A Novel Regulatory Cascade Involving BluR, YcgZ, and Lon Controls the Expression of Escherichia coli OmpF Porin

Valérie Duval*, Kimberly Foster, Jennifer Brewster and Stuart B. Levy*

Center for Adaptation Genetics and Drug Resistance, Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA, United States

In Escherichia coli, OmpF is an important outer membrane protein, which serves as a passive diffusion pore for small compounds including nutrients, antibiotics, and toxic compounds. OmpF expression responds to environmental changes such as temperature, osmolarity, nutrients availability, and toxic compounds via complex regulatory pathways involving transcriptional and post-transcriptional regulation. Our study identified a new regulatory cascade that controls the expression of OmpF porin. This pathway involves BluR, a transcriptional regulator repressing the expression of the ycgZ-ymgABC operon. We showed that BluR was responsible for the temperature-dependent regulation of the ycgZ-ymgABC operon. Furthermore, our results showed that independent expression of YcgZ led to a decreased activity of the ompF promoter, while YmgA, YmgB, and YmgC expression had no effect. We also determined that YcgZ accumulates in the absence of the Lon protease. Thus, mutation in bluR leads to de-repression of ycgZ-ymgABC transcription. With a second mutation in lon, YcgZ protein accumulates to reach levels that do not allow increased expression of OmpF under growth conditions that usually would, i.e., low temperature. With BluR responding to blue-light and temperature, this study sheds a new light on novel signals able to regulate OmpF porin.

Keywords: regulation, porin, Lon protease, E. coli, blue light, temperature

INTRODUCTION

The outer membrane of Gram-negative bacteria provides a physical barrier to hydrophobic and hydrophilic compounds including many toxic molecules (Pagès et al., 2008). Embedded in the outer membrane, the porins (outer membrane proteins or OMPs) have multiple functions: allowing the diffusion of small molecules, stabilizing the cell envelope, and acting as receptors for phages and bacteriocins or as virulence factors in pathogenic bacteria (Achouak et al., 2001; Nikaido, 2003; Galdiero et al., 2012). Three major porins are found in abundance in the outer membrane of Escherichia coli: OmpA, OmpC, and OmpF (Achouak et al., 2001). With only a small fraction of the porin forming open channels, OmpA seems to be mainly involved in maintaining the shape of the cell (Sugawara and Nikaido, 1994). Conversely, OmpF and OmpC form hydrophilic pores that allow the diffusion of small nutrients and toxic compounds (Chopra and Eccles, 1978; Yoshimura and Nikaido, 1985; Mortimer and Piddock, 1993; Nikaido, 1994, 2003). Although OmpF and
OmpF plays a crucial role in the accumulation of small hydrophobic antibiotics such as monoanionic cephalosporins, tetracyclines, and fluoroquinolones (Yoshimura and Nikaido, 1985; Cohen et al., 1989; Mortimer and Piddock, 1993; Duval et al., 2009). In order to respond to changes in environmental conditions, *E. coli* adjusts OmpF and OmpC expression through a complex regulatory network utilizing both transcriptional and translational regulation (for review, see Forst and Inouye, 1988; Pratt et al., 1996; Vogel and Papenfort, 2006). For instance, the osmolarity-dependent transcriptional control of ompF and ompC is exerted via the EnvZ/OmpR two-component signal transduction system in which EnvZ, an inner membrane histidine protein kinase, senses osmotic signals and transmits them to the transcription factor OmpR (Igo et al., 1989; Egger and Inouye, 1997; Yoshida et al., 2006). High osmosity leads to lower OmpF levels, while relative expression of OmpC is increased (Forst and Inouye, 1988; Mizuno and Mizushima, 1990; Pratt et al., 1996). In addition, expression of OmpF and OmpC is controlled at the post-transcriptional level by non-translated small RNAs such as MicF, MicC, and RybB (Mizuno et al., 1984; Chen et al., 2004; Gogol et al., 2011).

A major environmental parameter that affects OmpF porin expression is temperature. OmpF is abundant in *E. coli* outer membrane at ambient temperature, while growth at 37°C leads to decreased amount of OmpF. It is assumed that the downregulation of OmpF at body temperature limits the entry of toxic bile salts into the periplasm while the bacterium is grown at ambient temperature (Duval et al., 2009). BluR, a regulator harboring a MerR-like N-terminal domain, has been shown to directly repress the transcription of the adjacent ycgZ-ymgABC operon (ZABC operon, Figure 1; Tschowri et al., 2009). The lon locus encodes the Lon protease, an ATP-dependent serine protease involved in the degradation of unstable and misfolded proteins (Tsilibaris et al., 2006; Van Melderen and Aerts, 2009). Lon also plays a major role in regulating multiple biological processes by controlling the abundance of specific regulatory proteins such as MarA, RcsA, and SulA (Mizusawa and Gottesman, 1983; Torres-Cabassa and Gottesman, 1987; Griffith et al., 2004). With no available studies describing the regulation of OmpF by BluR and Lon, we investigated how BluR and Lon together control the abundance of the OmpF porin. Our study identified the ZABC operon as an intermediate in the regulation of OmpF by BluR and Lon. Precisely, we identified YcgZ as a novel repressor of ompF expression. Using a transcriptional fusion of the ompF promoter with lacZ (PompF-lacZ), we showed that YcgZ acted on ompF promoter. Finally, our study showed that the amount of YcgZ, when expressed from a plasmid, was amplified and highly stable in a lon mutant of *E. coli*, identifying YcgZ as a novel substrate of the Lon protease.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

The bacterial strains used in this study are listed in Table 1. *E. coli* strains were cultured under agitation in LB medium (tryptone 10 g l\(^{-1}\), yeast extract 5 g l\(^{-1}\), NaCl 5 g l\(^{-1}\)). The same medium containing 1.5% agar was used for growing bacteria on plates. Selection of *E. coli* after transformation with pBAD and pMPM vectors was performed using ampicillin 100 µg ml\(^{-1}\) and tetracycline 15 µg ml\(^{-1}\), respectively. Kanamycin 25 µg ml\(^{-1}\) and chloramphenicol 12 µg ml\(^{-1}\) was used for selection of chromosomal insertion of *kan* and *cat* genes.

