Journal of Experimental Microbiology and Immunology (JEMI) Vol. 13:67-70

Effects of the PitA Phosphate Uptake System and Calcium Ion Channel on Transformation Efficiency in *Escherichia coli* K-12 Cells

Amy Prangnell, Kali Romano, Casey Wong and Gary Yip
Department of Microbiology & Immunology, UBC

The Hanahan protocol is widely used to transform bacterial cells with a plasmid of interest. However, little is known about the mechanism by which bacterial cells, particularly *Escherichia coli*, take up these plasmids. It was hypothesized that the PitA phosphate uptake system, which has been shown to transport divalent metal cations including Ca^{2+}, may be involved in the Ca^{2+}-mediated plasmid uptake in the Hanahan protocol. The model on which this hypothesis is based involves the binding of plasmid DNA by Ca^{2+}, followed by the localization of the Ca^{2+}-plasmid complex to the PitA system and subsequent complex uptake into the cell. In this study, the transformation efficiencies of a *pitA* mutant and a *pitA*$^+$ wild type control were compared. A modified version of the Hanahan protocol was used to transform these strains with plasmid pBR322. Transformation efficiencies were determined by comparing ampicillin-selective with non-selective plate counts. This study indicated that the presence or absence of *pitA* has little impact on plasmid uptake in these transformations. Future studies on the basis of this study's data must be done at plasmid-saturating conditions for both the mutant and parental strains in order to support these results.

Successful transformation of *E. coli* cells has been accomplished since the early 1980s using the Hanahan protocol in addition to modifications of the Hanahan protocol to accommodate constraints of specific experimental questions (4). Essentially, the protocol enables the uptake of plasmid DNA by rendering *E. coli* cells competent by incubation with an ice cold calcium chloride solution and subsequently heat-shocking the competent cell and plasmid DNA solution to allow the transport of DNA into the cell.

Exactly how the transport of DNA is mediated and what role calcium plays in transformation as a divalent cation is still unclear. Studies by Chen and Dubnau (2) identify natural competence for transformation as a transient physiological state in bacteria, common to bacteria from all taxonomic divisions. This inspired curiosity to determine which bacterial membrane proteins may be involved in the uptake of DNA and if these membrane components were involved with divalent cations like calcium. According to van Veen *et al.*, PitA of *E. coli* is a constitutively expressed phosphate uptake system requiring divalent cations such as Mg^{2+} and Ca^{2+} for activity and transport of soluble neutral metal phosphate complexes (6). It was from these observations that we developed our experimental question of whether or not the PitA system is involved in the solubilization and neutralization of plasmid DNA complexes for uptake into the cytoplasm of *E. coli* cells.

As alluded to previously, the mechanism of how transformation occurs via the Hanahan protocol is unclear. It was hypothesized that the successful transformation of cells may involve Ca^{2+}-binding membrane proteins. The PitA inorganic phosphate system has been identified as a binder and transporter of Ca^{2+} and was hypothesized to play a role in transformation (6). If this were the case, then transformation efficiencies would be decreased in the *pitA* mutant strain JW3460-5. The hypothesis was that the binding of plasmid DNA by Ca^{2+} followed by the localization of the Ca^{2+}-plasmid complex to the PitA system, would lead to subsequent complex uptake into the cell. Although there is no evidence of DNA transport through the PitA system, it was hypothesized that in this protocol, Ca^{2+} would strongly bind the negatively charged phosphate back bone of plasmid DNA allowing the DNA to be 'dragged' through the channel and co-transported with the divalent cation.

Through transformation and selective plating on ampicillin-containing media of both *pitA* mutant strain JW3460-5 and *pitA*$^+$ parent strain BW25113, transformation efficiencies were determined in order to assess whether the *pitA* mutant strain experienced a significant loss in transformation efficiency compared to the parental strain. This loss in efficiency would translate into a loss in the ability of the cells to be

transformed through the PitA system.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* strain DH5α was obtained from the MICB 421 culture collection at the University of British Columbia Department of Microbiology and Immunology and was used as a transformation competency control (Table 1). Both *E. coli* K-12 substrains BW25113 (*pitA*$^+$ positive control) and JW3460-5 (*pitA*$^-$ mutant) were obtained from the Coli Genetic Stock Center at Yale University (Table 1). The DH5α, BW25113, and JW3460-5 *E. coli* strains were all cultured at 37°C in Luria-Bertani (LB) broth (1% w/v tryptone, 0.5% yeast extract, and 0.5% w/v NaCl, adjusted to pH 7). 1.5% w/v agar was added for all LB plates and 100 µg/mL of ampicillin (Sigma, St. Louis, MO) was used to supplement the LB ampicillin plates.

