gels showing ligation of pBSKS plasmid at 4°, 16° and 37°C. ***a***, Migration pattern of the ligated pBSKS plasmids at the respective incubation temperatures on agarose gel, in which ethidium bromide (EtBr) was incorporated prior to electrophoresis. Lane 1, 1 kb DNA ladder (Gene ruler, Fermentas, USA); Lanes 2, 4 and 6, Migration pattern of the ligated pBSKS plasmid at 4°, 16° and 37°C respectively. In all the lanes equal amount of DNA has been loaded. Ligated plasmids are running as large-sized bands towards the upper end of the gel and also as bands between 1.5 and 2.0 kb linear DNA. The former class belongs to the cocatemeric DNA moving slowest due to larger size and the latter class belongs to the supercoiled plasmid DNA which is running faster than the linear control DNA in lane 8. pBSKS ligated at 4°C loaded in lane 2 is migrating faster than the ligated plasmids at 16° and 37°C, loaded in lanes 4 and 6 respectively. In fact, pBSKS ligated at 37°C is running the slowest followed by plasmid ligated at 16°C, in this case; lane 8, The digested pBSKS plasmid as control. ***b***, Migration pattern of the ligated pBSKS plasmids at the respective incubation temperatures on agarose gel, stained with EtBr post-electrophoresis. Lane 1, 1 kb DNA ladder; lanes 2, 4 and 6, Migration pattern of the ligated pBSKS plasmid at 4°, 16° and 37°C respectively. In all the lanes equal amount of DNA has been loaded. Ligated plasmids are running as large-sized bands towards the upper end of the gel, few in the middle and the lowermost bands are running as supercoiled plasmids corresponding to 2.0–2.5 kb linear DNA ladder and are moving faster than the linearized control DNA in lane 8. The former class at the topmost front of the gel is concatemeric DNA running slowest of all due their larger size. pBSKS ligated at 37°C loaded in lane 6 is migrating faster than the ligated plasmids at 4°C and 16°C, loaded in the lanes 2 and 4 respectively. In fact, pBSKS ligated at 4°C is running the slowest followed by ligated plasmid at 16°C, in this case; lane 8, Digested pBSKS plasmid (control). The experiment was performed thrice and the best of three results is shown here.

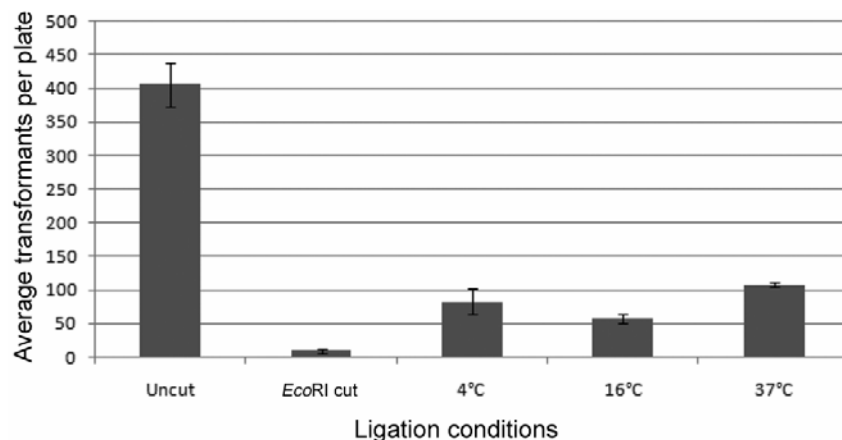

Figure 4. A histogram depicting the average transformants per plate obtained. In the histogram, horizontal axis depicts the state of pBSKS used for heat shock transformation such as temperature conditions kept as 4°, 16° and 37°C for ligation, and also the uncut and *Eco*RI cut plasmid. Vertical axis shows the average number of transformants per plate obtained after the transformation event. Transformation with the intact undigested plasmid yields the maximum average transformants per plate as shown by its highest stature followed by plasmid ligated at 37°, 4° and 16°C respectively. This result is the best of the three sets of experiments carried out.