**Plasmids**

Expression plasmids used in this study are listed in Table 1 and were constructed as follows. The nucleotide sequence of *ycgZ*, *ymgA*, *ymgB*, and *ymgC* was amplified by polymerase chain reaction (PCR) using the primers listed in Supplementary Table S1. The fragments were cloned into the pBAD/HisA and pMPM vectors using restriction sites indicated in Table 1. We constructed the pDVMBluR by PCR amplification of *bluR* nucleotide sequence, the 200 bases upstream of start codon GTG, and the 100 bases downstream of its stop codon TAA using the primers listed in Supplementary Table S1. The PCR fragment was then ligated into the pMPM plasmid using the restriction sites indicated in Table 1. All nucleotide sequences were verified at the Tufts University Core Facility (Tufts University School of Medicine, Boston, MA, United States).

![Figure 1](https://example.com/figure1.png)

**FIGURE 1** | Organization of ycgZ-ymgABC, ycgE, and ycgF ORFs in Escherichia coli. The bent arrows indicate the transcription start for each gene. ycgZ, ymgA, ymgB, and ymgC genes are expressed as one transcript. ycgE, ycgF, and ymgB have been renamed bluR, bluF, and arlB, respectively.
### TABLE 1 | Bacterial strains and plasmids used in this study.

| Strain or plasmid | Genotype/relevant characteristics | Reference or source |
|-------------------|-----------------------------------|---------------------|
| **Strains**       |                                   |                     |
| TOP10             | *E. coli* F− mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15Δ lacZ4 recA1 araD139 Δ[araA-lexu]7697 galUgAlK rpsL1 endA1 supG | Invitrogen, United States |
| AG100             | *E. coli* argE3 thi-1 rpsL1 | lysogen            | Oethinger et al., 1998 |
| M113R             | AG100 Jon3::IS186               |                     | Duval et al., 2009    |
| AGEZ3             | AG100 ycgE::Tn5                 |                     | Duval et al., 2009    |
| M113REZ3          | AG100 Jon3::IS186 ycgE::Tn5    |                     | Duval et al., 2009    |
| BW25113           | F− Δ[araD-araB]567 ΔlacZ4787::rrnB-3 ϕ, pro−1, Δ(rhaD-rhaB)568, hsdR514 | CGSC (Baba et al., 2006) |
| JW0419-1          | BW25113 Δlon-725::kan           |                     | CGSC (Baba et al., 2006) |
| VD101             | BW25113 ΔbluR::cat              | This study          |
| VD102             | BW25113 Δlon-725::FRT ΔbluR::FRT | This study          |
| VD103             | BW25113 ΔycgZ::ymgAB::cat       | This study          |
| VD104             | BW25113 Δlon-725::FRT ΔbluR::FRT ΔycgZ::ymgAB::cat | This study          |
| VDL25113          | BW25113 λ att ompFp-lacZ [AmpR] | This study          |
| VDL0419           | JW0419-1 λ att ompFp-lacZ [AmpR] | This study          |
| VDL101            | VD101 λ att ompFp-lacZ [AmpR]   | This study          |
| VDL102            | VD102 λ att ompFp-lacZ [AmpR]   | This study          |
| VDL103            | VD103 λ att ompFp-lacZ [AmpR]   | This study          |
| VDL104            | VD104 λ att ompFp-lacZ [AmpR]   | This study          |
| **Plasmids**      |                                   |                     |
| pBAD/HisA         | Expression cloning vector; [AmpR]; pBR322 ori; the araBAD promoter initiates the transcription of the target gene | Invitrogen          |
| pDV9B             | pBAD/HisA carrying ycgZ nucleotide sequence cloned between Ncol and PstI restriction sites; expression of native protein | This study          |
| pDV9B-XP          | pBAD/HisA carrying ycgZ nucleotide sequence cloned between Sacl and PstI restriction sites; allow the expression of an XPress-tagged YcgZ | This study          |
| pDV9A             | pBAD/HisA carrying ymgA nucleotide sequence cloned between Ncol and PstI restriction sites; expression of native protein | This study          |
| pDV9B             | pBAD/HisA carrying ymgB nucleotide sequence cloned between Ncol and PstI restriction sites; expression of native protein | This study          |
| pDV9C             | pBAD/HisA carrying ymgC nucleotide sequence cloned between Ncol and PstI restriction sites; expression of native protein | This study          |
| pMPM              | Expression cloning vector; [TetR]; ori p15A; low copy; the araBAD promoter initiates the transcription of the target gene; used in strains carrying λ att ompFp-lacZ [AmpR] | Mayer, 1995         |
| pDV9Z             | pMPM carrying ycgZ nucleotide sequence cloned between EcoRI and Xhol restriction sites; expression of native protein | This study          |
| pDV9M             | pMPM carrying ymgA nucleotide sequence cloned between EcoRI and Xhol restriction sites; expression of native protein | This study          |
| pDV9MB            | pMPM carrying ymgB nucleotide sequence cloned between EcoRI and Xhol restriction sites; expression of native protein | This study          |
| pDV9MC            | pMPM carrying ymgC nucleotide sequence cloned between EcoRI and PstI restriction sites; expression of native protein | This study          |
| pDV9MbluR         | pMPM carrying bluR nucleotide sequence, as well as the 200 bp upstream of the start codon and 100 bp downstream of the stop codon; expression of native protein | This study          |
| pRS415            | ori colE1 lacZ fusion vector; [AmpR] | Simons et al., 1987 |
| pDV915O           | pRS415 ompFp-lacZ                | This study          |
Gene Deletion

