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Growing *Escherichia coli* at a Range of Temperatures to Change Expression of *omp*A did Not Affect the Efficiency of Transformation

Martin (Chien Hao) Chen, Jacky Li, Jonathan Ng, Joshua (Ting Hua) Wu
Department Microbiology & Immunology, University of British Columbia

The outer membrane protein A (OmpA) is a major protein in *Escherichia coli* outer membrane. The expression of *omp*A is tightly correlated to temperature stress, with lower expression at higher growth temperature. Combined with the observation that transformation of *E. coli* growing at a lowered temperature has higher transformation efficiency when using $CaCl_2$, OmpA could potentially play a role in chemical transformation. In order to determine the effect of OmpA on the transformation efficiency, two strains of *E. coli,* BW25113 (WT *omp*A) and JW0940-6 intended *om*pA deletion/insertion mutation), were grown at different temperatures. The relative levels of OmpA were compared after a membrane protein isolation step. SDS-PAGE and PCR were used to confirm the expression of *omp*A and genotypes of both strains respectively. SDS-PAGE analysis revealed that both strains had *omp*A expression and PCR revealed that the JW0940-6 strain contained the entire *omp*A coding region. Both WT and intended Δ*omp*A strains showed higher transformation efficiency when grown at 42℃ compared to 37℃. However, 20℃ growth temperature promoted transformation efficiency in BW25113 but lowered transformation efficiency in the JW0940-6. Overall, the results implied that, in the JW0940-6 strains obtained, either the kanamycin resistance cassette was inserted into a different gene or the strain had intrinsic kanamycin resistance. Without *omp*A deletion strain and accurate protein assay for protein gel loading, the data were not sufficient to support the original hypothesis that *omp*A expression affected transformation efficiency.

Transformation using plasmid vector is a common technique to incorporate and express exogenous DNA in bacteria for industrial and research purposes. This process involves chemical treatments using $CaCl_2$ or charge stabilization of genetic materials using electroporation, followed by the disruption of the membrane allowing uptake of extracellular DNA into cell cytoplasm. Chemical transformation is done by treating *Escherichia coli* with cold $CaCl_2$, and following a heat-pulse shock at 42℃ to improve the cells competency for plasmid uptake (1).Artificial transformation must overcome electrostatic repulsion and size exclusion. Since both the phosphate groups of the DNA helix and the phosphate groups of the lipid membrane are negatively charged, the electrostatic repulsion force blocks the movement of DNA across membranes. The outer-membrane porins, responsible for diffusion of hydrophilic molecules, also has a size limitation of around 500 daltons (2).

Two models of $CaCl_2$-mediated transformation have been proposed. The first model hypothesized that divalent calcium ions neutralize the charges of plasmids and facilitate the binding of extracellular plasmids to the cell surface. The heat-shock step then destabilizes both the outer-membrane and the plasma membrane by releasing lipids from the cell membrane into the environment and forming pores on cell surface. At the same time, the heat-shock lowers the membrane potential, which facilitates the uptake of negatively-charged molecules such as DNA (2). The thermal imbalance created by the heat-shock on two sides of the *E. coli* membranes helps to physically pump DNA into cytoplasm. The second model suggested that plasmids bind to surface proteins to be transported through transmembrane channel rather than temporary holes on phospholipids membranes (3).

Recently, the expression of *omp*A was shown to be correlated with competency in JW0940 *E.coli* strain. Deletion of *omp*A significantly improved transformation in solid medium without the use of $CaCl_2$ but lowered transformation efficiency in liquid medium with the use of $CaCl_2$ (4). OmpA is an outer-membrane protein pore, which is permeable to ions and small solutes. During bacteriophage infection and conjugation, it transfers DNA cross the outer-membrane into cell (5). However, the effect of OmpA on DNA transfer during transformation is still unclear.

