January 2012

Sigma factor N (σN): A Novel Regulator of Extreme Acid Resistance in Enterohemorrhagic E. coli O157:H7

Pamela Ann Fay
University of South Florida, pfay@mail.usf.edu

Sigma factor N (σ^N): A Novel Regulator of Extreme Acid Resistance in Enterohemorrhagic *E. coli* O157:H7

by

Pamela A. Fay

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
Department of Cell Biology, Microbiology, Molecular Biology
College of Arts and Sciences
University of South Florida

Major Professor: James T. Riordan, Ph.D.
Lindsey N. Shaw, Ph.D.
Kathleen M. Scott, Ph.D.

Date of Approval:
June 20, 2012

Keywords: GDAR, ADAR, *rpoN*, qRT-PCR, EHEC

DEDICATION

I dedicate this thesis to my husband, David, for the support and encouragement he has offered me over the past few years. My appreciation goes beyond anything I can express in words, and the sacrifices you have made for me are endless. You inspire me to work towards my dreams, especially when they require more than most can handle. I would also like to thank my mom, dad, family, friends and best friends for constantly reassuring me that I can make this happen. Specifically I need to thank my sister, Samantha, who is always there for me at any time of need. Although I am the only one receiving this particular degree, it is because of my support team that I was able to make it happen. And for this, I thank all of you in my life.

ACKNOWLEGEMENTS

First I would like to express infinite gratitude to my mentor, Dr. James Riordan, whose guidance and patience throughout this process was selfless. His experience and intellect for scientific research has provided me with an amazing learning experience that I will carry throughout my career. Above all I would like to thank him for giving me the opportunity to truly experience the meaning of research through his passion and dedication to the field molecular and microbiology.

I gratefully want to thank Dr. Lindsey Shaw who believed in me in the early stages of this degree. I appreciate the honesty and support he has provided as a committee member to make me a better prepared scientist. I would like to kindly thank Dr. Kathleen Scott for the time and effort she has given me to better understand the dynamics of prokaryotes in regards to my research. Along with my other advisors, her passion to teach and mentor is obvious and it does not go unnoticed.

To Nada, Avi, and Jason, I want to thank you for always lending a helping hand, your collaboration, and friendship. I have enjoyed working with all of you from Riordan lab as I worked toward this degree. I appreciate all the laughs we have shared along with the complaining we did about edits and such. Once again I would like to thank my family and friends that support me although they still try to understand what I do. Together, it takes more than just one person to achieve this degree and I am truly grateful to all of you.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Extreme acid resistance contributes to the successful transmission of enterohemorrhagic *E. coli* (EHEC) through acidic food matrices and the stomach, allowing it to gain access to the intestine and elicit disease in humans. Alternative sigma factor N (σ^N, encoded by *rpoN*) was previously identified as a novel regulator of extreme acid resistance in EHEC. This study investigated the role for σ^N and co-expressed products of the *rpoN* operon in the acid resistance phenotype of EHEC. The results revealed that σ^N primarily controls acid resistance through repression of the glutamate-dependent acid resistance (GDAR) system through control of the σ^S-directed GadXW pathway. σ^N was also determined to repress additional acid resistance systems, including arginine-dependent acid resistance, and an anaerobic acid resistance mechanism. Two gene products of the *rpoN* operon, *hpf* and *ptsN*, were also determined to negatively affect GDAR, as well as expression of the σ^N dependent genes *glnA*, *astA*, and *pspA*. Mutation of *hpf* and *ptsN* did not however alter the transcription of *rpoN*. Transcript levels of *rpoN* operon genes were observed to be differential, and inconsistent with the hypothesis of expression as a single transcriptional unit. Together this data signifies the importance of *rpoN* operon genes in the negative regulation of extreme acid resistance systems, and suggests that the products of *hpf* and *ptsN* control the activity of σ^N at its promoters

CHAPTER 1

Introduction

Characteristics of enterohemorrhagic *Escherichia coli* O157:H7

Enterohemorrhagic *Escherichia coli* (EHEC) is an enteric pathogen associated with major outbreaks and sporadic cases of severe gastrointestinal illness worldwide. The O157:H7 serotype of EHEC was first recognized as a food-borne pathogen in 1982 following two outbreaks in the U.S. where patients presented with severe bloody diarrhea (hemorrhagic colitis, HC) uncommon to other disease-causing *E. coli* [1]. Since then, there have been over 350 documented outbreaks of EHEC O157:H7 in the U.S., with an average of 73,000 cases and 60 deaths per year [2]. Approximately 80-90% of infected patients will present with HC and 5-10% of these patients develop hemolytic uremic syndrome (HUS), a toxin-associated kidney disease [3].

EHEC is a subgroup of Shiga toxin-producing *E. coli* (STEC) that can produce Shiga toxin (Stx), and also possess the ability to intimately attach to the intestinal epithelia and elicit diarrheagenic disease [4]. Colonization of the intestine is dependent on a type III secretion system (TTSS), encoded on a laterally acquired pathogenicity island termed the locus of enterocyte effacement (LEE) [5,6]. The TTSS translocates the EHEC-encoded receptor Tir into host colonocytes, which in-turn, interacts with the afrimbrial adhesin intimin [7]. Hemorrhagic colitis results from damage to intestinal epithelial cells, and the cytotoxic activity of Stx to the intestinal microvasculature. Shiga

toxin is an AB_5-type toxin shown to have an affinity for glycosphingolipid globotriaosylceramide (Gb_3) receptors on endothelial cells of the vasculature [8] and on the renal glomerular endothelial cells of the kidneys [9,10]. The toxic A1 subunit of Stx is an N-glycosidase that cleaves a specific essential adenine residue located on the 28S ribosomal subunit to inhibit protein synthesis [11]. Depending on the severity of infection, release of Stx will cause HUS, which is pathologically characterized by acute renal failure, hemolytic anemia and thrombocytopenia. Development of HUS can lead to permanent kidney damage, organ failure, and death [3,12].

Current treatment and prevention for EHEC

Prophylaxis and therapy for EHEC infections is severely limited and has been an ongoing medical dilemma. Case studies testing antibiotic regimens independently on infected patients over multiple outbreaks revealed that antibiotic treatment often fails to alleviate the primary infection and, in some cases, may have increased the risk of developing HUS [13,14]. In addition, *in vivo* studies with mice have demonstrated that fluoroquinolones actually increase production of Shiga toxin [15]. Currently, antibiotics are contraindicated for EHEC infections, and patients are treated with supportive care, and dialysis if the infection progresses to HUS [4].

Considerable efforts have been made in the development of a cattle vaccine for EHEC that has shown some efficacy, although it has not gained practicality in industry. In 2003, Potter *et al*. developed a vaccine using EHEC attachment proteins and found that shedding significantly decreased in cattle; with similar findings in later studies

[16,17,18]. Recently, a two-dose vaccine against type III secreted proteins was administered to commercially fed cattle and revealed that colonization of EHEC was significantly reduced by 92% [19]. Although this form of treatment against EHEC is in its infancy, studies are proving that inhibiting transmission of this pathogen is possible.

Standard operating procedures for contamination control have been developed by the United States Department of Agriculture (USDA) to be used in the meat processing industry to reduce the risk of EHEC transmission to foods from the bovine reservoir. All slaughterhouses and processors must comply with these federal regulations, which include pre-harvest intervention strategies, random sampling for *E. coli* from cattle carcasses, temperature control during processing and transportation, and sanitation procedures (USDA). The estimated cost of these preventative measures is $1.3 billion over the last 20 years (USDA 1997). Consequently, the financial impact of EHEC outbreaks is estimated at $178.8 billion over the same time frame without implementation of these regulations.

Outbreaks and transmission of EHEC

EHEC is a food-borne human pathogen transmitted primarily by the fecal-oral route through contamination of various food matrices, water, and through direct contact [20,21,22]. Most EHEC outbreaks have been linked to the ingestion of contaminated meat of bovine origin, a notorious example of which was the U.S. Jack in the Box outbreak in 1993 reporting 501 total cases that lead to 45 patients developing HUS and 3 deaths [23]. There have also been many outbreaks associated with tainted vegetables,

including one in Sakai City, Japan from radish sprouts and recently in 2006 from contaminated spinach [24,25]. Many EHEC outbreaks have also been linked to acidic and cured foods that would normally destroy microbes, such as apple juice, apple cider, and salami [26]. EHEC has been the responsible agent for infections and death with outbreaks associated with contaminated recreational, municipal, and well water [27,28,29].

The ability of EHEC to survive in acidic food matrices and travel through the harsh acidic gastric barrier of the stomach contributes to its success as an enteric pathogen. This trait is mainly attributed to the expression of multiple acid resistance (AR) systems allowing for gastric passage and intestinal colonization, and is a major determinant of low oral infectious dose (ID_{50} <100 cells) [30,31]. Evidence suggests that multiple AR systems have evolved to allow for survival in different acidic environments [32,33,34]. Moreover, exposure to moderately acidic environments prior to ingestion has been shown to lead to acid habituation of EHEC, further enhancing survival at low pH [35,36,37,38].

Acid resistance systems of *E. coli*

EHEC, and many other pathogenic and nonpathogenic subtypes of *E. coli*, have several distinct extreme acid resistance (XAR) systems, which allow for protracted survival in various acidic environments. These include the oxidative, glutamate- and arginine-dependent acid resistance systems [39]. Multiple AR systems may seem redundant in terms of genetic sustainability, but expression of each system is dependent on the type of environmental exposure [36]. Price *et al.* discovered that while the

glutamate dependent acid resistance system provided protection within the bovine intestinal tract (pH 2.0 – 2.5), this system did not protect against acidity in apple cider (~pH 3.5) [36]. This group also determined that the oxidative acid resistance system offers protection within the bovine intestinal tract and apple cider, whereas the arginine dependent acid resistance system had no impact on survival under either of these conditions [36,40].

The oxidative system is induced following exposure to moderate acidity (pH 5.0) and is repressed by glucose [32,41]. The only known genetic determinant of the oxidative system is sigma factor S (σ^S) [34], and the mechanism that drives this system is unknown in *E. coli*, *Shigella flexneri* or *Salmonella typhimurium*. EHEC also utilizes two amino acid dependent decarboxylase systems for AR: glutamate-dependent acid resistance (GDAR) and arginine-dependent acid resistance (ADAR). The decarboxylase AR systems are very robust and important to survival of *E. coli* under aerobic, anaerobic, and low pH conditions [34,42]. In contrast to the oxidative AR system, σ^S in not essential to fully activate ADAR or GDAR [34]. GDAR and ADAR rely on a proton-scavenging mechanism that requires exogenous glutamate or arginine, respectively [32,41,43]. Each system has a specific decarboxylase enzyme that converts glutamate and arginine to γ-aminobutyric acid (GABA) and agmatine by exchanging the α-hydroxyl group with a proton recruited from the cytoplasm. Both GABA and agmatine are exported from the cell by a specific membrane antiporter, which will increase cytoplasmic pH to levels permissive for survival (see Chapter 1). **Figure 1.1** illustrates the structural pathways and complex regulation of the ADAR and GDAR systems.

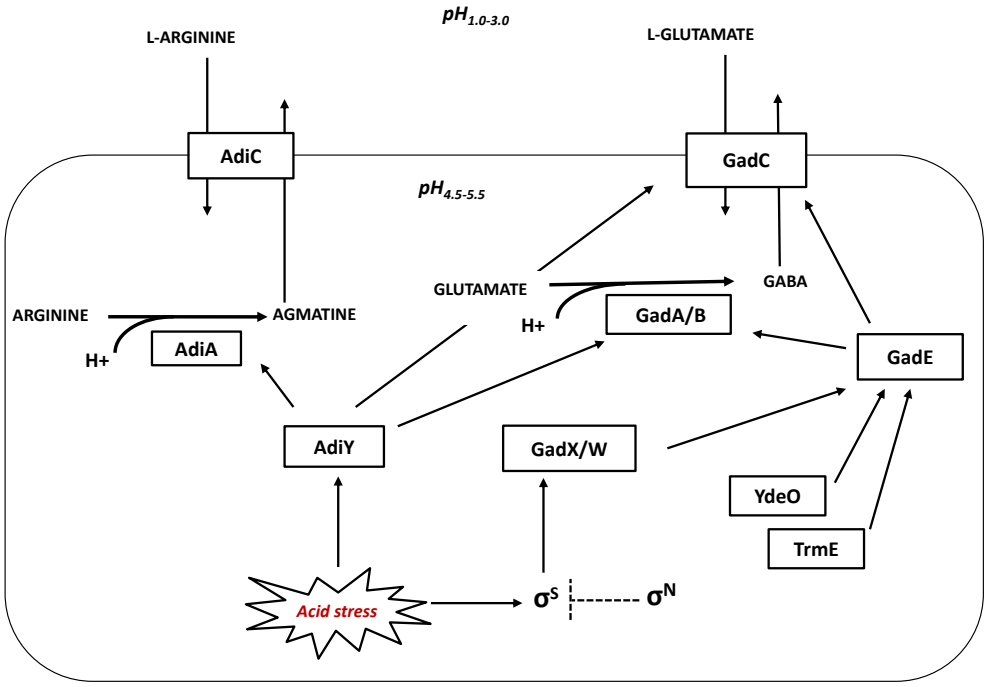

Figure 1.1 Illustration of arginine- and glutamate-dependent acid resistance systems. This model illustrates the σ^N- σ^S connection to the molecular regulation of GDAR and ADAR in response to acidic pH. Protons are consumed by arginine decarboxylase (AdiA) and glutamate decarboxylases (GadA, GadB) to produce agmatine and GABA, respectively, to be exported from the cell by the membrane associated antiporter AdiC and GadC.

Alternative sigma factors regulate acid resistance in *E. coli*

The Exposure to harmful environmental conditions, such as acidity, is detrimental to bacterial fitness. To survive these conditions, bacteria must be prepared to rapidly adapt at the genetic and physiological level. Alternative sigma factors play a significant role in this adaptive response by directing the RNA polymerase to condition-specific promoters. *E. coli* has six alternative sigma factors which function in response to

stationary phase and generalized stress (σ^S), nitrogen limitation (σ^N), heat shock (σ^H), and membrane stress (σ^E); or are important for iron metabolism (σ^{FecI}) and motility (σ^F) (for review see reference [44]).

σ^S is a global regulator, influencing the transcription of >400 genes, many of which are important for resistance to acid and other stressors such as oxidative stress, starvation, hyperosmolarity, heat shock, and UV-irradiation [45,46]. σ^S (encoded by *rpoS*) is important for the full or partial expression of all three major acid resistance systems in *E. coli* [34], and for GDAR in *Shigella flexneri* [47]. *In vivo* studies have confirmed the importance of σ^S in gastric passage and fecal shedding using both murine and bovine models [40].

Regulation of acid resistance systems by σ^S-dependent pathways occurs as cells enter stationary phase [32], which correlates with σ^S being a known stationary phase alternative sigma factor [48]. Unsurprisingly mutation of *rpoS* reduces AR in stationary phase cultures in acidified rich and minimal media [32,34]. Due to the importance of σ^S for acid resistance and other general stress responses in the cell, σ^S is tightly controlled at multiple regulatory levels [45,49]. At the transcriptional level *rpoS* is positively regulated by guanosine tetraphosphate (ppGpp), which is an alarmone that responds to amino acid starvation [49]. Normal rates of *rpoS* translation are fully dependent on the Hfq RNA binding protein [50]. Following translation, the RssB response regulator can sequester σ^S to the ClpXP protease complex for degradation [51]. This strict regulation of *rpoS* in growing cells can be interrupted if the cells experience stress that can be regulated by the σ^S regulon.

Another alternative sigma factor recently identified to control acid resistance is sigma N (σ^N, encoded by *rpoN*). This unique alternative sigma factor requires an activator protein(s) to initiate transcription from its promoters [52]. In addition, σ^N binds to a unique -24/-12 consensus at the core promoter region, in contrast to σ^{70}-family sigma factors that recognize a -35/-10 site. Originally σ^N was identified as being involved in nitrogen scavenging under limiting conditions, however not all σ^N-dependent genes function in nitrogen metabolism [53]. For example, σ^N has been shown to positively regulate motility by inducing flagellar genes [54,55], and upregulate expression of LEE genes necessary for attachment and pathogenesis in EHEC [56]. In *Borrelia burgdorferi and Pseudomonas aeruginosa*, σ^N was found to be essential for mammalian infection [57] and virulence [58], respectively. Expression of the phage shock operon is also regulated by $\sigma^{N,}$, and is important for survival during extracellular stress, such as high temperature, alkaline stress, and the dissipation of proton-motive force [59].

Specific aims of this study

Recently, a strain mutated for *rpoN* was demonstrated to significantly increase expression of the GDAR central regulator gene (*gadE*) and structural genes (*gadA, gadBC*) [56]. Moreover, a mutation in *rpoN* resulted in glutamate-dependent acid resistance during exponential phase, whereas wild-type cells were acid susceptible. This phenotype was abrogated when a mutation of *rpoS* (encoding σ^S) in a Δ*rpoN* background reconstituted wild-type levels of acid susceptibility. This finding, suggests that σ^N is a novel repressor of exponential phase GDAR, and that this negative regulation is

dependent on the presence of an intact σ^S. The mechanistic basis for σ^N-dependent control of GDAR, and its influence on other important XAR systems is unknown. *rpoN* (encoding σ^N) is the first gene of a five-gene operon (see Chapter 3, Fig 3.1). The precise function of these gene products is not known, however there is evidence to suggest that they may regulate *rpoN*/σ^N and thus influence *rpoN*-dependent XAR [60,61,62]. This study investigates the role for σ^N and *rpoN* operon genes in the XAR phenotype of EHEC.

