J Chem Biol (2013) 6:135–140
DOI 10.1007/s12154-013-0095-4

ORIGINAL ARTICLE

Development of rapid microwave-mediated and low-temperature bacterial transformations

Valerie T. Tripp · Johnathan C. Maza · Douglas D. Young

Received: 19 December 2012 / Accepted: 5 May 2013 / Published online: 21 May 2013

Abstract The introduction of exogenous DNA into *Escherichia coli* is a cornerstone of molecular biology. Herein, we investigate two new mechanisms for bacterial transformation involving either the use of microwave irradiation or a freeze–thaw protocol in liquid nitrogen. Ultimately, both methods afforded successful transfer of plasmid DNA into bacterial cells, with the freeze–thaw technique yielding efficiencies of ~10^5. More importantly, both techniques effectively eliminated the need for the preparation of competent cells.

Keywords Microwave irradiation · Bacterial transformation · Molecular biology

Introduction

The introduction of exogenous DNA into bacterial cells is a cornerstone of molecular biology and thus has become a fundamental technique in innumerable laboratories [1]. Bacterial transformation has been the subject of a variety of investigations as it is essential for applications involving molecular cloning, mutagenesis, protein expression, and many others [2]. Currently, there are several known methods for efficient introduction of the exogenous DNA into a variety of bacterial species; however, each has distinct advantages and disadvantages, requiring the necessity for further studies to improve this prevalent technique [1]. Transformation protocols generally involve a three-step procedure: the preparation of competent cells, a physical shocking of the cells, and a recovery. The two most common methods for transformation of *E. coli* cells are chemotransformation and electroporation, each differing in their cell preparation and method of physical shock. We set forth to develop new methods of rapid transformation of bacterial cells using either low temperatures or microwave irradiation and shortening the protocol by the removal of a time-consuming competent cell preparation.

Chemotransformation involves the pre-treatment of cells with multiple washings/incubations containing $CaCl_2$ to aid in the uptake of plasmid DNA [3–5]. It is speculated that the divalent cation serves as a mechanism to shield the negatively charged DNA backbone from the charge of the phospholipid bilayer, increasing its cellular membrane permeability [6]. Typically, these chemically competent cells are then incubated on ice to solidify cellular membranes, exposing pores for DNA transport. In order to shock the cell to increase uptake, chemotransformations utilize a brief pulse of heat (often 42 °C for 45–60 s) followed by a recovery prior to growth on agar [7]. Chemotransformations have been demonstrated to have transformation efficiencies ranging from 10^5 to 10^9 cfu [1].

Electroporation requires a competent cell preparation that removes many of the conducting ions from the cell media and generates highly concentrated cells. Cells are typically concentrated and washed with a glycerol solution several times in order to increase transformation efficiency. Once prepared, cells are subjected to an electrical pulse (5–10 kV/cm for 5–10 μs), resulting in the transient formation of pores within the membrane for the transport of plasmid DNA. This technique is highly efficient (ranging from 10^3 to 10^{10} cfu), but requires specialized equipment for the delivery of the electrical pulse [8, 9]. Moreover, the requisite of both approaches for lengthy and variable preparation of competent cells warrants further investigation of other mechanisms for transformations to yield

Electronic supplementary material The online version of this article (doi:10.1007/s12154-013-0095-4) contains supplementary material, which is available to authorized users.

V. T. Tripp · J. C. Maza · D. D. Young (✉)
Department of Chemistry, The College of William & Mary, Williamsburg, VA 23187, USA
e-mail: dyoung01@wm.edu

a more rapid, more practical, and more robust methodology for the introduction of foreign DNA into bacterial cells.

