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Development of a System to Monitor *omp*C Transcription in *Escherichia coli* Using a Green Fluorescence Protein Reporter System

Angie Hui, Grace Lai, Jaclyn Lam, Fiona Wong
Department of Microbiology and Immunology, University of British Columbia

Transient resistance to antibiotics is believed to be achieved through regulation of outer membrane proteins like OmpC. OmpC is upregulated in response to sublethal doses of the aminoglycoside kanamycin. The ompC promoter is regulated by the CpxA-CpxR system; here, we examine the necessity of CpxR regulation in OmpC expression following treatment with sublethal doses of streptomycin. Our study assessed ompC gene and protein expression in Escherichia coli wild type and cpxR mutant strains following 1 hour treatments with sublethal streptomycin, 3.5% NaCl or no treatment. To measure ompC expression, we used a reporter plasmid in which the ompC promoter was fused to the gene encoding green fluorescent protein (GFP). We intended to monitor ompC expression levels by measuring luminescence in treated and untreated cultures. Our results suggest that neither CpxR nor sublethal streptomycin treatment has an effect on expression of ompC. Since no difference in luminescence was observed between the three treatment groups for wild type and cpxR mutant E. coli, further optimization of the GFP reporter system is required.

There has been an increase in antibiotic resistant bacteria, partly due to the use of low antibiotic doses and the unnecessary prescription of antibiotic treatments (1). Despite demand for new antibiotics and knowledge of the specific cellular targets of antibiotics, little is known about how antibiotics cross the outer membrane (2). Equally important is the need to better elucidate the bacterial mechanisms that confer such resistance. Bacterial adaptive resistance to antibiotics may involve regulating pore sizes in outer membrane porins to reduce the passage of antibiotics from the environment into the cell (3). In particular, Escherichia coli can alter the proportions of OmpC and OmpF porins in its outer membrane (4, 5, 6). OmpC has a smaller pore diameter of 1.08 nm while OmpF has a larger pore with a diameter of 1.16 nm (7). In this case, antibiotic resistance may involve the increased expression of ompC in relation to ompF such that antibiotics which could easily pass through the OmpF porin channels, but not OmpC, are hindered by increased proportions of OmpC (8, 9).

E. coli employs a two-component system to detect environmental stress and to regulate itself accordingly (10). There are various mechanisms for regulating the expression of OmpC and OmpF porins, so that E. coli is able to develop transient antibiotic resistance following exposure to sublethal doses (9). As a result, an aminoglycoside antibiotic, such as kanamycin, could enter the cell through these porin channels more slowly. The EnvZ-OmpR system was deemed to play a non-essential role in kanamycin-induced porin regulation (11). However, the CpxA-CpxR system is involved in porin regulation and may be essential in resisting aminoglycoside antibiotics (12). This system responds to cell envelope stress, such as protein distress, heat shock, oxidative stress, and high pH (13). When this system is activated, CpxA phosphorylates CpxR, which binds upstream of ompC and ompF promoters to increase OmpC and decrease OmpF expression (14). The total amount of OmpF and OmpC porins stays consistent in the E. coli membrane, with only the proportional levels changing in response to the environment (15).

Our study investigated the use of a green fluorescence protein (GFP) reporter system to determine whether CpxR was necessary for the upregulation of OmpC expression following treatment with sublethal doses of the aminoglycoside streptomycin. By transforming E. coli with the ompC promoter-fused GFP plasmid, transcriptional activity on the ompC promoter will result in production of the GFP reporter. The fluorescent GFP can be measured as an indicator of gene expression levels. While the CpxA-CpxR system has been shown to regulate OmpC in response to envelope stress and changes in osmolarity (14), it is unclear whether this system is involved in regulation of OmpC in response to aminoglycosides. Since EnvZ was previously determined to be non-essential to kanamycin-induced porin regulation (11), and the CpxA-CpxR system was shown to contribute to resistance against aminoglycoside antibiotics (12), we hypothesized that CpxR is necessary for upregulation of OmpC and downregulation of OmpF porin proteins following treatment with sublethal streptomycin. Our preliminary results suggest that sublethal streptomycin (1 hour exposure) and CpxR may not be necessary for regulation of OmpC in E. coli. However, further work must be done to optimize the ompC promoter-fused GFP reporter system to detect statistically significant differences in gene expression levels.

