Genetic Regulation of the Locus of Heat Resistance in *Escherichia coli*
by

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Abstract

During food processing, *Escherichia coli* are exposed to stress from oxidation, osmolarity and temperature, and have developed mechanisms to survive. A highly heat resistant strain of *E. coli* AW1.7 was isolated from a beef carcass after thermal processing and it has a genomic island called the locus of heat resistance (LHR) that has 16 predicted open reading frames. The LHR has been reported in *E. coli* isolated from raw cheese milk and treated wastewater and contributes to high pressure and chlorine resistance. Most studies on the LHR has focused on genetic function or its role on the survival under different types of stress, but there has been limited research on its genetic regulation. Previous studies predicted that EvgA is a regulator of the LHR because it induces *yfdX* expression and the LHR contains two hypothetical proteins from the YfdX family. The current research aims to investigate the regulatory mechanisms of the LHR as its expression of genes in response to different stressors remains unclear. The objectives were to evaluate EvgA as a regulator of the LHR; investigate the presence of additional promoters using *in-silico* methods and validate their role using gene expression; and to examine the induction of promoters using stress. The constructed *evgAS*-pRK complement was not able to restore phenotype of the EvgA deficient Keio strain, *E. coli* MG1655 Δ*evgA.* Further investigation found that the pLHR plasmid was absent from *E. coli* MG1655 Δ*evgA* (pLHR), explaining its loss of heat resistance. CNNPromoter was used to predict six promoter sequences regulated by Crp, OmpR, PurR, FadR, RpoD or OxyR within the LHR. Overexpression of regulatory proteins using plasmids from the ASKA library and gene expression using RT-qPCR of genes downstream of the predicted promoters were used to determine promoter activity. EvgA, OxyR, and RpoD affected the expression of LHR genes. Crp, FadR, PurR, and OmpR had minor effects on gene expression. When the OxyR regulated promoter was induced by chlorine or hydrogen peroxide, expression of *trx* increased, supporting that OxyR regulates its respective promoter. In conclusion, there are regulatory sites within the LHR that may account for the differential expression of genes in response to stress.

Preface

This thesis is an original work by Oanh Nguyen. No part of this thesis has been previously published.

Dedication

To my mother.

Thank you for your words of wisdom, encouragement and above all, your love.

Acknowledgments

Thirty years ago, my mother was one of many boat people who fled Vietnam and ended up in a refugee camp in Malaysia. Shortly after I was born, Canada welcomed both my mother and me through the private sponsorship of refugees program, and we became Canadian citizens. I grew up listening to stories of the life my mother had escaped in Vietnam and realized how lucky I am to be Canadian. Since I was a child, the value of education is what my mother instilled in me the most. She was robbed of her chance at an education during the Vietnam War but continually pushed me to become a diligent, hard-working student. It was her words of encouragement and support through all the years that I was able to attend University and graduate with a master's degree. Thank you, mom, for your love and being my pillar of strength.

I am fortunate to be given the opportunity by my supervisors, Lynn McMullen, and Michael Gänzle to study in a field that I am passionate about. I am grateful to have supervisors who cared so much about my work made time for me whenever I needed. Without both of your guidance, patience, and encouragement, I would not be able to finish my degree. Most of all, I am thankful to you both for giving me the opportunity to work in 2-50 when I was an undergraduate student. At the time, my grades did not reflect who I was, and you took a gamble on me without even looking at my transcript. Thank you for giving me the chance to excel in academic research and finding my passion in Microbiology.

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CFU	colony forming unit
C_T	cycle threshold
DNA	deoxyribonucleic acid
EGFP	enhanced green fluorescent protein
gDNA	genomic DNA
GFP	green fluorescent protein
HGT	horizontal gene transfer
ITPG	isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani
LHR	locus of heat resistance
OD_{600}	optical density at 600 nm
orf	open reading frame
PEC	Profiling of *E. coli* Chromosome
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RFU	relative fluorescence unit
RNA	ribonucleic acid
RT	reverse transcriptase
TBE	tris borate EDTA
sHSp	small heat shock protein
SOC	super optimal broth with catabolite repression
WT	wildtype

1. Introduction

Escherichia coli are versatile bacteria and can be found in diverse habitats including the gastrointestinal tract of animals and the environment (Ishii and Sadowsky, 2008). *E. coli* can be grown readily in the laboratory making it a well-studied model organism and widely exploited host organism for recombinant DNA. Most strains of *E. coli* are harmless; however, there are pathogenic isolates that can cause foodborne illness in humans (Kaper et al., 2004; Smith et al., 2007; Vogt and Dippold, 2005). For example, *E. coli* O157:H7 produces Shiga toxin which causes gastroenteritis, or in some cases, progresses to hemolytic-uremic syndrome (Besser et al., 1999). To enter the gastrointestinal tract, *E. coli* must have strategies to persist in food as well as survive exposure to host defenses such as gastric acid, bile salts, and organic acids after ingestion (Chekabab et al., 2013; Boor, 2006). To adapt to changing environments, *E. coli* has the ability to sense the environment and respond with changes in gene expression and protein activity (Boor, 2006).

Mechanisms employed by *E. coli* to alter gene expression include two-component regulatory systems, transcription factors and sigma factors. Two-component systems consist of a sensor kinase, which is often an integral membrane protein that responds to external stimuli such as chemical and/or physical signals (Mitrophanov and Groisman, 2008). The second component is a cytoplasmatic response regulator that can bind DNA or RNA, produce enzymatic activity, or have protein-protein interactions (Mitrophanov and Groisman, 2008). The sensor kinase communicates to the response regulator through a series of phosphorylation events (Mitrophanov and Groisman, 2008). Response regulators often function as transcription factors by binding to DNA to interact with other factors or the RNA polymerase to modulate transcription (Latchman, 1993). Transcription factors can be negative regulators that bind to the promoter and physically prevent binding of the RNA polymerase or positive regulators that bind to the upstream region of a promoter to help recruit the polymerase (Balleza et al., 2008).

In *E. coli,* the core RNA polymerase is comprised of five subunits: αI and αII, β, β′ and ω. The core RNA polymerase combined with the sigma/σ factor forms the active holoenzyme that recognizes specific DNA

sequences called promoter sites located upstream of a gene (Borukhov and Nudler, 2003; Boor, 2006). This allows the expression of genes under certain environmental conditions and creates the mRNA transcripts that are translated into proteins (Borukhov and Nudler, 2003; Boor, 2006). In non-stress conditions, sigma70 (RpoD), the house keeping sigma factor is responsible for transcription of most gene promoters (Jishage et al., 1996). Under stress conditions, alternative sigma factors are induced and compete for binding to the RNA polymerase (Chung et al., 2006; Kazmierczak et al., 2005). The ability of RNA polymerase to associate with alternative sigma factors allows recognition of different promoter sequences and therefore the expression of different target genes or regulon (Boor, 2006). There are several known sigma factors associated with specific stress regulons (Table 1).

Table 1. Sigma factors of *Escherichia coli*

Sigma Factor	Gene	Function	References
σ70	*rpoD*	Exponential phase, house keeping	Helmann and Chamberlain, 1988
σ38/σ^S	*rpoS*	Starvation, general stress response	Lange and Hengge-Aronis, 1991
σ28/σ^F	*rpoF*	Flagellum biosynthesis	Arnosti and Chamberlain, 1989
σ32/σ^H	*rpoH*	Heat shock	Erickson et al., 1987
σ24/σ^E	*rpoE*	Extra-cytoplasmic stress, extreme heat	Erickson et al., 1989 Raina et al., 1995
σ54/σ^N	*rpoN*	Nitrogen limitation	Merrick, 1993; Magasanik, 1982

E. coli faces multiple stresses during food processing and relies on stress response pathways and resistance mechanisms to survive through different environmental conditions (Gunasekera et al., 2008). The first line of defense that *E. coli* has against environmental stress is its cell envelope which maintains optimal cytoplasmatic conditions while sensing and protecting against changes from the external environment (Delhaye et al., 2016). In addition, the ability to adapt the cell membrane in response to stress and maintain cell integrity is crucial for survival (Rowlett, et al., 2017). Two important envelope stress response pathways are the σ^E and Cpx regulatory pathways (Raivio and Silhavy, 1999). The alternative sigma factor σ^E monitors and responds to changes in folding of outer membrane proteins

(OMP) and activates genes involved in OMP folding and degradation and expression of alternative sigma factors (Raivio and Silhavy, 1999). The Cpx regulon is a two-component regulatory system comprised of CpxR, the response regulator and CpxA, the histidine kinase (Raivio, 2013). In response to envelope stress from misfolded inner membrane proteins, the Cpx regulon increases the production of chaperones and proteases that aids in protein refolding and degradation (Raivio, 2013; Guest and Raivio, 2016). The Cpx-mediated envelope stress response is diverse and leads to the activation of additional genes involved in varied functions such as energetics and transport in the inner membrane (Guest and Raivio, 2016). In addition to envelope stress, *E. coli* encounters a variety of other stresses such as oxidation, osmolarity, and temperature and has developed specific regulons to counter these stresses (Gunasekera et al., 2008).

During processing of fresh fruits and vegetables, chlorine, hydrogen peroxide or peroxyacetic acid can be applied to reduce bacterial contamination (Capozzi et al., 2009). These disinfectants cause an imbalance in oxidant concentration and generate reactive oxygen species (ROS) that damage bacterial DNA, RNA, proteins and lipids (Cabiscol et al., 2000). *E. coli* has acquired multiple defensive mechanisms against oxidative stress with the main one being the OxyR regulon (Demple and Halbrook, 1983; Storz et al., 1990). In the presence of hydrogen peroxide, OxyR, induces the transcription of genes for catalase and hydroperoxide reductase (Storz et al., 1990; Cabiscol et al., 2000). These enzymes catalyze the decomposition of hydrogen peroxide into non-toxic by-products to eliminate toxicity (Capozzi et al., 2009).

E. coli encounters osmotic stress when salt is added to meat or fermented foods such as salami or cheese (Taormina and Sogos, 2014; Burgess et al., 2016). Salt disrupts the osmotic balance between cytoplasmic and intracellular environments and damages bacterial cells (Burgess et al., 2016). To sense and respond to the changing osmotic environment, *E. coli* uses the EnvZ/OmpR two-component system to differentially express different outer membrane porins (Feng et al., 2003). OmpR is the response regulator and is phosphorylated by the sensor kinase EnvZ (Feng et al., 2003). At low osmolarity, the major outer membrane porin is OmpF, whereas, at high osmolarity, OmpC is predominant (Feng et al., 2003). Phosphorylated OmpR binds to *ompF and ompC* promoters differentially to change the porin composition and adjust the diffusion of small molecules across the outer membrane (Aiba and Mizuno, 1990).

E. coli may encounter thermal stress during pasteurization, meal preparation, or during hot water washes of beef carcasses, thermization of raw cheese milk, or cereal fermentations that generate high temperatures (Dlusskaya et al., 2011; Boll et al., 2017; Wang et al., 2018). In *E. coli,* high temperatures induce the expression of genes for alternative sigma factors, heat shock chaperones, and proteases that help in protein folding and degradation (Li and Gänzle, 2016). *E. coli* AW1.7, a slaughter plant isolate was discovered to have exceptional heat resistance (Dlusskaya et al., 2011). Its heat resistance was not mediated by the σ^S or σ^H heat stress regulon but attributed to a 15 to 19 kb genomic island called the locus of heat resistance (LHR) (Ruan et al., 2011; Pleitner et al., 2012; Mercer et al., 2015). The LHR has 16 open reading frames (orfs), encoding for putative heat shock proteins, proteases, transporters and hypothetical proteins related to envelope and oxidative stress (Mercer et al., 2015). In addition to heat resistance, the LHR contributes to high-pressure resistance and chlorine resistance (Garcia-Hernandez et al., 2015; Liu et al., 2015; Mercer et al., 2015; 2016; Wang et al., unpublished). The LHR has been identified in strains of *E.* coli from other sources. Isolates from raw cheese milk and wastewater plants have been discovered to contain the LHR (Boll et al., 2017; Zhi et al., 2016) The LHR is not found exclusively in *E. coli,* but can be found in other *Enterobacteriaceae* such as *Klebsiella pneumoniae, Cronobacter sakazakii,* and *Salmonella enterica* suggesting horizontal gene transfer (Mercer et al. 2017). Additionally, *E. coli* isolates were screened by PCR for the presence of the LHR and it was reported in Shiga toxin producing *E. coli* (STEC) which raises concern for pathogen survival in cooked meats (Ma and Chui, 2017).