Based on the rapidly advancing field of microwave technologies, we became interested in exploring the use of microwave irradiation for bacterial transformation [10]. While microwaves have previously been utilized to enhance the rate, yield, and purity of organic reactions, they have more recently been employed in biological systems ranging from PCR [11, 12] and DNA hybridizations [13] to the activation of enzymatic catalysis [14, 15]. The oscillating electromagnetic wave is thought to directly interact with dipoles and ions to induce molecular motion, resulting in highly efficient heating. Due to the prevalence of significant dipoles within many biomacromolecules (including DNA, proteins, and phospholipid bilayers) [10], we speculate that these molecules should be susceptible to the effects of microwave irradiation. Specifically, due to the involvement of nucleic acids, membrane-associated proteins, carbohydrates, and phospholipids in the process of bacterial transformation, the effect of microwaves on this important biological technique appeared optimal for investigation. Additionally, due to the direct interaction of microwaves with the cells and plasmid DNA, we considered that it might be feasible to obtain high transformation efficiencies without any special preparation of the cells associated with other techniques, eliminating the significant amount of effort associated with competent cell preparation.

Materials and methods

Optimized preparation of competent cells 2xYT media (4 mL) was inoculated with *Escherichia coli* Novagen BL21(DE3) strain of cells and then incubated for 16 h at 37 °C to stationary phase. The starter culture (1 mL) was then added to fresh 2xYT media (100 mL) and incubated with vigorous shaking at 37 °C and monitored by routine optical density measurements on a spectrophotometer at 600 nm to assess bacterial growth. At the desired density, the culture was centrifuged at 4,000 rpm for 20 min. The resulting cell pellet was resuspended in either deionized water or glycerol, for a final cell optical density of 27.7. The resuspended cells were then aliquoted (100 μL each) and kept on ice.

Liquid nitrogen transformation An aliquot of BL21(DE3) cells was kept on ice, and plasmid DNA (1 μL, 70 μg) was added. The cells were incubated on ice for 5 min and then submerged in liquid nitrogen for 20 s. After thawing, the cells were either subjected to a second freeze cycle or recovered in 2xYT media (500 μL), followed by 1 h of incubation at 37 °C. Cells were then plated on Luria-Bertani (LB) plates containing appropriate antibiotics and incubated overnight at 37 °C. Colonies were counted to assess transformation efficiency, and individual colonies from these plates were selected to ensure the presence of plasmid DNA.

Microwave CoolMate transformation An aliquot of BL21 (DE3) cells was kept on ice, and plasmid DNA (1 μL, 70 μg) was added. The cells were incubated on ice for 5 min and then placed in a Discover CoolMate vessel. Each sample was cooled in the CoolMate to a specific temperature (typically −30 °C) before it was subjected to microwave radiation and simultaneous cooling. The temperature was monitored using a fiber optic probe, and upon the conclusion of the microwave pulse, the sample was removed from the vessel and thawed to room temperature. The cells were recovered in 2xYT media (500 μL), followed by 1 h of incubation at 37 °C. Cells were then plated on LB plates containing appropriate antibiotics and incubated overnight at 37 °C. Colonies were counted to assess transformation efficiency, and individual colonies from these plates were selected to ensure the presence of plasmid DNA (see Supplementary Data).

Results and discussion

Development of microwave-mediated bacterial transformations Recent reports investigated the ability of traditional microwaves to substitute for a heat shock during chemotransformations and found a three-fold increase in transformation efficiency when employing a 1-min pulse at 180 W [16, 17]. However, these studies utilized prepared calcium chloride competent cells which already displayed transformation efficiencies of ~10^7 cfu. Thus, the water bath heat shock was simply replaced with a brief microwave heat shock. Moreover, the use of a conventional microwave does not rely on focused microwaves, potentially making the use of a microwave reactor more advantageous as a mechanism to harness the microwave energy and afford more reproducible results. Based on these studies, we hypothesized that it may be feasible to further enhance the utility of these transformations and potentially eliminate the need for competent cell preparation by employing focused microwave irradiation. Initially, an *E. coli* BL21(DE3) strain of cells was grown to either log phase (OD_{600} 0.5) or stationary phase (OD_{600} 3.2) followed by centrifugation and resuspension in either 1:1 LB/water or 1:1 LB/glycerol. The cells were then incubated on ice for 10 min with a pET plasmid containing ampicillin resistance and subjected to brief pulses (2 or 5 s) of microwave irradiation (50, 100, 150, 200, or 300 W) in a CEM Discover microwave reactor. Due to the intensity of the

