Loss of Osmoregulation of OmpC Porin Occurs if *Escherichia coli* are Deficient in cAMP Receptor Protein

Seo Hyung Joo, Haozhong Situ, Jimmy Wang, and Li Zhu
Department Microbiology & Immunology, University of British Columbia

In *Escherichia coli*, the regulator OmpR and EnvZ respond to cyclic AMP and are directly responsible for the regulation of outer membrane porin proteins OmpC and OmpF. We observe that increasing levels of OmpC expression in wildtype strains exposed to increasing osmotic pressure. However, *Δcrp* strains deficient in cyclic AMP receptor protein (CRP) showed significantly lower levels of OmpC and the OmpC protein level remained stable with increased osmotic pressure. This observation is consistent with previously proposed osmoregulation pathway, and confirm the importance of cAMP receptor protein in osmoregulation.

The ability to cope with osmotic stress is a fundamental biological process that protects the organism against fluctuations in solute content of their environment (1). The Cyclic adenosine monophosphate (cAMP) receptor protein (CRP) is a global regulator in bacteria that controls many target genes, including genes that are involved with osmoregulation in *Escherichia coli* (2, 3). The cAMP-CRP complex directly regulates the expression of *ompB* operon, which in turn controls production of the porin proteins OmpC and OmpF in response to changes in environment osmolarity (4). OmpC with a smaller porin size is preferentially synthesized under high osmolarity to limit the amount of solute entering cells (5).

The cAMP-CRP complex directly affects the expression of OmpB operon through both activation and inhibition of multiple OmpB promoters (4). Through this interaction, cAMP-CRP complex controls the levels of OmpR and EnvZ in the cell, both of which are proteins coded for by the *ompB* operon (4). Molecular cAMP binds to the cAMP receptor protein to form a complex that can activate expression of the OmpB operon via protein-protein interaction with the RNA polymerase σ70, increasing expression of EnvZ (6). The transmembrane sensor kinase in the cytoplasmic membrane that detects change in extracellular osmolarity and relays the signal via phosphorylation of the response regulator OmpR, a DNA binding protein (7, 8). When phosphorylated, OmpR is responsible for controlling transcriptional expression of genes involved in the adaptation to changes in osmolarity, including the outer membrane porin proteins OmpC and OmpF (9).

In an environment with high osmolarity, EnvZ efficiently autophosphorylates and transfers its phosphate onto the n-terminus of OmpR, and the large quantity of OmpR-P allows it to bind to low affinity sites in the promoters of *ompF* and *ompC* genes, repressing OmpF expression and activating OmpC expression (9, 10). Under low osmolarity, only low levels of OprR-P is present, and as such OmpR-P only binds to high affinity sites in the *ompF* promoter, resulting in increased expression of OmpF, and decreased expression of OmpC. The differentiation between the expression of OmpF and OmpC under low osmolarity can be attributed to the fact that OmpF has a larger pore diameter relative to OmpC, resulting in an increased rate of diffusion and an increased rate of nutrient take-up, which has a positive selective pressure in a low osmolar environment, (9)

This study investigated the role of the cAMP-CRP complex in the regulation of OmpC expression when under osmotic stress using a mutant strain of *E. coli* that lacks the cAMP receptor protein genes. The decrease in CRP level will result in a deficient production of OmpC.

**MATERIALS AND METHODS**

**Strains and media.** *Escherichia coli* strain BW25113 (regarded as wild type, CGSC #7636), JW2203-4 (*ΔompC* mutant, CGSC #9781), and JW5702-4 (*Δcrp* mutant, CGSC #11596) from the Keio collection (Coli Genetic Stock Centre), LB-lennox media featuring three different additional levels of NaCl concentration: 0.0M, 0.3M, and 0.6M. Each medium was prepared using 1% tryptone (Becton Dickinson #211701), 0.5% yeast extract (DIFCO #210929), and 0.5% NaCl (Fisher Scientific #BP258-1), with additional 0.3M or 0.6M NaCl added.

**Osmotic pressure.** Each of the three strains were incubated in LB-Lennox media overnight. Each overnight culture was then diluted to an OD<sub>600</sub> reading of 0.3, then allowed to grow back up to an OD<sub>600</sub> reading of 0.5 to ensure that each sample is in exponential phase of growth. Exponential phase cultures were centrifuged at 6000 xg for 10 minutes using a Sorvall RC-5B superspeed centrifuge and the Sorvall SLA-1500 rotor. The supernatant was discarded and the pellets were resuspended in LB-Lennox with an additional NaCl supplement, and incubated in the shaking water bath at 37°C bath for 2 hours before cell harvest.