Methods

Strains, plasmids, and media

The strains and plasmids used for this study are listed in **Table 1.2**. All strains were stocked at -80°C in glycerol (15% v/v final) diluted in Lysogeny Broth (LB) broth. Unless otherwise noted, strains were grown from single colonies at 37°C overnight in MOPS (50 mM) buffered Dulbecco's Modified Eagle's Medium (DMEM) (cat. #D2902, St. Louis, MO) containing 4g/l glucose (pH 7.2) in a rotary shaker (200 RPM) as described [56]. Overnight cultures (18-20 h) were used to inoculate fresh DMEM to an initial OD_{600}=0.05, then cultured to a final of OD_{600}=0.5 for exponential phase. When required, the antibiotics ampicillin (100 μg/ml), or kanamycin (50 μg/ml) were added to growth media

E-minimal glucose (EG) media was used for acid resistance assays as described by Large *et al.* [33], and contains 0.4% glucose, 73 mM potassium phosphate (K_2PO_4), 17 mM sodium ammonium phosphate ($NaNH_4PO_4$-$4H_2O$), 10 mM sodium citrate

($NaNH_4PO_4$-$4H_2O$), 12 mM magnesium sulfate ($MgSO_4$-$7H_2O$) and is then supplemented with 5.7 mM L-glutamic acid or 0.6 mM L-arginine. EG was acidified by titrating with 10N HCl to pH 2.0 for GDAR, or pH 2.5 for ADAR, followed by sterilization using vacuum filtration (Millipore 0.22 μm), and stored at 4°C.

All growth experiments in minimal media used 1X MOPS minimal media containing 0.2% NH_4Cl (nitrogen source), 4 g/l glucose (carbon source), 1.32 mM K_2HPO_4 (phosphate source), and 0.5 g/l L-glutamine [63]. To test for each nutrient limiting condition the concentrations were adjusted to 0.8 g/l glucose for carbon limitation and 0.264 mM K_2HPO_4 for phosphate limitation. For nitrogen limiting conditions, NH_4Cl was excluded from 1X MOPS with 0.5 g/l of L-glutamine as the sole nitrogen source. To determine limiting concentrations, each nutrient was reduced by 1/10 over a range of five concentrations and evaluated on growth curves. The pH was adjusted to 7.2 using 5N NaOH then filter sterilized using a 0.22 μm filter (Millipore) and stored at 4°C. 1X MOPS minimal media is derived from 10X MOPS minimal media as described by Neidhart *et al.* (1974) that was filter sterilized using a 0.22 μm filter (Millipore, cat. #SCGPU05RE Billerica, MA), aliquoted into 50 ml sterile tubes, and stored at -20°C.

Acid resistance assays

Methods to measure aerobic glutamate- and arginine-dependent acid resistance were adapted and modified from Riordan *et al.* [56]. For stationary phase glutamate-dependent acid resistance (GDAR), cells were grown overnight (18-20 h) at 37°C in

DMEM and directly resuspended at 1×10^6 CFU/ml in acidified (pH 2.0) E minimal glucose (EG), with or without 5.7 mM L-glutamate, for 2 h. Initial cell density was determined using EG at neutral pH, 7.0. For exponential phase, GDAR cultures were grown as described and cells were harvested after 1 h challenge in EG test environment following the same procedure described for stationary phase. To test for arginine-dependent acid resistance (ADAR), cells were harvested for stationary and exponential phase as described for GDAR and resuspended at 1×10^6-10^7 CFU/ml in acidified (pH 2.5) EG test environment using 0.6 mM L-arginine. All EG test environments were incubated at 37°C in a rotary shaker (200 RPM), followed by serial dilution in phosphate buffer solution (PBS, pH 7.0), which were then plated on Lysogeny Broth with 1.5% agar (LBA), followed by overnight incubation at 37°C.

Anaerobic acid resistance was measured using methods adapted from Noguchi *et al.* 2010 for exponential phase cultures. Cells were grown in LBK (10 g/l tryptone, 5 g/l yeast extract and 7.45 g/l KCl) buffered with 2-(N-morpholino) ethanesulfonic acid (MES) acidified with KOH to pH 5.5. To attain anaerobic conditions, cultures were grown overnight in LBK-MES, pH 5.5 without rotation in closed screw capped 15 ml tubes. On day two, overnight cultures were added to fresh LBK-MES (pH 5.5) at an initial OD_{600}=0.05 then cultured to exponential phase (OD_{600}=0.25). Cells were then challenged in acidified unbuffered LBK (pH 2.0) for 1 h followed by serial dilution in LBK-MES, pH 7.0 then immediately plated to LBK agar. To determine percent survival, CFU/ml at t=1 was compared to CFU/ml at t=0, which used LBK-MES pH 7.0. Similar conditions were used to test for anaerobic GDAR except acidified (pH 2.0) EG media

supplemented with 5.7 mM of L-glutamate was used for acid challenge, and EG-minimal pH 7.0 was used for t=0 counts.

RNA extraction and purification

For transcript analysis DMEM cultures were harvested for total RNA extraction for exponential, late exponential, and stationary phase, determined by OD_{600} readings of 0.5, 1.0, and 2.0, respectively. RNA was purified according to the manufacturers protocol for Qiagen RNeasy Mini Kit, including initial treatment with RNA Protect (cat. #76506). The yield and purity was determined by UV reading using Nanodrop 2000 (Thermo Scientific, USA). RNA integrity was confirmed on a formaldehyde-agarose gel using 500 ng RNA added to DEPC H_2O in a PCR tube to a final volume of 15 µl. Samples were denatured at 65ºC for 10 min, while the gel was pre-warmed for 5 min at 70V in 1X MOPS running buffer.

Primer Optimization for qRT-PCR

All oligonucleotides for qRT-PCR were designed using the Primer 3 software within the range of 80-100 nucleotides. They were analyzed using Oligo Analyzer (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/) and aligned with other *E. coli* 0157:H7 strains through NCBI. To optimize primer sets, optimal annealing temperatures were determined by gradient PCR using temperatures ranging from 57°C to 59°C. The following was added to each PCR tube for a 20 µl reaction: 1X Taq Buffer, 2

mM Taq Magnesium Solution, 0.05U Taq Polymerase, 0.2 mM dNTPs, 0.2 M forward and reverse primers and 2 µl genomic DNA to total 100 ng/µl. To determine optimal cDNA concentrations, 10-fold dilutions of genomic DNA were prepared and analyzed by qRT-PCR using the optimal annealing temperature determined by gradient PCR. The slopes were analyzed for each dilution and chosen based on an amplification efficiency of >90% (or E=1.9).

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was used to determine expression of genes for the GDAR (*gadE, gadX, gadW, trmE,* and *ydeO*) and ADAR (*adiC, adiA*, and *adiY*) systems. cDNA was synthesized from 1 µg RNA samples using the iScript cDNA synthesis kit (Bio-Rad, USA). Gene specific primers were used along side those for *rrsH* (encoding a 30S ribosomal subunit) as a standard cDNA normalizing gene for all experiments. The qRT-PCR method used was adapted from Riordan *et al.* (2009) using SYBR Premix Ex Taq II (Takara Biotechnology Co. Ltd.) and BioRad 96 well plates (cat. #2239441) sealed with BioRad Microseal 'B' Film in an Eppendorf Realplex2 Mastercycler (Eppendorf, Germany). Reactions were performed in triplicate with two technical replicates using sterile ddH_2O in place of cDNA template as a negative control for each primer set.

Directed gene deletion for construction of isogenic mutants

Deletion strains for this study were constructed by the one-step inactivation method using a λ Red recombinase-assisted approach, described by Datsenko and Wanner (2000), which replaces target gene sequence with a kanamycin resistance (Km^R) cassette [64,65]. WT strains TW14359 (*E. coli* 0157:H7 U.S. 2006 Spinach Outbreak) and TW08264 (*E. coli* 0157:H7 Sakai City, Japan 1996 Outbreak) were originally provided by the STEC Reference Center (T. S. Whittam, Mich. State Univ., East Lansing MI) and were used to construct all mutants in this study (Fig. 1.2). Homology primers (H1, H2) were designed by selecting 40 bp regions within target genes for inactivation (Table 1.2). Priming sequences (P1, P2) from the pKD4 plasmid were added to the 3' end of the selected sequence to create a 60 bp oligonucelotide that amplified the Km^R sequence from pKD4 by PCR. A 10 ml starter culture of TW14359 transformed with the pKM208 helper plasmid was grown overnight at 30°C (200 RPM) in Lysogeny broth (LB) supplemented with 10 µg/ml ampicillin. On day two, 250 µl of overnight culture was passaged to 25 ml fresh LB plus 10 µg/ml ampicillin (1:100), followed by incubation at 30°C until an OD_{600} was reached of 0.50. To induce the Lambda Red recombinase encoded on pKM208, 1 mM IPTG was added to an OD_{600}=0.50 culture, followed by incubation at 30°C with shaking (200 RPM) until OD_{600}=0.60 was reached. Cultures were then heat-shocked for 15 min in a 42°C waterbath, followed by a 10 minute cold-shock on ice, with mixing intervals every 2 min. Cells were pelleted by temperature controlled (4°C) centrifugation for 5 min at 3,500 RPM. Cells were washed with 1 ml chilled electroporation buffer (EB) 3-4 times followed by final resuspension using 100 µl

EB. Using a fresh tube, 40 µl was transferred plus 1 µg of transforming product (containing the *kan* resistance cassette from pKD4 flanked by 40-mer oligos homologous to 5' and 3' regions proximal to the target gene for Km replacement). The entire mixture was added to a chilled electroporation cuvette and electroporated at 2.5 kV for 5.6 msec. using a BioRad MicroPulser™. Cells were immediately resuscitated in 5 ml pre-warmed LB (37°C) and further incubated for 1.5 h 37°C at 200 RPM. Aliquots of 0.1ml, 0.2ml, and 0.4ml were then plated to pre-warmed LB agar plus 20 µg/ml Km, and incubated overnight at 37°C. Putative transformants were passaged to LB agar plus 50 µg/ml Km, then incubated at 37°C for 24 h. Putative transformants were passaged to LB agar plus 50 µg/ml Km for isolation to prepare master stocks as described.

Removal of the Km cassette was accomplished using the 30°C temperature sensitive pCP20 helper plasmid. The pCP20 plasmid expresses Flp recombinase that will recognize Flp recombinase target (FRT) sequences for intramolecular recombination between P1/P2 regions at 5' and 3' regions of the Km cassette.

Two-hundred and fifty microliters of an overnight culture grown in LB with Km (50 ug/ml) (1:100) was transferred to fresh LB with Km (50 µg/ml) and incubated at 37°C, 200 RPM until OD_{600} = 0.60 was reached. Cultures were then heat-shocked for 15 min at 42°C followed by cold-shock for 10 min on ice with careful attention to gently mix culture at all times. Cells were pelleted by temperature controlled (4°C) centrifugation for 5 min at 3,500 RPM then washed with 1ml chilled EB3-4 times followed by a final resuspension in 100 µl of EB. Cells were electroporated by adding 50 µl of resuspension mixed with 300-600 ng of purified pCP20 to a chilled electroporation

cuvette. Following electroporation cells were immediately resuscitated in pre-warmed (30°C) LB, incubated at 30°C for 1.5 h, then plated to pre-warmed LB agar containing 10 µg/ml ampicillin. Plates were incubated at 30°C for 24 h, and on day three, possible transformants were selected and passaged to LB containing 10 µg/ml ampicillin. To cure cells containing pCP20, colonies were transferred to LB agar and incubated at 42°C overnight. Mutants were then plated onto LB containing 50 µg/ml Km and LB containing 100 µg/ml ampicillin to confirm KmS AmpS, colonies as the *kan* cassette and plasmid bearing *amp* resistance gene should be absent. Validation primers were designed outside of the ORF to amplify entire gene by PCR (Table 1.2). DNA was extracted from each mutant and validation primers were used to amplify PCR product. PCR product size was confirmed by gel electrophoresis and further by Restriction Fragment Length Polymorphism (RFLP).

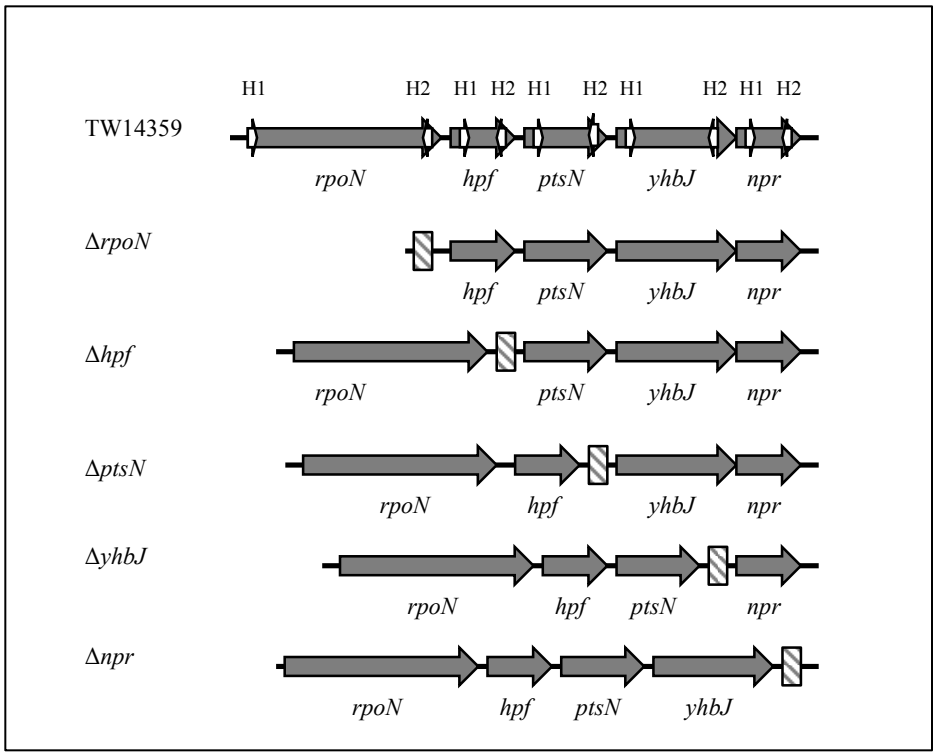

Figure 1.2 Genetic schematic of constructed isogenic mutants. Illustration depicts sites (H1, H2) of homologous recombination for removal of functional unit for each target gene within the ORF. Hashed lines illustrate scar sequence after removal of Km^R gene by recombination at FRT sites located within the H1/H2 sequences.

PCR and Restriction Digest Conditions

Isogenic mutants were confirmed by PCR and RFLP. Resistant mutants (Km^R) were first confirmed by whole cell PCR method using K1/K2 primers that amplify Km^R sequence. A single colony was selected and resuspended in 3ml LB containing 50 µg/ml Km for 8 h at 37°C, with cells pelleted by centrifugation at 10,000 g for 5 min. The pellet was resuspended in 1ml sterile water and washed 2 times, followed by a final resuspension in 100 µl water. Unless otherwise noted, PCR reactions used 0.05U of Taq DNA Polymerase (5 Prime, Gaithersburg, MD), 10x Taq Buffer, 2.5 mM Taq magnesium

solution, 0.25 mM dNTPs, 0.25 mM forward and reverse primers (Table 1.2) and 100-200 ng/ µl DNA template in 25 µl or 50 µl reactions using an Eppendorf VapoProtect Mastercycler. Transformants were then confirmed by extracting total DNA and amplifying the target gene using validation primers outside of the ORF (Table 1.2). The amplified target sequence was then digested for 6 h at 37°C in 30 µl mixture of 1xNEBuffer, 0.3 µg Bovine Serum Albumin (BSA), 0.5U restriction enzyme (*Bcc*I) and 6-10 µg DNA Template (New England BioLabs #R0704S, USA). Virtual digest products were analyzed using Restriction Mapper (http://www.restrictionmapper.org/) to determine fragments, which were further analyzed on 1% agarose gel.

PCR Purification

All PCR products were purified using the Qiagen QIAquick PCR Purification Kit (cat. #28104, Maryland) according to manufacturer's protocols. PCR product yields and purity were determined by taking UV readings using a Thermo NanoDrop 2000; with fragment sizes confirmed on 1% agarose gel.

Extraction of Genomic DNA

Genomic DNA was extracted using a Qiagen Gentra PureGene kit for bacteria (cat. #158222, Maryland) according to the manufacturer's protocol. To increase DNA yield an additional 1 ml of culture was used for a final volume of 2 ml. The purity and yield of DNA was determined by nanodrop (UV) reading and samples were stored at -20°C.

Miniprep Plasmid Extraction

Plasmids were extracted from strains using a Qiagen QIAprep Spin Miniprep Kit (#27104, Maryland) according to manufacturer's protocol. The yield and purity of plasmids was determined by nanodrop (UV) reading, and size was confirmed by gel electrophoresis on 1% agarose. Plasmids were stored at -20°C, and integrity was verified before use.

Nutrient limitation assays

For nutrient limitation experiments single colonies (n≥3) were inoculated into 10 ml LB for 6 h to resuspend cells at 37°C, 200 RPM. Cultures were transferred into 10 ml 1X MOPS to a starting OD_{600}=0.05 and grown for 18 h at 37°C, 200 RPM. Overnight cultures were then inoculated at 1:200 into 10 ml fresh 1X MOPS limiting test environments containing reduced glucose (0.8 g/l) or phosphorous (0.264 mM K_2HPO_4). For nitrogen limiting conditions NH_4Cl was excluded from media, leaving glutamine as the sole nitrogen source. All cultures started with an initial OD_{600} of 0.05 at t=0, then OD_{600} readings and plate counts were recorded every hour for 8 h. Cell viability was determined over five days by plate counts at 24, 48, 72, 96, and 120 h. Since TW14359 was growth impaired in the control 1X MOPS environment, a generation time lag (G_L) was calculated for each strain after normalizing to control conditions in MOPS using by subtracting test generation time (g) from control ($g=[(Log_{OD2} - Log_{OD1})/0.301]/T_{(mins)}$).