TABLE 1. *E. coli* strains used in transformations.

E. coli Strains	Strain Characteristics	Source
DH5α	F-; *deoR; endA1; gyrA96; hsdR17(rk-mk+); recA1; relA1; supE44; thi-1; D(lacZYA-argFV169); f80lacZ; DM15*	MICB 421 culture collection
BW25113	F-; *DE(araD-araB)567; lacZ4787(del)(::rrnB-3); LAM-; rph-1; DE(rhaD-rhaB)568; hsdR514*	Yale University Coli Genetic Stock Collection
JW3460-5	F-; *DE(araD-araB)567; lacZ4787(del)(::rrnB-3); LAM-; rph-1; DE(rhaD-rhaB)568; hsdR514; pitA749(del)::kan*	Yale University Coli Genetic Stock Collection

Plasmid. Purified plasmid pBR322 (Fermentas, Burlington, ON) in 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA was obtained from the University of British Columbia Department of Microbiology and Immunology and was used to transform *E. coli* strains DH5α, BW25113, and JW3460-5. Plasmid pBR322 is a 4361 bp plasmid and contains *bla* which codes for beta-lactamase and confers resistance to ampicillin. It also *tet* which encodes a tetracycline resistance protein.

Preparing Competent Cells. The DH5α, BW25113, and JW3460-5 *E. coli* strain cultures were grown to an OD_{600}=0.2 in 10 mL of LB broth. For each bacterial culture, a 1 mL portion was transferred into three separate chilled 1.5 mL microfuge tubes. These microfuge tubes were then spun at 10,000 X g for 10 minutes at 4˚C. The supernatant was discarded from each microfuge tube and 50 µL of ice cold 100 mM $CaCl_2$ was added to each cell pellet and pipetted up and down to resuspend the pellet. The contents of the three tubes for each strain were combined into one 1.5 mL microfuge tube and were set on ice for 30 minutes. This method was adapted from the Hanahan protocol (4).

Transformation. The competent cells for each strain were split into three 1.5 mL microfuge tubes – each to be transformed with a different plasmid concentration. Plasmid pBR322 was added at final concentrations of 0.1, 0.5, and 1.5 µg/mL. The tubes were set on ice for 10 minutes. Each tube was heat shocked in 42˚C for exactly 30 seconds and then placed back on ice for 2 minutes. After incubation on ice, 0.95 mL of LB broth was added to each cell and DNA mixture before transferring the contents to a test tube for incubation at 37˚C for 1 hour. After recovery, sterile saline was used as a diluent to generate dilutions of 10^{-1} to 10^{-6} by intervals of 10. One hundred µL of resuspended and diluted transformants were plated on LB agar supplemented with 100 µg/mL of ampicillin. All plates were incubated at 37˚C for 18-24 hours, or until sufficient growth was observed. Plates containing between 30 and 300 colonies were counted. This method was adopted from the Hanahan protocol (4). Transformation efficiency was calculated by dividing the concentration of colony forming units plated on LB agar supplemented with ampicillin by those plated on LB agar alone.

RESULTS

The DH5α strain was used as a positive control to confirm that the modified Hanahan transformation protocol employed was functioning adequately to allow for a comparison of the mutant JW3460-5 and parental BW25113 strains, as it is a readily transformable strain of E.coli.

The DH5α, BW25113, and JW3460-5 strains yielded reliable plate counts (i.e. between 30 and 300 colonies) on both LB and ampicillin-containing LB at all three plasmid concentrations. Table 2 summarizes the transformation efficiencies and their respective 95% confidence intervals of all *E. coli* strains at the various plasmid concentrations to facilitate comparisons of transformation efficiency between each strain. With a plasmid concentration of 0.1 µg/mL, the transformation efficiency of DH5α was 4x greater than BW25113 and 60x greater than JW3460-5. At a plasmid concentration of 0.5 µg/mL, the difference between the transformation efficiency of DH5α and the parent and mutant *pitA* strains was more distinct – efficiency was approximately 35x and 28x greater in DH5α than BW25113 and JW3460-5 respectively.