**Outer membrane protein isolation.** Outer membrane proteins were isolated using protocol described by Carson et al (11). Each sample was briefly centrifuged at 10,000 xg for 5 minutes in the Sorvall RC-5B centrifuge with the SLA-1500 rotor, and each pellet was then resuspended in 7 ml Resuspension Buffer 1 (10 mM Tris-HCl pH 8.0, 50 μg/mL Deoxyribonuclease I, 20% sucrose). The resuspended samples were then lysed by French pressing cultures at 10,000 psi, then centrifuged at 2,500 xg for 25 minutes at 10°C to remove cell debris and unbroken cells in the pellet. Each supernatant was then diluted with 0.1M Na<sub>2</sub>CO<sub>3</sub> solution and incubated on ice for 1 hour with occasional swirling. Following incubation, each sample was centrifuged using the Beckman J2-21 centrifuge with a JA-30.50 rotor at 35,000 xg for 20 minutes. The pellets were then washed twice with 10 ml of 10 mM Tris-HCl pH 8.0, and finally resuspended in 1 ml OMP Resuspension Buffer 2 (2% Triton X-100, 10 mM Tris-HCl) and stored at -20°C.
Analysis of outer membrane protein samples. The buffer in each sample was replaced with an SDS replacement buffer (8 mM Tris-HCl, pH 8.0 with 2% SDS) in an effort to remove the Triton X-100 molecules in the original buffer without diluting the protein sample. 500 µl of each sample was loaded into separate Millipore Amicon Ultra-0.5 ml 30K filters (Millipore #C82301), and spun at 14,000 x g for 5 minutes in Eppendorf Centrifuge 5424 to reach 42 µl of concentrated sample. 458 µl of SDS replacement buffer was added to each filter, and centrifugation was repeated to dilute the Triton-X content to less than 1/100. In the end, the filter was inverted and spun at 1,000 xg for 3 minutes to collect the resulting samples, which could subsequently be stored at -20°C.

BCA protein concentration assay. Concentrations of the recovered outer membrane proteins were determined using the bicinchoninic acid (BCA) assay, using a BCA reagent composed of a mix of 1 part 4% cupric sulphate (Sigma #C-7631) and 49 parts bicinchoninic acid (Sigma #B-9643). Bovine serum albumin (Sigma #P-0914) standards were prepared with known concentrations ranging from 31.25 µg/ml to 500 µg/ml beforehand. Then, 10 µl of each sample or their dilutions was mixed with 200 µl of BCA reagent and incubated at 37°C for 45 minutes. After the samples and standards cooled down to room temperature following incubation, the absorbance at 562 nm was measured using Ultraspec 3000 UV/Visible spectrophotometer (Pharmacia Biotech #80-2106-20).

SDS-PAGE analysis of outer membrane proteins. The outer membrane proteins of each strain was separated based on molecular weight (12). 12% separating gels (2 ml 1.5 M Tris pH 8.8, 2.6 ml dH2O, 5.2 ml 30% acrylamide, 80 µl 10% Ammonium Persulfate, 80 µl 1% SDS, 6 µl TEMED) and 6% stacking gels (1.25 ml 0.5M Tris pH 6.8, 2.6 ml dH2O, 1 ml 30% acrylamide, 50 µl 10% Ammonium Persulfate, 50 µl 1% SDS, 5 µl TEMED) were used. 2.75 µg of each protein sample was mixed with 2× SDS sample loading buffer (50 mM Tris-HCl (Sigma #096K5405) pH 6.8, 10% glycerol, 2% SDS, 0.05% DTT (Sigma # 3483-12-3), 0.1% bromophenol blue (Sigma #B-8026)) at 1:1 (v/v) ratio, and run at 170 volts for 60 minutes. The protein bands were then visualized with Coomassie Blue staining as described by Borejdo and Flynn in order to compare their band intensity (13).

Quantification of protein content in stained gels. Digital photos were taken from each gel, and analyzed using the Image J version 1.440 program as described by Miller to quantify the intensity of the bands (14). The intensity of each band was calculated by the program and subsequently compensated for the background. We subtracted each band intensity value with the background in the ΔompC control, and divided each result by that of the 0M WT sample to obtain the relative band intensities.

RESULTS

Outer membrane proteins expression. Identification of the 3 dominant outer membrane proteins (OmpA, OmpC, OmpF) was based on their molecular weight as well as comparison with the knockout mutant (Fig. 1). Overall, the lanes displayed comparable band intensity (Fig. 1). The OmpC bands in the Δcrp strains appeared fainter, even though the protein loaded to each lane was the same. Concentration in each sample and normalized accordingly, the total amount of proteins in each lane was supposed to be roughly equal. As a matter of fact, a supporting observation was that the uppermost band had a comparable intensity across most lanes.