Table 1.1 List of O157:H7 strains and plasmids used for this study

Strain	Genotype	Source
TW14359	*E. coli* O157:H7 (Spinach 2006)	T. Whittam, MSU
EcRPF1	TW14359Δ*rpoN::kan*	This study
EcRPF6	TW14359Δ*rpoN*	This study
EcRPF2	TW14359Δ*hpf::kan*	This study
EcRPF7	TW14359Δ*hpf*	This study
EcRPF3	TW14359Δ*ptsN::kan*	This study
EcRPF8	TW14359Δ*ptsN*	This study
EcRPF4	TW14359Δ*yhtJ::kan*	This study
EcRPF9	TW14359Δ*yhtJ*	This study
EcRPF5	TW14359Δ*npr::kan*	This study
EcRPF10	TW14359Δ*npr*	This study
EcRPF12	TW14359Δ*ydeN:kan*	This study
TW08264	*E.coli* O157:H7 (Sakai 1996)	T. Whittam, MSU
EcJR7	Sakai*ΔrpoS::kan*	Riordan *et al*. (2009)
EcJR8	SakaiΔ*rpoN::kan*	Riordan *et al*. (2009)
EcJR9	SakaiΔ*rpoN*Δ*rpoS::kan*	Riordan *et al*. (2009)
EcJR5	SakaiΔ*rpoN::kan pCR2.1:rpoN*$^{+}$	Riordan *et al*. (2009)
EcRPF11	SakaiΔ*rpoN*	This study
EcRAM13	Sakai:pSC-B*::rpoS*$^{+++}$	Riordan lab
EcRAM14	Sakai:pSC-B*::rpoN*$^{+++}$	Riordan lab
EcJR1	TW14359 *pKM208*	T. Whittam, MSU
EcRPF14	TW14359Δ*rpoN::pKM208*	This study
EcRPF16	TW14359Δ*rpoN*Δ*gadX*	This study
Plasmids		
pKM208	Red-recombinase expression vector	T. Whittam, MSU
pKD4	Template plasmid for Km cassette	T. Whittam, MSU
pCP20	Flp recombinase expression vector	T. Whittam, MSU

Table 1.2 List of primers used for this study

Name	Type	5'-Sequence-3'	Tm	GC%	Source
rpoN -12/P2	Recombinant	ACG ATT CTG AAC ATG AAG CAA GGT TTG CAA CTC AGG CTT AGT CTA GGC TGG AGC TGC TTC	70.40	48.3	Riordan *et al.* (2009)
rpoN +1430/P2	Recombinant	ACG AGC TGT TTA CGC TGG TTT GAC GGC GGA ATG GAT AAA GCA TAT GAA TAT CCT CCT TAG	68.80	45.0	Riordan *et al.* (2009)
hpf +40	Recombinant	GAGGCACTGCGCGAATTTGTTACAGCCAAATTTGCCAAACGTG TAG GCT GGA GCT GCT TC	71.80	51.6	This study
hpf +336	Recombinant	GTTGTGCCGCACAACAGGCCGCCATGCACATGCTAATTGCCATATGAATATCCTCCTTAG	70.90	50.0	This study
ptsN +25	Recombinant	CAG CTT AGC AGT GTT AAC AGG GAA TGT ACG CGA AGC CGT GTA GGC TGG AGC TGC TTC	71.30	53.3	This study
ptsN +415	Recombinant	GGC GGC AGA TGG TTT TGT CCG CCA GAC GTT TCG CCA CCA GCA TAT GAA TAT CCT CCT TAG	71.50	53.3	This study
yhbJ ECs4084 +6	Recombinant	ACT GAT GAT CGT CAG CGG ACG TTC AGG TTC AGG TAA ATC TGT GTA GGC TGG AGC TGC TTC	71.00	51.6	This study
yhbJ ECs4084 +808	Recombinant	TAC CGC GCG AGC GGA AGT AGT CTG CCA GTT GCT CTG CAA TCA TAT GAA TAT CCT CCT TAG	70.20	50.0	This study
npr ECs4085 +9	Recombinant	CAA GCA AAC TGT TGA AAT GAC AAA CAA GCT GGG CAT GCA TGT GTA GGC TGG AGC TGC TTC	70.70	48.3	This study
npr ECs4085 +225	Recombinant	TGC TTC CTC TTC CTG TGG ACC GGT CGC TTC AAC TTC AAT CCA TAT GAA TAT CCT CCT TAG	69.00	46.6	This study
gadX +80/P1	Recombinant	GCTATTTTAATGGCGGTGACCTGGTTTTTGCGGATGCAAGGTGTAGGCTGGAGTTGCTTC	71.50	51.6	This study
gadX +774/P2	Recombinant	CGCAACAATACTTGCCGCCGAGTCACGGTTCGGCAATCCCCATATGAATATCCTCCTTAG	70.70	51.6	This study
ydeN-107	Validation	TGT CAA CGC TTT ATG GAC TGT T	54.80	40.9	This study
ydeN+1771	Validation	GCA TCG CTA TCT CGC TCA AT	55.10	50.0	This study
rpoN-45	Validation	GGG TAG AAG TTT GCG ACG TT	55.30	50.0	Riordan Lab
rpoN+1476	Validation	CCG GTA ATG TTG AGC TGC AT	55.60	50.0	Riordan Lab
hpf-51	Validation	CGT CAA ACC AGC GTA AAC AG	54.30	50.0	This study
hpf+363	Validation	TCG GAG CAT TAA CCG TAC AA	53.80	45.0	This study
ptsN-55	Validation	TTA ATC CTC CGA GCC TGT TC	55.00	50.0	This study
ptsN+589	Validation	GGG CGA CAG ATT TAC CTG AA	54.80	50.0	This study
npr-81	Validation	GCA GAG CAA CTG GCA GAC TA	57.30	55.0	This study
npr+312	Validation	GGG GAG TTT GAA GGG AGT TG	55.60	55.0	This study
yhbJ-64	Validation	ACT CCG GAT GAA GCG TAG TT	55.90	50.0	This study
yhbJ+893	Validation	GCC CAG CTT GTT TGT GAT TT	54.30	45.0	This study
gadX -470	Validation	GAACTGTTGCTGGAAGACTACAAAG	56.00	44.0	This study
gadX +1249	Validation	TTCATGATAAATATCGAATGAACGA	50.70	28.0	This study
KT	Validation	CGG CCA CAG TCG ATG AAT CC	58.40	60.0	Datsenko (2000)
K2	Validation	CGG TGC CCT GAA TGA ACT GC	59.20	60.0	Datsenko (2000)
gadE +309	Real Time	TGGTAAACACTTGCCCCATA	54.00	45.0	This study
gadE +419	Real Time	AGCGTCGACGTGATATTGCT	56.60	50.0	This study
gadX +319	Real Time	CGCTATGCAGAAATGCTACG	54.10	50.0	This study
gadX +413	Real Time	ACGTTCAGAAGCAGCGGTAT	56.70	50.0	This study
gadW+445	Real Time	ATCGCCAAACGTTGGTATCT	54.50	45.0	This study
gadW +536	Real Time	CAGGTGTTTTCATCCTGCAA	53.50	45.0	This study
adiC +987	Real Time	CGTCGGTATTTTGATGACCA	52.70	45.0	This study
adiC+1076	Real Time	ATGACCGACACGGAAGAAAC	55.10	50.0	This study
adiY +276	Real Time	CCTGACACCAGACGTCTTTC	55.40	55.0	This study
adiY+357	Real Time	GCGTGTTCGTTCCTTTTCTG	54.60	50.0	This study
adiA +1463	Real Time	CACAAACCGGCAAAACCTAT	53.70	45.0	This study
adiA+1542	Real Time	ATGCATTACCCAGCAGTCCT	56.50	50.0	This study
trmE +991	Real Time	ACCGTGGTACGCAATAAAGC	55.70	50.0	This study
trmE+1083	Real Time	AGTCTTTGCCGAGAGACGAA	56.00	50.0	This study
ydeO +548	Real Time	TAGATGCCAGAATGCAGCAC	55.40	50.0	This study
ydeO+631	Real Time	TGGCATAACCACATTGTTCG	53.50	45.0	This study
rpoN +735	Real Time	GCTGAAAGAAGCCGTCAATC	59.96	50.0	Riordan Lab
rpoN +845	Real Time	TTATGCTTACGCACCAGCAC	59.90	50.0	Riordan Lab
hpf +93	Real Time	TGACCGAATCAACCAGGTCT	56.00	50.0	This study
hpf +173	Real Time	CCGTTTACATGCAGTGTTGC	55.00	50.0	This study
ptsN +138	Real Time	GCCTCAGGTGGTTTTTGAAG	54.30	50.0	This study
ptsN +235	Real Time	CTTCTTCCAGTTTGCCATGC	54.60	50.0	This study
yhbJ +386	Real Time	CAGCGGATCTGATTGTCGAT	54.50	50.0	This study
yhbJ +479	Real Time	AGTTCGCGTTCACGTTTACC	55.70	50.0	This study
npr +82	Real Time	CAGGGTTTTGACGCTGAAGT	55.70	50.0	This study
npr+179	Real Time	CCTTTGGCAGAATCCAACA	53.10	47.3	This study
astA+420	Real Time	GTCGAAATCGCGCTTTATGT	53.70	45.0	This study
astA +519	Real Time	TGAATAGCCGTGTTCGTCAA	54.30	45.0	This study
pspA +472	Real Time	AGTGGCAAACTGGATGAAGC	55.80	50.0	This study
pspA +565	Real Time	TACCGAAGCTGTGGCTTTTC	56.50	50.0	This study
glnA +890	Real Time	ACTACATTGGCGGCGTAATC	59.90	50.0	Riordan Lab
glnA +1064	Real Time	CGTGCTTTCGGAGAAGAAAC	59.90	50.0	Riordan Lab

CHAPTER 2

The role for σ^N in the extreme acid resistance phenotype of EHEC

Background

E. coli depends on acid resistance systems for survival and transmission through acidic food matrices, gastric acidity, and volatile fatty acids common to fermentative conditions in the intestinal tract [37,66,67]. These systems maintain a sustainable internal pH of 4.0-4.7, and protect *E. coli* cells against extreme acid conditions when exposed to an external pH environment ranging from 2.0-2.5 [34,43]. Under these conditions, survival ≥10% of initial cell volume is representative of extreme acid resistance [35].

The oxidative acid resistance (ODAR) system offers the least protection and requires acid adaptation in order to survive in low pH [34]. The exact mechanism to increase cellular pH of this system is not known, however it is fully dependent on expression of σ^S, and is partly controlled by the cyclic AMP receptor protein (CRP) [32,34]. The ODAR system is induced when cells approach stationary phase as the abundance and stability of σ^S increases. Trace amounts of σ^S are present during exponential growth, but levels increase significantly in stationary phase to effectively respond to the stress associated with limited nutrient availability [49].

The glutamate dependent acid resistance (GDAR) system is well characterized, and confers the most acid protection [32,33,34] (Fig. 1.1). Although σ^S is not essential for full expression of GDAR, it is a strong regulator of *gadE* during stationary phase [68,69]. A decrease in cytoplasmic pH will activate the GDAR system to import exogenous glutamate through the membrane-associated antiporter GadC [70]. As glutamate accumulates in the cell, the glutamate specific decarboxylase isoenzymes GadA and GadB replace the α-carboxyl group on glutamate with a hydrogen proton (H+), producing γ-aminobutyrate acid (GABA), which is then exported by GadC in exchange for another glutamate molecule [69]. The GDAR system is completely dependent on the AraC-type transcriptional regulator GadE to transcribe the structural genes (*gadA*, gad*BC*) of this system [32,68]. The multifactorial transcriptional regulation of *gadE* is complex and occurs through σ^S-dependent and σ^S-independent circuits in response to endogenous signals, such as low pH. σ^S is an important regulator of *gadE* which acts both directly on the *gadE* promoter, and indirectly by activating transcription of promoters driving expression of the *gadE* regulators, *gadX* and *gadW* [68,71,72,73,74]. Expression of *gadE* also occurs independent of σ^S through the two-component regulatory system (EvgSA). The membrane bound sensor kinase EvgS responds to an unidentified signal and phosphorylates the cytoplasmic response regulator EvgsA, which activates the AraC/XylS-family transcriptional regulator *ydeO* to induce *gadE* [71]. In an alternative cascade, *gadE* is upregulated in a glucose-dependent manner by TrmE, which is a GTP-binding protein [75,76]. The exact mechanism or signal by which these σ^S-independent systems are induced is yet to be discovered.

The regulation of the arginine dependent acid resistance (ADAR) system is not completely defined, but like GDAR, there is a partial dependence on σ^S, and a putative transcriptional regulator referred to as *adiY* [34,77]. Expression of the arginine-specific decarboxylase (AdiA) is essential for this system to function, and its regulation is partially dependent on the LysR-family transcriptional regulator CysB [32,78]. Low pH induces the arginine specific antiporter AdiC, which is an arginine-specific membrane-associated protein that imports arginine and exports agmatine [79]. As arginine is shuttled into the cell, AdiA replaces the carboxyl group with a proton to produce agmatine, which can be exported from the cell. Although acidic pH drives induction of this system, the transcriptional regulator AdiY along with AdiA, can also be induced under anaerobic conditions, which is common to the gastrointestinal tract [80].

Some acid resistance systems are expressed strictly under anaerobic conditions. One example involves σ^N expressed hydrogenase-3 (Hyd-3) and formate dehydrogenase (FDH-H), which forms formate hydrogen lyase (FHL) complex, to breakdown formate [81]. Of the four known hydrogenase systems in *E. coli,* Hyd-3 was shown to contribute to anaerobic extreme acid resistance (AXAR) by consuming protons and producing molecular hydrogen, H_2 [82]. There is no evidence that σ^S controls Hyd-3 AXAR, however, it is likely dependent on σ^N for expression of Hyd-3 and other FHL structural genes.

Previous evidence indicates that σ^N regulates at least one of the XAR systems: GDAR. A strain lacking *rpoN* had significantly increased survival when compared to WT in a

cidified minimal (pH 2) media, and a key regulatory gene, *gadE*, of the GDAR system was correspondingly upregulated upon transcription analysis [56]. Furthermore, when *rpoS* (encoding σ^S) was inactivated in a Δ*rpoN* background, cells were acid susceptible in exponential phase cells [56]. This data implies that the acid resistance phenotype of *rpoN* mutants is at least partly dependent on a σ^S directed pathway of GDAR, however, the mechanism by which this occurs, and the effect of σ^N on other AR systems, is unknown. This study focused on determining the mechanism by which σ^N regulates glutamate-dependent acid resistance (GDAR) and further investigated the importance of σ^N to other systems of extreme acid resistance.

Results

Control of the GDAR system by σ^N

Multiple regulatory pathways for GDAR control converge on the central regulator, GadE (see Fig. 1.1). These pathways include EvgSA-YdeO,TrmE, and GadXW; the latter being dependent on σ^S [32,71,75,83]. For the EvgSA-YdeO two-component pathway, both EvgA and YdeO activate *gadE* by directly binding to its promoter [71]. For TrmE, the mechanism of *gadE* regulation is unknown, but is dependent on growth in glucose [75]. σ^S-dependent regulation of *gadE* is more defined, controlling its expression indirectly through the transcriptional regulators GadX and GadW [73]. Previous work demonstrated that a strain lacking *rpoN* had increased exponential phase GDAR [56]. Furthermore, upregulation of GDAR was dependent on an intact *rpoS*. Since control of the GadXW circuit of GDAR is dependent on σ^S

(encoded by *rpoS*), it was hypothesized that σ^N is regulating GDAR through the σ^S-GadXW pathway. This was tested by measuring the expression of the σ^S dependent regulators *gadX* and *gadW,* along with two other known *gadE* regulators not controlled by σ^S, referred as *ydeO* and *trmE*. Transcriptional analysis using qRT-PCR for these *gadE* regulators was examined for exponential, late exponential and stationary phases cultures in Sakai, SakaiΔ*rpoN*, and SakaiΔ*rpoN*Δ*rpoS*. The expression of *gadE*, *gadX* and *gadW* was significantly higher in SakaiΔ*rpoN* compared to Sakai during exponential growth ($p<0.05$) (Fig. 2.1). As expected, minimal expression was observed for *gadE*, *gadX*, and *gadW* in SakaiΔ*rpoN*Δ*rpoS* (Fig. 2.1) and for *gadX* in SakaiΔ*rpoS* (Fig. 2.2).

Transcript analysis for σ^S-independent regulators of *gadE* expression (*trmE* and *ydeO*) revealed that σ^N is not working through either pathway since expression levels did not differ statistically between the strains (Fig 2.2). This data supports the hypothesis that σ^N is regulating GDAR expression in a σ^S-dependent manner.

