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Transcriptional Regulation of *ompC* by Calcium Chloride May Involve *envZ*

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The standard method of preparing chemically competent *E. coli* involves two steps: (1) resuspension and incubation of cells in ice-cold calcium chloride and (2) short heat-shock at 42ºC. Exactly how DNA crosses the cell wall and enters the cytoplasm during this treatment is not understood. DNA entry may be expedited through lipid permeabilization and pore production in the outer membrane. The channel formed by the porin OmpC has also been proposed as a route of DNA entry. Differential expression of OmpC is regulated by transcription factor OmpR, which becomes phosphorylated by EnvZ in response to environmental changes, such as osmolarity. In high-osmolarity conditions, OmpC production is upregulated, while in low osmolarity, expression is suppressed. Overproduction of OmpC in $CaCl_2$-mediated transformation has previously been reported. Based on this observation, we hypothesized that *ompC* transcription would also increase in response to increasing $CaCl_2$ concentrations. To test this hypothesis, we used RT-qPCR to measure *ompC* transcription in *E. coli* treated with calcium chloride. Results showed a slight increase of *ompC* transcription following exposure to calcium chloride in *E. coli* strain BW 25113. In *ΔenvZ* strain JW 3367-3, *ompC* transcription did not increase following treatment with calcium chloride. This may indicate that the EnvZ-OmpR pathway is involved in the regulation of *ompC* in response to calcium chloride.

Calcium chloride ($CaCl_2$) treatment is a commonly used approach to prepare competent *E. coli* (14). The basic protocol requires the incubation of bacterial cultures in ice-cold 0.1 M $CaCl_2$, followed by a brief heat-shock at 42ºC (11). Although this procedure has been used since 1970, the specific mechanisms and proteins involved in mediating DNA uptake remain unknown. It has been suggested that the process involves the release of lipids and the regulated production of pores on the cell surface to facilitate DNA entry (11,14).

OmpC and OmpF are the two major porin channels in *E. coli* that facilitate the passive diffusion of small hydrophilic molecules across the outer membrane (13). Production of both proteins is mediated by the EnvZ-OmpR two-competent system. EnvZ is a sensory kinase which phosphorylates the cytosolic transcription factor OmpR (4). Phosphorylated OmpR (OmpR-P), upregulates or represses *ompC* and *ompF* transcription in response to environmental factors such as osmolarity, pH, and presence of antibiotics (4). This regulation under different conditions can provide a means of balancing nutritional needs and protection from toxins (12).

In its natural environment, the intestinal tract, the high osmolarity of bile salts requires *E. coli* to minimize osmotic pressure. As a form of defense, OmpC production is favored due to its narrow channel to counteract diffusion across the outer membrane (13). Under these conditions of high-osmolarity, OmpC upregulation is also correlated with high levels of OmpR-P (19). Moreover, OmpC is shown to be required for hyperosmotic adaptation in alkaline medium (17). Conversely, in low-osmolarity conditions, such as lake water, low levels of OmpR-P are associated with OmpC downregulation to favour production of the larger porin channel, OmpF, which can uptake scarce nutrients at the greatest rate of diffusion (9).

Aside from the EnvZ-OmpR system, recent studies have found other proteins with a regulatory role in porin expression. For example, the CpxA-CpxR system responds to extracytoplasmic stress, and Lrp responds to low nutrient conditions (2, 6). Furthermore, small RNAs (sRNAs) have been demonstrated to be involved in post-transcriptional processes to fine-tune outer membrane composition (6). At low temperature and starvation conditions, elevated levels of MicC sRNA bind to *ompC* mRNA and prevent translation, thereby negatively regulating *ompC* expression (5). Due to the diversity of regulatory proteins, the complete mechanism of transcriptional regulation of *ompC* has yet to be elucidated (4).

OmpC is an important facilitator in $CaCl_2$-mediated transformation. In a recent study, OmpC overproduction was observed in $CaCl_2$ -treated *E. coli* cells (1). Compared to its wildtype counterpart, an OmpC mutant strain was almost 40% less efficient in transformation (1). Due to a higher number of charged residues, OmpC is generally less permeable in neutral conditions; however, high salt concentrations enhance OmpC permeability through electrostatic interactions (8). Large numbers of charged residues stabilize the structure within the channel, which slows down solute penetration (8). High salt concentrations thus neutralize and provide shielding from these residues accelerating diffusion across the channel (8).

The aim of this study is to investigate whether OmpC upregulation in response to $CaCl_2$ is regulated through *envZ* at the transcriptional level. We hypothesized that *ompC* transcription would increase under increasing concentrations of $CaCl_2$ and that this induction would require *envZ.* Using RT-qPCR of *E. coli* treated with $CaCl_2$,

we found that there was a positive trend in *ompC* transcription with increasing $CaCl_2$ concentration. An *ΔenvZ* strain exhibited different expression levels than its wildtype counterpart, suggesting that EnvZ plays a role in *ompC* transcription. This study provides preliminary results into fully understanding the role of $CaCl_2$ in DNA uptake through modifying levels of *ompC* transcription.