MATERIALS AND METHODS

Bacterial strains and plasmids. E. coli JW3883-1 (cpxR mutant) and E. coli BW25113 (parental, wild type) were obtained from the MICB 421 bacterial strain collection at the University of British Columbia. The ompC promoter-fused GFP reporter plasmid was kindly provided by Ivan Villanueva, from the Davies Lab at the University of British Columbia, and contains a kanamycin resistance gene for selection. All strains were cultured in lysogeny broth (LB) in shaking flasks at 37oC prior to use in experiments.

Streptomycin minimum inhibitory concentration assay. Wild type and cpxR mutant strains were cultured in 50 mL LB and grown to log

phase (0.2 to 0.4 at OD600). Concentrations of streptomycin from 0 to 10 µg/mL were tested in a 96-well plate. 5 µL of 10-4 diluted cells were inoculated into each well. After incubating overnight at 37oC, the presence or absence of turbidity in each well was visually assessed. The sublethal streptomycin concentration was taken to be 1/4 of the averaged minimum inhibitory concentrations.

Isolation of ompC promoter-fused GFP reporter plasmid. E.coli K-12 MG1655 cells harboring the ompC promoter-fused GFP reporter plasmid were inoculated in 10 mL LB with 50 µg/mL kanamycin and grown overnight. Extraction of ompC promoter-fused GFP reporter plasmid DNA was performed using Thermo Scientific's PureLink Quick Plasmid Miniprep Kit. The concentration of the reporter plasmid was measured using the NanoDrop 2000C (Thermo Scientific).

Preparation of competent cells and transformation. Competent wild type and cpxR mutant cells were prepared via the calcium chloride method. Isolated colonies of each strain were inoculated in 50 mL LB and grown to log phase. Cells were incubated on ice for 10 minutes and centrifuged at 10,000 rpm for 5 minutes using the Avanti J30-I Centrifuge, JA-14 rotor (Beckman Coulter). The supernatant was removed and pellets were resuspended in 25 mL of ice cold CaCl2 (0.1M). Cells were incubated on ice for 20 minutes and centrifuged again at 10,000 rpm for 5 minutes to pellet cells. After removal of supernatant, pellets were suspended in 1 mL freezing media (0.1 M CaCl2 with 15% glycerol) and incubated at 4oC overnight. Competent E. coli wild type and cpxR mutant cells were transformed with the ompC promoter-fused GFP reporter plasmid via the heat shock method. 0.1 mL of competent cells of each strain were transformed with 1 ng of reporter plasmid. Cells were incubated on ice for 30 minutes and heat shocked in a 42oC water bath for 60 seconds followed by incubation on ice for 1 minute. 1 mL of LB was added to each transformation reaction and cells were incubated in shaking incubator at 37oC for 1 hour. 0.1 mL of transformed cells were plated on LB agar plates containing 50 µg/mL kanamycin and incubated overnight at 37oC.

Treatment of wild type and cpxR mutant E. coli with sublethal streptomycin or 3.5% NaCl. Transformed wild type and cpxR mutant cells were inoculated in 5 mL containing 50 µg/mL kanamycin and grown until log phase. Three sets of 1 mL cells were aliquoted into Eppendorf tubes for each strain. Cells were spun at 13,000 rpm for 5 minutes using the Eppendorf 5415D centrifuge. Cell pellets were resuspended in 1 mL LB with 50 µg/mL kanamycin (negative control), 1 mL LB with 50 µg/mL kanamycin and 3.5% NaCl (positive control) or 1 mL LB with 50 µg/mL kanamycin and 0.313 µg/mL streptomycin (sublethal streptomycin treatment) and incubated at 37oC for 1 hour. High salt is known to upregulate OmpC via a different pathway and thus acts as a positive control. Kanamycin was included in all treatments to apply selective pressure for cells to maintain the reporter plasmid. 200 µL of cells were plated in duplicate onto a flat bottom 96-well fluorescence plate (Corning Costar®). Fluorescence and OD600 were measured using Varioskan (SkanIt Software with excitation wavelength of 485 nm and emission wavelength of 535 nm).