Many of the stress regulons that allow adaptation to change in the environment involve a shock response that consists of rapid responses or a prolonged stress response that persists and aids in exponential growth (Gunasekera et al., 2008). Mounting a response to stress requires a significant amount of resources and is only activated when necessary in order to save cellular energy and allocate it towards more essential processes (Gunderson et al., 2010). However, it appears that the LHR is a protective stress regulon that is constitutively expressed during both exponential growth and stationary phase (Ruan et al., 2011, Mercer et al., 2017). This constitutive expression provides *E. coli* with higher fitness in

situations when responsive expression may not be fast enough; for example, rapid exposure to heat (Geisel, 2011).

Most studies on the LHR focuses on genetic function and its role on survival, but there has been limited research on its genetic regulation. OmpR was strongly predicted to regulate the promoter upstream of *orf1*; however, LHR expression in the OmpR deficient strain was comparable to the wildtype (Mercer et al., 2015, Mercer et al., 2017). EvgA was predicted to have a role in regulation because the expression of the YfdX family proteins (*orf8 and orf9*) are regulated by the two-component regulatory system EvgA/EvgS (Nishino et al., 2003; Mercer et al., 2017). YfdX is a hypothetical protein predicted to be involved in periplasmic chaperone activity, antibacterial stress and virulence (Liu et al., 2019; Lee et al., 2019). It was discovered that in an EvgA deficient strain, LHR expression was reduced and heat resistance was lost (Mercer et al., 2017). Therefore, LHR expression is dependent on a chromosomal copy of *evgA.* Other target genes under the control of EvgA/EvgS include drug efflux, acid resistance osmotic adaptation and drug resistance (Eguchi et al., 2003; Nishino et al., 2003). Additionally, heat resistance increased when EvgA was overexpressed (Christ and Chin, 2008).

This research aims to investigate the regulatory mechanisms of the LHR. In addition, how LHR expression is regulated in response to different stressors remains unclear. Therefore, it is hypothesized that additional regulatory components regulate LHR expression in response to multiple stressors.

The objectives of this study were to:

1. evaluate EvgA as a regulator of the LHR
2. investigate additional promoters using *in-silico* methods and validate using gene expression
3. examine the natural induction of promoters using stress.

2. Materials and Methods

Bacterial strains and plasmids.

Strains and plasmids used in this study including isolates and mutants are listed in Table 2 and 3, respectively. The Keio mutants were previously generated using the lambda red recombination system in a *E. coli* MG1655 derivative strain (Baba et al., 2006) and ASKA plasmids were generated by cloning each open reading frame from *E. coli* K-12 into the vector plasmid, pCA24N (Kitagawa et al., 2005). The two plasmids, pUC::eGFP and pUCp2::eGFP were obtained from a previous study in which an enhanced green fluorescent protein (EGFP) promoter fusion was constructed to visualize the activity of the LHR promoter 63 bp upstream of *orf1* (Mercer et al., 2017).

Table 2. Bacterial strains used in this study

Strains	Description	Reference
E. coli		
DH5α	Chemical competent cells and cloning	ATCC
Top10	Source of pUC19	ATCC
JM101	Source of pRK	ATCC
MG1655	K-12 lab strain; LHR negative	ATCC
AW1.7	LHR-positive food isolate	Dlusskaya et al., 2011
AW1.3	LHR-positive food isolate	Dlusskaya et al., 2011
7039	LHR-positive food isolate	Webster, 2018
Δ*evgA* (Keio: JW2366)	EvgA deficient derivative of MG1655	Baba et al., 2006
Δ*ompR* (Keio: JW3368)	OmpR deficient derivative of MG1655	Baba et al., 2006
Δ*fadR* (Keio: JW1176)	FadR deficient derivative of MG1655	Baba et al., 2006
Δ*crp* (Keio: JW5702)	Crp deficient derivative of MG1655	Baba et al., 2006
Δ*purR* (Jeio: JW1650)	PurR deficient derivative of MG1655	Baba et al., 2006
Δ*oxyR* (Keio: JW3933)	OxyR deficient derivative of MG1655	Baba et al., 2006
S. enterica		
ATCC 13311	serovar Typhimurium; LHR negative	ATCC
ATCC 43845	serovar Senftenberg; LHR positive	ATCC

Table 3. Plasmids used in this study

Plasmid	Description	Reference
pUC19	High copy number plasmid	Sigma
pRK767	Low copy number plasmid	Gill and Warren, 1988
evgAS-pRK	*evgA* and *evgS*, including promoter cloned into pRK767	This study
pLHR	full length LHR including promoter cloned into pRK767	Mercer et al., 2015
pUC::eGFP	*egfp* cloned into pUC19	Mercer et al., 2017
pUCp2::eGFP	*egfp* cloned in-frame with the promoter sequence upstream of *orf1*	Mercer et al., 2017
pkD46	red recombinase expression plasmid	Datsenko and Wanner, 2000
pCA24N	high copy number; IPTG inducible plasmid	Kitagawa et al., 2005
fadR-pCA24N	*fadR* cloned into pCA24N	Kitagawa et al., 2005
purR-pCA24N	*purR* cloned into pCA24N	Kitagawa et al., 2005
crp-pCA24N	*crp* cloned into pCA24N	Kitagawa et al., 2005
ompR-pCA24N	*ompR* cloned into pCA24N	Kitagawa et al., 2005
rpoD-pCA24N	*rpoD* cloned into pCA24N	Kitagawa et al., 2005
evgA-pCA24N	*evgA* cloned into pCA24N	Kitagawa et al., 2005

Growth conditions, media and antibiotics.

All cultures were stored at -80 °C in Luria-Bertani (LB) medium (BD Difco, Fisher Scientific, Ottawa, CA) with 25% glycerol (v/v) in 2 mL screw cap tubes (Starstedt, Montreal, Quebec). Bacterial cultures were inoculated using sterilized plastic loops (INO-LOOP, Daigger Scientific, USA) or a metal loop from a -80 °C stock culture or a single colony. Strains were grown in LB medium with antibiotics for plasmid maintenance when necessary, at 37 °C for 16 h with agitation at 200 rpm. LB agar plates were prepared with LB broth and 1.5% granulated agar (BD Difco, Fisher Scientific, Ottawa, CA). Based on different antibiotic resistance genes in the chromosome or plasmid, strains were selected on LB agar supplemented with antibiotics. Antibiotics were dissolved in nuclease-free water (Invitrogen, Carlsbad, USA) or 100% ethanol, filter sterilized, and stored at -20 °C for up to a year. Antibiotics, solvents, and concentrations are listed in Table 4. The Keio strains were grown in LB with kanamycin-sulfate. Strains with the pCA24N plasmids from the ASKA collection were selected using chloramphenicol. Strains with

pRK767 based plasmids, including pLHR were selected using tetracycline-HCl. Strains with pKD46 and pUC19 based plasmids were selected using ampicillin.

Table 4. Antibiotic concentrations and solvents

Antibiotic	Solvent	Stock concentration (g/L)	Working concentration (mg/L)
Chloramphenicol	ethanol	34	34
Kanamycin sulfate	water	50	25
Tetracycline-HCl	ethanol	15	15
Ampicillin	water	100	50

Construction and transformation of *evgAS*-pRK plasmid.

To construct an *evgAS*-pRK plasmid to complement *E coli* MG1655 Δ*evgA*, primers were designed (Benchling, San Francisco, USA) to amplify the DNA sequence containing the promoter, *evgA* and *evgS*. All primers used in this experiment are listed in Table 5. The Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) was used to extract genomic DNA from 5 mL of *E. coli* MG1655 culture that had been incubated at 37 °C for 16 h with agitation at 200 rpm. The genomic DNA was subsequently used as the PCR template with the following PCR conditions: 98 °C for 10 s (initial denaturation); 30 cycles of 98 °C for 10 s (denaturation); 55-65 °C for 30 s (annealing); 72 °C for 30 s/kb (extension); followed by 72 °C for 10 min (final extension) and 4 °C hold. PCR amplification was carried out using Phusion™ High Fidelity DNA polymerase (Thermo Scientific, Ottawa, CA) according to manufacturer's guidelines and a PCR machine (Mastercycler® epgradient, Eppendorf, Hamburg, Germany). The products from the PCR reaction were confirmed using gel electrophoresis at 115V for 60 min on a 1% agarose 0.5X TBE (Tris, borate, EDTA) and visualized by UV transillumination. The PCR amplicon was purified using the GeneJET Gel Extraction and DNA Cleanup Microkit (Thermo Scientific, Ottawa, CA) according to manufacturer's guidelines and quantified using the UV-Vis spectrophotometer (NanoDrop™ One, Thermo Fisher, Madison, USA). by measuring light absorption at 260 nm. The purified PCR amplicon and pRK767 were

digested with KpnI and HindIII (Thermo Fisher Ottawa, CA) at 37 °C for one hour and the restriction enzymes were inactivated at 80 °C for 5 min. Ligation of the PCR amplicon into pRK767 was done using T4 DNA Ligase (Thermo Scientific) according to the manufacturer's guidelines and a 3:1 molar ratio of vector to insert. The ligation mixture was incubated at 4 °C for 16 h and then directly used for downstream transformation. The cloning scheme is illustrated in Figure 1.

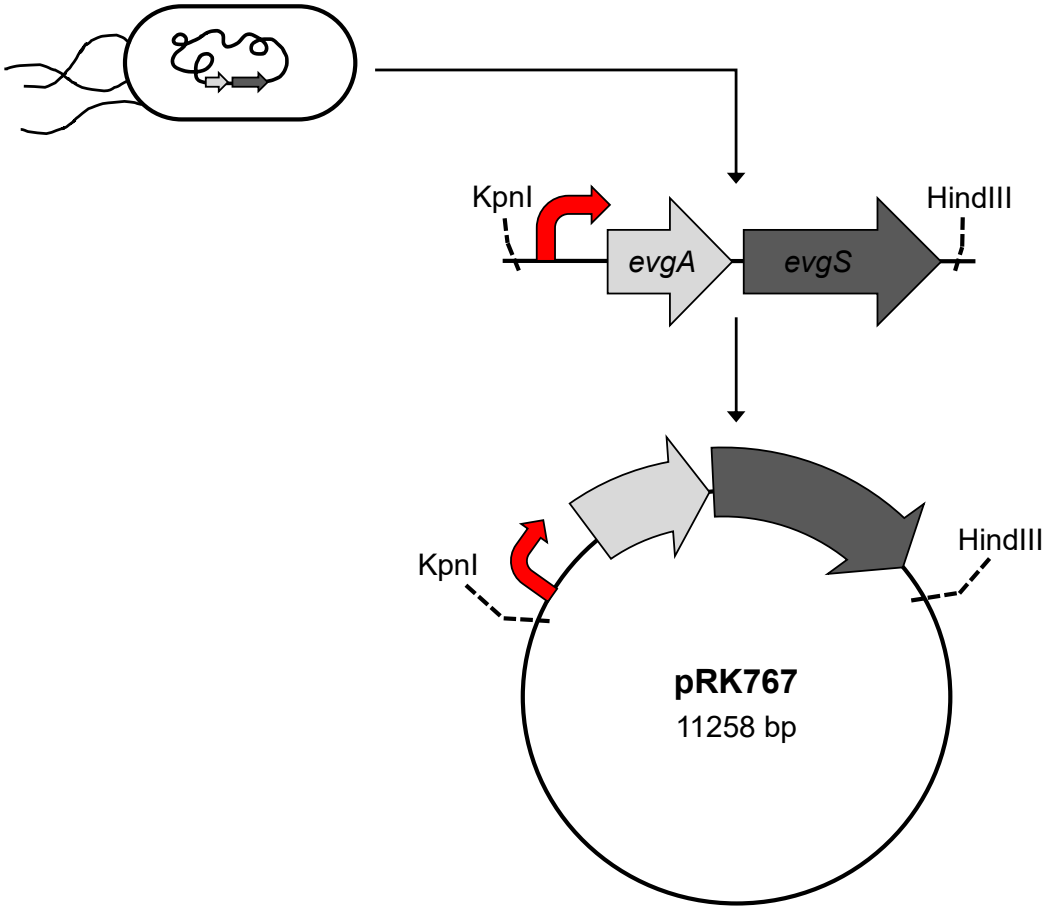

Figure 1. The cloning schematic of *evgAS*-pRK construction. PCR amplification from *E. coli* MG16655 gDNA produced a DNA fragment with *evgAS* and its promoter region. The directional arrows show the transcription orientation. The red represents the native promoter; the light grey is *evgA* and the dark grey is *evgS*. The dashed lines indicate the restriction enzyme sites. The DNA fragment was ligated into a pRK767 vector using KpnI and HindII.