Earlier studies have shown that growing *E. coli* at a lower temperature (18℃) prior to the $CaCl_2$ treatment can greatly improve the transformation efficiency (7). This experiment focused on the exploration of the role OmpA plays during $CaCl_2$-mediated transformation in different temperatures (20℃, 37℃ and 42℃) by using both a wild type strain of *E. coli* (OmpA+) and an intended *omp*A deletion strain (OmpA-). This investigated whether the temperature-sensitive effects on chemical transformation are caused by the fluctuation in *omp*A expression at different growth temperature. To confirm the presence and the absence of *omp*A gene in the two *E. coli* cell lines, SDS-PAGE and PCR amplification were performed and OmpA levels of cells grown at different growth temperatures were compared.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* BW25113 and JW0940-6 (a reported ΔompA) cells were obtained from the Coli Genetic Stock Center (CGSC), Yale University (8). Luria-Bertani

(LB) media contained 10 g/L of tryptone, 5 g/L of yeast extract and 10 g/L of NaCl was used to grow the cultures. Samples of overnight culture grown at 37°C, 42°C and 20°C were inoculated into LB broth allowing growth to reach exponential phase with the indicated temperature and shaker specification. A prior study found that *E. coli* strains were in exponential phase around 0.5 OD_{600} with the cell density of 5 x 10^7 cells per ml (4). Turbidity readings were read at OD_{600} using UV spectrophotometer before proceeding to outer membrane extraction and transformation experiments.

Outer membrane protein isolation. The outer membrane isolation was done as described in the previous studies with minor modifications (10, 11). 200 ml of bacterial samples were centrifuged for 5 minutes at 10,000 x g to separate supernatants and cell pellets. Supernatants were discarded. Pellets were resuspended in 10 ml of Tris HCl, pH 8.0, 1 mM EDTA, 20% sucrose and 1 mg/ml lysozyme. A French Press was used to lyse sample cells at 15,000 psi for two rounds. Lysed cells were centrifuged at 2,500 x g at 10°C for 25 minutes to remove cell debris. Supernatants were transferred to clean centrifuge tubes and centrifuged at 35,000 x g for 20 minutes. The resultant supernatants were discarded. Pellets were resuspended in 10 ml of 10 mM Tris HCl, pH 8.0 and centrifuged at 35,000 x g for 20 minutes for two times. Pellets were resuspended in 1 ml of sterile deionised water. Samples were stored at -20°C until use.

Protein concentration assay. The Bradford protein assay was performed in a 96 well plate. The bovine serum albumin standard was used to prepare a standard series at 0, 0.025, 0.050, 0.100, 0.150, 0.200, and 0.250 µg/µl. 20µl of each standards and samples were loaded on the plate followed by addition of 200 µl of Bradford dye reagent (BioRad) to all wells contained sample. The plate was set for 15 minutes and it was immediately taken for the absorbance readings at A_{595}.

SDS-PAGE. The SDS-PAGE gels were prepared as previously described with minor modifications (9). The separating and stacking gels with the addition of 300 µl of 10% APS and 31.5 µl of TEMED were prepared and stored at 4°C until the membrane protein samples were analysed. A total of 20 µl of each undiluted protein sample and SDS sample buffer was incubated at 90°C for 5 minutes and loaded on each well. SDS-PAGE molecular weight standards, low range, were used (Bio-Rad #161-0304). The gels were run at 200 volts for 30 minutes, stained with a 0.1% w/v coolmassie blue solution in buffer containing 40% v/v methanol and 10% v/v acetic acid and microwaved for 1.5 minutes. The gels were then placed in destaining solution, microwaved for 1.5 minutes, and gently agitated a shaker platform for 45 minutes or until bands are clearly visible.

***E coli* competence induction via $CaCl_2$ and subsequent transformation of pUC19.** Transformation was done as described in the previous study with minor modifications (12). After reaching 0.5 OD_{600}, the cells were chilled on ice for 10 minutes and centrifuged at 4000 x g for 5 minutes at 4 °C. Pellets were resuspended in 50 ml of LB with 60 mM $CaCl_2$. The cells were chilled on ice for 30 minutes and centrifuged again at 4000 x g for 5 minutes at 4°C. The pellets were gently resuspended in 5 ml of sterile cold $CaCl_2$ containing 10% glycerol. The competent cells were stored at -80°C in microfuge tubes, each tube having 250uL aliquots. For transformation, 30 ng of pUC19 plasmid was added to 250 µl aliquot of competent cells. Concentrated stock plasmid DNA was diluted with TE buffer. The cells were incubated on ice for 30 minutes with pUC19 plasmid, and then heat shocked in a 42°C water bath for 2 minutes. The cells with pUC19 were put on ice for 5 minutes. The Recovery step was done by adding 1 ml of LB to the cells and incubating the cells 37°C shaker at 250 rpm for 60 minutes. 50 µl of diluted transformed cells were spread plated on the LB agar plates with and without 100ug/ml ampicillin.