Targeted deletion of bluR and ycgZ-ymgABC, and subsequent marker removal were made using the λ.Red recombinase method previously described (Datsenko and Wanner, 2000). The Flp recombination target (FRT)-flanked chloramphenicol resistance gene (cat) has been amplified by PCR from plasmid pKD3 using primers listed in Supplementary Table S1. bluR-PA/bluR-PB and ycgZ-PA/ymgC-PB primers contain sequences upstream and downstream of bluR and of ycgZ-ymgABC operon, respectively. The PCR product was gel-purified and concentrated by ethanol precipitation. Transformants carrying the Red helper plasmid pKD46 were then grown in LB medium with 100 μg ml⁻¹ ampicillin and 10 mM l-arabinose at 30°C to an optical density at 600 nm (OD₆₀₀) of 0.6 and then made electro-competent. Electroporation was done using 200 ng of PCR product. Chloramphenicol resistant clones were selected on LB agar plates containing chloramphenicol 12 μg ml⁻¹. Correct integration of the cat gene in the targeted genes was verified by PCR using the primers listed in Supplementary Table S1. Appropriate chloramphenicol resistant clones were subsequently transformed with the pCP20 plasmid and ampicillin resistant clones were selected at 30°C on LB agar plates containing ampicillin 100 μg ml⁻¹. The transformants were then colony-purified non-selectively at 42°C on LB agar and then tested for loss of ampicillin and chloramphenicol resistance. Deletions were further verified by PCR using the primers listed in Supplementary Table S1.

LacZ Transcriptional Fusion and β-Galactosidase Assays

To construct the plasmid pDV415O, amplification of ompF promoter (PompF) was carried out by PCR using chromosomal DNA from strain Escherichia coli AG100 as template and the primers ompF1 and ompF2 listed in Supplementary Table S1. The PompF fragment was 273 bp long (from −273 to +1 relative to the transcription start) and was cloned into the pGEM-T Easy vector (Promega) following the manufacturer instruction. The resulting plasmid was digested with EcoRI and BamHI and the fragment corresponding to PompF was ligated to the similarly cut vector pRS415 yielding the plasmids pDV415O. The sequence of the PompF-lacZ fusion in pDV415O was then verified at the Tufts University Core Facility. Insertion of PompF-lacZ into E. coli chromosome was realized as followed. Recombination between the pDV415O and λ.RZ5 (Silhavy et al., 1984; Simons et al., 1987) resulted in a lysate bearing λ.RZ5 (PompF-lacZ). This was used to infect strains BW25113, a λ− and lac− strain of E. coli. AmpR Lac+ lysogens were selected and purified on LB agar containing ampicillin 20 μg ml⁻¹ and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) 40 μg ml⁻¹. Lysates from these lysogens were then used to infect at low multiplicity of infection (MOI = 0.005) strain BW25113 and derivative mutants. AmpR Lac+ lysogens were again isolated and the resulting strains were confirmed by PCR, as previously described (Powell et al., 1994), to have a single copy of the transcriptional fusion located in the λatt site on the chromosome. To assess the β-galactosidase (LacZ) activity, overnight cultures of fresh colonies of E. coli carrying a chromosomal λ.PompF-lacZ fusion were grown in LB medium containing ampicillin 20 μg ml⁻¹ and were subsequently diluted to an OD₆₀₀ of 0.05 in identical medium for growth with no antibiotic added. When the OD₆₀₀ reached 0.6, β-galactosidase (LacZ) activity was assayed by first rendered the cells permeable with 0.005% sodium dodecyl sulfate and 0.05% chloroform. LacZ activity was expressed in Miller units as previously described (Miller, 1972). All assays were carried out at least in three independent experiments.

RNA Isolation

Fresh colonies of E. coli strains were grown overnight (14–16 h) in 3 ml of LB medium. The cultures were then diluted to an OD₆₀₀ of 0.05 in 3 ml of identical fresh medium and cells were grown until an OD₆₀₀ of ~0.6. The total RNAs were isolated from 0.5 ml of culture using the Trizol Reagent (Thermo Fisher Scientific) following the manufacturer protocol. Directly after the preparation of the RNAs, the integrity of the RNAs was evaluated on a bleach gel stained with ethidium bromide (Aranda et al., 2012). The RNAs amount was quantified by absorbance at 260 nm (A260) and purity was evaluated by a A260/A280 ratio > 1.9 and a A260/A230 ratio > 1.7 using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). RNAs were stored for no more than 3 days at −80°C before DNase treatment and cDNA synthesis (see Reverse Transcription and Quantitative PCR Analysis).

Reverse Transcription and Quantitative PCR Analysis

Two micrograms of purified RNAs were treated using the Turbo™ DNase (Thermo Fisher Scientific) following the manufacturer instructions. A total of 500 ng of RNAs was then used to synthesize cDNA by reverse transcription using the Quantitect Reverse Transcription kit (Qiagen) and following the manufacturer instructions. To control for chromosomal DNA contamination, the reverse transcription step was also performed with reaction mixtures containing no reverse transcriptase and was used as a negative control in subsequent quantitative PCR (qPCR) reactions. Primers used for the qPCR were designed using the online PrimerQuest tool from Integrated DNA Technologies and are listed in Supplementary Table S1. Amplification efficiency and specificity for each set of primers are reported in Supplementary Figure S1. All qPCR reactions were performed using a Roche LightCycler 480 instrument II. The following experimental run protocol was used: UDP activation (50°C for 2 min), denaturation (95°C for 2 min), quantification program repeated 40 times (denaturation at 95°C for 5 s, anneal/extend 60°C for 30 s with a single fluorescence measurement), melting curve program (60–95°C with a heating rate of 0.15°C s⁻¹ and a continuous fluorescence measurement). Reactions were carried out in 20 μl with 2 μl of diluted cDNA, 0.4 μl of 10 μM forward primer (0.2 μM final concentration), 0.4 μl of 10 μM reverse primer (0.2 μM final concentration), 7.2 μl H₂O and 10 μl of the Power Up SYBR Green Master Mix (Applied Biosystems by Life Technologies). After the reverse transcription step, the cDNA samples were diluted fivefold and
used as templates for qPCR amplification of gapA, ompF, and ycgZ. A mix with no cDNA was also prepared (non-template control, NTC) and was run in parallel. NTC wells did either give a Ct > 35 or no Ct at all. We quantified the relative expression of ompF and ycgZ transcripts to the reference gene gapA. Using the Pfaffl method (Pfaffl, 2001), we determined the ratio R of a transcript expressed in a sample versus that expressed in the wild type strain grown at 37°C. We report the average (mean) and the standard deviation (SD) ± SD1 of two numbers R1 ± SD1 and R2 ± SD2, with R3 = R2/R1 and SD3 = √((SD1/R1)² + (SD2/R2)²). The statistical significance of differences between two averages was determined by a Student’s t-test (two independent samples, with two-tailed distribution) using GraphPad Prism software.¹