For chemical competent cells, a starter culture of *E. coli* DH5α was incubated for 16 h at 37 °C with agitation at 200 rpm. The starter culture was diluted 1/100 into 100 mL LB broth and incubated at 37 °C with 200 rpm until the OD_{600} was between 0.5 and 0.7. The culture was divided into two 50 mL conical tubes (Starstedt, Montreal, Quebec), incubated on ice for 5 min, centrifuged at 8000 *x g* for 5 min and resuspended with 30 mL of 80 mM $MgCl_2$-20 mM $CaCl_2$. After 15 min incubation on ice, the cultures were centrifuged at 8000 *x g* for 5 min and resuspended with 1 mL 0.1 M CaCl2-15% glycerol. The cells were dispensed into 50 µL aliquots to be used immediately or stored at -80°C. For the chemical transformation, 5 µL of the ligation reaction mixture was added to 50 µL *E. coli* DH5α chemical competent cells. After 30 min incubation on ice, the mixture was placed in a 42 °C water bath for 60 s, an immediately transferred to ice. SOC (super optimal broth with catabolite repression) medium (2% tryptone, 0.5% yeast extract, 8.56 mM NaCl, 250 mM KCl, 2M $MgCl_2$, 1M glucose) was added (950 µL) and cells were recovered by incubation at 37 °C for 1 h with 200 rpm. One-tenth of the recovered cells were spread on agar plates with tetracycline to select for transformants and the remaining cells were pelleted and spread onto another agar plate. Colonies grown on the antibiotic selective LB agar were subjected to plasmid isolation using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Edmonton, CA) and confirmed by restriction digests. Additionally, the plasmid construct was sent to the Molecular Biology Service Unit (MBSU, University of Alberta, Edmonton, CA) and verified by Sanger DNA Sequencing using six primer pairs throughout *evgAS* (Table 5).

Electroporation was used to transform the plasmid into other strains of *E. coli.* For electrocompetent cells, a starter culture of *E. coli* was incubated for 16 h at 37 °C with agitation at 200 rpm. The starter culture was diluted 1/100 into 5 mL LB broth and incubated at 37 °C with agitation at 200 rpm until the OD_{600} was between 0.4 and 0.6. The cells were incubated on ice for 15 min and harvested at 14 000 *x g* and 4 °C for 5 min. The pelleted cells were washed three times with 1 mL of cold 10% glycerol with a centrifugation step at 14 000 *x g* and 4 °C for 1 min between each wash. After the last wash, cells were resuspended in 500 µL of cold 10% glycerol and subsequently, 100 µL was transferred into a pre-chilled 0.1 cm gap electroporation cuvette (Gene Pulser®/MicroPulser™, Bio-Rad, Mississauga, CA). Plasmid DNA (100 ng) was added to the electrocompetent cells and the mixture was electroporated with the transformation

apparatus (*E. coli* Pulser™, Bio-Rad, Mississauga, Canada) at 25 µFD, 200 Ω, 2.5kV. Electroporated cells were transferred into 950 µL SOC media and recovered at 37 °C for 1 h with 200 rpm. One-tenth of the recovered cells were spread on an agar plate with tetracycline to select for transformants and the remaining cells were pelleted and spread onto another agar plate.

Table 5. Primers used in constructing and sequencing of *evgAS*-pRK

Product	Primer	Sequence (5'-3')	Size (bp)	Reference
evgAS	evg-F (KpnI)	TGCAGGTACCACTATAAATCATCGGTAC	4457	This study
	evg-R (HindIII)	CGTAAGCTTTCCCACATTTGAACATTG		
	evg-F1	CCGGTTTGTTGGAAGTTTAACG		This study
	evg-R1	GCTGGTCGGACGTTAAACTT		This study
	evg-F2	GTAACCACCCTTCACGACTC		This study
	evg-R2	ATTGAGTCGTGAAGGGTGG		This study
	evg-F3	GGGATATAATACCTGGCGCTA		This study
	evg-R3	GGCGTTGTTATGAAGGCTTCA		This study
	evg-F4	TACCTGTTACGCTCAGTTCG		This study
	evg-R4	CCCTGAATGACTTTACGACGA		This study
	evg-F5	TCACTCCTCGGCTTAATTGG		This study
	evg-R5	GTAGGGATATCGACCCATTG		This study
	evg-F6	GCTATTACTCAAACGCCAGC		This study
	evg-R6	GCATACTGACTTTGTGTAGCGC		This study

Measuring fluorescence using promoter reporter assay.

To complement the EvgA deficient strain and determine promoter activity, the *evgAS*-pRK plasmid was transformed into electrocompetent *E. coli* MG1655 Δ*evgA* (pUCp2::eGFP) and *E. coli* MG1655 Δ*ompR* (pUCp2::eGFP) as described above. To measure EGFP fluorescence, cultures were incubated at 37 °C for 16 h with agitation at 200 rpm, then diluted to an OD_{600} of 1 and placed into a 96-well microtiter plate in

duplicate. The relative fluorescence of cultures was quantified using a fluorescent plate reader (Varioskan Flash, Thermo Scientific) at an excitation wavelength of 488 nm and an emission wavelength of 509 nm. A photometric reading at 600 nm was obtained to measure the OD_{600} values of the samples. The LHR promoter expression was evaluated by calculating the fluorescence intensity of EGFP in *E. coli* carrying pUCp2::eGFP relative to the promoter-less pUC::eGFP. The relative fluorescent units per OD was replicated three times for each strain.

Construction of *evgA* knock out mutants.

To construct EvgA deficient *E. coli* AW1.7 and 7039, both strains were transformed with the pKD46 plasmid (Datsenko and Wanner, 2000) using electroporation as previously described above and selecting transformants on LB agar with ampicillin. PCR primers were designed (Benchling, San Francisco, USA) to amplify 700 bp outside of the deleted *evgA* region of *E. coli* MG1655 Δ*evgA* (Table 6). The DNA template was gDNA isolated from *E. coli* MG1655 Δ*evgA* using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA). PCR amplification was done as described above. Both strains carrying the pKD46 plasmids were grown in SOC medium with ampicillin and 1 mM arabinose at 30 °C until an OD_{600} of 0.4-0.6 was reached. Production of electrocompetent cells and electroporation of the Δ*evgA* DNA fragment were done as described above with the exception of using SOC medium supplemented with 1 mM arabinose. DNA was isolated from colonies to confirm successful recombination with PCR.

Table 6. Primers used in construction of *evgA* knock out mutant in *E. coli* AW1.7 and 7039

Product	Primer	Sequence (5'-3')	Size (bp)	Reference
Δ*evgA*	evgA-KO-F	GTATCTTTGGTATGCTCCAGCG	2754	This study
	evgA-KO-R	TTATATTGACGCGGCGAGTTAT		

Determination of heat resistance of strains.

To determine the heat resistance of strains with overexpressed EvgA and EvgS, electrocompetent LHR positive and negative strains of *E. coli* and *Salmonella* were transformed with *evgAS*-pRK using electroporation as described above. For the heat treatment, cultures were grown in 5 mL LB medium with

tetracycline-HCl at 37 °C for 16 h with agitation at 200 rpm. Each culture (100 µL) was transferred into 0.2 mL PCR tubes (Axygen, Corning, USA) and heated using a PCR machine (Mastercycler® epgradient, Eppendorf, Hamburg, Germany). Both LHR positive and negative strains of *E. coli* and *Salmonella* were subjected to heat at 60 °C for 5, 10, and 20 min. Two LHR negative strains, *E. coli* MG1665 and *S.enterica* ATCC 13311 were treated at 55 °C and 50 °C respectively, for 5, 10, 20, and 40 min. Strains with empty vector pRK767 served as controls. After heat treatment, serial 10-fold dilutions were made of each strain in LB broth, strains were plated onto LB agar and incubated at 37 °C for 16 h. The reduction of cell counts was determined by comparing the colony forming units (CFUs) from the untreated strains to the heat-treated strains. Experiments were replicated three times for each strain.

To determine the heat resistance of strains with overexpressed Crp, OmpR, EvgA, PurR, FadR, OxyR and RpoD, strains from the ASKA collection with the pCA24N plasmids were obtained and plasmids were isolated using GeneJET Plasmid Miniprep Kit (Thermo Scientific, Edmonton, CA) (Kitagawa et al., 2005). Electrocompetent *E. coli* MG1655 (pLHR) were transformed with the pCA24N plasmids using electroporation as described above. Strains carrying pCA24N ASKA plasmids were grown in LB broth with chloramphenicol and 0.01 mM or 0.1 mM IPTG at 37 °C for 16 h with agitation at 200 rpm. Strains were subjected to heat at 60 °C for 5 min and dilutions, plating and cell counts were done as described above.

Identification of Keio strains using PCR.

To identify the Keio strains, DNA was isolated from *E. coli* MG1655 Δ*evgA* and Δ*ompR* with and without pLHR and the other Keio strains using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA). The pLHR plasmids were isolated from the strains using GeneJET Plasmid Miniprep Kit (Thermo Scientific, Edmonton, CA). Primers were designed to amplify from a middle of the deleted region (P2-F) to 500-700 bp downstream (Table 7). Recombinant Taq polymerase (Thermo Scientific, Ottawa, CA) was used according to the manufacturer's guidelines with the following gradient PCR conditions: 95 °C for 1 min (initial denaturation); followed by 30 cycles of 95 °C for 30 s (denaturation); 54 °C, 57 °C, and 60 °C for 30 s (annealing); 72 °C for 1 min/kb; followed by 72 °C for 10 min (final extension) and 4 °C hold.

To confirm the presence of the pLHR in the Keio strains, multiplex PCR was used to amplify three fragments of LHR using three primer pairs (Table 7). DreamTaq Green PCR Master Mix was used according to manufacturer's guidelines with the same PCR conditions as recombinant Taq polymerase but an annealing temperature of 66°C. The pLHR and pRK plasmids were included as the positive and negative controls, respectively. PCR amplicons were analyzed using gel electrophoresis. Additionally, the plasmid miniprep sample was sequenced (MBSU, University of Alberta, Edmonton, Canada) using M13 primers (Addgene, Watertown, USA).