Colony PCR. The colony PCR was done as described in the California State University website with minor modifications using (http://www.csun.edu/~mls42367/Protocols/Colony%20PCR.pdf) . PCR reaction was prepared on ice by adding 1 µl of 10 mM dNTPs, 5µl of 10X PCR buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of 20 µM forward and reverse primers, 39 µl of sterile distilled water and 1 µl of 2.5U Taq polymerase to each tube. Two primers were designed to flank *ompA* gene. The forward primer sequence, 5'-CGCCAGGGGTGCTCG-3', located at 119 bp upstream of the *ompA* start codon , and the reverse primer sequence, 5'-TGATTGAAGATGCGG-3', located at 149 bp downstream of *ompA* stop codon. A small amount of colony was added to each tube by using a yellow pipette tip.Cells were mixed by pipetting up and down. PCR conditions were 5 minutes at 95°C for 1 cycle, 1 minute at 95°C for 35 cycles, 1.5 minutes at a temperature gradient of 35°C, 40°C and 45°C for 35 cycles, 1 minute at 72°C for 35 cycles, and 5 minutes at 72°C for last cycle, using Biometra T-Gradient thermocycler. The expected size of *ompA* PCR product was 1339 bp, and according to previous experiments, the expected size of ompA knock-out/kanamycin resistance cassette insertion had 284 additional basepairs more than the WT (4), which means that our expected PCR product should be 1623 bp long.

TABLE 1 NCBI reference sequence, forward and reverse primer sequences used for amplifying *ompA*, with gene size and product size specified

	Reference sequence	Primer sequence (5' to 3')		Gene size (bp)	Product size(bp)
ompA	NC_000913.3	Forward	CGCCAGGGGTGCTCG	1041	1339
		Reverse	TGATTGAAGATGCGG		

Agarose gel electrophoresis. The 1% agarose gels and TBE buffer were prepared as described in (13). The gels were run at 200 volts for 30 minutes and stained in ethidium bromide for 20 minutes before taking pictures of gels.

RESULTS

SDS-PAGE gel of BW25113 (OmpA+) and JW0940 (OmpA-) strains of *Escherichia coli* to confirm Δ*ompA* mutant. The BW25113 positive control showed the expected phenotype for both of the SDS-PAGE gels. The SDS-PAGE gels showed a clear band at 30 kDa; previously identified as the size OmpA runs at by Afonyushkin et al. (14). The cleanliness of the gel also indicated that the outer membrane extraction protocol was working as intended. If there were cytosolic proteins present, a much larger number of bands would show up as the coomassie blue stain targets the positive amine groups of any protein. However, even though the expected band for OmpA existed for BW25113, this 30 kDa band also unexpectedly showed up for the *ompA*-knockout mutant, JW0940-6 strain. This data indicates that the JW0940-6 strain obtained from CGSC (listed as an *ompA* knockout), still had the *ompA* in some form, either the knockout failed or there had been a duplication of the gene.