MATERIALS AND METHODS

Bacterial strains. Three different *E. coli* strains were used: a) BW 25113, as the background strain; b) JW 2203-1, a *ΔompC* mutant in BW 25113 background; and c) JW 3367-3, a *ΔenvZ* mutant in BW 25113 background (Table 1).

TABLE 1 *E. coli* K-12 strain names.

Parent strain	Deletion	Strain name
BW 25113	N/A	BW 25113
BW 25113	Δ*ompC*	JW 2203-1
BW 25113	Δ*envZ*	JW 3367-3

Primer design and selection of target sequence for RT-qPCR. Two genes were chosen for the quantitative reverse transcription PCR (RT-qPCR) in this study. DNA sequences of *ompC* and *gapA* were retrieved from EcoGene. NCBI primer design program was used for the primer design (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer pairs capable of amplifying a DNA fragment of about 100 bp were chosen and ordered from Integrated DNA Technologies. The primers used are listed in Table 2.

TABLE 2 Primers used for RT-qPCR.

Primer		Sequence (5' to 3')
ompC	forward	TGGCATTCGCAGGTCTGAAA
	reverse	AGTACGTCGGTCCAGGAAGT
gapA	forward	GCAAACTGACTGGTATGGCG
	reverse	AGACGAACGGTCAGGTCAAC

Cell culturing and $CaCl_2$ treatment. Cultures of BW 25113, JW 2203-1, and JW 3367-3 were cultivated in lysogeny broth (LB) overnight at 37°C (15). The cells were spun down to remove media, then incubated in $CaCl_2$ concentrations of 0 M, 0.05 M, and 0.1 M. Cells were incubated in $CaCl_2$ for 7 minutes, as this was the duration observed by Aish et al. to have the highest production of OmpC (1).

RNA isolation. Following $CaCl_2$ treatment, total RNA was isolated from cells using a phenol-free, filter-based RNA isolation system, RNAqueous Total RNA Isolation kit (Ambion) according to the manufacturer's protocol for RNA isolation. Total RNA was eluted in 50 µl diethylpyrocarbonate (DEPC)-treated deionized water and stored at −80°C. The RNA concentration and purity were determined by spectrophotometry (Nanodrop 2000, Thermo Scientific).

cDNA synthesis and quantitative PCR. For mRNA expression analysis, cDNA was obtained by reverse transcription of RNA samples using the Invitrogen SuperScript II RT kit according to the manufacturer's instructions. The resulting cDNA was quantified by qPCR using the CFX96 real time PCR instrument (BioRad). Relative gene expression was calculated using CFX Manager™ Software (BioRad) using internal equations. The quantification cycle (C_q) values were determined based on the qPCR cycle number at which fluorescence emission reaches a threshold above baseline emission. Amplification of the expected single qPCR product was confirmed using 3% agarose gels stained with SYBR® Safe DNA gel stain.

RESULTS

***ompC* expression induced by $CaCl_2$ concentrations requires regulation by *envZ*.** To investigate the expression of *ompC* under varying $CaCl_2$ concentrations, *E. coli* strains BW 25113, JW 2203-1, and JW 3367-3 were treated with $CaCl_2$ and then analysed with RT-qPCR. Primer pairs designed to amplify a region of *ompC* and a region of control gene *gapA* were used. *gapA* coding for GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was chosen as an internal positive control since it is involved in a metabolic pathway and is not expected to respond to $CaCl_2$ treatments (16). A summary graph of the mean C_q values is shown in Fig. 1. The C_q values for *ompC* amplification of each sample was normalized by CFX Manager™ software using *gapA* amplification as a reference gene to show relative abundance of *ompC* transcript. As shown in Fig. 2a, the wild type strain BW 25113 showed increasing *ompC* expression with increasing $CaCl_2$ concentrations. From 0.05 M to 0.1 M $CaCl_2$ there was a two-fold increase in *ompC* expression. For the *envZ* mutant strain JW 3367-3, we see no clear trend of *ompC* expression with increasing $CaCl_2$ concentration; in fact, there was high expression of *ompC* with no $CaCl_2$ treatment, and lower expression of *ompC* with $CaCl_2$ treatment (Fig. 2b). As expected, there was very little expression of *ompC* in the *ΔompC* strain JW 2203-1 (Fig. 2c). These results suggest that *ompC* expression is induced by $CaCl_2$, but requires regulation by *envZ*.