TABLE 2. Transformation efficiency using the Hanahan protocol and plasmid pBR322 of various *E. coli* strains.

Strain	Transformation efficiency (10^{-3} %) at various plasmid concentrations[a]		
	0.1 µg/mL	**0.5 µg/mL**	**1.5 µg/mL**
DH5α	13.6 ±4.2	76.5±8.5	42.0±11.2
BW25113	3.4±0.6	2.2±0.1	13.0±1.7
JW3460-5	0.2±0.1	2.7±2.2	8.2±4.2

[a] ± values indicate the limits of the 95% Poisson confidence interval

The transformation efficiencies varied between the BW25113 and JW3460-5 *E. coli* strains. At the various plasmid concentrations, transformation efficiency was

15x and 1.6x greater in BW25113 at concentrations of 0.1 μg/mL and 1.5 μg/mL respectively. However, at 0.5 μg/mL, efficiency was 1.2x greater in JW3460-5.

With respect to the change in transformation efficiency due to a change in plasmid concentration, two cases are noted. DH5α transformation efficiency at a plasmid concentration of 1.5 μg/mL decreased to approximately 0.5x the efficiency at 0.5 μg/mL. In BW25113, transformation efficiency decreased to about 0.7x between 0.1 μg/mL and 0.5 μg/mL.

DISCUSSION

The expectation of our experiment was that the transformation efficiency would be significantly decreased in the JW3460-5 strain compared to the BW25113 strain as a result of the mutation of *pitA* interfering with the inorganic phosphate uptake system which we hypothesized to have a role in DNA uptake in transformation. This hypothesis was extrapolated from studies by van Veen *et al.* who uncovered evidence for the transport of divalent metal cations including Ca^{2+} via the Pit inorganic phosphate system in *E. coli* (6). Although there is no evidence of DNA transport through the PitA system, using evidence from the studies of van Veen *et. al.* (6), we proposed that the negatively charged phosphate back bone of plasmid DNA would form a strong association with divalent cation Ca^{2+} in solution, and thus could be co-transported into the *E.coli* cell through the Pit A system during transformation with Ca^{2+} (6). In addition, because the PitA system naturally enables the transport of inorganic phosphate, and has proven to transport divalent cations (6), we hypothesized that the complex of the phosphate backbone to Ca2+ should be equally as efficient at transforming into the *E.coli* cells. The limitation to our hypothesis was that we were assuming that the effect of the large organic molecules associated with the phosphate backbone of the DNA would not affect the efficiency of our hypothesized transport. However, the difference in transformation efficiencies across all plasmid concentrations between BW25113 and JW3460-5 was not consistent with any general trends – our data indicates that at plasmid concentrations of 0.1 and 1.5 μg/mL, BW25113 shows higher efficiency while JW3460-5 does so at 0.5 μg/mL. This suggests that the *pitA* uptake system did not have as significant of a role in transformation as expected, and had little impact on plasmid uptake during transformation.

It is reasonable to hypothesize that there may be additional Ca^{2+} channels involved in the uptake of DNA in *E.coli* to compensate for the loss of the PitA system in the JW3460-5 strain. For example, Das *et al.* (1997) proved the existence of a non-proteinaceaous calcium selective channel in *E. coli* composed of poly(3-hydroxybutyrate) and inorganic polyphosphate which is currently being investigated for its role in the membrane and upregulation as a result of temperature increases (3). It is plausible to suggest that perhaps the temperature shock of the Hanahan protocol may lead to an upregulation in this non-proteinaceous channel, which subsequently may play a role in the transformation of plasmid across bacterial membranes.

As the concentration of DNA increases, the transformation efficiency is subsequently expected to increase as well, as long as the calcium chloride concentration is consistent (1). Our datum show a decrease in efficiency for DH5α between 0.5 and 1.5 μg/mL as well as a decrease for BW25113 between 0.1 and 0.5 μg/mL, which were unexpected. The decrease in efficiency for DH5α between 0.5 and 1.5 μg/mL could be due to the fact that the plasmid concentration has already reached saturating conditions and differences observed can be due to random background noise in measurements. In the JW3460-5 strain, we see an increase between 0.1 and 0.5 μg/mL which is expected. This leads us to the suspicion that the value measure for BW25113 may have been due too high. A possible explanation for this result could be that the *pitA* gene that is present in BW25113 is responsible for assisting transformation at low plasmid concentration. It may be that at low plasmid concentration, the PitA plays a role in assisting the binding of DNA to cell membrane surface but at saturating plasmid concentrations, its importance becomes less significant.