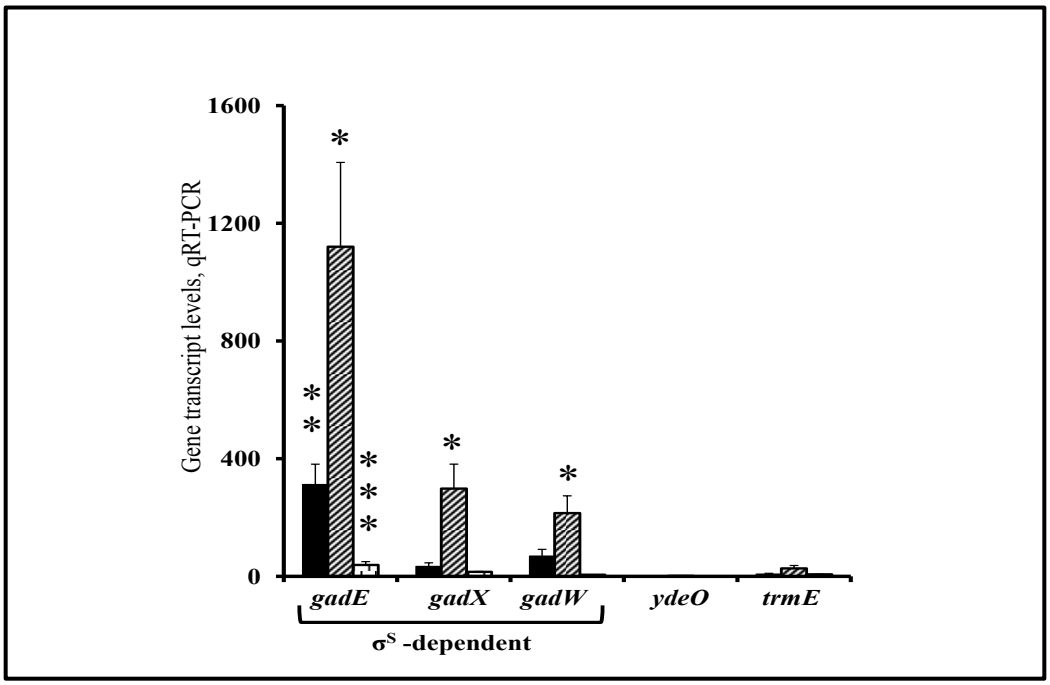

Figure 2.1 Transcriptional analysis of glutamate-dependent acid resistance (GDAR) regulatory genes. Gene transcript levels determined by qRT-PCR are plotted for GDAR regulatory genes *gadE*, *gadX*, *gadW*, *trmE* and *ydeO*. Mean transcript levels are normalized to the 16S rRNA gene *rrsH* and are plotted for Sakai (filled), Sakai Δ*rpoN* (hatched), and Sakai Δ*rpoN* Δ*rpoS* (stippled). Plots which differ in the number of asterisks differ significantly by Tukey's HSD following a significant F-test ($p<0.05$, $n\geq3$).

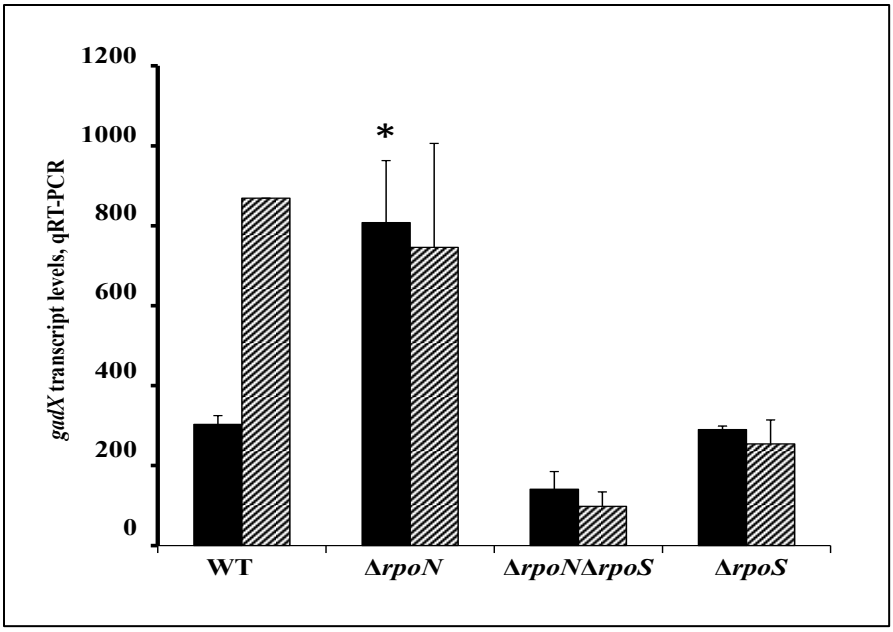

Figure 2.2 qRT-PCR analysis for *gadX*. Gene transcript levels determined by qRT-PCR are plotted for the *gadX* central regulator of GDAR. Mean transcript levels are normalized to the 16S rRNA gene *rrsH*. Transcript levels are plotted for exponential (filled) and late exponential (hatched) phases for Sakai, Sakai Δ*rpoN*, Sakai Δ*rpoNrpoS*, and Sakai Δ*rpoS*. Asterisks represent significance of $p<0.05$ ($n\geq3$) in comparison to WT using student's t-test and error bars indicate standard error of the mean.

The increased expression of *gad* genes correlated with increased survival by GDAR in Sakai Δ*rpoN* (13.4%), when compared to Sakai (0.06%) and Sakai Δ*rpoN*Δ*rpoS* (<0.01%) (Fig. 2.3a). These results suggest that *rpoN* mutation leads to the de-repression of σ^S-GadX/GadW dependent control of GDAR. It was thus predicted that the overexpression of *rpoS* in a WT strain (Sakai*rpoS*$^{+++}$) during exponential growth would lead to a similar level of GDAR seen in Sakai Δ*rpoN*. However, Sakai*rpoS*$^{+++}$ was still highly susceptible to acid, only 1.2% survival (Fig. 2.3a), suggesting that overexpressing σ^S alone does not increase acid resistance. Likewise, it was expected that if σ^N is

suppressing GDAR through σ^S, then *rpoN* overexpression in Sakai during stationary phase, when σ^S-dependent GDAR is normally observed, should interfere with the acid resistance phenotype. GDAR was thus measured during stationary phase in a strain overexpressing *rpoN* (Sakai*rpoN*$^{+++}$). Interestingly, survival of Sakai*rpoN*$^{+++}$ was reduced to 42.2% compared to Sakai (81.62%) ($p<0.02$), but did not differ from SakaiΔ*rpoN* at 47.54% (Fig. 2.4). This result suggests that both the absence and abundance of σ^N negatively affects survival of stationary phase cells in acid.

Both the expression of GDAR regulatory genes, and GDAR results, suggest that σ^N negatively controls acid resistance during exponential growth in a manner dependent on σ^S. A microarray study identified 103 ORFs upregulated and downregulated in a strain mutated for *rpoN* [56]. One gene of interest downregulated in SakaiΔ*rpoN* was *ydeN,* which is a putative sulfatase encoded on the Acid Fitness Island (AFI). The AFI is an area on the genome that contains genes necessary to tolerate acid stress [56,84]. Since *ydeN* is clustered with these genes then it may have some influence on acid stress dependent on RpoN. To determine if this gene is involved in σ^N dependent GDAR, both exponential and stationary phase cells were challenged in acidified media using an isogenic mutant for *ydeN* (SakaiΔ*ydeN*). Stationary and exponential GDAR was similar between SakaiΔ*ydeN* and Sakai. Stationary phase GDAR yielded 81.6% and 64.8% for Sakai and SakaiΔ*ydeN,* respectively, whereas <1.3% survival was detected for either strain during exponential phase (data not shown). This data indicated that the product for this gene is not likely to contribute to the σ^N- σ^S dependent acid resistance mechanism.

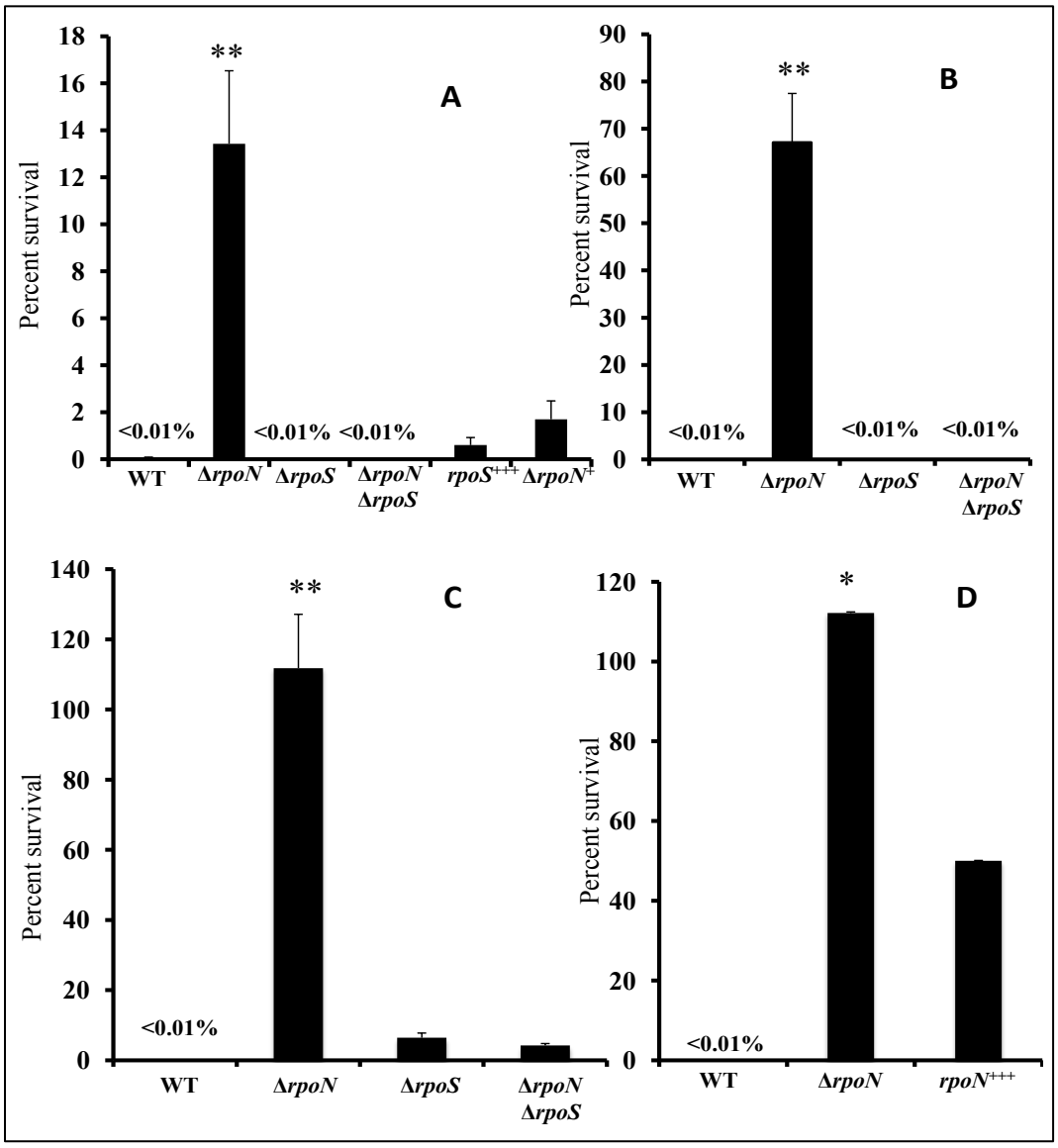

Figure 2.3 Extreme Acid Resistance. Exponential phase acid resistance for (A) GDAR, (B) ADAR, (C) AXAR and (D) AGDAR systems. Cells grown to exponential phase in DMEM (pH 7.2) or LBK (pH 5.5) media then challenged in acidified EG minimal media at pH 2.0 for GDAR and pH 2.5 for ADAR or LBK pH 2.0 for AXAR. For GDAR and ADAR cultures were aerated at 200 RPM in baffled flasks; anaerobic cultures for AXAR and AGDAR were grown and tested in closed capped tubes without rotation. Results shown indicate survival after 1h from starting cultures at ca. 1 x 10^6 CFU/ml. Experiments were repeated two or three times and bars shown represent an average of calculated from CFU/ml at t=1 divided by CFU/ml at t=0. Asterisks indicates significance of *p<0.05 and ** p<0.005 using student's t-test and error bars represent standard error of the mean.

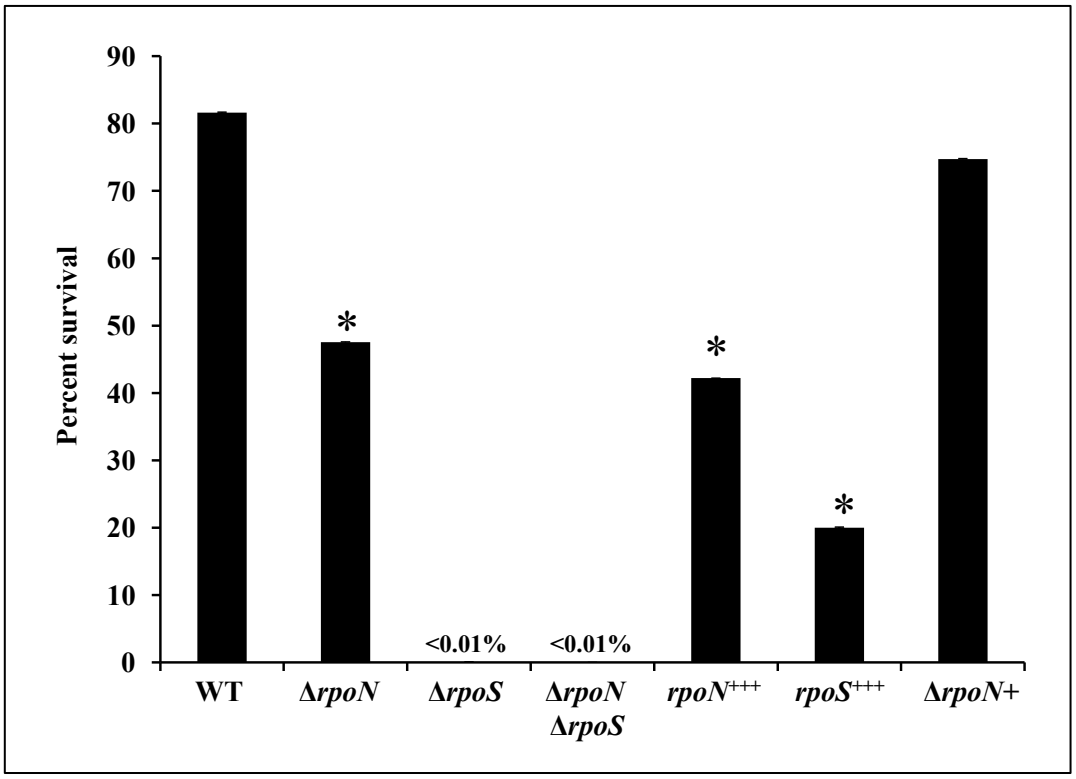

Figure 2.4 Stationary phase glutamate dependent acid resistance. Cells grown to over night in DMEM then challenged in acidified (pH 2.0) EG minimal media supplemented with 5.7mM glutamate. Results shown indicate survival after 2 h from starting cultures at ca. 10^6 CFU/ml. Experiments were repeated two or three times to determine average calculated from CFU/ml at t=1 divided by CFU/ml at t=0. Asterisks indicate $p<0.05$ using student's t-test in comparison to WT ($n\geq3$) and error bars represent standard error of the mean.

Role for σ^N in arginine-dependent acid resistance

E. coli depends on many AR systems to ensure survival in acidic environments. Similar to GDAR, the arginine-dependent acid resistance (ADAR) system uses decarboxylase activity to increase cellular pH via an arginine-specific decarboxylase (AdiA) and a membrane-associated antiporter (AdiC). σ^S has shown to regulate ADAR although the mechanism is not as defined as σ^S regulation of GDAR. Since σ^N was

observed to control GDAR in a σ^S-dependent manner the following experiments investigated if σ^N also regulates ADAR by a similar mechanism. To test if σ^N influences exponential phase ADAR, cells were challenged in acidified media supplemented with L-arginine. Unlike GDAR, mutation of *rpoN* did not lead to survival by the ADAR mechanism; the number of CFU/ml recovered being <0.01% for Sakai and SakaiΔ*rpoN*. Interestingly, increasing the inoculation density from 1×10^6 to 1×10^7 CFU/ml into acidified EG test environments resulted in a 67.1% survival for SakaiΔ*rpoN* following 1 hour acid challenge whereas there was still no detectable survival for Sakai, SakaiΔ*rpoS* or SakaiΔ*rpoN*Δ*rpoS* ($p<0.005$, n≥6) (Fig. 2.3b).

Survival by the ADAR mechanism during stationary phase was unexpectedly low for both Sakai (≤0.03%) and SakaiΔ*rpoN* (≤0.06%). Although ADAR has been shown to vary substantially by media type and acid adaptation, percent survival typically ranges from 65% - 73% in stationary phase cultures [32,41]. This lack of survival suggests that some component of DMEM media is prohibitive to stationary phase expression of ADAR.

In contrast to the GDAR system, the regulation of the ADAR system is not fully understood, although some of the essential genes have been characterized [34,77,79]. Lin *et al.* (1996) suggested that ADAR may have some dependence on σ^S based on reduced survival in acidified EG media supplemented with arginine [34]. The results from this study imply that σ^N may be suppressing the ADAR system since the *rpoN* mutant has significantly more survival at 67% compared to Sakai and *rpoS* mutants with <0.01%. To determine if σ^N regulates transcript expression for the ADAR regulatory genes, qRT-PCR

was used to measure mRNA levels of the transcriptional regulator *adiY*, arginine decarboxylase *adiA* and arginine-agmatine specific antiporter *adiC*. Expression for *adi* genes during exponential phase (OD_{600} = 0.5) was higher in SakaiΔ*rpoN* than for Sakai by nearly 2-fold (Fig. 2.5). To test if σ^N regulates ADAR expression in a σ^S-dependent manner, transcripts were analyzed in a SakaiΔ*rpoN*Δ*rpoS* strain and expression was similar to that observed in Sakai for all three *adi* targets. Thus the increased expression of *adi* genes in SakaiΔ*rpoN* (Fig. 2.5) correlates with increased survival by ADAR (Fig. 2.3b), and is dependent on an intact *rpoS* gene, as for GDAR.