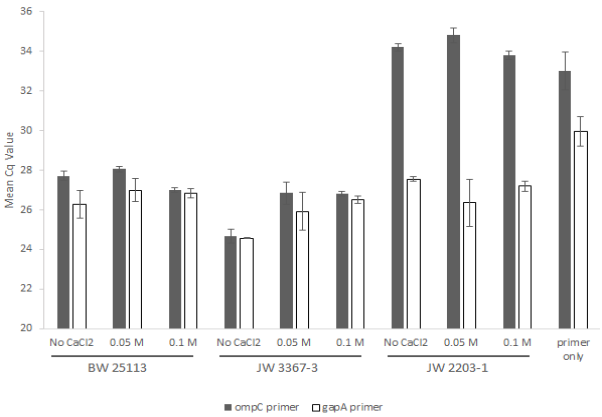

FIG 1 Mean C_q values of RT-qPCR run. Data represent means ± standard deviation between triplicate qPCR wells. For primer only samples, only duplicates were run. Of note is the late amplification of OmpC mutant strain with *ompC* primer, as well as primer only samples. Blank samples containing only qPCR mix and water as well as controls using pre-RT RNA with *ompC* primer were also run, with no amplification observed for any of them.

Gel electrophoresis of RT-qPCR products show expected single band per sample. To assess whether the RT-qPCR product size matched with the expected size from primer design, a 3% agarose gel was run for all RT-qPCR products (Fig. 3). The expected RT-qPCR product sizes for samples with *ompC* and *gapA* primers are 97 bp and 70 bp respectively. The observed RT-qPCR product size roughly matched with the expected, with fragments running at 120 bp and 90 bp for samples amplified with *ompC* and *gapA*

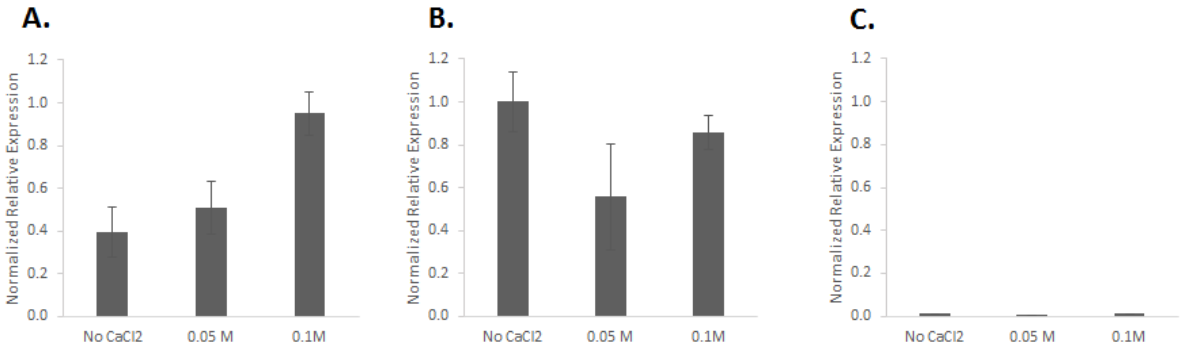

FIG 2 Normalized *ompC* expression level using *gapA* as reference gene. Normalization calculated by CFX Manager™ software using *gapA* amplification data as the reference gene. Data are means ± SEM between triplicate RT-qPCR wells. (a) Wildtype strain BW 25113 showed increasing trend of *ompC* expression with higher $CaCl_2$ concentration. (b) *ompC* expression in *envZ* mutant strain JW 3367-3. (c) o*mpC* mutant strain JW 2203-1 showed very low expression of *ompC* relative to wild type and *envZ* mutant strains.

primers respectively (Fig. 3a, b). A single band was observed for each sample, which indicated the specificity of amplification and confirmed that there was only one product present in each RT-qPCR reaction (Fig. 3).

DISCUSSION

Calcium chloride transformation for competent *E. coli* preparation may expedite DNA uptake through outer membrane pore formation (11). Based on previous studies, it is proposed that transcription of the porin OmpC may increase in response to increasing $CaCl_2$, and that the EnvZ-OmpR pathway may be involved in the regulation of *ompC* in response to $CaCl_2$ (4, 13).

Based on our RT-qPCR result, there is a trend of increasing *ompC* expression with increasing $CaCl_2$ concentration; however, there is little difference in *ompC* expression between no $CaCl_2$ treatment and treatment with 0.05 M $CaCl_2$ (Fig. 2). This suggests that 0.05 M $CaCl_2$ is too low to affect *ompC* expression level. We see a more significant effect on *ompC* expression with higher $CaCl_2$ concentrations closer to 0.1 M, which is the concentration generally used in $CaCl_2$ transformation (3).