Statistical Analysis
We report the average (mean) and the standard deviation (SD) from at least three experimental values. In Figures 2B and 4B, we report the ratio R3 ± SD3 of two numbers R1 ± SD1 and R2 ± SD2, with R3 = R2/R1 and SD3 = √((SD1/R1)² + (SD2/R2)²). The statistical significance of differences between two averages was determined by a Student’s t-test (two independent samples, with two-tailed distribution) using GraphPad Prism software.¹

Steady-State Levels of Protein and Stability Assays
Overnight culture of E. coli wild type (BW25113) and lon (JW0419-1) strains carrying plasmids pBAD/HisA and derivative pDVBZ, pDVBZ-XP, pDVBA, pDVBB, and pDVBC were grown in LB medium in the presence of 100 µg ml⁻¹ ampicillin. Fresh identical medium supplemented with l-arabinose was then inoculated to an optical density measured at 600 nm of 0.05 and cells were grown at 37 or 25°C to OD₆₀₀ of 1. Whole cell extracts were prepared for analysis of the steady-state levels of protein. To assess the intracellular stability of YcgZ, 150 µg ml⁻¹ chloramphenicol was added to stop the proteins synthesis and the cultures were kept at the indicated temperatures. Samples to be used for preparing whole cell extracts were removed at indicated times (0–60 min). Cell extracts were prepared as follows: 1 ml of culture was centrifuged and the cells pellet suspended in 200 µl of lysis buffer per OD₆₀₀ of 1 [Tris–HCl 10 mM pH 8.0, EDTA 0.5 mM, CaCl₂ 10 mM, and 1 unit ml⁻¹ of DNase (Promega)]. Cells were sonicated on ice twice 20 pulses using a Branson Sonifier 250 and the following parameters: output control = 1 and a duty cycle = 50%. Protein concentration was determined using the Pierce 660 nm Protein Assay Reagent and bovine serum albumin as a standard (Thermo Fisher Scientific). Eight micrograms of proteins were separated on a 16% acrylamide gel in denaturing condition (100 mM Tris, 100 mM Tricine, and 0.1% SDS). The gels were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich) or used for western blot analysis.

Detection of XPress-Tagged YcgZ by Western Blot
After separation on a 16% acrylamide gel, the proteins were electro-transferred to a nitrocellulose membrane (Millipore, Billerica, MA, United States). The membrane was incubated overnight at 4°C in Tris-borate-saline (TBS) buffer supplemented with 3% milk powder. The membrane was then incubated for 2 h at room temperature with monoclonal anti-XPress antibodies (Thermo Fisher Scientific) diluted in TBS (1/6000). After three 15 min washes with TTBS (TBS supplemented with 0.05% Tween 20), the membrane was incubated for 2 h with alkaline phosphatase-coupled anti-mouse IgG antibodies (Promega) diluted 1/10,000 in TBS followed by three 15 min washes with TTBS and two 5 min washes with TBS. XPress-YcgZ was visualized by adding 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium following the manufacturer’s instructions (Promega).
E. coli were grown at 37 and 25 °C, revealing that OmpF expression is sixfold when compared to that of wild type AG100 (Figure 2B). The mRNA for both temperatures (lon and bluR) in the wild type strain, our results show a ∼3-fold increase in OmpF mRNA level, while a lon mutation slightly decreases the expression of OmpF at both temperatures (Figure 2A). Nevertheless the fold change in OmpF mRNA for both lon and bluR mutants grown at 25 versus 37°C is similar to that of wild type AG100 (Figure 2B). However, a lon bluR mutant carrying both mutations fails to increase ompF expression when grown at 25°C.

To further characterize the role of Lon and BluR in regulating OmpF expression, we constructed a transcriptional reporter fusion of ompF promoter with lacZ (PompF-lacZ). The resulting fusion did not carry ompF 5′-UTR affected by MicF (Andersen and Delibas, 1990), but instead carried lacZ 5′-UTR. In this case, we prevented post-transcriptional effects on ompF 5′-UTR. A single copy of the reporter fusion was then integrated at the att(l) site of wild type E. coli BW25113, a lac– λ– strain, and of derivative mutants lon, bluR, and lon bluR. LacZ activity was then assayed in cells grown at 37 and 25°C. Our results from the β-galactosidase assays show that transcription of PompF-lacZ increases by approximately threefold in the wild type strain grown under low temperature conditions (Figure 3). In contrast, the lon bluR double mutant fails to increase PompF-lacZ expression at 25°C and displays similar PompF-lacZ activities at 25 and 37°C (Figure 3). We suspect that the decreased OmpF mRNA level observed for the lon mutant (see above) is likely due to a lowered stability of the messenger in the absence of the Lon protease since no difference is detected at the transcriptional level.

The simplest model illustrating the effect of both lon and bluR mutations on ompF transcription under the growth conditions used in our experiments implies that BluR represses a locus coding for an intermediate protein that is a substrate of the Lon protease. This intermediate protein would act as a repressor of the ompF promoter. In this model, the inactivation of bluR leads to an increased transcription of the intermediate locus, and only in the absence or with a reduced activity of Lon, can the intermediate protein accumulate and considerably repress ompF expression. In an otherwise direct activation of lon by BluR, deletion of bluR alone would lead to decreased ompF transcription.