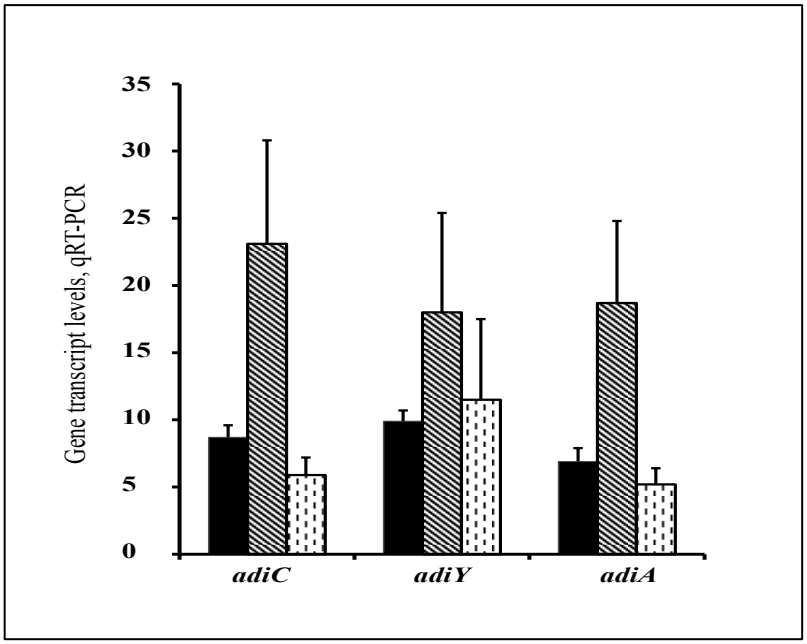

Figure 2.5 qRT-PCR analysis of XAR regulatory genes for the ADAR system. Gene transcript levels as determined by qRT-PCR are plotted for regulatory and structural genes of the ADAR system. Mean transcript levels are normalized to the 16S rRNA gene *rrsH*. Transcript levels are plotted against Sakai (filled), SakaiΔ*rpoN* (hatched), and SakaiΔ*rpoNrpoS* (stippled). Error bars indicate standard error of the mean (n≥3).

Dependence on σ^N for anaerobic acid resistance

Fermentative conditions are common to the gastrointestinal environment and *E. coli* is known for its ability to survive as a facultative anaerobe. Anaerobic conditions induce activity of an extreme acid resistance system that uses Hydrogenase-3 (Hyd-3) to increase cellular pH. Hyd-3 is part of the formate hydrogen lyase (FHL) complex, which breaks down the fermentative product formate to H_2 and CO_2 [81,82]. The genes encoding the Hyd-3 complex are located in a polycistronic operon directed by a σ^N promoter. Therefore it would be expected that a mutation in σ^N would cause cells to become acid susceptible similar to that observed for a strain lacking Hyd-3 [82]. To date, there is no evidence of dependence on σ^S for transcription. To determine if σ^N controls this anaerobic extreme acid resistance (AXAR) system, survival was measured by challenging exponential phase cells in acidified rich media in the absence of oxygen. This experiment yielded unexpected results in that no reduction in survival was observed for SakaiΔ*rpoN,* whereas Sakai and SakaiΔ*rpoNrpoS* were completely susceptible during exponential growth ($p<0.005$) (Fig. 2.3c), suggesting that AXAR in the *rpoN* null background is working independent of the Hyd-3 mechanism suggested by Noguchi et al [82]. One complication of using rich media to test for AR is that it is not controlled to select for a particular AR system. For example, the GDAR system functions under anaerobic conditions [43], which correlates with increased expression of the *gad* structural and regulatory genes [85].

Discussion

Acid resistance (AR) is required for transmission of EHEC, and contributes to successful colonization and infection. Although alternative sigma factors are generally considered to be non-essential to the cell they are important regulators of survival, and respond to inclement conditions such as low pH, salinity, nutrient limitation, heat stress [52]. The σ^N regulon includes genes linked to motility [55], biofilm formation [86], nitric oxide stress [87], type III secretion [56], and GDAR [56]. σ^N clearly functions in nitrogen metabolism since cells mutated for *rpoN* are growth impaired under nitrogen-limiting conditions in *E. coli* and other bacteria [88,89,90]. Specifically, *rpoN* mutants are glutamine auxotrophs since σ^N is needed to direct transcription of glutamine synthetase (*glnA*) under nitrogen limiting conditions [88,91]. It seems contradictory that the *E. coli* would have a mechanism to suppress glutamate dependent acid resistance during exponential phase growth. However, an explanation for this regulation could be that cells prioritize the use of nitrogenous compounds for nitrogen assimilation over decarboxylation. It would be interesting to investigate this further and determine if σ^N regulation of acid resistance has dependence on the nitrogen status of the cell. The results of this study reveal that σ^N represses the σ^S dependent GadX/GadW pathway at the transcript level, which in turn, would reduce expression of the central regulator *gadE*, and *gad* structural genes. σ^S is a known regulator of GDAR regulatory genes and no survival is detected for O157:H7 strains mutated for *rpoS* [92], or *rpoN/rpoS* during exponential or stationary phase growth [56]. One explanation for this regulation is that the presence σ^N negatively controls σ^S, restricting GDAR activity. This study attempted to test this by

overexpressing *rpoS* to overcome this mode of regulation, however this did not reproduce the levels of GDAR observed in the *rpoN* null background. Literature has shown that when *rpoS* is expressed on a high copy vector, only trace amounts of σ^S protein can actually be detected due to rapid proteolytic turnover [93] (REF 46), which could explain the unexpected results shown here.

The decarboxylase AR systems are induced by low pH but are also triggered by low oxygen levels [42,94]. This study determined that σ^N represses anaerobic acid resistance since there was no reduction in survival using acidified rich media. However, rich media cannot select for a particular AR system, and other systems operate in low oxygen environments, such as the GDAR system. Challenging anaerobic cultures for GDAR tested this and there was no difference in survival. Together these results suggest that the GDAR system may be the main source of acid resistance under anaerobic conditions and not the Hyd-3 system previously shown by Noguchi *et al.* [82].

Inactivation of *rpoN* was also shown to affect arginine dependent acid resistance (ADAR), which operates in a similar manner to GDAR. Lin *et al*. (1996) suggested that there might be a slight dependence on σ^S for ADAR and the results of this study agree with those findings [35]. Furthermore, transcript analysis suggests that σ^N represses *adi* genes essential for this system. The mechanism by which σ^N is suppressing exponential phase ADAR is yet to be discovered but it requires σ^S since survival was undetectable in the SakaiΔ*rpoN*Δ*rpoS* strain, similar to what was observed for GDAR. In contrast to GDAR, this phenotype was only observed when the initial cell density was increased from 10^6 to 10^7 CFU/ml. The influence of substances released as a result of cell density

was investigated by Cui *et al*. (2001) and they determined that ADAR is not affected by higher initial cell densities [95]. The disparity in survival may be attributed to other AR systems functioning in parallel with ADAR. One such example would be AR due to a change in membrane potential, which is a known mechanism of acid survival [43]. A mutation in *rpoN* could influence changes to the cell membrane that could in turn alter the membrane charge. The colony morphology of *rpoN* mutant strain was smaller than the wild-type and other mutant strains used in this study. Additionally, the higher cell density could have influenced the quorum sensing capabilities that induce such changes as described by Eboigbodin *et al.* (2006)[96]. This group determined cells deficient in quorum sensing abilities were also impaired with changing their surface chemistry which could also be a factor in membrane charge suggested here.

Intermediates and products of biochemical pathways link systems together that may otherwise seem unrelated. For example, the arginine used for ADAR can also be used as a nitrogen source by *E. coli* and its catabolism through the arginine succinyltransferase (AST) pathway or arginine decarboxylase (ADC) pathway generates glutamate as a byproduct [97,98]. Similarly, exogenous glutamate used for GDAR can be metabolized through the glutamine synthetase pathway to assimilate nitrogen. Metabolism of arginine and glutamate, along with amino acids, demonstrate the uncanny ability of *E. coli* to scavenge for nitrogen when sources are limited by expressing genes of the nitrogen response system (NTR-system). Future studies to investigate the relationship between the NTR-system and glutamate- and arginine-dependent acid resistance systems may elucidate intermediates responsible for driving the AR phenotype.

CHAPTER 3

Molecular and Functional Analysis of the *rpoN* Operon in Enterohemorrhagic *Escherichia coli* (EHEC)

Background

E. coli has several alternative sigma factors that belong to the σ^{70} family, but only one belonging to the σ^{54} family. referred to as sigma factor N (σ^{N}) encoded by *rpoN*. To initiate σ^{N} -RNA polymerase (RNAP)-dependent transcription, σ^{N} dependent promoters require bacterial enhancer binding proteins (bEBP) that facilitate formation of the open promoter complex through hydrolysis of ATP [99]. *rpoN* is the first gene located on a polycistronic operon comprised of *rpoN, hpf, ptsN, yhbJ,* and *npr* (Fig. 3.1) [100]. σ^{N} was originally identified as a nitrogen stress regulator and many of the operon genes have putative functions that relate to nitrogen regulation [62,101]. The importance of σ^{N} in the control of metabolism, motility and pathogenesis has been shown [54,56,102], but the regulation of σ^{N} activity and expression is unclear. The downstream genes of this operon have not been fully characterized, although it is predicted that the products of *ptsN* and *npr* are structural proteins of a nitrogen specific phosphotransferase system (PTS^{Ntr}) [103]. The role for these products in PTS^{Ntr} has been inferred from research in other bacterial species with homologous *rpoN* operons, such as *Klebsiella pneumoniae*, *Azobacter vinelandii, and Rhizobium meliloti* [11,62,104,105].

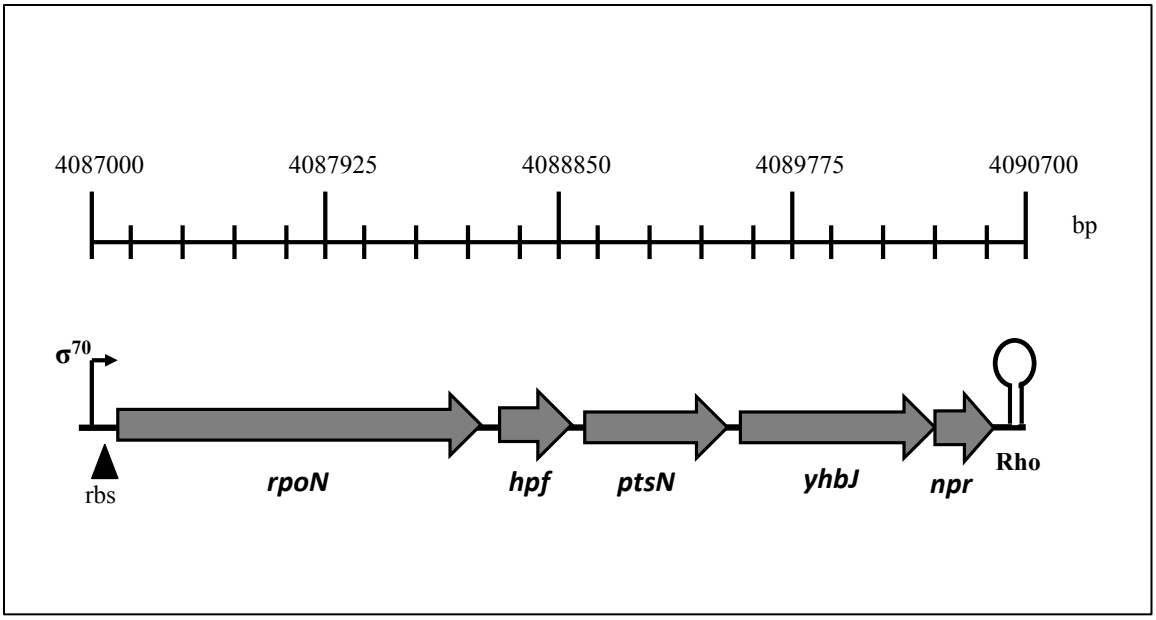

Figure 3.1 The *rpoN* operon for EHEC (Sakai NCBI Genbank accession number NC_002695). Genes are co-transcribed from a σ^{70} promoter with a Rho-dependent terminator downstream of *npr*.

Studies in *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* revealed that the *hpf* and *ptsN* products negatively regulate σ^{N}-dependent promoters involved in nitrogen assimilation [60,61,62]. Jin *et al.* (1994) also found that *rpoN* and a *ptsN* homolog jointly function in glutamine assimilation in *P. aeruginosa* [60], which is consistent with the role of σ^{N} in regulation of nitrogen metabolism. The products for *ptsN* and *npr* share similarity with those of phosphotransferase systems (PTS) common to enteric bacteria [100]. The product of *ptsN* shares homology to the A subunit of the enzyme II (EII) fructose complex $EIIA^{Fru}$ in *Salmonella typhimurium* and EII mannitol complex ($EIIA^{Man}$) in *E. coli* [100,106,107]. PtsN is similar to the sugar PTS systems in that it receives phosphate donated from Npr, however unlike these systems PtsN does not transfer phosphate to a membrane bound EIIB complex since the PTS^{Ntr} system lacks the

EIIB-EIIC components (for review see [103]). The product for *npr* shows sequence similarity to HPr of the glucose specific PTS (PTS^{Glc}) which transfers a phosphoryl group received from phosphophenylpyruvate (PEP) to $EIIA^{Glc}$ [100]. Collectively *ptsN* and *npr* function in phosphotransfer for the PTS^{Ntr} system to maintain cellular homeostasis in parallel with other PTSs, potentially bridging the regulation of nitrogen and carbon metabolism in the cell [108].

Of the five genes located within the *rpoN* operon, least is known about the fourth gene, referred to as *yhbJ,* which is predicted to encode an ATPase-like protein. In 2007, Kalamorz *et al*. determined that *yhbJ* negatively regulates GlmZ, which is a small regulatory RNA that activates glucosamine-6-phosphate synthase (GlmS) [109]. GlmS is an enzyme that catalyzes frucotose-6-phosphate and glutamine to glucosamine-6-phosphate and glutamate [110]. Glucosamine-6-phosphate is a precursor amino sugar used in cell wall synthesis, and Kalamorz *et al.* (2007) found that the YhbJ may function in sensing this molecule.

The products of genes encoded on polycistronic operons often contribute to similar metabolic functions since they are clustered together. Products of these operons may act as positive or negative regulators to control their own expression, as in the *E. coli glnALG* regulation by GlnG [91,111]. Since *rpoN* is located on a polycistronic operon, the downstream co-transcribed genes may function to directly regulate *rpoN* expression, or indirectly alter σ^N-regulated pathways and associated phenotypes. There is precedent for this in *E. coli* sigma factor regulation. For example, the membrane stress sigma factor E (*rpoE*) is negatively regulated by the product of a gene encoded directly downstream,

rseA. RseA is an anti-sigma factor, and interferes with the ability of σ^E to form the holoenzyme with RNAP, which inhibits autoregulation from one of the promoters of this operon [112].

This study investigated the importance of *rpoN* operon genes in the regulation of *rpoN*/σ^N, and explored the phenotypic similarities of *rpoN* operon mutants during nutrient limitation and extreme acid stress. To test this, isogenic mutants were constructed for each of the downstream genes and phenotypically tested for glutamate-dependent acid resistance (GDAR) and viability under nutrient limitation. Results indicate that several of the *rpoN* operon mutants phenocopy Δ*rpoN* for survival under nutrient limiting conditions and for GDAR, and that *hpf* and *ptsN* may have a role in the post-transcriptional control of σ^N.

Results

The genes of the *rpoN* operon are highly conserved

The coding region of the *rpoN* operon is 3521 bp with a σ^{70} promoter located 70 bp upstream of the *rpoN* start codon. The *rpoN* operon is highly conserved among other Enterobacteriaceae such as *Klebsiella pneumonia*, *Salmonella enterica* serovar *tymphimurium*, and *Yersinia pestis* with 81%, 85%, and 76% nucleotide sequence identity, respectively (NCBI BLAST). Alignments were performed using ClustlW with MEGA 5 software to compare the *rpoN* operon to pathogenic and non-pathogenic *E. coli* and *Shigella* species. For the aligned strains, no single nucleotide polymorphisms (SNPs)

were identified within the *hpf* gene located downstream of *rpoN*. Strains were grouped based on similarities of SNPs and compared to Sakai. For all strains, *rpoN, ptsN, yhbJ*, and *npr* show 99.9% homology whereas *hpf* has 100% (Fig. 3.2). The entire aligned operon consists of five ORFs totaling 3,347 bp of coding region. Aside from one synonymous mutation between *hpf* and *ptsN*, there were no identified mutations within the non-coding intergenic regions of the operon including the promoter region upstream of *rpoN*. Two point mutations, however, were identified within the Rho-dependent terminator region following the stop codon for *npr*, one being a transversion (C/A) and the other a transition (A/G).