focused beam of microwave irradiation, the irradiation time was dramatically reduced when compared to previous literature settings in conventional kitchen microwaves. The temperature achieved with each pulse was recorded, and appropriate controls were performed in the absence of microwave irradiation (see Supplementary Data). All transformations were recovered in 2xYT media for 1 h at 37 °C and then plated on LB agar containing ampicillin. Unfortunately, neither the microwave samples nor the controls yielded colonies that would signify a successful transformation. In order to assess if the microwave irradiation decreased the viability of the cells, the same pulses were performed without plasmid DNA and plated on agar in the absence of any antibiotic. No differential in colony growth was observed between microwave-irradiated samples and nonirradiated controls, indicating that the brief pulses of microwave irradiation were not affecting cellular viability and were not sufficient in transforming noncompetent cells.

Due to the requisite for aqueous media and the presence of various ions during transformations, the samples were found to rapidly heat when subjected to short microwave pulses. In order to optimize transformation conditions and maximize the power of the microwave input, the experiments were transitioned to a CEM CoolMate system. The instrument utilizes a jacketed reaction vessel, allowing for the continuous flow of pre-chilled microwave transparent solvent (at −50 °C), providing simultaneous cooling in the presence of microwave irradiation. Additionally, the CoolMate allows for a rapid cooling of the sample immediately after the microwave pulse in a fashion that mimics the post-heat shock transfer of cells to ice during chemotransformations. Due to the ability to modulate temperatures while maintaining microwave irradiation, this technique affords the ability to more directly observe the role of the microwaves within the transformations. Cells were grown to log phase (OD_{600} 0.5) or early stationary phase (OD_{600} 1.0) and then centrifuged and resuspended in either 1:1 LB/water or 1:1 LB/glycerol and transferred directly to the CoolMate with a pET plasmid containing an ampicillin-resistance marker. The transformation was then cooled to −30 °C in the CoolMate (~1 min) and subjected to microwave irradiation (100, 200, or 300 W) for a brief amount of time (10–30 s). After a 1-h recovery in 2xYT media at 37 °C, the cultures were plated on ampicillin LB agar and incubated overnight. These conditions afforded viable colonies harboring the ampicillin resistance plasmid, signifying a transformation event, albeit at low transformation efficiency (Table 1). The highest transformation efficiencies were observed using 15 s pulses with 300 W of power, yielding efficiencies of 213 cfu. Lower efficiencies were observed at shorter and longer pulses and at lower microwave powers. In order to ensure that colony growth was a result of the introduction of the desired plasmid, a colony was selected, grown, and the plasmid DNA was reisolated. Its presence was confirmed via a restriction enzyme digest and visualization on an agarose gel (Fig. 1a). Moreover, to ensure that the cells were still capable of protein expression following microwave irradiation, a green fluorescent protein (GFP) gene in the plasmid was expressed, yielding functional fluorescent protein (Fig. 1b). Additionally, when microwave conditions were replicated using a −30 °C bath followed by transfer to a conventional heat block to recapitulate the microwave temperature profile, the transformations yielded no

Table 1 Microwave-assisted transformation efficiencies

Power (W)	Time (s)	Initial temperature (°C)	Transformation efficiency
0	10	0	0
0	30	0	0
0	10	−30	0
100	10	−30	24
200	10	−30	94
300	10	−30	94
300	15	−30	213
300	30	−30	101
300	60	−30	24
300[a]	30	−30	0
300[b]	2	25	0

For microwave transformations, all cultures were grown to an OD_{600} of 0.5

[a] Sample was resuspended in 10 % glycerol instead of water

[b] Sample was irradiated in standard CEM Discovery system without cooling, reaching a temperature of 50 °C