To investigate the role of the EnvZ-OmpR two-component system in the signalling and regulation of *ompC* transcription, we also treated an *ΔenvZ* strain, JW 3367-3, with varying $CaCl_2$ concentrations. Based on previous research showing the role of this pathway in osmolarity, we hypothesized that the deletion of *envZ* would inhibit the ability of the cell to monitor changes in osmolarity (4, 7). RT-qPCR results on *E.coli* cells which were: untreated, treated at 0.05 M $CaCl_2$, or treated at 0.1 M $CaCl_2$ show that the levels of *ompC* transcription in JW 3367-3 were different from that of its parental strain BW 25113 (Fig. 2). A trend with increasing $CaCl_2$ was not observed, which may be due to the altered regulation of *ompC* transcription within the cell. This suggests that EnvZ plays a role in the transcriptional regulation of *ompC.*

Gel electrophoresis confirmed the specificity of the *ompC* and *gapA* primers by showing only single band per RT-qPCR. The fragments amplified from both the *ompC* and *gapA* primers showed slightly larger than expected product sizes, with observed sizes of 120 bp and 90 bp compared to expected sizes of 97 bp and 70 bp respectively. The reason for this remains unknown, and one possibility is that the DNA ladder does not resolve well within this range.

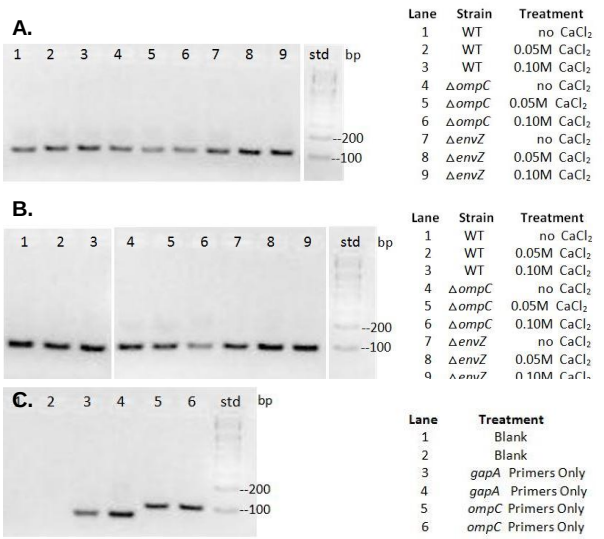

FIG 3 Gel electrophoresis results for RT-qPCR products. Samples A1-A9 showed band size of 120 bp. Samples B1-B9 showed band size of 90 bp. The DNA ladder used was TrackIt 1 kb Plus (Invitrogen). Amplification with (a) *ompC* primers, (b) *gapA* primers and (c) negative controls. Blank lanes are qPCR sample mixes run without cDNA templates and primers. Primers only lanes refer to qPCR sample mixes run without the cDNA templates.

Blanks without primers or cDNA templates showed no amplification during qPCR which indicated that the reagents and equipment were not contaminated. Both the *ompC* and *gapA* negative controls containing primers but lacking template showed late amplification (mean C_q

value ≥ 30). Subsequent gel electrophoresis showed RT-qPCR product size at 120 bp and 90 bp for *ompC* and *gapA* primers respectively, which is similar to that from the cDNA template containing samples (Fig. 3c). Late amplification in the *ompC* mutant samples, *ompC* and *gapA* primers, therefore, may suggest trace contamination of templates due to aerosols during pipetting.

Treating wild type strain BW 25113 with different $CaCl_2$ concentrations showed a general trend of increasing *ompC* expression with increasing $CaCl_2$ concentration. The *ΔenvZ* mutant strain JW 3367-3 showed no clear trend of *ompC* expression level when treated with increasing $CaCl_2$ concentrations. We observed late amplification of primer only negative controls as well as unexpected band size for qPCR products. Both of these issues remained unresolved due to time constraint. Since there is increased *ompC* expression under treatment of higher $CaCl_2$ concentration while the same trend is not seen in the *ΔenvZ* mutant, we conclude that the expression of ompC induced by $CaCl_2$ concentration may be mediated through the EnvZ-OmpR pathway.

FUTURE DIRECTIONS

Since cells from log growth phase are normally used for competent cells preparation, it would be beneficial to isolate RNA samples from log phase cultures instead of overnight cultures for future experiment to get better representation of the cellular physiology. Cells in the log growth phase are more actively dividing compared to those from overnight cultures, thus they would mimic the $CaCl_2$ transformation condition better. This will allow us to more confidently apply the experimental results to be representative of what takes place during the transformation process. A logical continuation of this project would be to include more $CaCl_2$ concentrations for cell treatments to confirm the currently observed positive correlation between increasing *ompC* expression and increasing $CaCl_2$ levels. Finally, the larger than expected RT-qPCR product size should also be checked by designing a new set of primers to test whether primer design is indeed the cause of unexpected product size.

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