Cell lysis. Following treatment of wild type and cpxR mutant E. coli, the cultures were centrifuged at 10,000 rpm for 5 minutes at 4°C using the JA-20 rotor on the Avanti J-30I (Beckman Coulter) centrifuge. For each treatment group, cells were resuspended in 6 mL LB and aliquoted into 4 FastPrep tubes. A truncated PCR tube was used to transfer approximately 50 µL of 500 µm diameter glass beads into each tube for lysis using the mP FastPrep 24 (MP Biomedicals) bead beater. Cells were lysed in three 1 minute cycles at 5.0 M/S, with a rest period of 5 minutes between each cycle. Following lysis, the FastPrep tubes were spun using the benchtop centrifuge at 13,200 rpm for 5 minutes. The glass beads and cell debris were spun down into a pellet and the supernatant was collected as the total protein sample.

Isolation of membrane protein. The membrane protein fraction was isolated using an adapted protocol based on methods described by Carson et al. (16) and Joo et al. (17). The protein samples were fractionated via ultracentrifugation at 30,000 rpm for 25 minutes at 4°C with the JA-30.50 rotor on the Avanti J-30I centrifuge. The cytosolic protein fraction in the supernatant was separated from the membrane fraction in the pellet. The membrane proteins were resuspended in 2 mL of 2% Triton X-100 (Bio-Rad) in 10 mM Tris, pH 8.0 and incubated for 30 minutes at room temperature for solubilization. Spin-X 10k MWCO Concentrators (Corning) were used to dilute out the Triton-X by concentrating the sample volume to 100 µL and washing 3 times with 10 mM Tris pH 8.0. Proteins were continuously added to the concentrator unit at 4°C and spun down at 13,200 rpm for either 20 minutes or until all samples were concentrated. Following the last wash, the concentrator unit was topped up with 2% SDS in 10 mM Tris pH 8.0 and spun down again. The bicinchoninic acid (BCA) assay (G-Biosciences) was used to quantify the concentration of total protein.

SDS-PAGE. Protein samples were prepared for loading onto a 12% polyacrylamide gel for separation based on relative molecular weight. The gel consisted of a 4% stacking gel (3 mL 30% acrylamide/bis-acrylamide, 75 µL 10% SDS, 1.8 mL 1.5M Tris, pH 8.8, 2.5 mL distilled water, 37.5 µL ammonium persulfate (APS) and 3.75 µL TEMED) on top of a 12% resolving gel (1 mL 30% acrylamide/bis-acrylamide, 75 µL 10% SDS, 1.9 mL 0.5M Tris, pH 6.8, 4.5 mL distilled water, 37.5 µL APS and 7.5 µL TEMED). The gel was polymerized in 0.75 mm glass plates with a 10-well comb. Calculations for each sample were performed to load 0.5 µg of total protein per lane in 30 µL sample volumes. Samples were prepared in 2x SDS-PAGE Laemmli sample buffer (25% glycerol, 2% SDS, bromophenol blue in 0.5M Tris. pH 6.8). Prior to loading, 5% β-mercaptoethanol was added and the samples were heated for 5 minutes at 90°C. Samples were loaded alongside 10 µL of prestained low-range SDS-PAGE standard (Bio-Rad). The gel was run in an electrophoresis cell containing tris/glycine/SDS running buffer; the power supply was set to 200V and ran for approximately 60 minutes or until the protein front reached the bottom of the gel.

Protein staining and imageJ gel analysis. The polyacrylamide gels were stained with Coomassie Brilliant Blue R-250. The dish was microwaved at full power for 1 minute and left on a rotary shaker at room temperature for 15 minutes. The gel was then rinsed with distilled water and placed in destain solution (10% ethanol, 7.5% acetic acid) over three days at 4°C. Images of the stained gel were processed using ImageJ software to assess the relative intensity of each band.