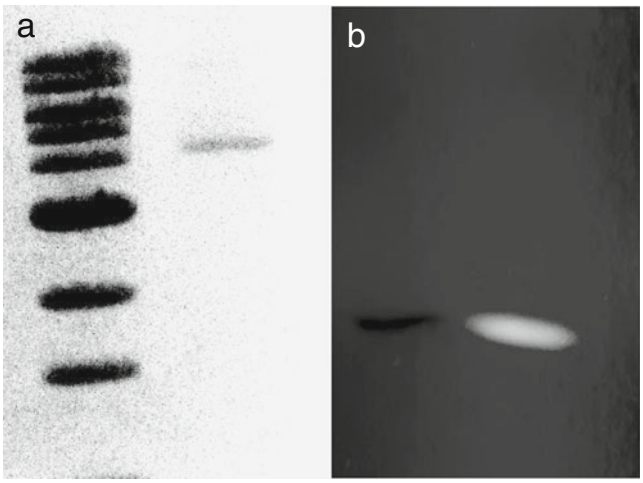

Fig. 1 Validation of DNA transformation. **a** Following either a liquid nitrogen or a microwave transformation of a pET22-GFP plasmid, a culture of cells was grown and the plasmid was isolated and digested with EcoRI. Analysis of the digest on 1 % agarose gel confirms the presence of the plasmid DNA. **b** To further confirm the transformation and the competence of the cells, the GFP protein was expressed, purified, and analyzed via SDS-PAGE. Successful transfer of plasmid DNA while retaining cellular function is demonstrated by the presence of GFP fluorescence

bacterial colonies (see Supplementary Data). This result confirms that the introduction of the plasmid DNA is a result of the microwave exposure and not just due to a cryogenic effect that has previously been reported [18]. Moreover, the increasing transformation efficiency with increasing power settings strongly indicates an active role of the microwave field on the bacterial transformation. These results suggest the importance of the microwaves, as they potentially disrupt bacterial membranes, affording the effective transfer of foreign DNA into the cell. While the transformation efficiencies are low, these results demonstrate the potential utility of microwave irradiation within complex biological systems and indicate that the microwaves themselves are the active agent in these transformations.

Optimization of low-temperature transformations In the process of performing various controls in the absence of microwave irradiation to find an optimal protocol to mimic the microwave temperature profile, a rapid freezing of the cells was attempted using liquid nitrogen (−196 °C), cooling the cells dramatically below −30 °C. Following a recovery, this protocol yielded a surprisingly high transformation efficiency of ~10^4 cfu. Low-temperature transformations in bacteria have been previously reported, originally by Dityatkin et al. in 1972 [18]; however, they have only found widespread use in *Agrobacterium tumefaciens* transformations [19–21], and examples in *E. coli* are rare. This is most likely due to relatively low transformation efficiencies compared to other methods and the overall prevalence of both electroporation and heat shock protocols. Most notably, a report by Takahashi et al. attempted a similar freeze–thaw transformation with non-chemically competent cells, but only obtained efficiencies of ~100 with optimized conditions [22]. Further research in the same report and by other laboratories elucidated that substantially higher freeze–thaw efficiencies can be obtained by using chemically competent cells and substituting a freezing procedure for the heat shock. While under optimal condition efficiencies of ~10^6 were reported, most of these methods resulted in efficiencies that were at least 1–2 orders of magnitude lower and still required a degree of competent cell preparation [21–24]. Thus, the ability to eliminate the time and use of sometimes expensive reagents associated with competent cell preparation while still maintaining relatively high transformation efficiencies represents an advance to the field.

