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

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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 LB ampicillin plates.

Strains

<table>
<thead>
<tr>
<th><em>E. coli</em> Strains</th>
<th>Characteristics</th>
<th>Source</th>
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<tbody>
<tr>
<td>DH5α</td>
<td>F−, deoR, endA1; gyrA46; hsdR17(rk-mk+); recA1; relA1; supE44; thi-1; D(lacZYA-argFV169); f80lacZ; BMI15</td>
<td>MICB 421 culture collection</td>
</tr>
<tr>
<td>BW25113</td>
<td>F−; DE(araD-araB)567; lacZ4787(del):(::rrnB-3); LAM-; rph-1; DE(rhaD-rhaB)568; hsdR514</td>
<td>Yale University Coli Genetic Stock Collection</td>
</tr>
<tr>
<td>JW3460-5</td>
<td>F−; DE(araD-araB)567; lacZ4787(del):(::rrnB-3); LAM-; rph-1; DE(rhaD-rhaB)568; hsdR514; pitA749(del)::kan</td>
<td>Yale University Coli Genetic Stock Collection</td>
</tr>
</tbody>
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Transformation. 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Transformation efficiency (10⁷ %) at various plasmid concentrations *</th>
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<tbody>
<tr>
<td></td>
<td>0.1 µg/mL</td>
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<tr>
<td>DH5α</td>
<td>13.6 ±4.2</td>
</tr>
<tr>
<td>BW25113</td>
<td>3.4±0.6</td>
</tr>
<tr>
<td>JW3460-5</td>
<td>0.2±0.1</td>
</tr>
</tbody>
</table>

* ± values indicate the limits of the 95% Poisson confidence interval

RESULTS

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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 Ca2+ 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 Ca2+ in solution, and thus could be co-transported into the *E.coli* cell through the Pit A system during transformation with Ca2+ (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 Ca2+ 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-proteinaceous 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 \( \mu \)g/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.

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REFERENCES