RESULTS

Assessment of OmpC expression in membrane protein using SDS-PAGE and imageJ analysis. The initial approach to assess OmpC regulation of E. coli wild type and cpxR mutant stains in untreated (no treatment as the negative control) and treated (1 hour incubation with either sublethal streptomycin or 3.5% NaCl as the positive control) conditions, was to use densitometry to compare OmpC band intensities between strains and conditions. The experimental method called for enrichment of membrane proteins in sufficient quantity and purity to be analyzed using SDS-PAGE. In a preliminary experiment, the membrane protein fraction was purified using ultracentrifugation and concentrated using size filtration. However, early observations noted issues with achieving ultracentrifugation speeds on the available centrifuges; in addition, other technical limitations made it difficult to attain sufficient quantities of membrane protein for subsequent quantification with a BCA assay and analysis via SDS-PAGE. An E. coli ΔompC (JW2203) was used as a control to verify the location of the OmpC band based on size (at 38.3kDa as indicated by a protein ladder) but further confirmation of the identity and purity of the assumed OmpC

band would have been required (Fig. S2). The gels (Figs. S1 and S2) showed high variability in amount of total protein loaded making it difficult to accurately conclude any reliable trends or observations between conditions or strains. We attempted to probe for trends between the OmpC bands using ImageJ densitometry analysis with relative expression but noted difficulties in making assumptions and identifying other proteins (Figs. S3 and S4). Therefore, protein assessment of OmpC regulation was not pursued and the project was re-focused to develop a gene assessment method for ompC using a GFP reporter assay system to detect ompC transcriptional activity.

One hour treatments of sublethal streptomycin and 3.5% salt may not induce OmpC regulation. Using an ompC promoter-fused GFP reporter construct, we measured ompC expression via fluorescence readings to determine whether treatment with sublethal levels of streptomycin leads to OmpC regulation. Results from the streptomycin minimum inhibitory concentration assays for the E. coli wild type and cpxR mutant strains determined the MIC to be 1.0 µg/mL and 1.5 µg/mL respectively. In this study, the sublethal dose is defined as ¼ of the average MIC, so the sublethal dose of streptomycin used in treatments was 0.31 µg/mL.

The GFP reporter was developed by fusion of the ompC promoter region to the gfpmut2 reporter gene in a low copy-number plasmid of pUA66 origin containing a kanamycin resistance gene (18). E. coli wild type and cpxR mutant transformants carrying this GFP reporter will fluoresce upon ompC promoter activity, potentially providing a high-resolution and accurate method for assaying ompC expression. Figure 2 shows the average of five trials with duplicates for each condition performed in each trial. The average fluorescence units for the wild type and cpxR mutant strains subjected to no treatment (negative control), or treated with 3.5% NaCl (positive control) or sublethal streptomycin were 49.4, 45.4, 53.3 and 41.0, 52.9, 44.5 respectively for each strain and condition (Fig. 1). However, major inconsistencies between each trial resulted in high standard deviations that made the differences in average fluorescence units for each strain or condition non-significant. It is important to note that the 3.5% NaCl positive control condition did not show the expected upregulation of ompC in either the wild type or cpxR mutant strains.

To account for variation in overall level of fluorescence between each trial, the fluorescence differences between induced (treated with 3.5% NaCl positive control or sublethal streptomycin) and uninduced (no treatment negative control) conditions in each trial were assessed (Fig. S5). However, analysis showed average fluorescence differences of the wild type strain treated with the positive control or sublethal streptomycin to be 3.93 ± 13.82 and -3.99 ± 14.01 respectively compared to the untreated condition (Fig. S5). The cpxR mutant showed treated conditions to have fluorescence differences of 3.41 ± 14.61 and 11.85 ± 11.99 in the positive control and sublethal streptomycin treatments compared to untreated (Fig. S5). As with the absolute average fluorescence values (Fig. 1), the average differences in fluorescence values of treated or untreated conditions in each trial for both E. coli wild type and cpxR mutant strains showed large standard deviations.