**RESULTS**

*Escherichia coli* Carrying Mutations in both lon and bluR Failed to Increased OmpF Expression at 25°C

When grown at 37°C, *E. coli* expresses low amount of OmpF in the outer membrane, while decreased temperatures lead to increased levels of the porin (Pratt et al., 1996; Nikaido, 2003). A previous study performed in our laboratory demonstrated that *E. coli* carrying mutations in both lon and bluR loci resulted in significantly lower amounts of the porin when the cells were grown under low temperature conditions (see protein level of OmpF in Duval et al., 2009). To better characterize the effect of lon and bluR on ompF expression, we performed reverse transcription and qPCR (RT-qPCR) and compared the level of OmpF messenger in wild type *E. coli* AG100 and derivative strains carrying mutations in lon, bluR, and lon bluR. All strains were grown at 37 and 25°C. Our data show that wild type *E. coli* grown at 25°C induces OmpF expression by approximately sixfold when compared to that of wild type *E. coli* grown at 37°C (Figures 2A,B). Our data also indicate that a single mutation in bluR has no significant effect on OmpF mRNA level, while a lon mutation slightly decreases the expression of OmpF at both temperatures (Figure 2A). Nevertheless the fold change in OmpF mRNA for both lon and bluR mutants grown at 25 versus 37°C is similar to that of wild type AG100 (Figure 2B). However, a lon bluR mutant carrying both mutations fails to increase ompF expression when grown at 25°C.

BluR Controls the Temperature-Dependent Expression of the ycgZ-ymgABC Operon

We subsequently aimed to identify the intermediate locus controlled by BluR and involved in the regulation of ompF expression. BluR has been previously described to directly repress the transcription of the ycgZ-ymgABC operon (ZABC; Tschowri et al., 2012). This operon encodes small proteins of 78–90 amino acid residues involved in biofilm formation through a mechanism that is yet to be determined (Tschowri et al., 2009). Using RT-qPCR, we measured the expression of the ZABC operon in AG100 (wt), AGEZ3 (bluR), and M113REZ3 (lon bluR) derivative strains. When comparing YcgZ mRNA levels at 37 and 25°C for the wild type strain, our results shows a ∼46-fold increased expression of ycgZ when the cells were grown at ambient temperature (Figures 4A,B). Our data also determine that a bluR mutant increases expression of the ZABC operon by...
Duval et al. Regulation of Escherichia coli OmpF

~100- and ~4-fold in cells grown at 37 and 25°C, respectively (Figure 4A), indicating a strong repression of the ycgZ promoter by BluR at 37°C. It was previously shown by Tschowri et al. (2009) that BluR–DNA interaction is released in the presence of BluF, a direct antagonist of BluR whose activity is induced by low temperature. We believe that the weaker repression of the ycgZ promoter observed at 25°C likely comes from the BluR inactivation by BluF under these growth conditions. At 37°C, BluR repressor is fully active and its deletion leads to a large de-repression of the ycgZ promoter. Our results in Figure 4A also show that the addition of a lon mutation to a bluR mutation (lon bluR) leads to a slight increased in ycgZ mRNA levels when compared to that of bluR. For AGEZ3 (bluR) and M113REZ3 (lon bluR), the low temperature mediated induction is only approximately twofold, overall indicating that bluR is mainly responsible for the temperature-dependent expression of ycgZ.

YcgZ Represses ompF Transcription

We then investigated whether the ZABC operon, which is de-repressed at 25°C, was involved in the control of ompF expression. In our model, if one of the proteins encoded by the ZABC operon accumulates in a lon bluR strain and represses ompF, we expect to see an increased ompF expression when the ZABC operon is deleted, i.e., in strain lon bluR ZABC. Using RT-qPCR, we compared OmpF mRNA levels in wild type, lon bluR and lon bluR ZABC strains grown at 37 and 25°C. When grown at 37°C, the three strains show similar OmpF mRNA levels (Figure 5A). Under low temperature growth conditions, the lon bluR ZABC strain expressed OmpF mRNA level similar to that of the wild type strain (Figure 5A). We further confirmed that the ZABC operon was involved in the control of ompF transcription using our PompF-lacZ reporter fusion; Figure 5B shows that deletion of the ZABC operon in a lon bluR background restores LacZ activity similar to that of the wild type. Taken together, our results suggest that the ZABC operon encodes a protein able to repress ompF expression. To further identify this repressor, we used a low copy pBAD vector allowing the expression of a target gene from the L-arabinose-dependent promoter araBAD. We independently expressed ycgZ, ymgA, ymgB, and ymgC in the lon bluR ZABC strain and subsequently measured the LacZ activity in the transformants grown at 25°C in the presence of L-arabinose. Our results show that the activity of PompF-lacZ decreases in response to increasing amount of YcgZ, while YmgA, YmgB, and YmgC have no significant effect on PompF-lacZ activity (Table 2 and see also Supplementary Figure S2). While our results identifies YcgZ as a repressor of the ompF promoter and our data show that ycgZ expression is high in a bluR mutant (Figure 4), a bluR mutant does not decrease ompF transcription (Figures 2, 3). However, a lon bluR strain grown at 25°C expresses less ompF transcript than the bluR mutant. These results strongly suggest that YcgZ is unstable in the presence of Lon under the growth conditions used in our experiments. That a lon bluR strain grown at 37°C does not decrease transcription of ompF is probably due

FIGURE 4 | BluR is controlling the temperature-dependent expression of ycgZ. (A) Fold change in YcgZ mRNA levels relative to the wild type strain AG100 grown at 37°C. (B) Fold change in YcgZ mRNA levels for all strains grown at 25 versus 37°C. The cells were grown to exponential phase (optical density at 600 nm ∼0.6) in LB medium. Total RNAs were prepared and used to measure the YcgZ mRNA levels by RT-qPCR. Numbers represent the means and standard deviations of expression levels from three independent experiments performed in duplicate. AG100, wt; AGEZ3, bluR; M113REZ3, lon bluR. See Supplementary Tables S4, S6 for details.
to growth conditions that inherently lead to a high repression of ompF expression. In this case, YcgZ amount may be too low to further repress the ompF promoter.