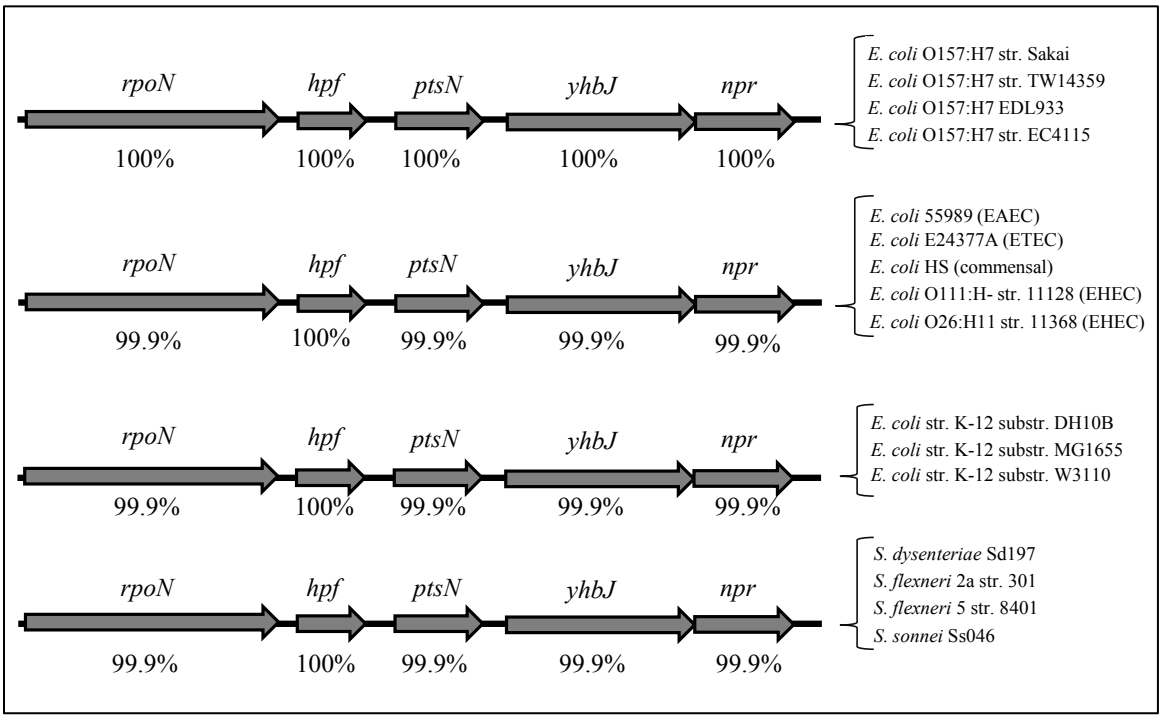

Figure 3.2 Alignment comparison of *rpoN* operon. Illustration depicts amino acid homology of *rpoN* operon genes in comparison to Sakai among listed strains. Percentage was determined by nonsynonymous mutations and strains are clustered into groups based on similarities. Sequences were obtained from NCBI (08/10/2010) and aligned by ClustlW using MEGA 5 software.

The influence of *hpf* and *ptsN* on GDAR

This study has already shown that the product of *rpoN* (σ^N) represses GDAR during exponential growth (Fig. 2.4a). It is known that *rpoN* is located in a polycistronic operon, and there is evidence to suggest that some downstream products of this operon may influence σ^N expression or activity in other bacteria [62,100]. If σ^N contributes to acid resistance then it is possible that other genes of this operon may also influence acid resistance through regulation of *rpoN*/σ^N. To determine if *hpf*, *ptsN*, *yhbJ* or *npr* contribute to GDAR, exponential phase cultures of Sakai and mutant derivatives isogenic for each gene of the operon were challenged for 1 h in acidified (pH 2.0) EG media with glutamate during exponential growth. The results revealed increased survival for both SakaiΔ*hpf* (6.91%) and SakaiΔ*ptsN* (5.49%) compared to Sakai (<0.01%) (Fig.3.3). Survival, however, was less than observed for SakaiΔ*rpoN* (12.38%). *hpf* and *ptsN* are located immediately downstream of *rpoN*, and encode a putative σ^N modulation protein referred to as hibernation promoting factor (HPF) [61] and a nitrogen specific enzyme IIA subunit (IIA^{Ntr}) [101], respectively. This data reveals that like *rpoN*, the mutation of *hpf* and *ptsN* de-represses GDAR during exponential growth, whereas the remaining mutants of the operon, Δ*yhbJ* and Δ*npr*, seem to have no effect on GDAR expression.

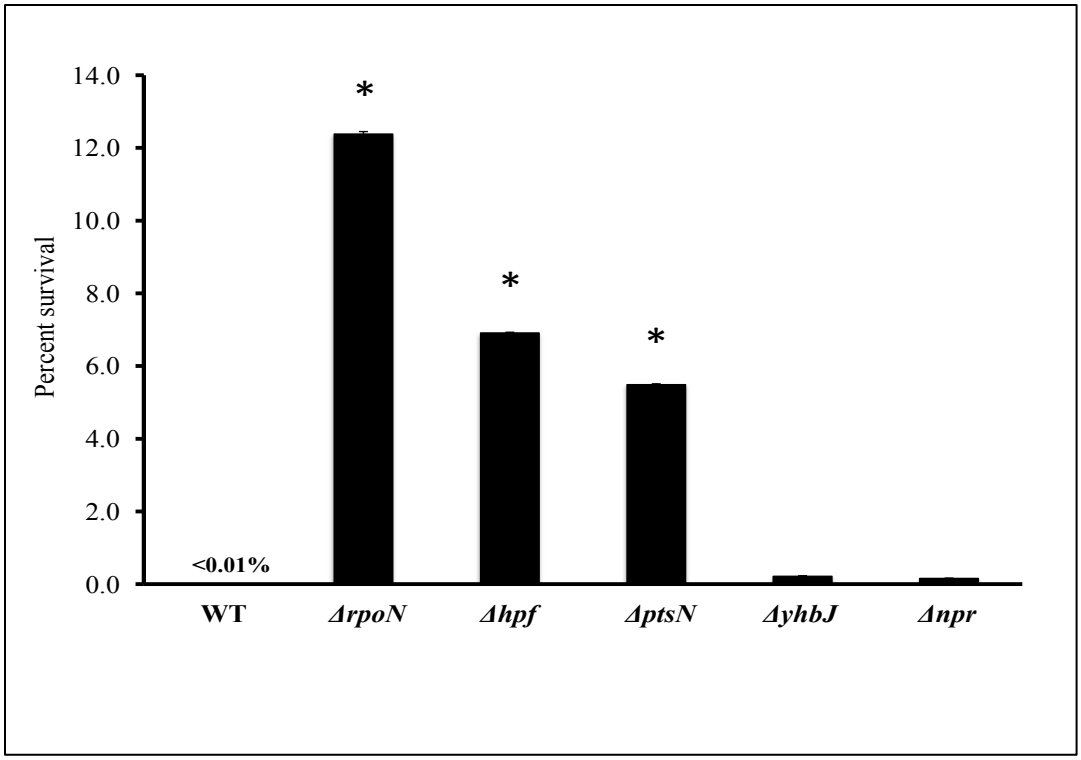

Figure 3.3 Exponential phase glutamate-dependent acid resistance. Cells were grown to exponential phase (OD_{600} = 0.5) in DMEM as described in materials and methods, then challenged in acidified (pH 2.0) EG minimal media supplemented with 5.7mM glutamate. Results indicate survival after 1 h from starting cultures at ca. 1 x 10^6 CFU/ml. The mean of three or more trials are shown, with error bars representing SEM. Asterisk indicates statistically significant differences ($p<0.05$)($n\geq3$) using student's t-test in comparison to WT. The strains tested were: TW14359, EcRPF6, EcRPF7, EcRPF8, EcRPF9 and EcRPF10.

The role of *rpoN* operon products in the transcription of *rpoN*

Since mutation of *hpf* and *ptsN* partially phenocopied TW14359Δ*rpoN* for GDAR, it was predicted that perhaps the products of these genes are in some way affecting *rpoN* expression. The next goal was to determine if the products of *hpf* or *ptsN* affect expression of *rpoN*.

Overall the abundance of *rpoN* transcript in Sakai was low, which agrees with the low cellular concentration of σ^N, averaging approximately 110 subunits per cell at all growth stages in *E. coli* [113]. Mutation of *hpf* led to a slight (1.6-fold) but significant increase in expression of *rpoN* ($p<0.03$) at $OD_{600} = 0.5$ compared to Sakai (Fig. 3.4). *ptsN* mutation increased *rpoN* transcript levels 1.4-fold, but not significantly. This finding indicates that HPF perhaps negatively regulates *rpoN* transcription and GDAR, which does not agree with a model in which HPF and σ^N are predicted to control GDAR through a common pathway of regulation.

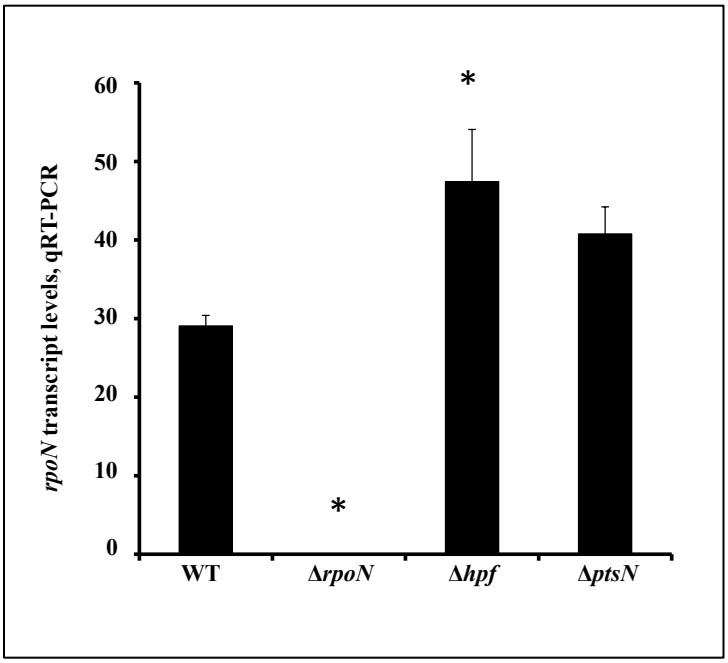

Figure 3.4 qRT-PCR analysis of *rpoN*. Transcript levels, as determined during exponential phase by qRT-PCR are plotted for expression of *rpoN* for *rpoN*, *hpf*, and *ptsN* mutants in TW14359. Mean transcript levels are normalized to the 16S rRNA gene *rrsH*. Asterisks represent significance of $p<0.05$ ($n\geq3$) in comparison to WT using student's t-test and error bars indicate standard error of the mean.

Hpf and PtsN influence expression of σ^N-dependent genes.

Previous work to characterize *hpf* and *ptsN* in *Klebsiella pneumonia* led to the hypothesis that these gene products alter σ^N activity, negatively regulating expression from σ^N-directed promoters [62]. To determine if these genes have a similar role in EHEC, we investigated their influence on the expression of three known σ^N dependent genes: *glnA*, *astA* and *pspA*. Thus, qRT-PCR was used to measure transcript levels of *glnA*, *astA*, and *pspA*, which are known to be downregulated in a strain mutated for *rpoN* during growth in DMEM [56]. The *glnA* product is glutamine synthetase, and its expression is driven by a strong σ^N promoter ($glnA_{P2}$) during growth in DMEM [114], and also has a σ^{70} promoter. Arginine succinyltransferase is the product of *astA* and functions in catabolizing L-arginine to succinate. The $astA_P$ promoter is also driven by σ^N in DMEM, but also has promoters for σ^{70} and σ^S. The *pspA* product is a transcriptional regulator of the phage shock operon [59,97], and is exclusively expressed from a σ^N promoter.

Mutation of *rpoN* was observed to significantly reduce expression of *glnA* by 70-fold ($p<0.05$) compared to TW14359 during exponential growth (Fig. 3.5). Trace levels of *glnA* expression in the Sakai*Δrpo*N background are likely due to transcription from *gln*Ap1 that is driven by σ^{70} [115]. Expression of *glnA* in TW14359Δ*hpf* and TW14359Δ*ptsN* was each reduced 2-fold in comparison to TW145359 ($p<0.05$), which suggests a partial dependence on either or both of these genes for expression from σ^N-dependent promoters (Fig. 3.5). Similarly, expression of *pspA* was reduced in Sakai*Δrpo*N by nearly 7-fold compared to TW14359. However, in Sakai*Δhpf* and

SakaiΔ*ptsN* the expression of *pspA* increased compared to TW14359 5-fold and 2-fold, respectively ($p<0.05$), which could imply that one or both of these genes negatively regulate the *pspA* σ^N-dependent promoter. A mutation in *rpoN* reduced expression of *astA* by 6-fold, and likewise, expression was reduced by 4-fold for SakaiΔ*hpf* and SakaiΔ*ptsN*. Both Hpf and PtsN appear to regulate σ^N dependent promoters, however the direction of regulation seems to vary depending on the target gene.

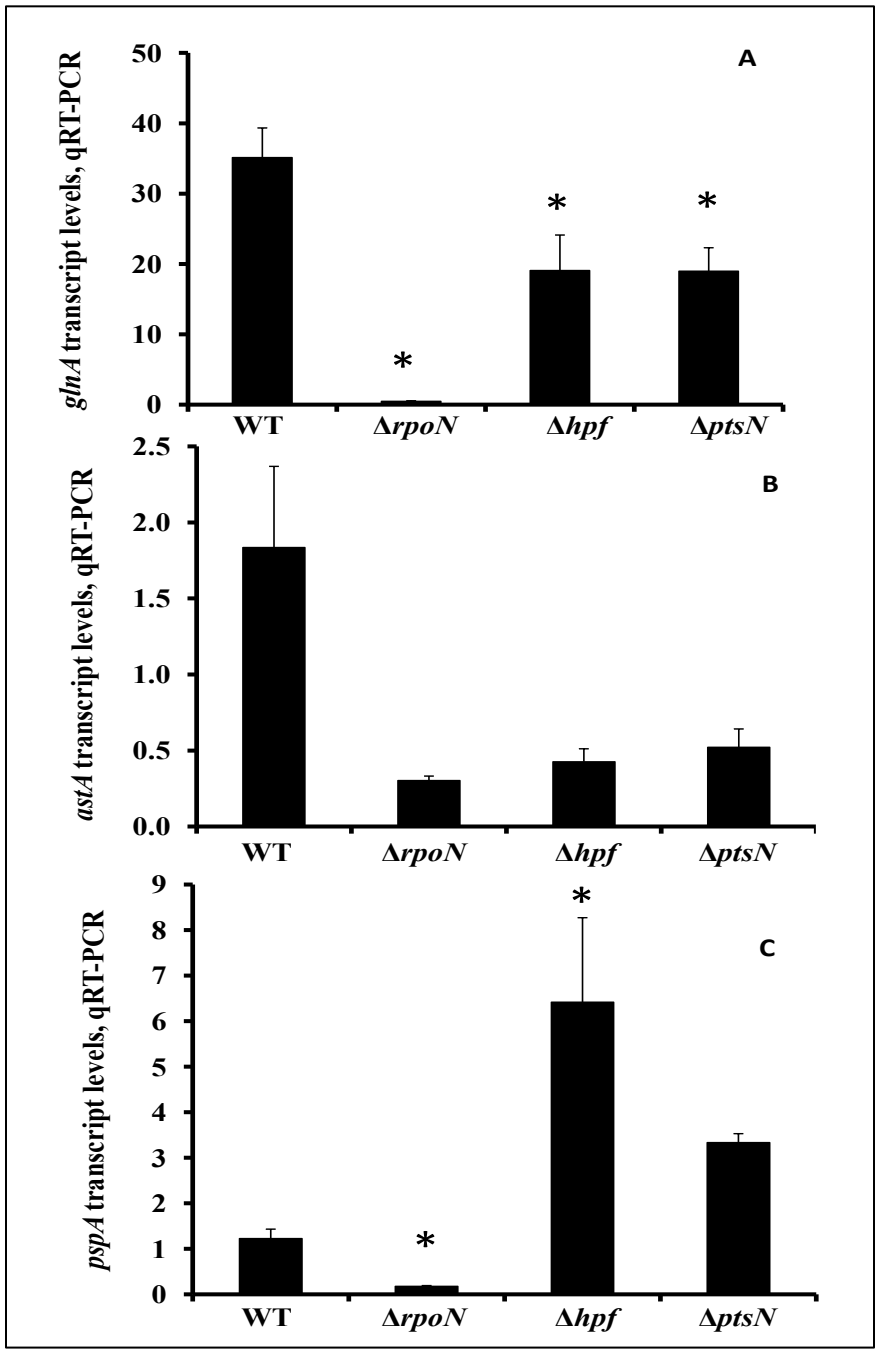

Figure 3.5 Transcript expression of *rpoN*-dependent genes. Transcript levels, as determined during exponential phase by qRT-PCR are plotted for (A) *glnA,* (B) *astA,* and (C) *pspA for* TW14359 isogenic mutants of *rpoN*, *hpf*, and *ptsN*. Mean transcript levels are normalized to the 16S rRNA gene *rrsH*. Asterisks represent significance of $p<0.05$ ($n\geq3$) in comparison to WT and error bars indicate standard error of the mean.

Expression profile of the *rpoN* operon

The data presented here indicates that both *hpf* and *ptsN* products are involved in the control of *rpoN* at the transcriptional level. It was of interest to determine if the pattern of expression of each gene of the *rpoN* operon was the same or in unequal amounts. To examine this, qRT-PCR analysis was used to measure transcript levels of the operon genes during exponential and late exponential/transition growth in DMEM.

Expression analysis revealed that *hpf* transcripts were higher in comparison to all the operon genes *ptsN*, *yhbJ* and *npr* by at least 2-fold. *hpf* expression was significantly higher that its preceding gene *rpoN* by 4-fold and 17-fold for exponential and late exponential phase, respectively ($p<0.04$, $p<0.02$) (Fig. 3.6). Two of the genes following *hpf* (*ptsN* and *yhbJ*) revealed an expression pattern similar to *rpoN* while transcripts of the final gene in the operon, *npr*, were barely detectable; expression levels being 300-fold ($p<0.001$) and 600-fold ($p<0.005$) less than *rpoN* for exponential and late exponential phase, respectively. The amplicons for *npr* were verified using gel electrophoresis since its expression was in such low abundance. To further confirm differential expression of the operon genes in EHEC, the experiment was repeated in the genetically distinct EHEC strain Sakai, and there were no differences in expression pattern between the two EHEC strains.