The large standard deviations represent major inconsistencies in overall fluorescence between trials as well as in each condition. As the 3.5% NaCl positive control did not show upregulation of ompC, this may suggest that the one hour treatment length was not sufficient for significant differences or

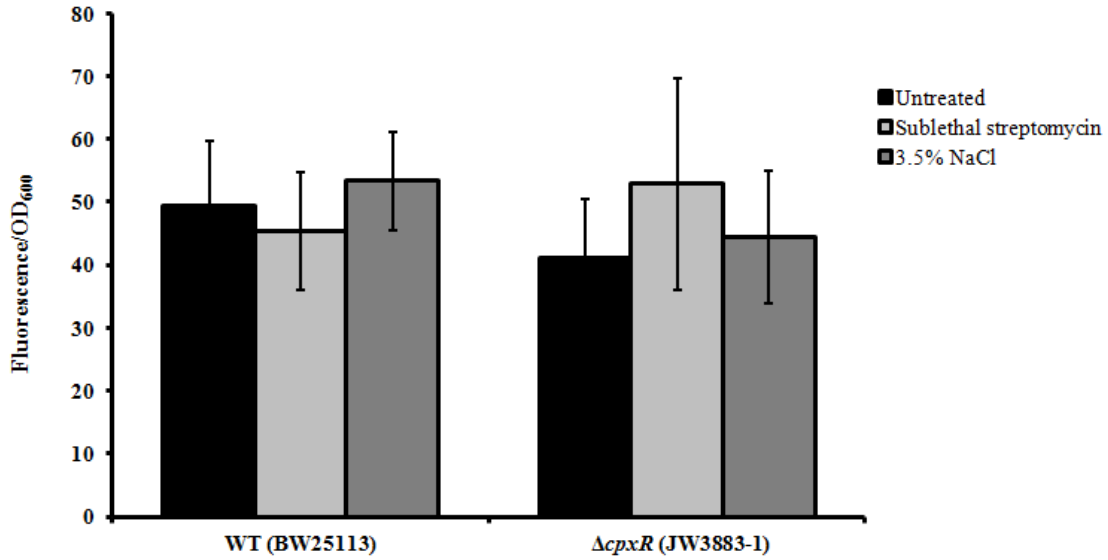

FIG 1. Normalized fluorescence of *E. coli* transformed with an *ompC* promoter-fused GFP reporter construct following treatment with sublethal doses of streptomycin or high salt. The error bars represent the standard deviation between five experimental trials (each condition in the trial was measured in duplicate).

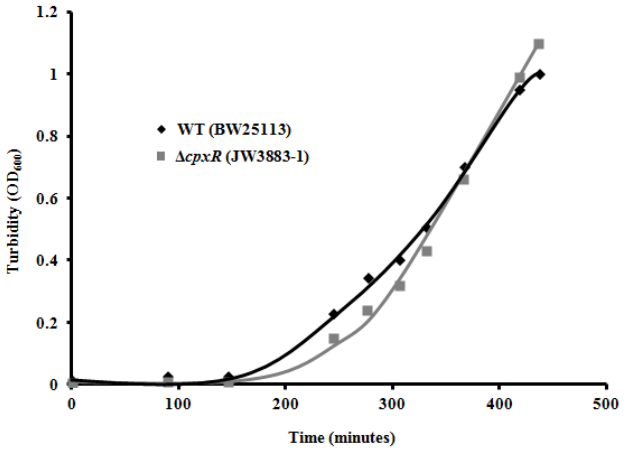

FIG 2. Growth curves constructed from OD_{600} measurements of 5 mL *E. coli* BW25113 and JW3883-1 transformants carrying the *ompC* promoter-fused GFP plasmid. Cultures were grown in LB supplemented with 50 µg/mL kanamycin.

that neither sublethal streptomycin nor 3.5% NaCl regulates ompC expression. However, since previous literature (17) has shown NaCl to be a positive regulator of ompC, it is more likely that the treatment length was insufficient for significant ompC regulation. Since no significant regulation of ompC was observed in either the absolute fluorescence units or in the difference in fluorescence units of treated compared to untreated conditions, we cannot definitively make conclusions but suggest that one hour treatments of sublethal streptomycin or 3.5% NaCl do not regulate ompC expression in either the wild type or cpxR mutant E. coli strains.