Table 7. Primers used to identify Keio mutants and pLHR screen

Product	Primer	Sequence (5'-3')	Size (bp)	Reference
	P2-F	CGAAGCAGCTCCAGCCTACA		Datsenko and Wanner, 2000
	evgA-R (KEIO)	GCTCTAGATTATATTGACGCGGCGAGTTAT	734	This study
	ompR-R (KEIO)	GGTGAGTCACGCCATCGTCG	531	This study
	oxyR-R (KEIO)	CGGACAGCTGAAGGTCAACAGC	514	This study
	fadR-R (KEIO)	TTCCGACTGGCTGGAAACGC	490	This study
	purR-R (KEIO)	GTTCAACGGTGTCTGGGATGCG	543	This study
	crp-R (KEIO)	CGTACCAGAGAGTGCCCAACGC	486	This study
LHR Fragment 1	1981-F	GCCCGGTGTCGAGGAGAAGG	900	This study
	2881-R	AAGAATGGCCGAGTTCATTGGAGG		
LHR Fragment 2	7561-F	GCGCGATGCCAAGCAGAACG	1500	This study
	9060-R	TGAACGCGCCATTGACCAAGG		
LHR Fragment 3	11281-F	GGAGACGCTGAGCTTTCTGTCCG	2000	This study
	13290-R	CGCAGCAGCCAGTAGGTCG		
	M13-F	TGTAAAACGACGGCCAGT		Addgene
	M13-R	CAGGAAACAGCTATGAC		

Using RT-qPCR to measure gene expression of LHR genes in strains with *evgAS*-pRK.

To determine the expression levels of LHR genes in response to the overexpression of EvgA and EvgS, primers were designed to target *orf1, shsp20, clpK, shsp, hdeD, orf11,* and *trx* (Table 3). Bacterial cultures were incubated at 37°C until mid-log phase growth corresponding to an OD_{600} of 0.5 or approximately 2 h.

RNAprotect cell reagent (Qiagen, Toronto, Canada) was added to the culture (2:1), incubated at 22°C for 10 min and centrifuged at 5000 *x g* for 15 min. The supernatant was decanted, and cell pellets were used to extract RNA with the RNeasy mini kit (Qiagen, Toronto, Canada). The RNA was eluted with 30 µL of nuclease-free water (Invitrogen, Carlsbad, USA) and quantified using the UV-Vis spectrophotometer (NanoDrop™ One, Thermo Fisher, Madison, USA). DNase digestion and first strand cDNA synthesis were carried out using a QuantiTect reverse transcription kit (Qiagen, Toronto, Canada). Quantifast Sybr Green PCR kit (Qiagen, Toronto, Canada) was used for qPCR with the following protocol: 95°C for 5 min (initial denaturation); followed by 40 cycles of 95°C for 30 s (denaturation); 60°C for 30 s (annealing); and 72°C for 30 s (extension); followed by a melting curve stage of 95°C for 15 s; 60°C for 1 min; and 95°C for 15 s. Each qPCR plate included controls containing no template or no reverse transcriptase. The C_T value is the number of cycles required for the fluorescent signal to cross the threshold or exceed the background level which was determined by the StepOnePlus™ software. The relative gene expression data was analyzed using *gapA* as a housekeeping gene. The relative gene expression was determined according to the following equation (Pfaffl, 2001):

$$\frac{E_{target}{}^{\Delta C_T(reference-sample)}}{E_{gapA}{}^{\Delta C_T(reference-sample)}}$$

where E_{target} refers to a PCR efficiency of 2 and ΔC_T is the threshold cycle for the samples obtained at sample and reference conditions. Gene expression in strains containing *evgAS*-pRK (sample) were quantified relative to strains containing pRK767 (reference). The relative gene expression was quantified from three biological replicates.

Identification of promoters within the LHR sequence.

To identify promoters in the LHR, promoter prediction was done using CNNPromoter_b, which predicts bacterial promoters using convolutional neural network models in genomic sequences (Bedoya et al., 2011). The query sequence was *E. coli* AW1.7, whole genome shotgun sequence, contig NZ_LDYJ01000141.1 and the model was *Escherichia coli.*

Overexpression of proteins to induce promoter.

To determine if overexpression of Crp, OmpR, EvgA, PurR, FadR, OxyR or RpoD would increase promoter activity, strains with pCA24N plasmids containing each protein from the ASKA collection were used. Each plasmid was isolated and their identities confirmed with primers (Table 8) and gradient PCR as previously described. Strains carrying the pCA24N plasmids were grown in LB media with chloramphenicol and 0.1 mM IPTG. RNA extraction, cDNA conversion, and qPCR conditions were the same as previously described. qPCR primers were designed to target *orf1, shsp20, clpK, shsp, hdeD, orf11,* or *trx* (Table 9). Gene expression of *orf1* and *shsp20* were quantified in strains containing Crp, OmpR and EvgA pCA24N plasmids. Gene expression of *shsp20* and *clpK* was quantified for the strains containing the PurR pCA24N plasmid; *hsp* was quantified in the strain with the FadR pCA24N plasmid; *hdeD* and *orf11* were quantified in the strain with the RpoD pCA24N plasmid and *trx* was quantified in the strain containing the OxyR pCA24N plasmid.

Table 8. Primers used to identify genes on the pCA24N plasmids from the ASKA collection

Product	Primer[1]	Sequence (5'-3')	Size (bp)
fadR	fadR-F	GCCGTCATTAAGGCGCAAAGCCC	720
	fadR-R	CCTCGCCCCTGAATGGCTAAATC	
crp	crp-F	GCCGTGCTTGGCAAACCGCAAAC	633
	crp-R	CCACGAGTGCCGTAAACGACGAT	
rpoD	rpoD-F	GCCGAGCAAAACCCGCAGTCACA	1842
	rpoD-R	CCATCGTCCAGGAAGCTACGCAG	
evgA	evgA-F	GCCAACGCAATAATTATTGATGA	615
	evgA-R	CCGCCGATTTTGTTACGTTGTGC	
ompR	ompR-F	GCCCAAGAGAACTACAAGATTCT	720
	ompR-R	CCTGCTTTAGAGCCGTCCGGTAC	
purR	purR-F	GCCGCAACAATAAAAGATGTAGC	1026
	purR-R	CCACGACGATAGTCGCGGAACGG	
oxyR	oxyR-F	GCCAATATTCGTGATCTTGAGTA	918
	oxyR-R	CCAACCGCCTGTTTTAAAACTTT	

[1] Kitagawa et al., 2005

Table 9. Primers used to measure expression of LHR genes using qPCR

Product	Primer	**Sequence (5'-3')**	Size (bp)	Reference
orf1	orf1-F	TTGAAAGTCGGCAAGCGGACG	152	This study
	orf1-R	CGCATCACCTCCTTCTTTGCTCC		
shsp20	orf2-F	GGACATCCAGGAAACCGACAAGC	102	This study
	orf2-R	ACCATCAGCACGTCGTTGTCG		
clpK	orf3-F	ACACCATCATCATCGCCACGTC	164	This study
	orf3-R	GATCTCGTCGATGCGGTTGAGG		
shsp	orf7-F	GTTCGGACGAATACCCCAGCGA	124	This study
	orf7-R	CAGATCCAGCTTGAGGAGGAATGC		
hdeD	orf10-F	GTTCAACTTGTTTGCAGCGGTGC	117	This study
	orf10-R	ACCCAAGGAGCAACCAGGACG		
orf11	orf11-F	AACCGGATGCCCAAGACGTG	164	This study
	orf11-R	CTCGCTCGTCCGATCAATCAGC		
trx	orf12-F	ATCAACCGCGTACCGACTGC	151	This study
	orf12-R	AGAAATCGACCAGCAGCGGC		

Using RT-qPCR to measure gene expression of LHR genes in Keio mutants.

To determine the level of gene expression of LHR genes in strains deficient in Crp, OmpR, EvgA, PurR, FadR, or OxyR, the pLHR plasmid was transformed into electrocompetent Keio mutants and selected for using kanamycin-sulfate and tetracycline-HCl as previously described above. The plasmids were isolated, and the presence of the regulatory gene was confirmed using PCR and gel electrophoresis. Keio strains with the pLHR constructs were grown in LB media containing tetracycline-HCl. The RNA extraction, cDNA conversion, and qPCR conditions were the same as previously described. Keio strains (sample) were quantified relative to the wildtype strain, *E. coli* MG1655 (reference). Additionally, *E.* coli MG1655 (pLHR), *E.* coli MG1655 Δ*fadR* and Δ*oxyR* Keio mutants were treated with 1mM of NaClO or 2.5 mM H_2O_2 for 40 min before RNA extraction. Treated Keio mutants (sample) were quantified relative to the untreated Keio mutants (reference). Gene expression of *shsp20, shsp,* and *trx* were measured. Cell counts before and after treatment with NaClO or H_2O_2 were performed on LB agar.

Statistical analyses.

Experiments were repeated at least three times with triplicate samples within each replicate for qPCR experiments. All microbiological counts were converted to $\log_{10}$ prior to data analysis. Data were subjected

to one-way or two-way analysis of variance using the PROC GLM procedure of Statistical Analysis Software (Version 3.4 and University Edition; SAS Institute. Inc., Cary, NC, USA). Differences among means were determined using Tukey's multiple range test with $P<0.05$.

3. Results

Measuring fluorescence using a promoter reporter assay.

It was previously demonstrated that heat resistance was lost in *E. coli* MG1655 Δ*evgA* (pLHR) compared to the wildtype strain (Mercer et al., 2017). Additionally, the GFP fusion of the promoter upstream of *orf1* (pUCp2::eGFP) had decreased fluorescence and promoter activity in the EvgA deficient strain compared to the wildtype strain (Mercer et al., 2017). To validate EvgA as the regulatory component of the promoter upstream of *orf1*, a plasmid complement was constructed to restore the fluorescence phenotype in *E. coli* MG1655 Δ*evgA*. The relative fluorescence of *E. coli* MG1655 Δ*ompR* was more than two times higher than in *E. coli* MG1655 Δ*evgA* (Figure 2). The relative fluorescence of *E. coli* MG1655 Δ*evgA* with *evgAS*-pRK complement was similar to that of *E. coli* MG1655 Δ*evgA* (Figure 2). The *evgAS*-pRK complement was not able to restore the fluorescence phenotype.

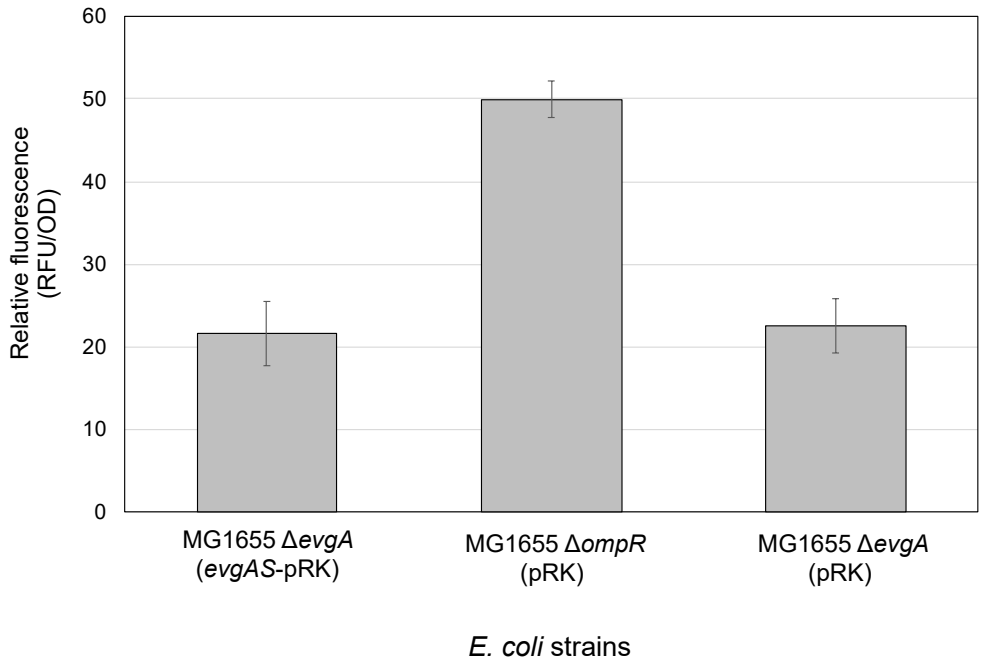

Figure 2. Effect of EvgA and EvgS plasmid complement on the fluorescence of the EGFP promoter fusion in Keio strains. Fluorescence of EvgA and OmpR deficient Keio strains carrying pUCp2::eGFP, a plasmid with EGFP expressed under the control of a LHR derived promoter fusion. The fluorescence was determined in strains carrying pUCp2::eGFP or the promoterless control pUC::eGFP, a EGFP plasmid. The EvgA deficient strain was complemented with *evgAS*-pRK and promoter activity was visualized with fluorescence and compared to the EvgA and OmpR deficient strain with an empty vector, pRK. Data are means ± standard deviation of three replicates.