YcgZ is a Substrate of the Lon Protease

To evaluate whether YcgZ was a substrate of the Lon protease, we compared YcgZ protein amount in E. coli wild type and lon mutant. For completion purpose, we also evaluated the stability of YmgA, YmgB, and YmgC. Lacking antibodies that can specifically interact with the proteins, we decided to evaluate the steady-state level of each native protein when overexpressed from an araBAD promoter using pBAD derivative plasmids (see Table 1). Protein expression was compared between BW25113 (wild type) and JW0419-1 (lon) carrying the plasmids and grown in the presence of L-arabinose. Our experiments indicated that the amount of YcgZ was significantly lower when expressed in the wild type strain, while a lon mutant accumulated a large amount of YcgZ (Figure 6A). Of note, we observed this phenomenon when the cells were grown at both 25 and 37°C. We verified that the lower amount of YcgZ in the wild type was not due to a lesser expression of ycgZ by quantifying ycgZ messenger levels. RT-qPCR experiments showed that ycgZ was similarly expressed in both strains (see Supplementary Table S8), suggesting that the higher level of YcgZ protein observed in the lon mutant is likely due to a higher stability of the protein in the absence of Lon. Our data illustrated in Figure 6A also shows similar amounts of YmgA and YmgC proteins when overexpressed in the wild type and in the lon strains. We could not detect significant amount of YmgB in any of the strains tested even though high level of ymgB messenger was measured by RT-qPCR in both strains (data not shown). Using an XPress-tagged YcgZ (XPYcgZ), we

| Plasmids          | L-Arabinose % | LacZ activity | % Controla |
|-------------------|---------------|---------------|------------|
|                   |               | Miller units  |            |
| pMPM              | 0             | 1513 ± 258    |            |
|                   | 0.005         | 1279 ± 201    |            |
|                   | 0.05          | 1066 ± 152    |            |
|                   | 0.5           | 1017 ± 145    |            |
| pDVMZ (+ycgZ)     | 0             | 1195 ± 190    | 79         |
|                   | 0.005         | 851 ± 135     | 66         |
|                   | 0.05          | 385 ± 49      | 36         |
|                   | 0.5           | 225 ± 16      | 21         |
| pDVMA (+ymgA)     | 0             | 1221 ± 273    | 81         |
|                   | 0.5           | 921 ± 57      | 91         |
| pDVMB (+ymgB)     | 0             | 1544 ± 69     | 102        |
|                   | 0.5           | 766 ± 140     | 75         |
| pDVMC (+ymgC)     | 0             | 1324 ± 98     | 88         |
|                   | 0.5           | 1068 ± 67     | 105        |

aPercentage of LacZ activity compared to the strain carrying the empty vector (pMPM).
confirmed an L-arabinose-dependent accumulation of YcgZ in the lon strains, while the protein was barely detectable in the wild type (Figure 6B). Additionally, we examined the stability of native YcgZ in the wild type and lon strains after stopping the protein synthesis with chloramphenicol and determining the amount of YcgZ in multiple samples taken over a certain period of time (Figure 6C). We observed a decline in YcgZ quantity in the wild type strain, while a high amount of the protein was maintained over time in the lon mutant, demonstrating a significant stability of YcgZ in the absence of Lon.

We further aimed to confirm the lon-dependent repression of ompF promoter by YcgZ. YcgZ was expressed in the ZABC and lon bluR ZABC mutant strains, both carrying a chromosomal PompF-lacZ fusion and providing both lon+/lon− backgrounds. In the absence of lon, expression of YcgZ significantly decreased LacZ activity with addition of 0.0025% of L-arabinose (Figure 7). In a lon+ background, similar repression of ompF promoter was reached by increasing the L-arabinose concentration by at least threefold (0.0075%). Ultimately, when a higher concentration of L-arabinose was used (>0.01%), both strains expressed a similar level of LacZ indicating that under those conditions, an abundant level of YcgZ is sufficient to repress ompF promoter even in the presence of the Lon protease (Figure 7). Of note, the level of ycgZ transcript measured when ycgZ is expressed from its own promoter is much lower than that expressed from the araBAD promoter with concentration of L-arabinose above 0.01% (compared Supplementary Tables S4, S5 with Table S8).

**DISCUSSION**

OmpF regulation in response to variable growth conditions provides a great example of bacterial adaptive response to the...
environment. In this work, we characterize a \textit{lon bluR} mutant of \textit{E. coli} and we show that transcription of \textit{ompF} is impaired when the cells are grown at 25°C. Our data reveal that both \textit{bluR} and \textit{lon bluR} strains expressed high levels of \textit{ycgZ} transcript, confirming a transcriptional repression by BluR on the \textit{ycgZ} promoter. We find that the repression of the \textit{ycgZ} promoter by BluR is strong when the cells are grown at 37°C, while growth at 25°C led to a weaker repression. We also show a high induction of \textit{ycgZ} expression at 25°C, which is essentially mediated by \textit{bluR}.

It was previously established that the BluR–DNA interaction is released in the presence of BluF, a direct antagonist of BluR whose activity is induced by low temperature. Inactivation of BluR by BluF consequently led to transcription of the \textit{ycgZ-ymgABC} operon (Tschowri et al., 2009). This effect is further increased upon exposure of BluF to blue-light irradiation in addition to the temperature downshift as blue-light activates BluF through conformational changes (Hasegawa et al., 2006; Nakasone et al., 2010).

While we do not describe the specific mechanism by which YcgZ regulates OmpF expression, our study clearly establishes that expression of YcgZ represses \textit{ompF} transcription. Moreover, our analyses demonstrate a high instability of YcgZ in wild type \textit{E. coli}, while the protein substantially accumulates and remains stable in a \textit{lon} mutant, identifying YcgZ as a substrate of the Lon protease. The high instability of YcgZ in the presence of Lon explains why, even though \textit{ycgZ} expression is large in a \textit{bluR} mutant at 25 and 37°C, \textit{ompF} transcription is not affected. These growth conditions are detrimental to the stability of YcgZ with the activity of Lon being high enough to keep the concentration of YcgZ below the threshold where it can inhibit the \textit{ompF} promoter.
In other words, when expressed from its own promoter and under the growth conditions used in our experiments, YcgZ will likely have an effect on the ompF promoter only if its stability is increased. Growth at temperatures below 25°C could reduce the proteolytic activity of Lon, leading to a higher level of YcgZ in the cell. For instance, Yersinia pestis YmoA is a substrate of the Lon protease. Jackson et al. (2004) found that YmoA stability increases as the growth temperature decreases to become stable at 17°C (half-life > 3 h). Alternatively, the interaction of YcgZ with another yet to be discovered protein could protect YcgZ from degradation by Lon. An example of such phenomenon in E. coli is illustrated with HU-α and HU-β proteins, two homologous proteins encoded by hupA and hupB, respectively and which form heterodimers. In the presence of HU-α, HU-β is fairly stable, while in a hupA mutant HU-β is degraded by Lon (Bonnefoy et al., 1989); HU-α seems to protect HU-β from degradation by Lon.