Growth rates do not differ between wild type and cpxR mutant E. coli. To assess for differences in growth rate between E. coli wild type and cpxR mutant strains, the cells were monitored via OD600 spectroscopy measurements and grown in liquid media conditions (LB containing 50 µg/mL of kanamycin as selective pressure for the GFP reporter plasmid). The E. coli cpxR mutant strain contains a non-functional CpxA-CpxR response system which may inherently subject the cells to be more stressed resulting in deleterious effects on growth. Since DNA replication and gene expression are critical components of the growth of E. coli, any significant differences in growth between the two E. coli strains may translate to differences in expression of genes like ompC. A growth curve experiment was simultaneously performed for each strain with one OD600 measurement at each time point. Although the cpxR mutant had a slightly longer lag phase (i.e., slower initial growth rate), both the wild type and cpxR mutant E. coli strains grew at overall relatively similar rates (Fig. 2). The growth curve results indicate that there are no significant growth differences between cpxR mutant and wild type E. coli.

DISCUSSION

In an attempt to evaluate the expression levels of ompC after exposure to sublethal doses of streptomycin, we evaluated ompC via protein and transcription levels. However, OmpC protein expression studies were difficult as yield was low and identification of OmpC was difficult. Although we used the BCA assay to normalize the amount of total protein loaded into each lane, there appeared to be varying amounts of protein in each lane (Fig. S1). With inconsistent amounts of protein loaded in each lane, it was not possible to determine whether OmpC levels increased or decreased in each condition. Hence OmpC protein studies did not provide further understanding regarding the expression of ompC after treatment with sublethal doses of streptomycin.

We have developed a system using a GFP reporter to evaluate ompC transcription in E. coli. The transformation of the GFP promoter-fused ompC plasmid of competent wild type and CpxR mutant E. coli allowed us to monitor ompC expression under various conditions. When ompC is expressed, GFP is produced. The level of fluorescence therefore indicates the amount of ompC expression. However, the ompC-GFP reporter system produced inconclusive results. No conclusion can be drawn as the standard deviations of the positive control (3.5% NaCl) and negative control (untreated) overlap considerably for both the wild type and cpxR mutant E. coli (Fig. 1).

In a study conducted by Roberto et al. (19), they evaluated a GFP reporter gene construct for detecting arsenic and other heavy metal contaminants in the environment. They constructed an arsenic-specific reporter gene construct in which the arsenic-binding regulatory protein gene arsR and its promoter were fused to GFP. When expressed in recombinant E.coli, the GFP reporter creates GFP in response to arsenic ion exposure (19). The normalized fluorescence intensity of cells induced with arsenite increased with increasing concentrations of arsenite. The fluorescence of uninduced cells was 0 units while fluorescence levels of up to 166 units were observed in cells induced with high concentrations of arsenite (19). The magnitude of difference in GFP fluorescence before and after induction was much greater compared to our GFP system which produced no significant differences in fluorescence before and after induction. Another study used an ompC-GFP reporter plasmid to determine ompC expression levels in E. coli after induction with malate (20). In this study, normalized fluorescence levels increased over 8 hours and with increasing concentrations of malate. The magnitude of increase in fluorescence at different time points ranged from approximately 2.5 fold to 5 fold, depending on the concentration of malate (20). These studies indicate that the GFP reporter is capable of showing significant differences in gene expression, but the sensitivity of the system appears to depend partly on the length and amount of induction as well as the specific promoter fused to the GFP reporter gene.