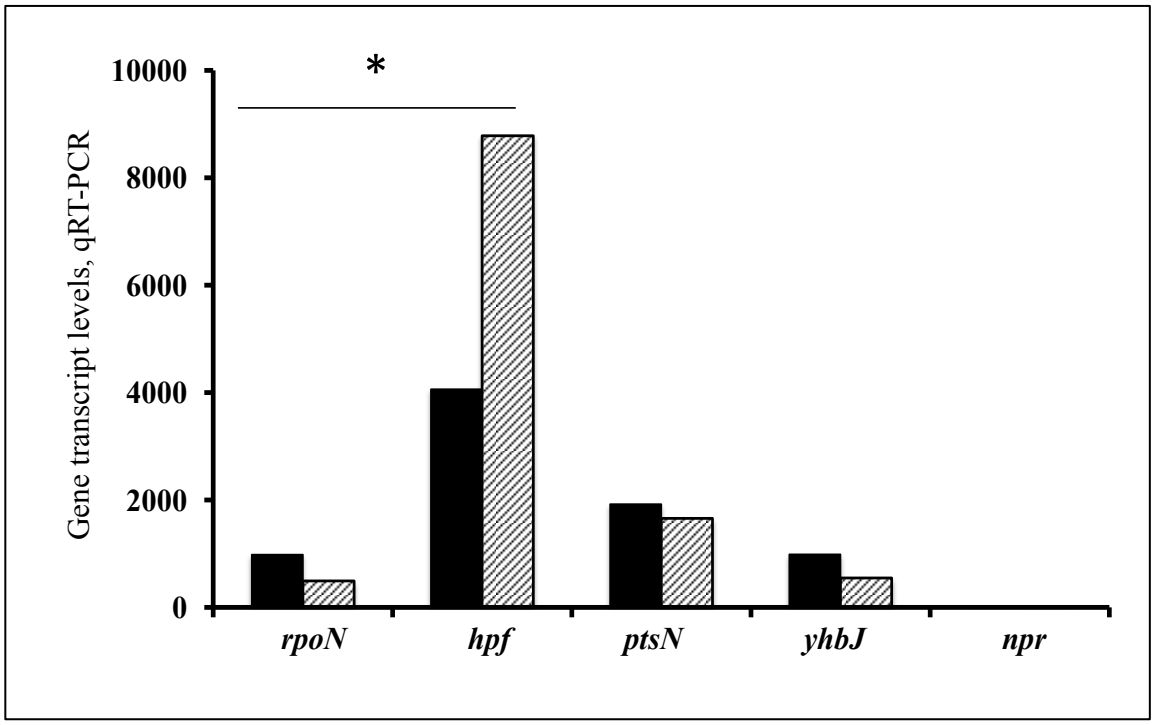

Figure 3.6 Transcript profile of *rpoN* operon genes. Transcript levels, as determined by qRT-PCR, are plotted for genes of the *rpoN* operon under normal conditions in DMEM media for: exponential (filled) and late exponential (hatched) phases. Mean transcript levels are normalized to the 16S rRNA gene *rrsH*. Transcript levels are represented from TW14359 for each of the isogenic mutants. Asterisks represent significance of $p<0.05$ ($n\geq3$) using student's t-test and error bars indicate standard error of the mean.

The effect of nutrient limitation on the growth of strains mutated for *rpoN* operon genes

To further explore the characteristics of operon genes in comparison to *rpoN*, we investigated the importance of *rpoN* operon genes for growth under nutrient limiting conditions by challenging each mutant in minimal media limited for carbon (MOPS-C^-), nitrogen (MOPS-N^-), or phosphate (MOPS-P^-). If any of the gene products regulate

rpoN or σ^N activity then growth in limited media may be similar to an *rpoN* mutant. Generation time was extrapolated from a linear standard curve plotting OD_{600} as a function of CFU/ml for each strain. The strength of linearity (R^2-value) was found to be >0.80 for all strains and conditions, except for TW14359Δ*rpoN* ($R^2 = 0.71$) in phosphate limited media. The generation time lag (G_L) was determined for WT and mutant strains as described in methods.

In the MOPS-C^- environment, cells mutated for *hpf* or *npr* actually had significantly reduced G_L compared to TW14359. Those mutated for *rpoN*, *ptsN* and *yhbJ* did not differ significantly under these conditions (Fig. 3.7). Therefore, inactivating *hpf* and *npr* increased the ability for cells to scavenge for carbon when sources are limited. For MOPS-N^-, G_L significantly increased for TW14359Δ*rpoN* and TW14359Δ*yhbJ* by 99.3- and 46.5-min, respectively, in comparison to 34.9 min for TW14359 ($p<0.03$) (Fig. 3.7); G_L did not differ significantly in TW14359Δ*hpf*, TW14359Δ*ptsN* and TW14359Δ*npr* compared to TW14359. For the MOPS-P^- environment, G_L was significantly increased compared to TW14359 for all of the mutants, except for TW14359Δ*hpf* (Fig. 3.7). In comparison to other limiting conditions tested, it appears that the majority of the genes in this operon are important to assimilating or transporting phosphorous or phosphorous containing compounds.

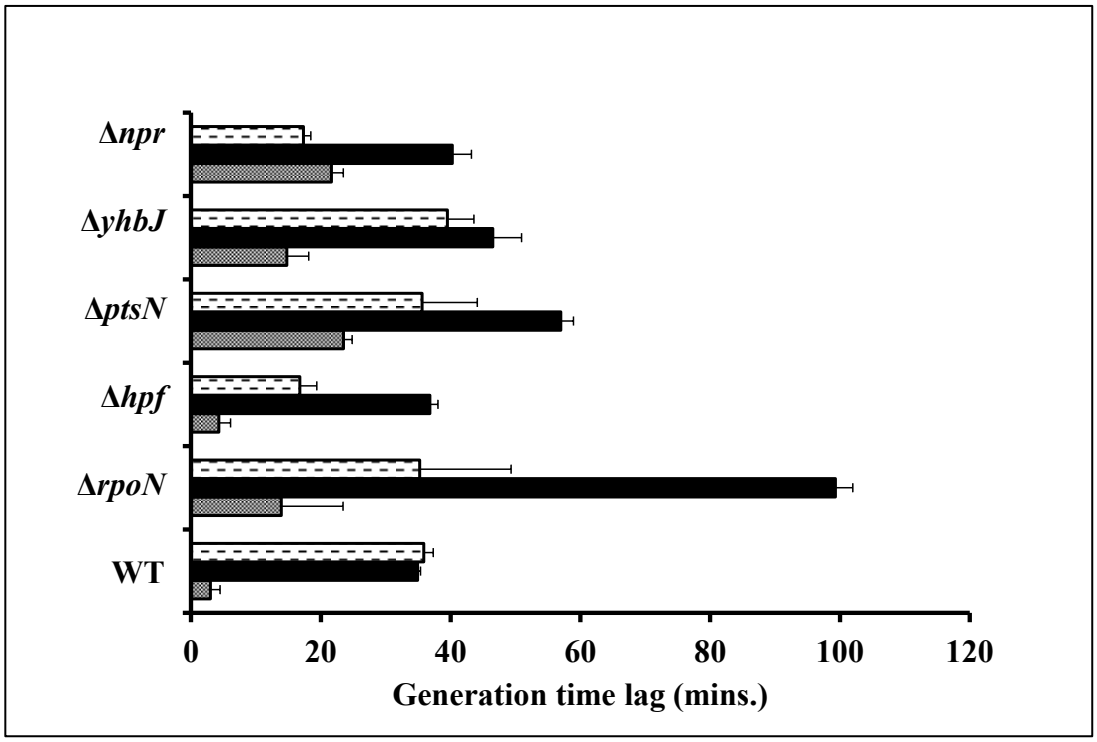

Figure 3.7 Generation lag time for operon mutants under different nutrient limiting conditions. Cells were grown overnight in MOPS passaged to limited media for carbon (dashed), nitrogen (filled), and phosphorous (shaded) using MOPS minimal media. $OD_{(600)}$ readings were recorded for 8 h during exponential growth. Generation time was calculated using the formula $G=[(Log_{OD2} - Log_{OD1})/0.301]/T_{(mins)}$ and generation time lag (G_L) time was calculated from generation time (g) normalized to data from MOPS media.

Role for *rpoN* operon genes during starvation under nutrient limiting conditions

Cell viability data was examined over a five-day period for nitrogen, phosphate, and carbon starvation for each of the constructed isogenic mutants to determine if any of the genes phenocopy *rpoN* mutants for survival. Determination of viability was carried out by plate counts at 24, 48, 72, 96, and 120 h following overnight growth in MOPS

limited media for carbon (MOPS-C^{-}), nitrogen (MOPS-N^{-}), or phosphate (MOPS-P^{-}). A mutation in *rpoN* slightly impaired survival in the MOPS-C^{-} environment, with a 2-fold decrease at 24 h compared to TW14359 (Fig. 3.8). Additionally, TW14359Δ*rpoN* exhibits a rapid decrease in viability after 24 h whereas viability of the operon mutants tend to fluctuate after this time point, and do not fully phenocopy TW14359Δ*rpoN* under these conditions. It was expected that the presence of *rpoN* will allow for cells to best survive in nitrogen limiting conditions and the results here confirm this hypothesis since cells lacking *rpoN* averaged nearly a 6-fold reduction in survival compared to TW14359 over the five day period. Initially a mutation in *hpf* allowed for a slight increase in viability compared to TW14359 at 24 h, whereas all other mutants showed nearly a 2-fold decrease. Mutations in *ptsN* and *yhbJ* allowed for cells to scavenge nitrogen for 48 h but cell counts decreased to levels similar to those of TW14359Δ*rpoN* following this time, whereas cells lacking *npr* exhibit static survival over time, similar to TW14359. Although the MOPS-P^{-} environment seemed to have the most impact on cell viability, it was interesting that the operon mutants averaged a 12-fold increase in survival at 24 h compared to TW14359 under these conditions. Mutations in the operon genes increased the ability of cells to initially scavenge phosphorous although over the 120 h time period all mutants had a reduction in viability similar to TW14349. Also, a mutation in *npr* led to a gradual reduction in survival in phosphate limiting conditions, whereas this mutation did not seem to impact viability in carbon or nitrogen limiting conditions. The results here indicate that the operon mutants have an advantage to survive under starvation conditions limited for phosphorous.

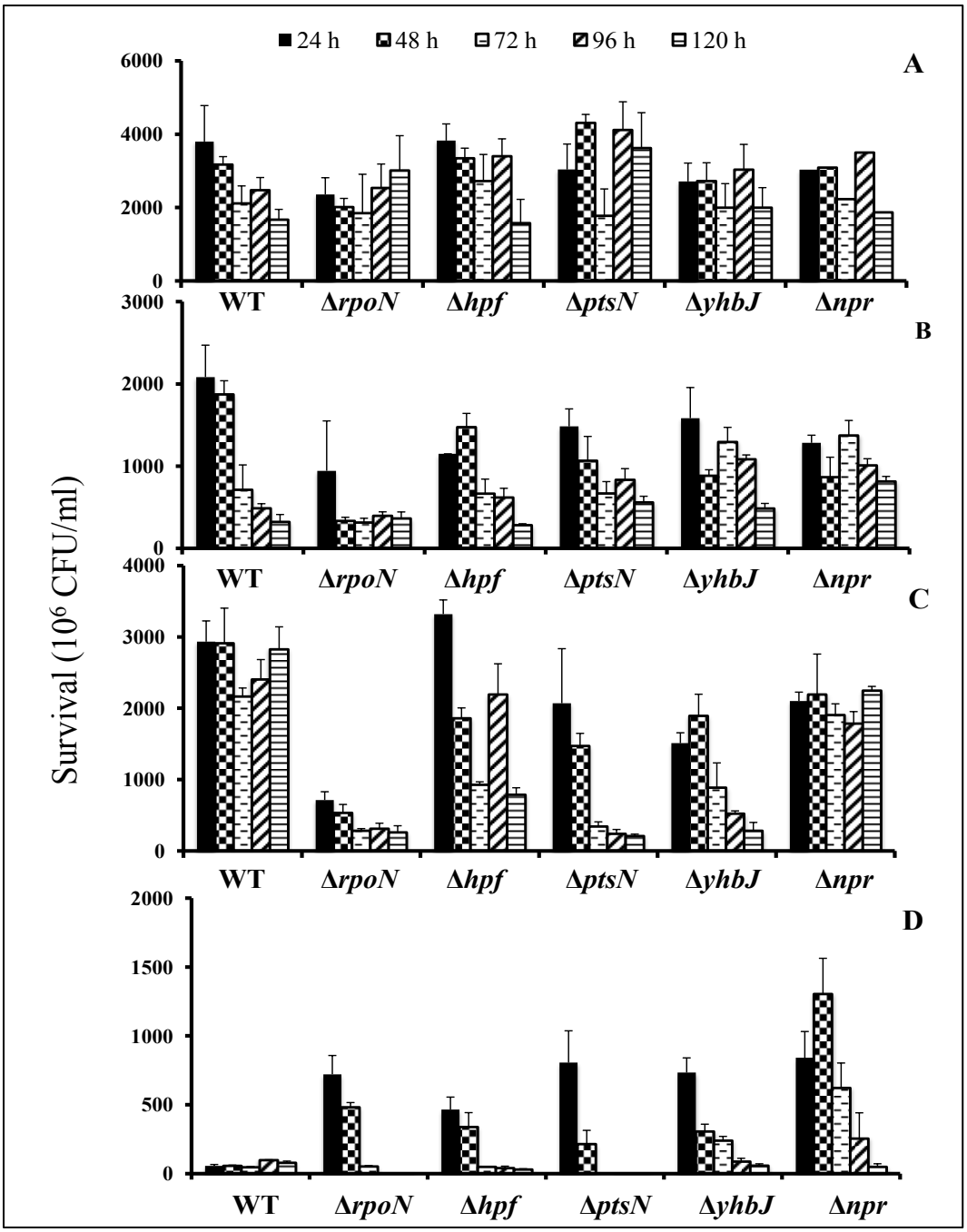

Figure 3.8 Survival during starvation under nutrient limiting conditions. Cell survival under starvation conditions for: (A) control, (B) carbon, (C) nitrogen, and (D) phosphorous in MOPS minimal media. Cell survival was determined by CFU/ml at 24, 48, 72, 96, and 120 hours for isogenic mutants of the *rpoN* operon genes in TW14359. Errors bars represent standard error of the mean (n≥3).

Discussion

The importance of σ^N and its operon is clearly evident in many species of bacteria. Specifically this sigma factor impacts motility [54,55,116,117], nitrogen metabolism [88,89,90], pathogenensis [56,57,58], and acid resistance [56]. The *rpoN* gene has even been shown to be an essential sigma factor in *Myxococcus xanthus* and the authors speculate *rpoN* null cells may be impaired at some point in the cell cycle [118]. This operon displays a high degree of genetic conservation among many species, which further contributes to the significance of these genes. Some differences in nucleotide sequence was observed within each of the genes, however, for *rpoN* there were no nucleotide mutations within the C-terminal RpoN-box DNA binding domain. This finding is in agreement with other studies that have found this to be true among many species such as *V. cholerae, M. xanthus, B. burgdorferi*, and *B. subtilis* [119]. The intergenic regions of the aligned EHEC strains lacked SNPs, which could imply the importance of maintaining conservation. It is plausible that these regions serve as regulatory domains if other promoters exist.

Evidence from this study supports the hypothesis that co-expressed gene products downstream of *rpoN* regulate σ^N, specifically *hpf* and *ptsN*. This regulation could be simply that one of these gene products bind to σ^N in a manner that will inhibit binding to core RNAP, as seen for RseA regulation of RpoE in *S. enterica* in the *rpoE-rseAB* operon [120]. Most likely this would be the mode of regulation for PtsN since it has recently shown to regulate sigma factor selectivity, specifically with σ^S and σ^{70} competition for core RNAP [121]. Alterations in intracellular K^+ levels trigger this activity by PtsN,

however the mechanism by which this occurs is unknown. Future studies to determine whether or not PtsN can bind σ^N or alter its ability to bind core RNAP may be informative. Hpf has shown to regulate σ^N dependent genes in a different manner, by either binding to ribosomes that change confirmation to the inactive 100S form, or by binding to σ^N specific promoters and inhibiting transcription in a more direct manner [62,122]. In this study Hpf altered expression of *rpoN*, however this may be ambiguous based on studies that confirmed levels of σ^N to be constitutively expressed and static throughout all phases of the cell cycle [100,123]. Hpf also regulated expression of σ^N dependent promoters; this agrees with findings in a similar study that investigated expression σ^N-dependent genes in *K. pneumonia* [62]. Merrick *et al.* (1989) found that a mutation in *hpf* led to increase expression of σ^N-dependent genes suggesting that Hpf negatively regulates these promoters. The mechanism by which Hpf and PtsN regulate σ^N dependent genes is speculative but based on other studies and data presented here it could be combinational effect of PtsN and Hpf. Perhaps Hpf is controlling PtsN translation by forming inactive 100S ribosomes. Yet in an active state, PtsN is regulating σ^N directly by phosphorylation or indirectly by interaction with σ^N as a result of changes in K^+ concentration. This study did show that these genes are differentially expressed, and that expression levels of *hpf* were significantly higher than others of the operon, which may suggest Hpf, has some regulatory function. It could also suggest that *hpf* has an additional promoter, which would allow for its differential expression an unequal amounts needed by the cell. Furthermore, expression for *astA* and *glnA* were low in the *hpf* and *ptsN* mutants compared to wildtype, and these are driven by σ^{70} and σ^N promoters

whereas *pspA* expression exceeded wildtype levels, and this gene is strictly driven by a σ^N promoter. The former could represent regulation by sigma factor competition, whereas the latter may represent direct regulation at the promoter region.