Confirmation of kanamycin resistance suggested that the kanamycin cassette was inserted into the genome of JW0940-6 strain. Due to the results obtained from Figure 1, both OmpA+ and OmpA- strains were inoculated into LB broth with 50 µg/ml kanamycin to grow at 37°C

overnight. The OmpA- strain grew, but the OmpA+ strain,had no growth at all. This indicates that the presumptive knockout did have the expected resistance for kanamycin that results from the lambda red mutagenesis procedure (http://www.biomedcentral.com/1471-2199/9/20)

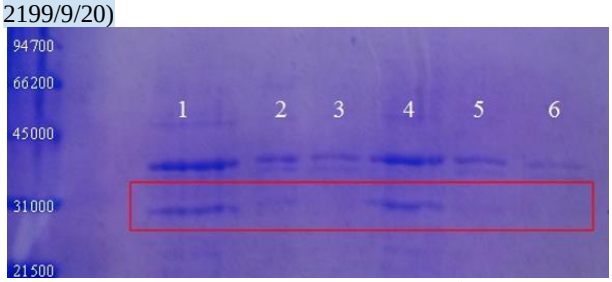

FIG 1 Coomassie Blue stained SDS-PAGE gel of cell membrane protein samples from OmpA+ and OmpA- strains. Lanes 1-3 are OmpA+ samples, undiluted, 5x diluted, and 25x diluted, respectively. Lanes 4-6 are OmpA- samples, undiluted, 5x diluted and 25x diluted, respectively. The numbers on the far left are MW standards, in units of kDa. The box indicates where the OmpAis predicted to be on the gel. A rough estimation of 20 µg of total protein to be loaded onto the wells was determined with a Thermo Scientific Nanodrop 2000c spectrophotometer.

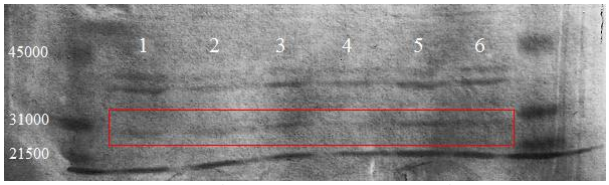

FIG 2 SDS-PAGE gel of cell membrane samples from OmpA+ and OmpA- strains. The image was taken by MultiImage (TM) light cabinet from Alpha Innotech Corporation. Lanes 1, 3 and 5 are samples from the OmpA+ strain, grown at 42 ˚C, 37 ˚C and 20˚C, respectively. Lanes 2, 4 and 6 are from the OmpA- strain, grown at 42 ˚C, 37 ˚C and 20˚C, respectively. The numbers on the far left are MW standards, in units of kDa. The box indicates where the OmpA should be on the gel.

SDS-PAGE gel of Outer membrane extraction of cells grown at 20, 37, and 42°C . All the samples were loaded onto the gel with roughly the same amount of total protein (1.6 µg) determined by a Bradford protein assay (15). Bradford assay cannot, however, be used quantitatively due to the fact that the is not accurate in representing the concentration of lipid-bound proteins dissolved in water. Therefore, the concentration of the samples loaded was insufficient to produce a clear band of OmpA. From the gel, we could see that the presumptive OmpA band was present in every lane, agreeing with the result from Figure 1 that the OmpA- strain was not *omp*A deletion and *omp*A was still being expressed in the cell. While the original goal was to identify the effect of OmpA on transformation efficiency, the experiment cannot be carried out due to the lack of an *omp*A-knockout strain. In order to adapt Bradford assay to membrane bound protein, OmpA, outer membrane extraction procedure should be modified. Instead of using water, detergent should be used to dissolve the protein pellets of the samples. According to Bradford Fanger's studies, 0.2 % of hexyl beta-D-glucopyranoside (HG) does not produce significant background absorbance at 595 (15). HG should be used for homogeneously dissolving hydrophobic OmpA and subsequent protein quantification

Colony PCR amplification of OmpA+ and OmpA- strains showed Δ*omp*A was not present in OmpA- strain. In order to confirm the presence or absence of *omp*A as well as to differentiate between a failed knockout and *ompA* duplication, PCR primers were designed to flank the WT *ompA* gene in the *E. coli* K-12 MG1655 strain (the parent strain to both the OmpA+ and OmpA- strains). The *omp*A gene itself should be 1041 bp long, from the start to stop codon. The predicted PCR product should be a total length of 1339 bp including the primers. According to Figure 3, all of the products were of the same length of 1322 bp, consistent with the protein gel (Fig.1, 2) that *omp*A was present in both OmpA+ and intended OmpA- strains.