In order to optimize the results observed in our freeze–thaw transformation, several experimental variables were altered including the number of freeze–thaw cycles, the temperature of the transformation, the concentration of divalent cations (which have previously been documented to serve as buffers to shield the negatively charged phosphodiester backbone of DNA), and the OD_{600} of the bacterial cells (Table 2). Overall, it appears that the optimal transformation conditions involved

Table 2 Freeze–thaw transformation efficiencies

Cell OD600[a]	Freeze–thaw cycles	Cation additive[b]	Freeze temperature (°C)	Transformation efficiency
0.1	1	–	−196	5.2×10^3
0.1	2	–	−196	2.9×10^4
0.2	1	–	−196	2.8×10^5
0.2	2	–	−196	2.8×10^5
0.4	1	–	−196	8.4×10^4
0.4	2	–	−196	2.3×10^5
0.6	1	–	−196	2.2×10^5
0.6	2	–	−196	3.7×10^5
0.8	1	–	−196	1.6×10^3
0.8	2	–	−196	3.5×10^4
1.0	1	–	−196	5.0×10^3
1.0	2	–	−196	4.8×10^4
3.0	1	–	−196	7.2×10^3
0.6	1	–	−94	1.17×10^3
0.6	1	–	−78	1.6×10^2
0.6	1	–	−42	38
0.6	1	–	−30	0
0.6	2	NH_4Cl	−196	8.9×10^4
0.6	2	$MgCl_2$	−196	1.5×10^5
0.6	2	$CaCl_2$	−196	1.1×10^5

[a] OD_{600} to which culture was grown and then resuspended to an OD_{600} of 27.7

[b] Growth media supplemented with 10 mM of the chloride salt

the growth of the cell culture to an OD_{600} of 0.5–0.6, followed by concentration and subjection to two freeze cycles directly in liquid nitrogen. The presence of 10 mM Mg^{2+} or Ca^{2+} cations in the growth media did not hinder the transformation, but also did not provide any significant advantage compared to the absence of the cation supplements. The cell density appears to have a moderate effect on the transformation (as the overall number of cells is normalized prior to freezing), with cells in early stages of replication and in stationary phase exhibiting lower overall efficiencies than cells in log phase. Increasing the freeze temperature negatively impacted the transformation efficiency, with any temperature above −42 °C resulting in no transformants. This appears to be a critical temperature for transformation success, as previously observed microwave controls provided no colonies when incubated at −30 °C. Several different plasmids of various sizes were examined harboring different origins of replications and antibiotic resistance, and consistently high transformation efficiencies were detected (see Supplementary Data). Moreover, the use of purchased chemically competent cells yielded no difference in transformation efficiency, demonstrating the lack of necessity for an additional procedure to prepare chemically competent cells. Isolation of plasmid and expression of a GFP reporter protein were also performed to

ensure the presence of the foreign DNA and the retention of cellular function under these conditions. Additionally, the ability to perform simultaneous double transformations of two plasmids was found to be possible with this methodology, albeit at the expected decreased efficiency associated with the uptake of two separate plasmids (~10^2). While none of the transformation efficiencies are comparable to that obtained by electroporation, transformations of ~10^5 are still highly efficient and useful for most practical purposes. Additionally, this approach obviates the requirement for substantial cell preparation prior to transformation and does not require additional equipment (e.g., an electroporator), making it applicable to virtually any laboratory.

Interestingly, the resuspension of cells in 10 % glycerol (a cryoprotectant), rather than water, resulted in no transformants under even the most optimized freeze–thaw condition. This suggests that a potential mechanism of transformation is the rapid formation of ice crystals, which disrupt the bacterial cell wall, providing a means for the uptake of plasmid DNA. This is corroborated by the fact that >2 freeze–thaw cycles substantially decrease transformation efficiencies as significant damage of the cell wall occurs, decreasing viability. Further investigations are currently underway to probe the mechanism of DNA uptake in both microwave and freeze–thaw transformations.