4°C). Apart from this, the role of plasmid conformation in expression of the antibiotic resistance gene cannot be ignored while drawing our conclusion about higher transformation efficiency of 37°C ligated product. It is known that the supercoiled state of the plasmid influences gene expression in bacteria[11,12]. As the natural negative supercoiled state of the plasmid is close to the relaxed state than the positive supercoiled state, the 37°C ligated plasmid may be able to multiply and express well after entering into the bacterium. On the other hand, it may be possible that the 16°C ligated product, which is positively supercoiled, experiences hindrance in multiplication and gene expression after entering into the host bacterium. Higher number of transformants obtained in our experiment with the 37°C ligated products was due to the high level of expression of the antibiotic resistant gene on the antibiotic supplemented medium (ampicillin in our case). In this connection, it is also important to note that a plasmid tends to attain the relaxed conformation from the supercoiled conformation as the cells approach the sta-

tionery phase from the exponential phase[13]. The above arguments regarding the difference in the antibiotic gene expression cannot support the observation of higher transformants with 37°C ligated products, because the 4°C ligated products also yielded higher transformants in comparison with the 16°C ligated products. The 4°C ligation products are more positively supercoiled than the 16°C products when shifted to 37°C. Therefore, explanation on the basis of the assumption of the difference in the antibiotic resistant gene expression by different states of the plasmid inside the host bacterium may not hold in our case.

Another explanation regarding the difference in transformation efficiency can be due to different levels of concatemeric DNA formation during ligation. A concatemeric DNA is made up of intermolecular ligation between the same plasmid molecules which results in direct repeats. After entry into the cell, intramolecular recombination within the concatemer (that may be circular or linear) results in the original plasmid, which can then multiply normally producing transformants. In our experiments, the three intense bands observed at the topmost front of the gel comprise this category of DNA because of their larger size. Does concatemeric DNA formation have any effect on the transformation efficiency we observed here? Entry of concatemeric DNA into competent *E. coli* cells during heat shock transformation cannot be ruled out. But in our case, even if the concatemeric DNA enters *E. coli* during transformation, possibility of its influence on the transformation is unlikely because the competent cells used are recombination deficient (*recA*-deficient). Therefore, in this study, the explanation for varied levels of concatemeric DNA leading to different transformation outcomes is irrelevant.

The findings obtained in this study with pBSKS, which is a small-sized plasmid of ~ 3.0 kb, might not hold for larger plasmids and this needs to be tested in future. The differences in transformation efficiency of the ligated sample in various other temperatures also need to be found. This is likely to give an insight into some of the interesting phenomena of DNA conformation during *in vitro* ligation and on the way through the cell envelope of *E. coli* during transformation and persistence of plasmids with variable conformations inside the *E. coli* cell. It might turn out in future that a relaxed state of a plasmid is more efficient for transformation than that of a supercoiled state, which will be completely different from our assumption made in Figure 1. Whether relaxed state of the plasmid is less efficient than the supercoiled state during transformation, is only a hypothesis proposed in this study considering the well-known fact that the relaxed plasmid migrates slower than the supercoiled plasmid during gel electrophoresis. Yet, none of the available literature has cited experimental evidence regarding the transformation efficiency of relaxed and supercoiled plasmid. In fact, the obvious practical limitations (e.g. linking number is an integer, the effect of salt concentration and temperature on supercoiling, etc.) for maintaining a covalently closed plasmid in relaxed state is most likely creating a hurdle for experimentation and critical analysis in this regard. In conclusion our study has opened up new challenges for exploring the mechanism behind the phenomenon of plasmid transformation during heat shock, which is not clearly understood.

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Declaration. Bacterial strains used were non-pathogenic and were sterilized after the experiment to avoid contamination in the environment. Ethidium bromide use and disposal was performed following statutory instructions.

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