In-silico analysis of predicted PCR product sizes suggested that *omp*A PCR products of OmpA+ and OmpA- should have different lengths that can be distinguished on a 1% agarose gel. According to Cherepanov and Wackernagel the kanR cassette that were inserted into the OmpA- mutant causing the Δ*omp*A genotype would have a PCR product of around 1800 bp (16) while our WT *omp*A will have a PCR product length of 1339 bp. Based on the calculations for the gel ran on Fig 3, a 478 bp difference should have a 3.8 mm difference on the gel (Fig 4)., a nd this difference was not observed.

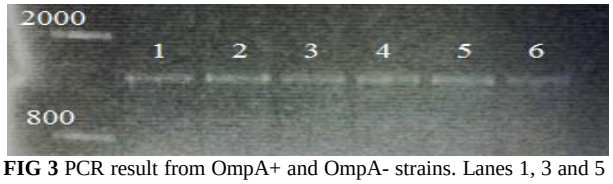

FIG 3 PCR result from OmpA+ and OmpA- strains. Lanes 1, 3 and 5 are samples from the OmpA+ strain, grown at 42 ˚C, 37 ˚C and 20˚C, respectively. Lanes 2, 4 and 6 are from the OmpA- strain, grown at 42 ˚C, 37 ˚C and 20˚C, respectively.

Transformation Efficiency of OmpA- and OmpA+ strains. We observed that the transformation efficiency of OmpA- strains decreased as the growth temperature decreased (Table 2). There were no difference in the transformation efficiency between our OmpA+ and OmpA- strains when the cells were grown at 37 ˚C; an increase in transformation efficiency in our OmpA- strain at 42 ˚C relative to our OmpA+ strain; and a decrease in transformation efficiency in our OmpA- strain at 20 ˚C relative to our OmpA+ strain

DISCUSSION

There are many potential reasons that the lack of OmpA knockout in the CGSC strain could have happened. First, the band was a cytosolic protein contamination that ran at the same size as OmpA. The chance of a cytosolic protein contaminant at exactly 30 kDa and expresses at the same level as our positive control strain's OmpA band is highly unlikely. However, it cannot be completely ruled out as a

TABLE 2 Transformation efficiency of JW0940-6 and BW25113 at different growth temperatures

Strain	Growth Temp (˚C)	Amp-			Amp+			Efficency (10^-4 %)
		cells/plate	Dilution (10^6)	Total cell (10^7)	cells/plate	Dilution	Total cell	
JW0940	42	48	1	4.83	TNTC	1	300*	>6.2
BW25113	42	41	1	4.10	152	1	152	3.7
JW0940	37	64	1	6.43	113.3	1	113	1.8
BW25113	37	63	1	6.33	112.3	1	112	1.8
JW0940	20	111	1	11.1	107	1	107	1
BW25113	20	77	1	7.70	TNTC	1	300*	>3.9

potential explanation. Second, the *omp*A-knockout strain obtained from CGSC does not actually have an *omp*A knockout. The CGSC method of knockout involved the substitution of the targeted gene with a gene encoding kanamycin-resistance through homologous recombination. A subsequent experiment involving growth in kanamycin was performed and the JW0940-6 strain was shown to be kanamycin-resistance while BW25113 was not. However, this does not rule out the possibility of a wild type, non-transformed cell developing kanamycin resistance through other means while keeping the *omp*A gene. Another possibility is that the kanamycin-resistance gene homologously matched to another region of the genome, inserting the kanamycin gene somewhere else. Lastly, the strain could have had a duplication of the *omp*A gene, resulting in one *omp*A gene being replaced by kanamycin resistance and the second one remaining the same.

In the PCR experiment, if the *omp*A gene was not replaced, there would be one band at around 1339 bp. Alternatively, the Δ*omp*A strain will have one band at around 1800 bp. If there was duplication in *omp*A, and one was replaced by the kanamycin resistance gene, then there would be two bands around 1339 and 1800 bp respectively. The agarose gel running the PCR products of both strains show a single band at 1339 bp. This indicated that the JW0940-6 strain we obtained did not have Δ*omp*A. This also eliminates the possibility that there was a duplication of the *omp*A gene and reinforces the idea that the 30 kDa band on the SDS-PAGE gels were most likely OmpA. Through elimination, the most likely explanations for the lack of *omp*A-knockout in the JW0940-6 strain obtained, despite having kanamycin resistance, are either that the kanamycin cassette was recombined into another part of the genome or that a non-transformed cell developed kanamycin resistance through mutation.