In Lane 8 (ΔompC) (Fig. 1), a clear loss of intensity in the lower band near the 35 kDa protein ladder was observed, this comparison of intensity as well as the relative size indicated the location of the OmpC band in the gel. It is noticeable that the ΔompC control was not completely colourless in the corresponding area, and actually had a relatively faint band. This fainter band was actually expected, and was likely to be a background due to some less abundant outer membrane proteins in E. coli. OmpD (38 kDa) and PhoE (36 kDa) might contribute to this background since they both have a similar molecular weight as OmpC, but there are also other candidates (15).

An increase of OmpC band intensity along with osmotic pressure was observed in wild type samples (Fig. 1, Lane 2, 3, and 4). It was noticeable that the next three bands observed in Lane 5, 6, and 7 had obviously lower intensity compared to the previous bands, indicating a lower OmpC protein level in Δcrp strains. Intensity difference among the three bands were also observable. Lane 7 had the
Data were collected from gel photos with Image J program. Figure shows mean relative band intensity ± 2 SD. Data shown is representative of one of two independent experiments. The salt concentration in all incubation from tryptone without addition of extra NaCl was calculated to be 0.08 M. Highest intensity, and Lane 6 seemed to have the lowest, however, the difference was less obvious than the wild type samples.

**Image J analysis of (relative) band intensity.** Band intensity from Image J analysis in (Fig. 2) were compatible with the preliminary observations. The trend in the result illustrated that wild type significantly upregulated OmpC under increasing osmotic pressure. The relative intensity of OmpC increased by 70% when 0.3 M NaCl was supplemented, and 120% when 0.6 M NaCl was supplemented. The Δcrp strain, on the other hand, did not display a similar trend. While the mean intensity of the +0.6 M sample was higher than that of the +0 M one, the 95% confidence interval of these values slightly overlapped, indicating the observed difference was insignificant and might not be genuine. It was also noticeable that OmpC expression level in Δcrp strains was generally lower when compared to wild type, roughly 20-35% lower than that of the wild type +0 M sample.

**DISCUSSION**

The identification of these 3 bands was also supported by previous publications (15, 16). Although the OmpA band seemed to be slightly fainter in the Δcrp lanes, it was reasonable since Δcrp E. coli strains were reported to express a lower level of OmpA compared wild type strains (17).

In previous studies, Scott and Harwood demonstrated that in some E. coli Δcrp strains, there is a significant reduction in OmpC protein levels relative to that of the wild type strains (18). In a later study, the cAMP-CRP complex was found to directly regulate the ompB promoter region in the expression of EnvZ and OmpR proteins (4). The sensor kinase EnvZ then phosphorylates the response regulator OmpR under high osmolarity, followed by the binding of OmpR-P to the promoter of porin proteins and the activation of expression of OmpC (9). In other words, CRP was found to indirectly regulate the expression of OmpC proteins. Our results were consistent with the outcome of the described pathway. For the wild type strain, an increased level of OmpC expression was displayed when high osmolarity was detected (Fig. 2) As the OmpC protein forms a porin with a smaller pore diameter than the OmpF protein, it is beneficial to the bacteria to upregulate the former when under high osmotic stress (9). Furthermore, a significantly lower level of OmpC was observed in the Δcrp strain when compared to a wild type strain (Fig. 2). The absence of cAMP-CRP regulation on OmpC expression as described by Huang et al. was likely to be responsible for this observation. In addition, under increased osmotic pressure, the Δcrp strain showed almost no increase in the level of OmpC. This phenomenon could also be explained by the lack of cAMP-CRP regulation. In the absence of a cAMP receptor protein, no cAMP-CRP complex is present to activate the ompB operon which is responsible for inducing OmpC expression under high osmolarity (4). As a result, there would be low levels of EnvZ and OmpR in the cell such that very little phosphorylation of these proteins can occur, causing the inability of the cell to stimulate ompC expression via the pathway described by Huang et al (4).