Finally, in order to assess the robust nature of the transformation protocols, each method was examined towards the transformation of *Mycobacterium smegmatis*. This mycobacterium represents a more significant challenge to the transformation process, as it possesses a complex cell wall and inefficient genetic transfer systems [25]. Thus, increasing the number of mechanisms available for genetic transfer in mycobacteria is desirable in that it has the potential to significantly advance research on a variety of diseases associated with these organisms. While some electroporation protocols have been established in *M. smegmatis*, foreign DNA is more commonly introduced via the formation of spheroplasts due to low transformation efficiencies [26–30]. Under identical low-temperature conditions as employed in the transformation of *E. coli*, a pMycVec1 plasmid was employed towards *M. smegmatis* freeze–thaw transformation [31]. Gratifyingly, the transformation was successful, yielding efficiencies of ~10^3 cfu (Table 3). This signifies a rapid and efficient method for the introduction of foreign DNA into *M. smegmatis* without requiring any significant preparation of cells and only employing liquid nitrogen. Although lower efficiencies were observed in *M. smegmatis* than for *E. coli* using the same technique, this result is expected due to the more challenging cell wall of the mycobacterium. Moreover, using an optimized literature-based electroporation protocol for *M. smegmatis*, a significant decrease in efficiency is observed when comparing to a similar electroporation in *E. coli* (Table 3) [30]. Unfortunately, the microwave transformations were not transferrable to *M. smegmatis*, as even under *E. coli* optimized conditions no transformants were observed. This is not necessarily unsurprising as even with *E. coli*, these methods led to lower transformation efficiencies than the corresponding liquid nitrogen transformations. However, current studies are underway to translate microwave transformations to mycobacteria using chemically competent cells and higher transformation temperatures. Overall, the ability to utilize simple transformations within a mycobacterium represents a significant advance to the field.

Conclusion

In conclusion, two mechanisms for the introduction of exogenous DNA into *E. coli* have been investigated, demonstrating the increased applicability of microwave irradiation on biological systems and providing rapid mechanisms (not involving significant competent cell preparation) for bacterial transformations. Overall, both of these techniques have general applicability to the fields of both molecular biology and chemistry. Perhaps most advantageous is the elimination of time-intensive preparation of bacterial competent cells and elimination of sometimes costly reagents used in competent cell preparation while (especially in the case of the freeze–thaw procedure) maintaining relatively high transformation efficiencies. The facile and streamlined procedure is feasible even for undergraduate researchers, is highly reproducible, and is both time- and cost-effective. Moreover, the expansion of these techniques to organisms that are more resilient to the transfer of genetic material represents a substantial advancement towards the utilization of mycobacteria in molecular biology and cloning.

Table 3 Summary of the various transformation techniques

Organism	Electroporation with competent cell	Electroporation with noncompetent cells[a]	Freeze–thaw transformation	Microwave transformation
E. coli	1.9×10^9	–	3.7×10^5	2.1×10^2
M. smegmatis	1.1×10^5	–	6.9×10^3	–

[a] High salt concentrations of cells/media resulted in arching of the sample and no viable cells.

Acknowledgments The authors would like to acknowledge the College of William & Mary for the funding, including a research award for D.D.Y. D.D.Y would like to thank the Jeffress Memorial Trust for the financial support. Additionally, J.C.M would like to thank the Howard Hughes Medical Institute for a research scholarship. Finally we would like to thank Dr. Mark Forsyth for providing the culture of *M. smegmatis* used in the experiments.