Identification of Keio strains.

To identify the Keio strains, the DNA region flanking the knock out gene location was amplified using PCR and multiplex PCR was used to screen for the presence of the three LHR fragments. Both *E. coli* MG1655 Δ*evgA* and *E. coli* MG1655 Δ*ompR* with and without pLHR had a DNA fragment corresponding to the amplified Δ*evgA* (700 bp) and Δ*ompR* region (1500 bp), respectively (Figure 3). The other Keio strains were confirmed to have the correct deletions (data not shown). To screen for the presence of pLHR, a plasmid miniprep sample and a gDNA sample were used as DNA templates. In both templates, *E. coli* MG1655 Δ*ompR* (pLHR) had three DNA fragments (900, 1500 and 2000 bp) corresponding to each fragment of the LHR (Figure 3). In both templates for *E. coli* Δ*evgA* (pLHR), the pLHR was not amplified (Figure 3). The sequence analysis of the plasmid miniprep sample showed no sequences corresponding to the LHR between the M13 cloning regions of pRK767 which suggests that the LHR is not located on the plasmid. Therefore, the pLHR plasmid was absent from the MG1655 Δ*evgA* (pLHR) strain either from improper plasmid transformation or loss of the plasmid from not using the selective pressure of antibiotics.

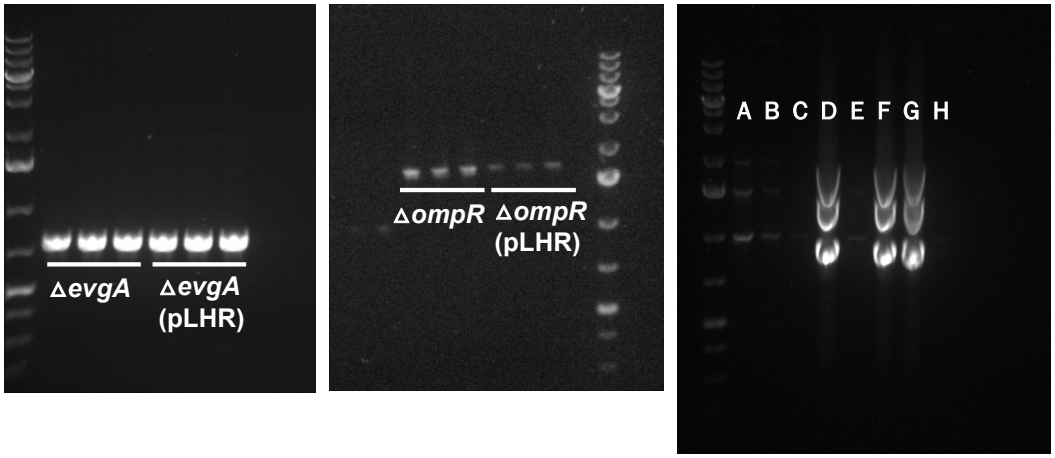

Figure 3. Identification and presence of pLHR in Keio strains. Gradient PCR amplification of deleted region of EvgA and OmpR deficient Keio mutants and LHR screening of strains carrying pLHR. Lanes A-D are plasmid templates, E and F are gDNA templates and G and H are plasmid controls. Templates in each lane are Δ*evgA* (A), Δ*evgA* (pLHR) (B), Δ*ompR* (C), Δ*ompR* (pLHR) (D), Δ*evgA* (pLHR) (E), Δ*ompR* (pLHR) (F), pLHR (G) and pRK (H). Amplicons were loaded on a 1% agarose 0.5X TBE gel, ran for 60 min at 115V and stained with SYBR™ Safe. GeneRuler™ 1kb plus DNA ladder was used to identify the approximate size of the bands (kb). EvgA deficient strains had DNA fragments corresponding to 700 bp and OmpR deficient strains had DNA fragments corresponding to 1500 bp. Strains with pLHR had DNA fragments corresponding to 900, 1500, and 2000 bp.

Construction of *evgA* knock out in *E. coli* AW1.3 and 7039.

To determine the effect of EvgA and EvgS on the expression of the native chromosomal LHR, the phage lambda-derived Red recombination system was used to generate a deletion mutation of *evgA*. Colonies were observed on ampicillin agar plates and therefore had taken up the pKD46 plasmid. After the recombination event, four colonies of *E. coli* 7039 were present on kanamycin agar plates. PCR amplification of the region containing the *evgA* deletion showed no visible band after gel electrophoresis. This indicated that the PCR fragment containing the *evgA* deletion did not recombine to replace the chromosomal copy of *evgA.*

Determination of heat resistance of strains with *evgAS*-pRK.

To investigate if overexpression of EvgA and EvgS had a phenotypic effect on heat resistance, *evgAS*-pRK was transformed into LHR positive and negative strains of *E. coli* and *Salmonella*. The strains were subjected to heat to determine survival. In LHR positive strains, *E. coli* AW1.3 and 7039 had more reduction of cell counts with *evgAS*-pRK compared to the strains with the empty vector after 5, 10, and 20 min of heating (Figure 4). *S. enterica* ATCC 43845 with *evgAS*-pRK had a similar reduction of cell counts after 5, 10, and 20 min compared to the strain with the empty vector (Figure 4). The cell counts of LHR negative strains, *E. coli* MG1655 and *S. enterica* ATCC 13311 with or without *evgAS*-pRK, were reduced to below the detection limit after 5, 10 and 20 min (Figure 4). Therefore, EvgA overexpression had an effect in *E. coli* because the EvgA regulon is present in *E. coli,* but EvgA had no effect on *Salmonella* because it lacks the EvgA regulon. In LHR positive *E. coli*, EvgA overexpression caused a loss of heat resistance.

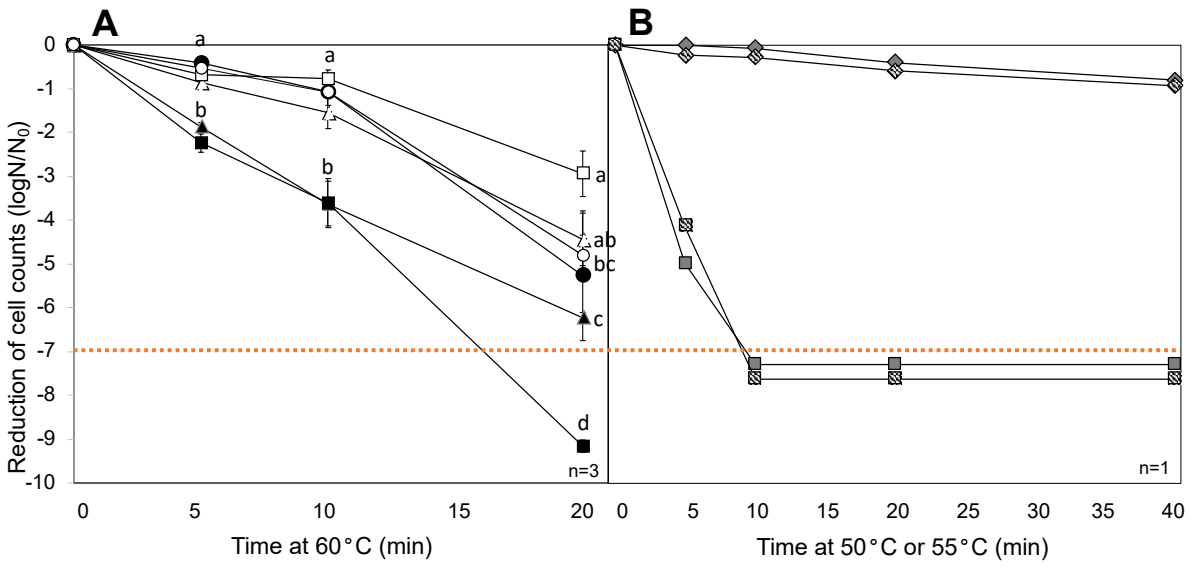

Figure 4. Effect of overexpressed EvgA and EvgS on heat resistance of LHR positive and negative strains of *E. coli* and *Salmonella.* Reduction of cell counts after heat treatment at A) 60 °C or B) 50 °C and 55 °C for 5, 10, 20, and 40 min. Strains carrying *evgAS*-pRK are *E. coli* AW1.3 (▲), *E. coli* 7039 (■), *S.enterica* ATCC 43845 (●), *E. coli* MG1655 (◆) and *S. enterica* ATCC 13311 (■). Strains carrying pRK are *E. coli* AW1.3 (△), *E. coli* 7039 (□), *S. enterica* ATCC 43845 (○), *E. coli* MG1655 (▨) and *S. enterica* ATCC 13311 (▨). Data are means ± standard deviation of three replicates (A). Letters indicate significant ($p<0.05$) differences of counts among strains at each time point. The red dashed line denotes the detection limit.

Gene expression of strains with *evgAS*-pRK.

To investigate if EvgA and EvgS overexpression had a genotypic effect on the LHR, expression of the first two orfs of the LHR were measured in strains of *E.* coli and *Salmonella* that contained *evgAS*-pRK (Figure 5). In *E. coli* AW1.3, overexpression of *evgAS* downregulated both *orf1* and *shsp20* (Figure 5). In contrast, both *orf1* and *shsp20* were upregulated in *E. coli* 7039 when *evgAS* was overexpressed (Figure 5). In *S. enterica* ATCC 43845, *orf1* and *shsp20* were not differentially expressed in response to *evgAS* (Figure 5). The reduced expression of *orf1* and *shsp20* in *E. coli* AW1.3 was consistent with its loss of heat resistance (Figure 4). Overexpression of EvgA in *S. enterica* ATCC 43845 had no effect on gene expression of *orf1* and *shsp20* which paralleled its effect on heat resistance (Figure 4). However, the upregulated gene expression of *orf1* and *shsp20* in *E. coli* 7039 was not consistent with its loss of heat resistance previously reported (Figure 4).