However, our data also showed that although the levels of ompC expression in Δcrp strains at all three salt concentrations were relatively low, they were not zero. This suggested a baseline level of ompC expression that was not subject to regulation by the cAMP-CRP system. This phenomenon could be explained by acetyl phosphate, a phosphodonor produced by cells as a metabolic intermediate, which was suggested to account for all EnvZ-independent OmpR phosphorylation reported to date (19). Acetyl phosphate could also be converted from acetyl coenzyme A through the enzyme phosphohydroxycetase (Pta) (20, 21). In fact, acetyl phosphate has been demonstrated to have the ability to phosphorylate OmpR directly both in vitro and in vivo (20). In addition, it has been proposed that acetyl phosphate can act as a signal for changes in porin content by providing a baseline level of phosphorylated response regulator protein, which can help facilitate a faster response upon a shift to the inducing conditions (22). The acetyl phosphate mechanism described above could provide enough phosphorylated OmpR to ensure expression of OmpC even in Δcrp strains that lack the cAMP-CRP complex.

The amount of samples loaded into each well was normalized according to the results of the BCA assay, this kind of adjustment has the potential to cause problems. For example, if the average amount of outer membrane proteins in the cells of a specific sample was a half of the others, then the amount of each protein would be doubled during this normalization step, which would in turn affect the results of our measurement. In this experiment, there was evidence of this kind of error: although the other samples generally had similar amounts of protein according to the relative band intensities (Fig. 1), the upper band of the ΔompC control had a significantly higher intensity than the other lanes. Image J analysis indicated that this band was around 30% denser than the
corresponding bands in other lanes. If the normalization error described above caused this exaggeration, an adjustment in the reading for ΔompC background may be necessary. When the adjustments were applied accordingly, relative band intensities for the +0.0 M, +0.3 M, and +0.6 M Δcrp samples became 0.73, 0.69, and 0.86, respectively. While the trends were similar to the trends in the non-adjusted values, the difference between Δcrp samples and wild type samples was lower, although they were still significantly different from a statistical point of view. On the other hand, although the OmpA band seemed to be slightly fainter in the Δcrp lanes, it was reasonable since Δcrp E. coli strains were reported to express a lower level of OmpA compared to wild type strains (15).

It is unfortunate that similar observations were found in other gels, and thus a more reliable source of data is unavailable. A possible consequence of this observation is that although the band appears to be narrower, protein was not lost from the lane, and thus it is likely that there is an exaggerated intensity in the band, as proteins were concentrated in a smaller area. This could cause an overestimation of the level of OmpC from the ImageJ analysis. However, even though the 0.0M wild type sample may be compromised due to this experimental error, a major observation concluded from this study remains unchanged. That is, there is still a significant osmotic pressure induced increase in OmpC levels in wild type strains relative to the low level of change in OmpC levels of the Δcrp samples. On the other hand, since we do not have an accurate measurement of the OmpC concentration in the 0.0M wild type sample, we cannot definitively conclude that the OmpC levels in the 0.0M Δcrp sample was in fact lower relative to the wild type sample.

This study has shown a decrease in the expression of OmpC protein in Δcrp E. coli strain compared to the wild type strain, and suggested the presence of a baseline level of OmpC which is not subject to osmoregulation.

**FUTURE DIRECTIONS**

To solve the issue with sample normalization, the gels should include a protein with a highly stable level of expression as a standard. Briefly, if that specific band has equivalent intensity among different lanes, it would mean that the samples loaded in each lane correspond to a very similar number of cells. Although the expression of OmpA was reported to be stable under different osmolarity, Δcrp strains of E. coli were found to have lower levels of ompA gene expression, hence OmpA is not suitable as a standard (15). The remaining major outer membrane protein candidate, OmpF, is also not suitable since its level would change according to osmotic pressure (4). Therefore, a solution may be to avoid isolating outer membrane proteins only, instead we could use whole membranes so there is a broader selection of potential membrane proteins.

To further investigate the effect of cAMP, the correlation between the level of cAMP-CRP and the level of OmpR-P should be studied. In this way, we can better understand how OmpR-P level would change with cAMP-CRP level. Importantly, it would also allow us to confirm whether there is a baseline level of OmpR-P in the absence of cAMP-CRP, which is supposed to account for the baseline level of OmpC in the Δcrp strains observed in this experiment.

Considering the important role of ΔompC strain in the experiment, it is essential that we interpret the control band correctly. Therefore, there is a need to confirm the validity of the mutant, and identification of the protein(s) in the control band would also be meaningful.

**ACKNOWLEDGEMENTS**

This project was supported by the Department of Microbiology and Immunology of University of British Columbia. We would like to thank Dr. William D. Ramey and Richard Allen White III for their advice and support during execution of the study. We would like to express our gratitude to Patrick Taylor for spending his time in the guidance for French Press technique as well as the staffs of the Westbrook media room for their patient support.

**REFERENCES**