Despite the lack of a Δ*omp*A mutant, preliminary tests on transformation efficiency were tested. The competent cells made from cells grown at various temperatures were transformed with pUC19 containing the ampicillin resistance gene. Previous studies have found that both *omp*A expression and transformation efficiency are affected by temperature changes with *omp*A expression decreasing at 47.5 ˚C (6) and transformation efficiency increased when cells were grown at 18˚C (9). OmpA was also found to be negatively correlated with transformation efficiency in liquid media using $CaCl_2$ (4). The expression of *omp*A at a lowered temperature (<37 ˚C) was not been studied. The BW25113 and JW0940-6 strains both had a transformation efficiency around $1.8x10^{-4}$ % at 37˚C and the transformation efficiency increased at 42˚C ($3.7x10^{-4}$ % and $>6.2x10^{-4}$ % respectively). This was as expected due to the decrease of *omp*A expression negatively correlating with transformation efficiency. However, due to the lack of a Δ*omp*A mutant, the significance of OmpA on transformation cannot be confirmed. The SDS-PAGE gel that was ran with outer membrane proteins made from the cells grown at various temperatures also cannot be used quantitatively due to the fact that the bradford assay did not accurately represent the concentration of lipid-bound proteins dissolved in water. However, these preliminary data are consistent with a possible correlation between *omp*A expression and transformation efficiency at 37˚C and 42˚C. A point of interest, however, was the difference in transformation efficiency change between BW25113 and JW0940-6 strains at 20˚C. In BW25113 strain, the transformation efficiency increased at 20˚C relative to the efficiency at 37˚C while the efficiency for JW0940-6 strain decreased. Since the JW0940-6 does not have Δ*omp*A mutation, the only potential difference between the two strains is the replacement of the kanamycin resistance gene into another part of the genome. Since there is a large difference between the two strains with regards to transformation efficiency at 20 ˚C, this could potentially indicate that another gene corresponding to a protein that affects transformation efficiency was disrupted. Studying the location of the kanamycin resistance gene homologously recombined could also yield the identity of another protein, potentially similar to *omp*A, which affects transformation efficiency.

In conclusion, the lack of Δ*omp*A in the JW0940-6 strain obtained was confirmed. Although the preliminary data showed a correlation between the levels of OmpA and the transformation efficiency, we were not able to draw a definite causal relationship

between *omp*A and transformation efficiency due to the absence of a Δ*omp*A.

FUTURE DIRECTIONS

This experiment should be repeated with a confirmed *ompA* because the Δ*omp*A JW0940-6 strain that we used did not have *omp*A deletion as we expected. Thus, this experiment did not provide a clear correlation between the level of OmpA and the transformation efficiency. We have shown that JW0940-6 strain was kanamycin resistant and *omp*A+. To locate the insertion site of kanamycin resistance cassette, PCR amplification of sequences around kanamycin resistance cassette should be done. DNA fragment in 5' direction of kanamycin resistance cassette can be sequenced by designing primers flanking the region from 5' end of the kanamycin resistance cassette to few hundred base pairs in 5' direction of the kanamycin resistance cassette. Random primers will be used for pairing with bacterial DNA at 5' direction of the KanR cassette. After PCR amplification, several bands are expected when the PCR products are run on the gel. Gel extraction of DNA with appropriate size should be used for DNA sequencing. We can blast the sequence against *E coli*. genome. Since *E. coli* had whole genome sequenced, the location of kanamycin resistance cassette in JW0940-6 strain can be located. As a result, this information might be useful for future cloning of mutant strains of *E. coli*. Since we have observed the differences in transformation efficiency between BW25113 and JW0940-6 strains, the unknown deletion gene in JW0940-6 strains should be further investigated.

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