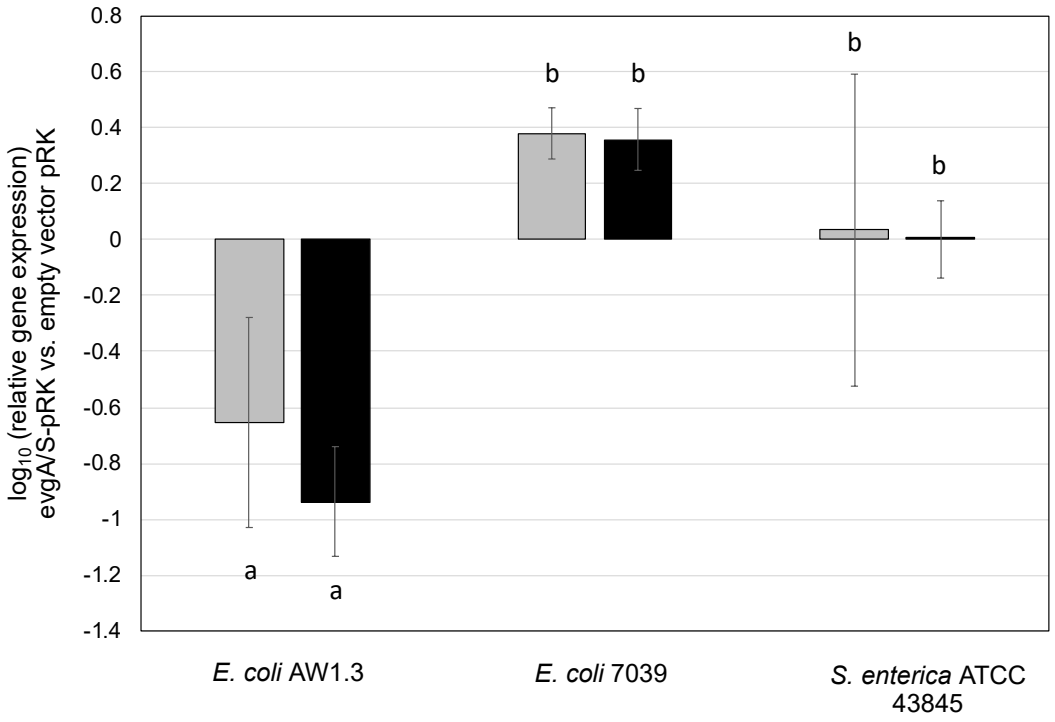

Figure 5. Effect of overexpressed EvgA and EvgS on LHR expression in LHR positive strains of *E. coli* and *Salmonella.* Relative gene expression of *orf1* (grey) and *shp20* (black) in strains with *evgAS*-pRK compared to those carrying pRK grown in LB broth and incubated at 37 °C until mid-log phase. Data are means ± standard deviation of three replicates. Significant ($p<0.05$) differences of each gene among strains are indicated by different letters.

Identification of promoters within the LHR sequence.

To determine the presence of additional promoters in the LHR, promoter prediction was done using CNNPromoter_b, which predicts bacterial promoters in genomic sequences. The program predicted Crp (456-486), OmpR (1279-1311), PurR (1662-1694), FadR (6272-6307), RpoD (8828-8867), and OxyR (10581-10641) (Table 10 Figure 6).

Table 10. Promoter sequences predicted in the Locus of Heat Resistance by CNNPromoter

Promoter	Sequence
Crp	AAATGTAAGGCCTTTGAATAAGACAAAACGC
OmpR	CTTGAAGTTATTCATAGTAGGTCTAATATTACA
PurR	TTCAAGAACGGGGTGCTCACGGTCACGATCGACAAG
FadR	TTCAAGAACGGGGTGCTCACGGTCACGATCGACAAG
RpoD	TTCACCCCATTAGATCTTTAGGAGATATAGCATGAATACA
OxyR	CGGAGTAAGCAAAAACGGGAACGATTGGCTCCAAGGCGTTGATTG GTTGGCATTGTCTGT

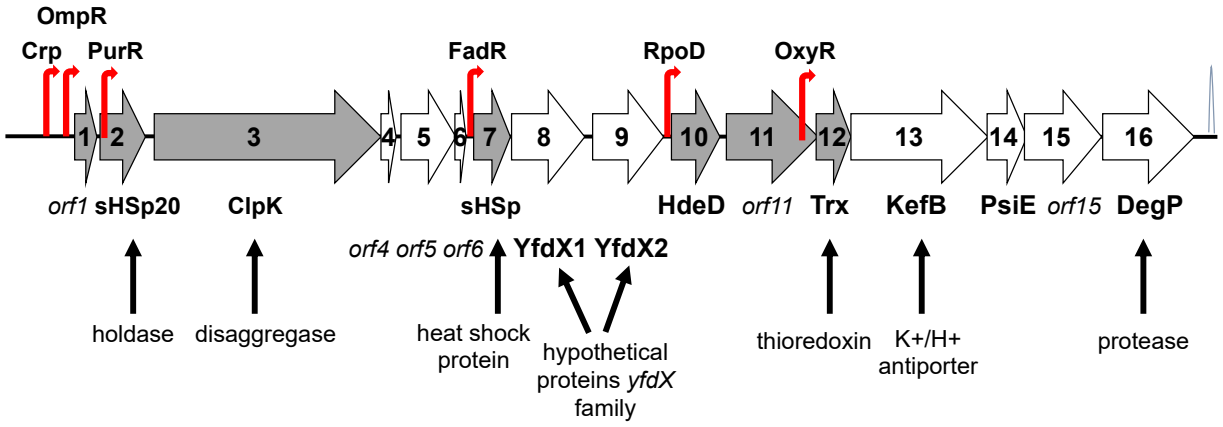

Figure 6. Schematic representation of the locus of heat resistance, putative functions and location of predicted promoters. The genes in grey have primers to measure gene expression. The diagram is based on data from Mercer et al., 2017.

Overexpression of regulatory proteins to induce the promoters in the LHR.

To determine if the overexpression of regulatory proteins would increase the activity of the predicted promoters, pCA24N plasmids from the ASKA library were used to overexpress the regulatory proteins and LHR gene expression was measured. The pCA24N plasmids were confirmed by observing the presence on an agarose gel and then used as a template for PCR to verify that the plasmids contained the correct gene (Figure 7). The promoter expression was determined by measuring the expression of the

downstream gene. Without induction by IPTG, plasmids from the ASKA collection generally did not change gene expression relative to the vector control, i.e., differences in gene expression to the vector control were less than 3-fold and error bars overlapped (Figure 8). EvgA, however, reduced expression of *orf1* and *shsp20* more than 10-fold even in un-induced conditions (Figure 8). For the IPTG induced strains, all the genes were upregulated more than 3 fold except for the EvgA- and OmpR regulated *shsp20* and the OxyR regulated *trx*. Some of the regulatory proteins were able to induce the expression of their promoter and subsequently upregulate the expression of the LHR genes.

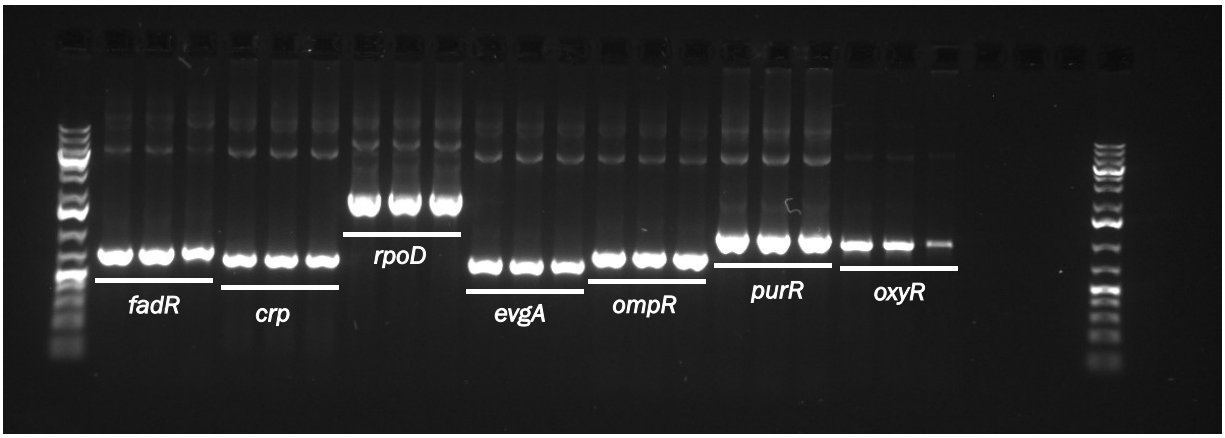

Figure 7. Identification of genes on the pCA24N plasmids from the ASKA library using gradient PCR. Primers designed to amplify each gene was used and DNA amplicons were loaded on a 1% agarose 0.5X TBE gel, ran for 60 min at 115 V and stained with SYBR™ Safe. GeneRuler™ 1kb plus DNA ladder was used to identify the approximate size of the bands (kb).

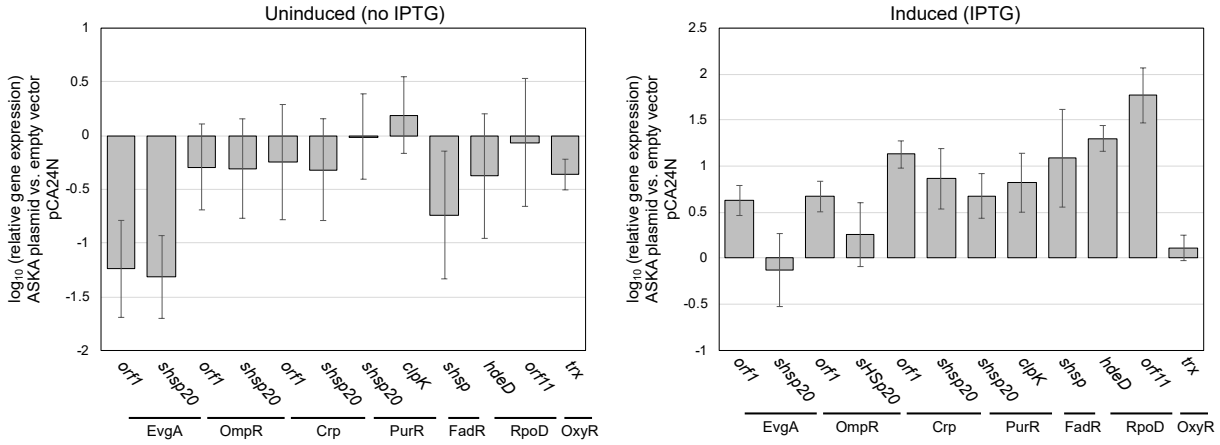

Figure 8. Effect of regulatory proteins on the expression of LHR genes. Relative gene expression of *orf1, hsp20, clpK, hdeE, orf11* and *trx* in *E. coli* MG1655 (pLHR) with pCA24N plasmids from the ASKA collection compared to the empty vector pCA24N grown in LB broth with or without IPTG incubated at 37 °C until mid-log phase. Data are means ± standard deviation of three replicates.

Determination of heat resistance of strains with pCA24N plasmids from the ASKA collection.

The effect of overexpressing regulatory proteins on the heat resistance was examined by treating the strains with the pCA24N plasmids with heat to determine survival. In both 0.01 mM and 0.01 mM IPTG concentrations, the cell counts for *E. coli* MG1655 with the empty vector pRK and pCA24N were reduced to below the detection limit (Figure 9). With the low concentration of IPTG, the difference between the reduction of cell counts for the induced and uninduced strains are smaller than the difference in the cells induced with a high concentration of IPTG with the exception of Crp. Therefore, an increased concentration of IPTG yielded higher expression of the regulatory proteins, which caused a higher reduction of cell counts.

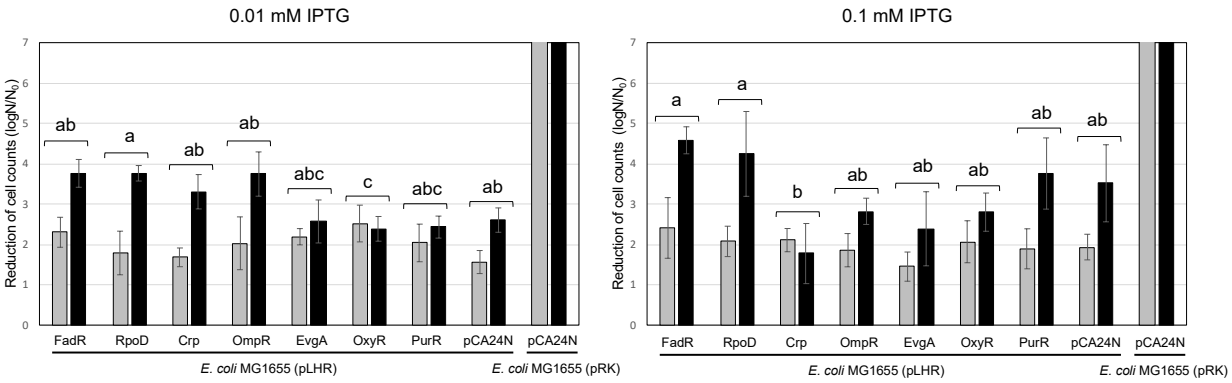

Figure 9. Effect of regulatory proteins on the heat resistance in *E. coli* MG1655. Shown is the reduction of cell counts in *E. coli* MG1655 (pLHR) with different pCA24N plasmids from the ASKA collection. Strains were grown in LB broth in presence of 0.01 mM or 0.1 mM IPTG to the exponential phase of growth prior to heat treatment at 60°C for 5 min. Grey bars are uninduced (no IPTG) and black bars are induced (IPTG). Data are means ± standard deviation of three replicates. For statistical analysis, the difference of cell counts between the uninduced control and the induced strains was calculated. Letters indicate significant ($p<0.05$) differences of the effect of induction between strain carrying different regulatory proteins. Data in the left and the right panels were evaluated separately.