The level of fluorescence between each treatment group was similar (Fig. 1), suggesting that the assay was not sensitive enough to show substantial differences among the treatments. The design of our experiment was most likely the cause of this

effect. The treatment time of 1 hour was assumed to be sufficient for cells to show substantial differences in regulation. However, this may not be the case. Longer incubation periods may be required to observe a greater difference in ompC expression between treatment groups. The study conducted by Roberto et al. (19) also notes differences in GFP expression following different incubation times with arsenite. They concluded that although GFP production was present 6 hours after exposure, steady state fluorescence occurs between 10 hours and 12 hours after exposure of arsenite.

The ompC-GFP reporter plasmid has not been analyzed to determine whether it is a "true" ompC promoter-fused GFP reporter plasmid and poses an experimental limitation to this study. It is crucial that GFP is only produced when ompC is expressed. In order to confirm this, the reporter plasmid should be sequenced. Additional methods can include polymerase chain reaction using primers that amplify the ompC promoter and gel electrophoresis. Another experimental limitation is that the plasmid copy numbers were not controlled for. To ensure that the GFP reporter is being expressed, the fluorescence of cells transformed with the GFP reporter plasmid should be compared with the fluorescence of cells containing an empty vector. If the GFP reporter is actively expressing GFP, there should be a significantly higher level of fluorescence in cells containing the reporter plasmid compared to the empty vector control.

The differences in growth patterns between the wild type and cpxR mutant E. coli can affect results. After cells have been transformed with the plasmid, the plasmid replicates to a level at which its copy number is regulated by its growth rate, cell replication and cell division. If high numbers of plasmid are present, a high fluorescence signal will be detected. Thus it is important to compare the growth patterns of the two strains to verify that the cell growth rate is not producing variation in plasmid copy numbers. Both E. coli strains must contain similar amounts of reporter plasmid in order to accurately compare ompC expression levels via GFP luminescence. The growth patterns of both strains were similar to typical bacterial growth curves. The wild type strain reached log phase slightly earlier than the cpxR mutant strain (Fig. 2). At approximately 450 minutes, the wild type strain appeared to be reaching the plateau stage (Fig. 2). Due to time constraints, additional readings were not obtained after 450 minutes. However, the current results suggest that subsequent optical density readings would yield a further extension into the plateau stage. Figure 2 shows that the cpxR mutant strain initially grows slightly slower than the wild type strain. We generally expect the cpxR mutant to grow slightly slower because this strain does not have a functional Cpx system, which normally allows the cell to respond to environmental stressors. If the growth rates were significantly different, it might suggest that the CpxR knockout affected another cell component or system that was not accounted for in this study. This might also decrease the amount of GFP reporter plasmid in cpxR mutant E. coli.

In conclusion, this study presents initial steps in establishing a GFP reporter system for monitoring ompC expression in the context of regulatory genes such as CpxR. The wild type and cpxR mutant E. coli strains harboring the GFP plasmid showed similar growth rates. In addition, the GFP reporter system is a simpler system to study ompC expression compared to outer membrane protein extraction of OmpC.

FUTURE DIRECTIONS

The results of this study did not provide conclusive evidence of the effects of streptomycin on OmpC regulation. In particular, while high salt is known to upregulate OmpC levels in both wild type and cpxR mutant E. coli strains, we observed no significant OmpC upregulation. It might be worthwhile to examine the effects of different treatment durations on ompC expression. This study only used one hour treatments of salt and streptomycin, but perhaps a longer treatment is necessary to observe upregulation of expression. In addition to treatment duration, the ompC GFP reporter plasmid may have contributed to the lack of significant difference between treatment groups. The reporter plasmid should be sequenced to confirm that it is a true ompC promoter GFP fusion. This would confirm that GFP fluorescence levels are representative of levels of ompC expression. It would also be useful to have cells transformed with an empty construct as a control for plasmid copy number.

While it appears that sublethal streptomycin does not upregulate OmpC in wild type or cpxR mutants, OmpC upregulation has been observed after sublethal kanamycin treatment (9). Streptomycin and kanamycin are both aminoglycosides and are expected to act through similar mechanisms. However, this was not supported by our results. Other aminoglycosides in addition to streptomycin and kanamycin could be examined for their effects on OmpC regulation. Levels of OmpC can be assessed again using an ompC promoter-fused GFP reporter construct used in this study.

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