How YcgZ, a protein of 78 amino acid residues, acts on ompF promoter is still under investigation. YmgB, a small three-helix protein of 88 amino acids encoded by the ZABC operon, was shown to display similarity to protein Hha (Lee et al., 2007) and to downregulate curli expression in an RcsB-dependent pathway (Tschowri et al., 2009). It was then proposed that YmgB acts as “connector” of the Rcs phosphorelay. YcgZ could likely work as a small protein connector as well. Interestingly, YmgA and YmgB proteins have been shown to activate colanic acid synthesis while decreasing curli synthesis (Tschowri et al., 2009). The only known role of YcgZ was to somehow alleviate the activity of both YmgA and YmgB.

CONCLUSION

Our study identifies OmpF promoter as a new target regulated by YcgZ, a small pleiotropic regulator, which expression is induced by low temperatures via BluR and is destabilized by the Lon protease. We believe our results shed a new light on novel signals able to regulate OmpF porin expression through complex regulatory pathways (see proposed model in Figure 8). The possibility of a more complex regulatory architecture involving other factors not considered in this study is under investigation.

AUTHOR CONTRIBUTIONS

VD and SL conceived the study. VD, KF, and JB performed the experiments. VD analyzed data. VD and SL prepared the manuscript and all the authors contributed to preparing the final version of the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

The research reported in this article was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award number R01 AI056021. The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

ACKNOWLEDGMENTS

The authors thank members of the Sonenshein and Belitsky’s laboratories for helpful suggestions and discussions during the course of this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01148/full#supplementary-material

REFERENCES

Achouak, W., Heulin, T., and Pagès, J. M. (2001). Multiple facets of bacterial porins. FEMS Microbiol. Lett. 199, 1–7. doi: 10.1111/j.1574-6968.2001.tb10642.x
Andersen, J., and Delilas, N. (1990). micF RNA binds to the 5′ end of ompF mRNA and to a protein from Escherichia coli. Biochemistry 29, 9249–9256. doi: 10.1021/bi00049a020
Aranda, P. S., LaJoie, D. M., and Jorcyk, C. L. (2012). Bleach gel: a simple agarose gel for analyzing RNA quality. Electrophoresis 33, 366–369. doi: 10.1002/elps.201100335
Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., et al. (2006). Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. 2, 2006.0008. doi: 10.1038/msb4100050
Basle, A. M., Qutub, R., Mehrazin, M., Wibbenmeyer, J. A., and Delcour, A. H. (2003). Functional studies on loop deletion mutants of E. coli OmpF porin. Biophys. J. 84, 533A–533A.
Bonnefoy, E., Almeida, A., and Rouvière-Yaniv, J. (1989). Lon-dependent regulation of the DNA binding protein HU in Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 86, 7691–7695.

Chen, S., Zhang, A., Blyn, L. B., and Storz, G. (2004). MicC, a second small-RNA regulator of Omp protein expression in Escherichia coli. J. Bacteriol. 186, 6689–6697. doi: 10.1128/JB.186.20.6689-6697.2004
Chopra, I., and Eccles, S. J. (1978). Diffusion of tetracycline across the outer membrane of Escherichia coli K-12: involvement of protein Ia. Biochem. Biophys. Res. Commun. 83, 550–557. doi: 10.1016/0006-291X(78)91025-2
Cohen, S. P., McMurry, L. M., Hooper, D. C., Wolfson, J. S., and Levy, S. B. (1989). Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) Escherichia coli selected by tetracycline or chloramphenicol: decreased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrob. Agents Chemotherapy. 33, 1318–1325. doi: 10.1128/AAC.33.8.1318
Cowan, S. W., Garavito, R. M., Jansonius, J. N., Jenkins, J. A., Karlsson, R., Konig, N., et al. (1995). The structure of OmpF porin in a tetragonal crystal form. Structure 3, 1041–1050. doi: 10.1016/S0969-2126(01)00240-4
Datsenko, K. A., and Wanner, B. L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. U.S.A. 97, 6640–6645. doi: 10.1073/pnas.120116297
Duval, V., Nicoloff, H., and Levy, S. B. (2009). Combined inactivation of lon and ycgE decreases multidrug susceptibility by reducing the amount of OmpF

Duval et al.
porin in *Escherichia coli*. Antimicrob. Agents Chemother. 53, 4944–4948. doi: 10.1128/AAC.00787-09

Egger, L. A., and Inouye, M. (1997). Purification and characterization of the periplasmic domain of EnvZ osmosensor in *Escherichia coli*. Biochem. Biophys. Res. Commun. 231, 68–72. doi: 10.1006/bbrc.1996.6007

Forst, S., and Inouye, M. (1988). Environmentally regulated gene expression for membrane proteins in *Escherichia coli*. Annu. Rev. Cell Biol. 4, 21–42. doi: 10.1146/annurev.cb.04.110188.000321

Galdiero, S., Falanga, A., Cantisani, M., Tarallo, R., Della Pepa, M. E., Gogol, E. B., Rhodius, V. A., Papenfort, K., Vogel, J., and Gross, C. A. (2011). Small proteins of the group 17 outer membrane lipoprotein family are involved in biofilm formation. Proc. Natl. Acad. Sci. U.S.A. 108, 12875–12880. doi: 10.1073/pnas.1109359108

Griffith, K. L., Shah, I. M., and Wolf, R. E. (2004). Proteolytic degradation of *Escherichia coli* transcription activators SoxS and MarA as the mechanism for reversing the induction of the superoxide (SoxRS) and multiple antibiotic resistance (Mar) regulons. Mol. Microbiol. 51, 1801–1816. doi: 10.1046/j.1365-2958.2003.03952.x