Gene expression of LHR genes in Keio mutants.

To investigate if the absence of the regulatory protein would affect the induction of the predicted promoters, Keio strains were used to measure the expression of the LHR genes. None of the genes was differentially expressed in the Keio mutants (Figure 10). Therefore, in the absence of the regulatory protein, the LHR proteins were still expressed.

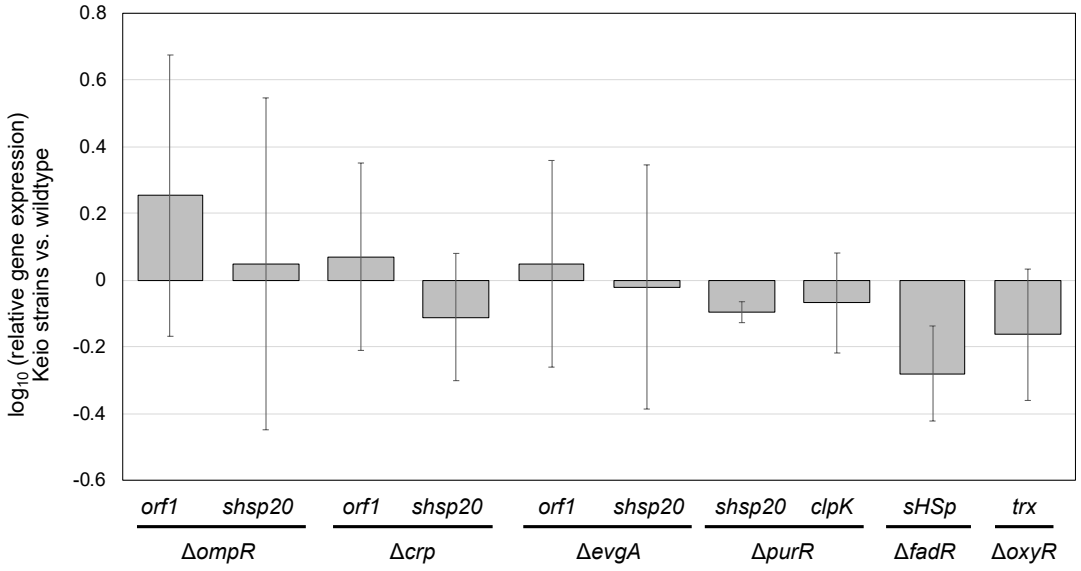

Figure 10. Effect of regulatory proteins on the expression of LHR genes in Keio strains. Relative gene expression of *orf1, hsp20, clpK, hdeE, orf11* and *trx* in Keio mutants compared to wildtype *E. coli* MG1655 grown in LB broth incubated at 37 °C until mid-log phase. Data are means ± standard deviation of three replicates.

Induction of promoters using stress.

To investigate if oxidative stress can induce FadR and OxyR predicted promoters, *E. coli* MG1655 (pLHR) was treated with NaClO and H_2O_2 and expression of *shsp20, shsp,* and *trx* were measured. Treatment with NaClO or H_2O_2 resulted in reductions between 0.05 and 0.2 log (cfu/mL) of untreated cells compared to treated cells (data not shown). In both treatments with H_2O_2 and NaClO, there was low expression of *shsp20*, some overexpression of *shsp* and significant expression of *trx* (Figure 11). To determine if the absence of the regulatory protein would have an effect on the expression of the oxidative stress induced promoter, *E. coli* Δ*fadR* and Δ*oxyR* Keio mutants were used to measure the expression of *shsp* and *trx*. In both treatments, *shsp* and *trx* were not differentially expressed (Figure 12). Both NaClO and H_2O_2 were able to induce the expression of FadR and OxyR predicted promoters; however, the absence of FadR and OxyR regulatory proteins did not abolish expression of the promoter.

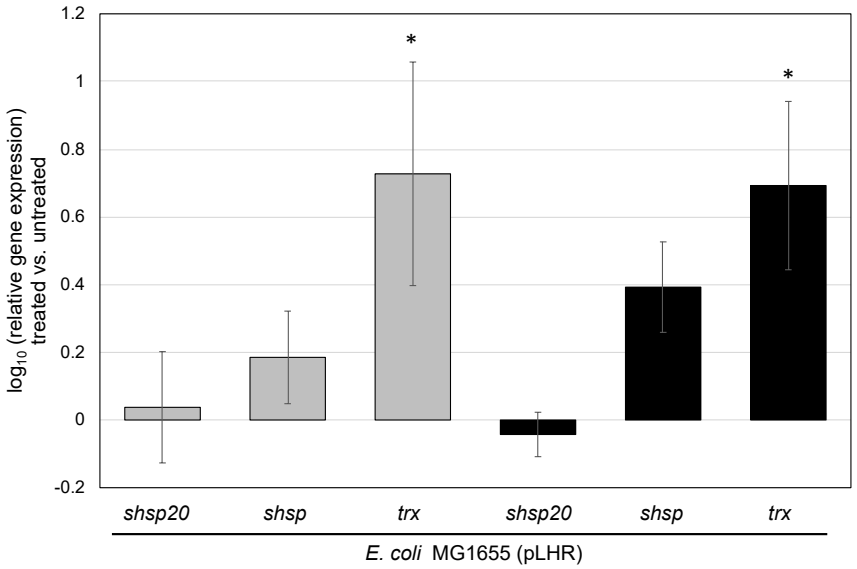

Figure 11. Effect of H_2O_2 and NaClO on relative gene expression of s*hsp20, shsp* and *trx* in treated *E. coli* MG1655 (pLHR) compared to untreated *E. coli* MG1655 (pLHR) grown in LB broth incubated at 37 °C until mid-log phase. Grey bars are treatment with H_2O_2 and black bars are treatment with NaClO. Data are means ± standard deviation of three replicates. Statistically significant ($p<0.05$) differences among genes of each treatment are indicated by an asterisk (*).

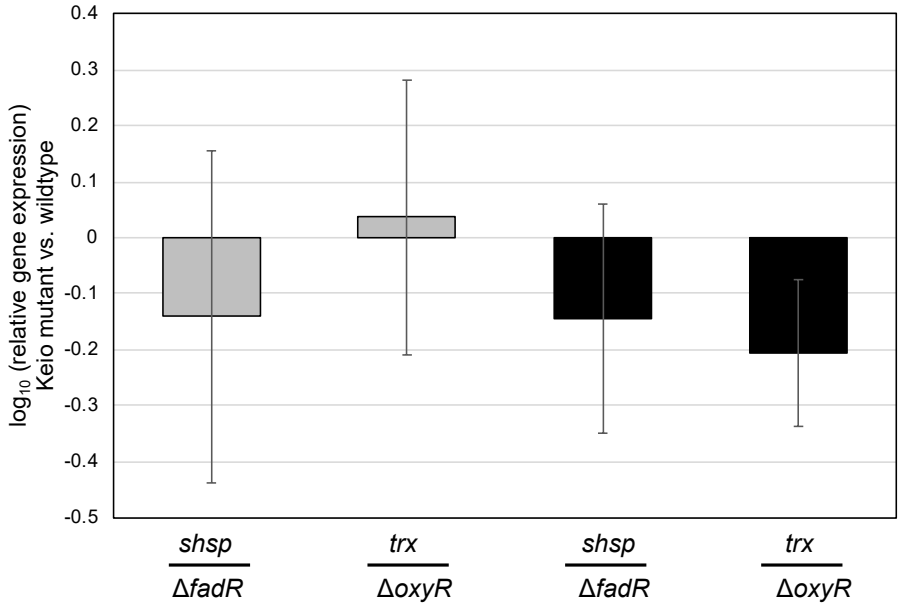

Figure 12. Relative gene expression of *shsp* and *trx* in FadR and OxyR deficient Keio strains grown in LB broth at 37 °C until mid-log phase compared with *E. coli* MG1655 WT grown in LB broth at 37 °C until mid-log phase. Grey bars are treatment with H_2O_2 and black bars are treatment with NaClO. Data are means ± standard deviation of three replicates.

4. Discussion

The LHR plays a role in *E. coli* survival of multiple stressors, but the genetic regulation in response to the different stressors remains largely unknown. To investigate the regulatory mechanisms of the LHR, the main objectives of this study were to 1) evaluate EvgA as a regulator of the LHR; 2) investigate additional promoters using *in-silico* methods and validate using gene expression and 3) examine the natural induction of promoters using stress.

EvgA was previously considered as a regulator because it induces the YfdX protein and the LHR contains two hypothetical proteins from the YfdX family (Mercer et al., 2015; Nishino and Yamaguchi, 2001). Thus, EvgA was proposed to play a role in regulating LHR-mediated heat resistance (Mercer et al., 2017). The EvgA deficient mutant had a phenotypic effect on *E. coli* by causing the loss of its heat resistance (Mercer et al., 2017). The first objective was to evaluate EvgA as a regulator, and this required plasmid complementation to restore heat resistance in the Δ*evgA* Keio mutant. Use of the EGFP promoter fusion plasmid and the *evgAS*-pRK complement plasmid failed to restore fluorescence comparable to the OmpR deficient strain that has a wildtype EvgA (Figure 2). The wildtype *E. coli* MG1655 should have been included and would have made a better comparison. However, it was discovered that EvgA deficient strain with pLHR did not actually have the pLHR which was the reason for its heat sensitivity (Figure 3). Despite the absence of the pLHR, the EvgA deficient strain had lower promoter activity compared to the OmpR deficient strain which possesses a wildtype EvgA (Figure 2). Therefore, EvgA has an effect on promoter activity and regulates LHR expression. One of the difficulties of complementing a gene involved in a two-component regulatory system is the complex and intertwined network of regulatory pathways. The EvgA/EvgS two-component system is involved in upregulating acid, osmotic, and drug resistance genes (Ninshino et al., 2003). It is possible that cross-talk among other regulatory components, such as other two-component systems, complicates the complementation (Eguchi et al., 2004). In addition, pRK767 is a low copy plasmid and expresses more than the native levels of the regulatory protein (Gill and Warren, 1988). As a result, complementation of a regulatory protein is not straightforward.