In an effort to elucidate factors in the mechanism of DNA entry during artificial transformation with *E. coli*, the PitA membrane transport system was analyzed using a modified version of the Hanahan protocol. According to our results, it appeared that the PitA channel does not play a major role in DNA transfer as no difference in transformation efficiencies between the mutant and parent strain was observed. However, we also speculate that PitA may only have a significant effect at low concentration of plasmid DNA. In conclusion, it may be possible that our technique was not accurate enough to determine the correlation between the absence of *pitA* and transformation efficiency.

FUTURE EXPERIMENTS

To fully determine the role of the PitA channel in *E.coli* additional, more direct tests need to be preformed before any specific statements can be made regarding this specific channel. In an effort to achieve this, repeated tests of the PitA channel at saturating and non-saturating plasmid concentrations are required to conclusively rule out its involvement in Hanahan transformation of *E. coli*.

In addition, experiments using inhibitors to block either the PitA transport system, or other channels believed to be associated with transformation could be very insightful in assessing the importance of that channel in transformation. Rather than observing differences in transformation efficiency, in working with an inhibitor one might expect to see a complete loss in transformation and therefore negligible transformation efficiency. Work of this nature has been recently explored by Miriuta and Pererva using inhibitors such as verapamil and furosemid to observe the effects on the yield of *E. coli* plasmid transformants in eukaryotes (5). Experiments of this nature have yet to be extrapolated to many bacterial models and could prove to yield insightful results.

As stated in our discussion, although there was no significant difference in the transformation efficiency between the BW25113 and JW3460-5 strains, the presence of the PitA channel may have an effect at lower plasmid DNA concentrations. Our data support this idea – there was a large difference in transformation efficiency between BW25113 and JW3460-5 at DNA concentration of 0.1 μg/mL. Thus, important parameters to test in future experiments would be the DNA concentration range where the presence of PitA would make a significant difference and where this effect would be neutralized by a saturating DNA concentration.

ACKNOWLEDGEMENTS

This work was funded and supported by the MICB 421 course within the Microbiology and Immunology Department at the University of British Columbia. We would like to thank the reliable advice and support provided by Dr. William Ramey and Shaan Gellatly through the planning and duration of our experiment. The media room has also provided greatly appreciated advice and experimental equipment that was essential to our experiment. We would also like to thank Ehleen Hinze for providing us with her pUC 19 plasmid which we did not end up using in our experiment as well as the Coli Genetic Stock Collection for providing us with *E. coli* BW25113 and JW3460-5 strains.

REFERENCES

1. **Baur, B., K. Hanselmann, W. Schlimme, and B. Jenni.** 1996. Genetic transformation in freshwater: *Escherichia coli* is able to develop natural competence. Appl. Environ. Microbiol. **62:**3673-3678.
2. **Chen, I., and D. Dubnau.** 2004. DNA uptake during bacterial transformation. Nat. Rev. Microbiol. **2:**241-249.
3. **Das, S., U. D. Lengweiler, D. Seebach, and R. N. Reusch.** 1997. Proof for a nonproteinaceous calcium-selective channel in *Escherichia coli* by total synthesis from (R)-3-hydroxybutanoic acid and inorganic polyphosphate. Proc. Natl. Acad. Sci. U. S. A. **94:**9075-9079.
4. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557-580.
5. **Miriuta, A. I., and T. P. Pererva.** 2008. Biological activity of *Ungernia victoris* extract in the *Escherichia coli* $CaCl_2$-transformation system in the presence of calcium channel modulators. Tsitol. Genet. **42:**45-49.
6. **van Veen, H. W., T. Abee, G. J. Kortstee, W. N. Konings, and A. J. Zehnder.** 1994. Translocation of metal phosphate via the phosphate inorganic transport system of *Escherichia coli.* Biochemistry **33:**1766-1770.