Hasegawa, K., Masuda, S., and Ono, T.-A. (2006). Light induced structural changes in the periplasmic domain of EnvZ osmosensor in *Escherichia coli*. Mol. Microbiol. 51, 231, 68–72. doi: 10.1006/bbrc.1996.6007

Jackson, M. W., Silva-Herzog, E., and Plano, G. V. (2004). The ATP-dependent ClpXP and Lon proteases regulate expression of the *Yersinia pestis* type III secretion system via regulated proteolysis of YmoA, a small histone-like protein. J. Mol. Biol. 345, 3785–3793. doi: 10.1016/j.ymolbi.2004.10.020

Kato, M., Hata, T., Ishii, A., Hasegawa, K., Masuda, S., and Ono, T.-A. (2006). Light-induced conformational changes in the periplasmic domain of EnvZ osmosensor in *Escherichia coli*. Mol. Microbiol. 61, 1077–1082. doi: 10.1111/j.1365-2958.2004.04353.x

Lee, J., Page, R., García-Contreras, R., Palermino, J.-M., Zhang, X.-S., Doshi, O., Jackson, M. W., Silva-Herzog, E., and Plano, G. V. (2004). The ATP-dependent ClpXP and Lon proteases regulate expression of the *Yersinia pestis* type III secretion system via regulated proteolysis of YmoA, a small histone-like protein. J. Mol. Biol. 345, 3785–3793. doi: 10.1016/j.ymolbi.2004.10.020

Mayer, M. P. (1995). A new set of useful cloning and expression vectors derived from pBlueScript. Gene 163, 41–46. doi: 10.1016/0378-1119(95)00389-N

Miyake, Y., Homma, H.-S., Ono, T.-A., Ishii, A., Masuda, S., and Terazima, M. (2010). Temperature-sensitive reaction of a photosensor protein YcgF: possibility of a role of temperature sensor. Biochemistry 49, 2288–2296. doi: 10.1021/bi100211z

Nakai, H. (1994). Porins and specific diffusion channels in bacterial outer membranes. J. Biol. Chem. 269, 3905–3908. doi: 10.1011/jbc.1365-2958.1992. MBBR.67.4.593–656.2003

Nakai, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. Microbiol. Mol. Biol. Rev. 67, 593–656. doi: 10.1128/MMBR.67.4.593–656.2003

Oethinger, M., Kern, W. V., Goldman, J. D., and Levy, S. B. (1998). Association of organic solvent tolerance and fluoroquinolone resistance in clinical isolates of *Escherichia coli*. J. Antimicrob. Chemother. 41, 111–114. doi: 10.1093/jac/41.1.111

Pagès, J.-M., James, C. E., and Winterhalter, M. (2008). The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nat. Rev. Microbiol. 6, 893–903. doi: 10.1038/nrmicro1994

Pfaff, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:e45. doi: 10.1093/nar/29.9.e45

Powell, B. S., Rivas, M. P., Court, D. L., Nakamura, Y., and Turnbough, C. L. (1994). Rapid confirmation of single copy lambda prophage integration by PCR. Nucleic Acids Res. 22, 5765–5766. doi: 10.1093/nar/22.25.5765

Pratt, L. A., Hsing, W., Gibson, K. E., and Silhavy, T. J. (1996). From acids to osmZ: multiple factors influence synthesis of the OmpF and OmpC porins in *Escherichia coli*. Mol. Microbiol. 20, 911–917. doi: 10.1111/j.1365-2958.1996.tb02532.x

Silhavy, T. J., Berman, M. L., Enquist, L. W., and Laboratory, C. S. H. (1984). *Experiments with Gene Fusions*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Simons, R. W., Hounam, F., and Kleckner, N. (1987). Improved single and multicopy lac-based cloning vectors for protein and operon fusions. Gene 53, 85–96. doi: 10.1016/0378-1119(87)90095-3

Sugawara, E., and Nakaido, H. (1994). OmpF protein of *Escherichia coli* outer membrane occurs in open and closed channel forms. J. Biol. Chem. 269, 17981–17987.

Torres-Cabassa, A. S., and Gottesman, S. (1987). Capsule synthesis in *Escherichia coli* K-12 is regulated by proteolysis. J. Bacteriol. 169, 981–989. doi: 10.1128/jb.169.3.981–989.1987

Tschouri, N., Busse, S., and Hengge, R. (2009). The BLUF-EAL protein YcgF acts as a direct anti-repressor in a blue-light response of *Escherichia coli*. Genes Dev. 23, 522–534. doi: 10.1101/gad.499409

Tschouri, N., Lindenberg, S., and Hengge, R. (2012). Molecular function and potential evolution of the biofilm-modulating blue light-signalling pathway of *Escherichia coli*. Mol. Microbiol. 85, 893–906. doi: 10.1111/j.1365-2958.2012.08147.x

Tsilibaris, V., Maenhaut-Michel, G., and Van Melder, L. (2006). Biological roles of the Lon ATP-dependent protease. Res. Microbiol. 157, 701–713. doi: 10.1016/j.resmic.2006.05.004

Van Melder, L., and Aerts, P. (2009). Regulation and quality control by Lon-dependent proteolysis. Res. Microbiol. 160, 645–651. doi: 10.1016/j.resmic.2008.11.021

Vogel, J., and Papenfort, K. (2006). Small non-coding RNAs and the bacterial outer membrane. Curr. Opin. Microbiol. 9, 605–611. doi: 10.1016/j.mib.2006.10.006

Yoshida, T., Qin, L., Egger, L. A., and Inouye, M. (2006). Transcription regulation of *ompF* and *ompC* by a single transcription factor. Ompr. J. Biol. Chem. 281, 17114–17123. doi: 10.1074/jbc.M602112200

Yoshimura, F., and Nakaido, H. (1985). Diffusion of organic solvent tolerance and fluoroquinolone resistance in clinical isolates of *Escherichia coli*. Antimicrob. Agents Chemother. 27, 84–92. doi: 10.1128/AAC.27.1.84

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.