To counter this problem, the *evgAS*-pRK complement plasmid was used to overexpress EvgA and investigate the phenotypic and genotypic effect on LHR expression. When EvgA was overexpressed, this caused a loss of heat resistance in LHR positive strains; the heat resistance of LHR-negative strains, however, remained unaffected (Figure 4). This effect was only detected in *E. coli* because *Salmonella* lacks the EvgA regulon (Ryan Mercer, personal communication), which was confirmed by BLAST analysis of 14,443 *Salmonella* genomes with EvgA from *E. coli* as query sequence. Therefore, there was a differential heat sensitive phenotype only when LHR and EvgA were present in the genome. This is also shown when there was no differential gene expression of the LHR in *Salmonella* (Figure 5). This might suggest a species-specific regulation of the LHR and the reason why LHR does not appear in strains of *Salmonella* as frequently as in *E. coli* (Mercer et al, 2015; Mercer et al., 2017). Conversely, when the pLHR was introduced into the heat sensitive *S. enterica* ATCC 13311, it conferred heat resistance to the same level as the LHR positive *S. enterica* ATCC 43845 (Mercer et al., 2017). *S. enterica* ATCC 43845 also harbors two versions of the LHR, and it may be possible that *Salmonella* has its own species-specific regulation (Nguyen et al., 2017). This decreased cell survival was unexpected because Christ et al. (2008) found that overexpression of EvgA increased heat resistance and survival. Furthermore, Matsuda and Church (2002) found that increasing *evgA* expression increased acid and multidrug resistance. The expression of the LHR does not match the phenotype of heat resistance in *E. coli*. In *E. coli* AW1.7, the LHR was downregulated but upregulated in *E. coli* 7039 (Figure 5). This variation in expression is also detected in EvgA/EvgS regulated acid resistance (Roggiani et al., 2017). There is considerable variation of responsiveness to mild acidity in different strains despite a high level of EvgA/EvgS conservation (Roggiani et al., 2017). Due to strain variation, the construction of an EvgA mutant in *E. coli* AW1.7 would allow for investigation of EvgA on the native chromosomal LHR. However, the targeted mutagenesis by recombination was not successful.

To investigate additional promoters in the LHR, a promoter prediction program, CNNPromoter (Bedoya and Bustamante, 2011) was used to predict promoter sequences controlled by regulatory proteins in *E. coli* AW1.7 (Table 5, Figure 6). With a total of seven regulatory proteins, including EvgA, gene expression and heat assays were used to investigate if overexpression or deletion of these proteins would affect LHR

expression and heat resistance. To characterize these effects, strains from the Keio and ASKA collections were used. The Keio collection consists of single-gene knockout strains of an *E. coli* MG1655 derivative strain (Baba et al., 2006). Strains deficient of Crp, OmpR, EvgA, PurR, FadR, and OxyR were used. RpoD was not used because it is reported to be essential in the Profiling of *E. coli* Chromosome (PEC) database (Yamamoto et al., 2009). The Keio strains were used to investigate if the absence of the regulatory proteins would affect LHR expression. The ASKA library is a set of ORFs cloned from *E. coli* K12 into a high copy number plasmid pCA24N with an IPTG inducible T5-lac promoter (Kitagawa et al. 2005). Plasmids from the ASKA collection containing genes for Crp, OmpR, EvgA, PurR, FadR, OxyR and RpoD were introduced into *E. coli* MG1655 (pLHR) to investigate if overexpressing the regulatory proteins would modify the expression of the LHR. Wong et al. (2013) used the ASKA library for overexpression in combination with a reporter gene to screen for genes that alter promoter activity. LHR expression was measured using reverse-transcriptase quantitative PCR (RT-qPCR) to determine the genotypic effect while heat assays were used to determine the phenotypic effect of heat resistance.

OxyR regulates the adaptive response to oxidative stress through a regulon of hydrogen-peroxide inducible genes (Christman et al.,1989; Gonzalez-Flecha and Demple, 1997). Overexpression of OxyR did not alter expression of *trx* after induction; *trx* was downregulated when uninduced. However, OxyR is an auto repressor and therefore overexpression may not necessarily increase gene expression (Storz et al., 1990; Michán et al., 1999). Additionally, the oxidized form of OxyR brings a conformational change to activate the regulatory protein therefore, just increasing the amount of OxyR without oxidation would not increase protein activity (Storz et al., 1990). When the OxyR regulated promoter was induced by chlorine and hydrogen peroxide, *trx* gene expression increased but not *shp20* or *shsp*. Because hydrogen peroxide is a known inducer of the OxyR regulon, this supports the interpretation that OxyR regulates *trx* expression (Figure 11; Gonzalez-Flecha and Demple, 1997). The presence of H_2O_2 oxidizes OxyR which in turn was able activate OxyR regulated genes Storz et al., 1990).

RpoD is the primary sigma factor promoting the attachment of RNA polymerase to specific initiation sites during exponential growth (Burgess et al., 1969; Fujita et al., 1994). Overexpression of RpoD resulted in the highest gene expression of both *hdeD* and *orf11.* This indicated that RpoD plays a role in regulating

the promoter upstream of *hdeD.* Even though cells in stationary phase are more heat resistant, the expression of the LHR in the exponential and stationary phases of growth are not significantly different (Mercer et al., 2017). However, previous studies reported that *E. coli* AW1.7 grown to late exponential phase are just as heat resistant as in stationary phase which suggests that RpoD may be involved in turning on genes during the exponential phase of growth that benefit survival in heat (Ruan et al., 2011).

FadR represses fatty acid degradation and activates the synthesis of unsaturated fatty acids (Black and DiRusso, 1993; Feng and Cronan, 2012). PurR represses purine nucleotide synthesis by regulating the transcription of enzymes involved in biosynthesis (Rolfes and Zalkin, 1988). Both of these proteins are involved in the biosynthesis of cell membrane components affecting membrane properties which could alter the ability of *E. coli* to counter stresses. When FadR is overexpressed, expression of *shsp* increases while PurR overexpression causes increased expression of both *shsp20* and *clpK*. The genes that are directly downstream of the FadR and PurR regulated promoters are heat shock proteins and a protease that are involved in protein folding and degradation (Beere, 2004; Li and Gänzle, 2016). Futher downstream of the FadR regulated promoter includes predicted periplasmic chaperones YfdX and HdeE which supports the role of this promoter in cellular envelope stress (Liu et al., 2019; Mates et al., 2007). PurR represses purine synthesis when *E. coli* is starved so it can allocate its energy into survival which would be beneficial for PurR to also be involved in regulation of genes in response to stress. The upregulation of the LHR genes in response to overexpression of FadR and PurR indicates it may play a role in regulation, but additional experimentation is required to confirm that FadR or PurR truly regulate the respective predicted promoters.

The cAMP receptor protein, Crp, is a transcription factor that responds to carbon nutrient conditions (Tagami and Aiba, 1995). It forms a CRP-cAMP complex that activates or represses transcription at promoters by bending DNA or through protein-protein interaction with RNA polymerase (Botsford and Harman, 1992). Crp was the only protein that altered heat resistance when induced with a high concentration of IPTG (Figure 9). Crp exists as a dimer and is involved in the transcription of many *E. coli* genes (Lawson et al., 2004). Therefore, cells are able to cope with excess Crp. Overexpression of Crp increased expression of *orf1* and *shsp20* which indicates it plays a role in the regulation of the promoter

upstream of *orf1* but its role should also be confirmed by additional experimentation such as promoter fusions to EGFP to measure promoter activity.

Both OmpR and EvgA are response regulators of a two-component system. The overexpression of both of these proteins lead to a slight increase in expression of *orf1* but little or no expression of the downstream *shsp20.* It is possible that the basal level of expression of the sensor kinase was not sufficient to phosphorylate and activate the high levels of the response regulators. However, it was shown previously that an OmpR deficient strain did not affect promoter activity of the LHR (Mercer et al., 2017). The current research demonstrates that OmpR has a minimal effect on the regulation of the promoter upstream of *orf1* but further work is needed to have a more conclusive answer such as promoter deletion of mutagenesis to verify the role of the promoter sequence on LHR expression.

The use of overexpressed regulatory proteins to determine if they influence survival after heat has a major caveat on metabolic burden. The expressed protein is present in higher amounts than normal and uses a portion of the host's resources to alter the physiology of the host (Glick, 1995). Plasmids require cellular energy to be maintained and the metabolic load increases with increasing plasmid copy number. As plasmid copy number increases, growth rates decrease and reduces the fitness of the host (Seo and Bailey, 1985; Smith and Bidochka, 1998). In the current research, this was observed when high induction of regulatory proteins caused a larger difference in the reduction of cell counts after heat treatment between induced and uninduced strains (Figure 9). This indicates that higher induction causes the cells to allocate more energy to producing more proteins which burden the cells and heat resistance suffers as a result. In addition, plasmid-encoded antibiotic resistance genes may also compromise the fitness of the host (Hong et al., 2016). The gene expression strategy used in the current research had the advantage of directly measuring the effect of the regulatory protein on expression of the LHR gene.

There is more than one promoter in the LHR operon and its regulation is not dependent on a single regulatory protein. This is similar to the glycerol phosphate transport in *E. coli* K-12, where the glpEGR operon has one promoter located upstream of the *glpE* start codon and three internal promoters that express the latter genes in the operon (Yang and Larson, 1998). The presence of multiple promoters could allow for differential expression in response to different stress. The LHR has multiple parts to aid in

multiple stressors, which requires multiple regulators to alter gene expression in response to the different stressors. The last objective of this research was to induce the promoters with stress. Selective LHR expression is demonstrated when the cells are subjected to oxidative stress from chlorine and hydrogen peroxide. There was upregulation of *trx* expression but not *shsp20* and *shsp* (Figure 11). However, the caveat of having multiple regulators is that they may overlap in function and produce a protective effect when one regulatory protein is absent. This explains why the absence of one regulatory gene did not have any significant effects on gene expression and consequently promoter activity (Figures 10 and 12).

E. coli are naturally present in a wide range of environments and can survive many different stresses (Ishii and Sadowsky, 2008). The LHR is expressed in both the exponential and stationary phase of growth which produces a higher metabolic load for the cell, but this baseline expression allows protection against any encountered stress in the environment (Mercer et al., 2017). The LHR is known to contribute to heat, high pressure and chlorine resistance (Mercer et al., 2015; Wang et al., 2019; Hu et al., 2018). Therefore, due to the lifestyle of constant adaptation, the LHR is advantageous because it allows cross resistance to many stressors.

Conclusion

It was hypothesized that there are regulatory components that regulate LHR expression in response to multiple stressors. My thesis research demonstrated that the LHR is regulated by multiple regulatory proteins, which accounts for its high expression at diverse growth and stress conditions, and matches its protective role against multiple stressors. EvgA, OxyR, and RpoD consistently affected the expression of LHR genes but more work is required to determine the precise mechanisms of regulation. Crp, FadR, PurR, and OmpR showed effects on the expression of LHR genes but further experiments are needed to confirm their relationship with the LHR. There are study limitations in studying genetic regulation because regulatory components are complicated considering proteins could be working together in a network making it difficult to differentiate the effect of one protein from another. Another limitation is that the regulatory proteins were not confirmed to be active. Therefore, measuring expression of genes known to be regulated by the proteins would have been useful to determine if the proteins expressed from the pCA24N plasmids were active and could influence transcription. For future direction, mutagenesis or

deletion of each promoter would allow direct assessment if the promoter contributes to transcription of LHR genes. To support gene expression from promoter activity, promoter reporter fusion using reporter genes such as luciferase or GFP should be constructed to visualize gene expression through fluorescence. This would allow for individual assessment of a single protein at a time which would establish a conclusive relationship between regulatory protein and promoter. It would be meaningful to use additional stressors such as osmotic or pressure to investigate its effect on promoter activity to further investigate the effect of stressors on differential LHR expression.

The LHR is like a shield that defends *E. coli* against many different stresses in the environment but maintaining the LHR comes with a fitness cost. In stable environments, *E. coli* allocates its energy towards expression of proteins important for growth and not towards constitutive expression of proteins for stress. However, the LHR benefits *E. coli* in situations when responsive expression may not be fast enough for survival. Therefore, depending on the environment *E. coli* is situated in, it might be beneficial to have constitutive LHR expression or it could be a poor investment as it comes with a fitness cost. The work done demonstrated that there are regulators of the LHR that may account for the differential expression of genes in response to stress. Determining how the LHR is regulated contributes to our understanding of the ecology of *E. coli* and its evolutionary biology. It may provide insight into the natural history of the LHR as well. The findings of this study pioneers the investigation of which regulators under what specific conditions are expressed to contribute to the protection of *E. coli*. Since the presence of the LHR has been discovered in a wide range of environmental sources, understanding regulation may be relevant to understanding how the LHR is beneficial to the cell and how it contributes to survival in an ecological niche.

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