References

1. Aune TE, Aachmann FL (2010) Methodologies to increase the transformation efficiencies and the range of bacteria that can be transformed. Appl Microbiol Biotechnol 85:1301–1313
2. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
3. Cohen SN, Chang AC, Hsu L (1972) Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA. Proc Natl Acad Sci U S A 69:2110–2114
4. Mandel M, Higa A (1970) Calcium-dependent bacteriophage DNA infection. J Mol Biol 53:159–162
5. Chung CT, Niemela SL, Miller RH (1989) One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc Natl Acad Sci U S A 86:2172–2175
6. Inoue H, Nojima H, Okayama H (1990) High efficiency transformation of *Escherichia coli* with plasmids. Gene 96:23–28
7. Norgard MV, Keem K, Monahan JJ (1978) Factors affecting the transformation of *Escherichia coli* strain chi1776 by pBR322 plasmid DNA. Gene 3:279–292
8. Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH (1982) Gene transfer into mouse lyoma cells by electroporation in high electric fields. EMBO J 1:841–845
9. Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557–580
10. Collins J, Leadbeater N (2007) Microwave energy: a versatile tool for the biosciences. Org Biomol Chem 5:1141–1150
11. Fermér C, Nilsson P, Larhed M (2003) Microwave-assisted high-speed PCR. Eur J Pharm Sci 18:129–132
12. Orrling K, Nilsson P, Gullberg M, Larhed M (2004) An efficient method to perform milliliter-scale PCR utilizing highly controlled microwave thermocycling. Chem Commun (Camb) 790–791.
13. Edwards W, Young D, Deiters A (2009) The effect of microwave irradiation on DNA hybridization. Org Biomol Chem 7:2506–2508
14. Young DD, Nichols J, Kelly RM, Deiters A (2008) Microwave activation of enzymatic catalysis. J Am Chem Soc 130:10048–10049
15. Leadbeater NE, Stencel LM, Wood EC (2007) Probing the effects of microwave irradiation on enzyme-catalysed organic transformations: the case of lipase-catalysed transesterification reactions. Org Biomol Chem 5:1052–1055
16. Fregel R, Rodríguez V, Cabrera VM (2008) Microwave improved *Escherichia coli* transformation. Lett Appl Microbiol 46:498–499
17. Roychoudhury A, Basu S, Sengupta DN (2009) Analysis of comparative efficiencies of different transformation methods of *E. coli* using two common plasmid vectors. Indian J Biochem Biophys 46:395–400
18. Dityatkin SY, Lisovskaya KV, Panzhava NN, Iliashenko BN (1972) Frozen-thawed bacteria as recipients of isolated coliphage DNA. Biochim Biophys Acta 281:319–323
19. Wise AA, Liu Z, Binns AN (2006) Three methods for the introduction of foreign DNA into *Agrobacterium*. Methods Mol Biol 343:43–53
20. Weigel D, Glazebrook, J Transformation of *Agrobacterium* using the freeze-thaw method. CSH Protoc 2006, 2006(7).
21. Chen H, Nelson RS, Sherwood JL (1994) Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. Biotechniques 16:664–668, 670
22. Takahashi R, Valeika SR, Glass KW (1992) A simple method of plasmid transformation of *E. coli* by rapid freezing. Biotechnique 13:711–712, 715
23. Merrick MJ, Gibbins JR, Postgate JR (1987) A rapid and efficient method for plasmid transformation of *Klebsiella pneumoniae* and *Escherichia coli*. J Gen Microbiol 133:2053–2057
24. Shokolenko IN, Alexeyev MF (1995) Transformation of *Escherichia coli* TG1 and *Klebsiella oxytoca* VN13 by freezing-thawing procedure. Biotechniques 18:596–598
25. Wards B, Collins D (1996) Electroporation at elevated temperatures substantially improves transformation efficiency of slow-growing mycobacteria. FEMS Microbiol Lett 145:101–105
26. Jacobs WR, Tuckman M, Bloom BR (1987) Introduction of foreign DNA into mycobacteria using a shuttle phasmid. Nature 327:532–535
27. Naser SA, McCarthy CM, Smith GB, Tupponce AK (1993) Low temperature protocol for efficient transformation of *Mycobacterium smegmatis* spheroplasts. Curr Microbiol 27:153–156
28. Zainuddin ZF, Kunze ZM, Dale JW (1989) Transformation of *Mycobacterium smegmatis* with *Escherichia coli* plasmids carrying a selectable resistance marker. Mol Microbiol 3:29–34
29. Zainuddin ZF, Kunze ZM, Dale JW (1989) Transformation of Mycobacterium smegmatis with E. coli plasmids. Acta Leprol 7(1):212–216
30. Snapper SB, Melton RE, Mustafa S, Kieser T, Jacobs WR (1990) Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol Microbiol 4:1911–1919
31. Kaps I, Ehrt S, Seeber S, Schnappinger D et al (2001) Energy transfer between fluorescent proteins using a co-expression system in *Mycobacterium smegmatis*. Gene 278:115–124