A mutation in *hpf* and *ptsN* phenocopy *rpoN* mutants for glutamate-dependent acid resistance and in nutrient limiting environments, specifically for nitrogen and slightly for phosphorous. Previous work demonstrated that σ^N suppressed GDAR and this study found that the σ^N-dependent pathway of GDAR is most likely regulated through the σ^S-GadXW pathway based on survival results, and expression of the key regulators for GDAR in a strain lacking *rpoN*. If Hpf and PtsN modulate σ^N then it should be within this same pathway of control. However, it could be that regulation is selective and occurring by affecting the ability of σ^S to bind to core RNAP, independently of σ^N. There is no evidence in this study that Hpf or PtsN are actually dependent on σ^N for GDAR and future work would need to be done to determine the direction of this regulation. Efforts towards characterizing the mode of regulation by *hpf* and *ptsN* to σ^N may delineate how σ^N-σ^S regulation occurs in EHEC. The determinant of σ^N activity in *E. coli* is most likely not due to fluctuating levels of this sigma factor, since the abundance of σ^N is relatively low and static throughout cell growth [113]; rather it is due to direct or indirect regulation.

Starvation induces genes that respond to nutrient scavenging systems and genes of the *rpoN* operon may influence expression or activity since each of the mutants exhibited more viability compared to wildtype when starved for phosphorous. Starvation alters expression of outer membrane proteins (OMPs)[124], and specifically the OmpE protein

is responsible for the intake of P_i and phosphorous containing compounds and is normally repressed by σ^S [125,126]. Taschner *et al.* (2004) found that σ^S does this by competing for core RNAP with σ^D, where expression of survival genes supersedes growth genes. The results presented in this study for phosphorous starvation were similar to that shown in GDAR, where inactivation of *rpoN* increases survival compared to wildtype. This survival may be attributed to a decrease in cellular pH as a result of an increase in glucose consumption producing glucose fermentative products [127]. Future work could investigate this by introducing glutamate in the media so cells respond using the GDAR system then determining if impairment for survival is attributed to pH or regulation. If addition of glutamate restores the ability to survive then it can be inferred that the low wildtype survival is due to acidic pH, otherwise it may be due to regulation by σ^N in some manner. The former would be contradictory to the ability of EHEC to survive under extreme low pH, however no studies have shown if the acid resistance occurs with starved cells.

It is well known that σ^N regulates genes that function in nitrogen scavenging and the viability results confirm that a strain lacking *rpoN* is significantly impaired in media containing glutamine as the only nitrogen source. Without *rpoN,* the cells were impaired in their ability to produce glutamine synthetase (*glnA*) and therefore were reduced in the ability to assimilate glutamine. However, cells lacking *rpoN* gained the ability to grow in nitrogen-limited media following 24 h of prolonged stationary phase, thus putatively creating a suppressor mutant that has gained the ability to survive in nitrogen limiting conditions in the absence of σ^N. To confirm if this is an actual suppressor mutant, cells

were grown in fresh nitrogen limited media and the results were comparable to wildtype, however the mutation that allows for this phenotype has not been identified. One possibility is that under starvation, a mutation was created in the *glnA* promoter region allowing for expression of its product, glutamine synthetase. This mutation allows for expression of *glnA* in the absence of σ^N and thus able to metabolize glutamate as a nitrogen source.

Jones *et al.* (1994) sequenced the *rpoN* operon in *E. coli* and found a potential σ^{70} promoter upstream of *rpoN* that transcribes a single mRNA transcript encoding five ORFs with a Rho-dependent terminator following the *npr* gene [100]. In the present study, genes of the *rpoN* operon were differentially expressed during exponential and transition growth phases in two EHEC wild-type strains. The *hpf* gene exhibited the most expression followed by *ptsN*. This could be due to differences in the mRNA stability or due to an uncharacterized promoter upstream of *hpf,* and within or downstream of the *rpoN*. In support of this hypothesis, Powell *et al.* (1994) predicted the presence of a promoter immediately downstream of *rpoN* and a weak transcriptional terminator within *yhbJ* upstream of *npr* [101]. The presence of these cis elements agrees with the increase expression observed for *hpf,* and the low abundance observed for *npr;* and future work could investigate this hypothesis by primer extension. Unequal amounts of downstream transcripts could suggest they control expression of upstream genes by a feedback mechanism, which has been suggested in previous studies [100,101]. Delineating the regulation of σ^N or σ^N-directed activity could essentially impact transmission of this pathogen. Understanding the mechanism by which σ^N suppresses AR, or how *rpoN* is

regulated, could lead to advances in developing synthetic agents to mimic this unknown determinant.

REFERENCES

1. Wells JG, Davis BR, Wachsmuth IK, Riley LW, Remis RS, et al. (1983) Laboratory Investigation of Hemorrhagic Colitis Outbreaks Associated with a Rare *Escherichia-Coli* Serotype. Journal of Clinical Microbiology 18: 512-520.

2. Rangel JM, Sparling PH, Crowe C, Griffin PM, Swerdlow DL (2005) Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. Emerging infectious diseases 11: 603-609.

3. Kaper JB, O'Brien AD (1998) *Escherichia coli* O157:H7 and other shiga toxin-producing *E. coli* strains. Washington, DC: ASM Press. xxii, 465 p. p.

4. Tarr PI, Gordon CA, Chandler WL (2005) Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. Lancet 365: 1073-1086.

5. Perna NT, Mayhew GF, Posfai G, Elliott S, Donnenberg MS, et al. (1998) Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157:H7. Infect Immun 66: 3810-3817.

6. McDaniel TK, Jarvis KG, Donnenberg MS, Kaper JB (1995) A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc Natl Acad Sci U S A 92: 1664-1668.

7. Kaper JB, Nataro JP, Mobley HL (2004) Pathogenic *Escherichia coli*. Nature Reviews Microbiology 2: 123-140.

8. Strockbine NA, Marques LR, Newland JW, Smith HW, Holmes RK, et al. (1986) Two toxin-converting phages from *Escherichia coli* O157:H7 strain 933 encode antigenically distinct toxins with similar biologic activities. Infect Immun 53: 135-140.

9. Louise CB, Obrig TG (1995) Specific interaction of *Escherichia coli* O157:H7-derived Shiga-like toxin II with human renal endothelial cells. J Infect Dis 172: 1397-1401.

10. Obata F, Tohyama K, Bonev AD, Kolling GL, Keepers TR, et al. (2008) Shiga toxin 2 affects the central nervous system through receptor globotriaosylceramide localized to neurons. Journal of Infectious Diseases 198: 1398-1406.

11. Ching JC, Jones NL, Ceponis PJ, Karmali MA, Sherman PM (2002) *Escherichia coli* shiga-like toxins induce apoptosis and cleavage of poly(ADP-ribose) polymerase via in vitro activation of caspases. Infect Immun 70: 4669-4677.

12. Ostroff SM, Tarr PI, Neill MA, Lewis JH, Hargrett-Bean N, et al. (1989) Toxin genotypes and plasmid profiles as determinants of systemic sequelae in *Escherichia coli* O157:H7 infections. J Infect Dis 160: 994-998.

13. Carter AO, Borczyk AA, Carlson JA, Harvey B, Hockin JC, et al. (1987) A severe outbreak of *Escherichia coli* O157:H7--associated hemorrhagic colitis in a nursing home. N Engl J Med 317: 1496-1500.

14. Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, et al. (1983) Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med 308: 681-685.

15. Zhang XP, McDaniel AD, Wolf LE, Keusch GT, Waldor MK, et al. (2000) Quinolone antibiotics induce shiga toxin-encoding bacteriophages, toxin production, and death in mice. Journal of Infectious Diseases 181: 664-670.

16. Potter AA, Klashinsky S, Li Y, Frey E, Townsend H, et al. (2004) Decreased shedding of *Escherichia coli* O157:H7 by cattle following vaccination with type III secreted proteins. Vaccine 22: 362-369.

17. Allen KJ, Rogan D, Finlay BB, Potter AA, Asper DJ (2011) Vaccination with type III secreted proteins leads to decreased shedding in calves after experimental infection with *Escherichia coli* O157. Can J Vet Res 75: 98-105.

18. Smith DR, Moxley RA, Peterson RE, Klopfenstein TJ, Erickson GE, et al. (2008) A two-dose regimen of a vaccine against *Escherichia coli* O157:H7 type III secreted proteins reduced environmental transmission of the agent in a large-scale commercial beef feedlot clinical trial. Foodborne Pathog Dis 5: 589-598.

19. Smith DR, Moxley RA, Peterson RE, Klopfenstein TJ, Erickson GE, et al. (2009) A two-dose regimen of a vaccine against type III secreted proteins reduced *Escherichia coli* O157:H7 colonization of the terminal rectum in beef cattle in commercial feedlots. Foodborne Pathog Dis 6: 155-161.

20. Bach SJ, McAllister TA, Veira DM, Gannon VPJ, Holley RA (2002) Transmission and control of *Escherichia coli* O157 : H7 - A review. Canadian Journal of Animal Science 82: 475-490.

21. Warshawsky B, Gutmanis I, Henry B, Dow J, Reffle J, et al. (2002) Outbreak of *Escherichia coli* 0157:H7 related to animal contact at a petting zoo. The Canadian journal of infectious diseases. Journal canadien des maladies infectieuses 13: 175-181.

22. Crump JA, Sulka AC, Langer AJ, Schaben C, Crielly AS, et al. (2002) An outbreak of *Escherichia coli* O157:H7 infections among visitors to a dairy farm. N Engl J Med 347: 555-560.

23. Bell BP, Goldoft M, Griffin PM, Davis MA, Gordon DC, et al. (1994) A multistate outbreak of *Escherichia coli* O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers. The Washington experience. JAMA 272: 1349-1353.

24. Watanabe Y, Ozasa K, Mermin JH, Griffin PM, Masuda K, et al. (1999) Factory outbreak of *Escherichia coli* O157:H7 infection in Japan. Emerg Infect Dis 5: 424-428.

25. Wendel AM, Johnson DH, Sharapov U, Grant J, Archer JR, et al. (2009) Multistate outbreak of *Escherichia coli* O157:H7 infection associated with consumption of packaged spinach, August-September 2006: the Wisconsin investigation. Clin Infect Dis 48: 1079-1086.

26. Nataro JP, Kaper JB (1998) Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 11: 142-201.

27. Olsen SJ, Miller G, Breuer T, Kennedy M, Higgins C, et al. (2002) A waterborne outbreak of *Escherichia coli* O157:H7 infections and hemolytic uremic syndrome: implications for rural water systems. Emerg Infect Dis 8: 370-375.

28. Bruce MG, Curtis MB, Payne MM, Gautom RK, Thompson EC, et al. (2003) Lake-associated outbreak of *Escherichia coli* O157:H7 in Clark County, Washington, August 1999. Arch Pediatr Adolesc Med 157: 1016-1021.

29. Jackson SG, Goodbrand RB, Johnson RP, Odorico VG, Alves D, et al. (1998) *Escherichia coli* O157:H7 diarrhoea associated with well water and infected cattle on an Ontario farm. Epidemiol Infect 120: 17-20.

30. Teunis P, Takumi K, Shinagawa K (2004) Dose response for infection by *Escherichia coli* O157:H7 from outbreak data. Risk Anal 24: 401-407.

31. Keene WE, McAnulty JM, Hoesly FC, Williams LP, Jr., Hedberg K, et al. (1994) A swimming-associated outbreak of hemorrhagic colitis caused by *Escherichia coli* O157:H7 and Shigella sonnei. N Engl J Med 331: 579-584.

32. Castanie-Cornet MP, Penfound TA, Smith D, Elliott JF, Foster JW (1999) Control of acid resistance in *Escherichia coli*. J Bacteriol 181: 3525-3535.

33. Large TM, Walk ST, Whittam TS (2005) Variation in acid resistance among shiga toxin-producing clones of pathogenic *Escherichia coli*. Appl Environ Microbiol 71: 2493-2500.

34. Lin J, Smith MP, Chapin KC, Baik HS, Bennett GN, et al. (1996) Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. Appl Environ Microbiol 62: 3094-3100.

35. Gorden J, Small PL (1993) Acid resistance in enteric bacteria. Infect Immun 61: 364-367.

36. Price SB, Wright JC, DeGraves FJ, Castanie-Cornet MP, Foster JW (2004) Acid resistance systems required for survival of *Escherichia coli* O157:H7 in the bovine gastrointestinal tract and in apple cider are different. Appl Environ Microbiol 70: 4792-4799.

37. Leyer GJ, Wang LL, Johnson EA (1995) Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. Appl Environ Microbiol 61: 3752-3755.

38. Bergholz TM, Whittam TS (2007) Variation in acid resistance among enterohaemorrhagic *Escherichia coli* in a simulated gastric environment. Journal of Applied Microbiology 102: 352-362.

39. Foster JW (2004) *Escherichia coli* acid resistance: Tales of an amateur acidophile. Nature Reviews Microbiology 2: 898-907.

40. Price SB, Cheng CM, Kaspar CW, Wright JC, DeGraves FJ, et al. (2000) Role of *rpoS* in acid resistance and fecal shedding of *Escherichia coli* O157:H7. Appl Environ Microbiol 66: 632-637.

41. Lin J, Lee IS, Frey J, Slonczewski JL, Foster JW (1995) Comparative analysis of extreme acid survival in *Salmonella typhimurium, Shigella flexneri,* and *Escherichia coli*. J Bacteriol 177: 4097-4104.

42. Diez-Gonzalez F, Karaibrahimoglu Y (2004) Comparison of the glutamate-, arginine- and lysine-dependent acid resistance systems in *Escherichia coli* O157:H7. J Appl Microbiol 96: 1237-1244.

43. Richard H, Foster JW (2004) *Escherichia coli* glutamate- and arginine-dependent acid resistance systems increase internal pH and reverse transmembrane potential. J Bacteriol 186: 6032-6041.

44. Helmann JD (1991) Alternative sigma factors and the regulation of flagellar gene expression. Mol Microbiol 5: 2875-2882.

45. Hengge-Aronis R (2002) Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. Microbiology and molecular biology reviews : MMBR 66: 373-395, table of contents.

46. Weber H, Polen T, Heuveling J, Wendisch VF, Hengge R (2005) Genome-wide analysis of the general stress response network in *Escherichia coli*: sigmaS-dependent genes, promoters, and sigma factor selectivity. J Bacteriol 187: 1591-1603.

47. Waterman SR, Small PLC (1996) Identification of σ^S-dependent genes associated with the stationary-phase acid-resistance phenotype of *Shigella flexneri.* Molecular Microbiology 21: 925-940.

48. Lange R, Hengge-Aronis R (1991) Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Mol Microbiol 5: 49-59.

49. Lange R, Hengge-Aronis R (1994) The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. Genes Dev 8: 1600-1612.

50. Muffler A, Traulsen DD, Fischer D, Lange R, Hengge-Aronis R (1997) The RNA-binding protein HF-I plays a global regulatory role which is largely, but not exclusively, due to its role in expression of the sigmaS subunit of RNA polymerase in *Escherichia coli*. J Bacteriol 179: 297-300.

51. Schweder T, Lee KH, Lomovskaya O, Matin A (1996) Regulation of *Escherichia coli* starvation sigma factor (sigma s) by ClpXP protease. J Bacteriol 178: 470-476.

52. Reitzer L, Schneider BL (2001) Metabolic context and possible physiological themes of sigma(54)-dependent genes in *Escherichia coli*. Microbiol Mol Biol Rev 65: 422-444, table of contents.

53. Hirschman J, Wong PK, Sei K, Keener J, Kustu S (1985) Products of nitrogen regulatory genes *ntrA* and *ntrC* of enteric bacteria activate *glnA* transcription in vitro: evidence that the *ntrA* product is a sigma factor. Proc Natl Acad Sci U S A 82: 7525-7529.

54. Dong T, Yu R, Schellhorn H (2011) Antagonistic regulation of motility and transcriptome expression by RpoN and RpoS in *Escherichia coli*. Molecular microbiology 79: 375-386.

55. Zhao K, Liu M, Burgess RR (2010) Promoter and regulon analysis of nitrogen assimilation factor, sigma54, reveal alternative strategy for E. coli MG1655 flagellar biosynthesis. Nucleic acids research 38: 1273-1283.

56. Riordan JT, Tietjen JA, Walsh CW, Gustafson JE, Whittam TS (2010) Inactivation of alternative sigma factor 54 (RpoN) leads to increased acid resistance, and alters locus of enterocyte effacement (LEE) expression in *Escherichia coli* O157 : H7. Microbiology 156: 719-730.

57. Fisher MA, Grimm D, Henion AK, Elias AF, Stewart PE, et al. (2005) *Borrelia burgdorferi* sigma54 is required for mammalian infection and vector transmission but not for tick colonization. Proc Natl Acad Sci U S A 102: 5162-5167.

58. Comolli JC, Hauser AR, Waite L, Whitchurch CB, Mattick JS, et al. (1999) *Pseudomonas aeruginosa* gene products PilT and PilU are required for cytotoxicity in vitro and virulence in a mouse model of acute pneumonia. Infection and Immunity 67: 3625-3630.

59. Weiner L, Brissette JL, Model P (1991) Stress-induced expression of the *Escherichia coli* phage shock protein operon is dependent on sigma 54 and modulated by positive and negative feedback mechanisms. Genes Dev 5: 1912-1923.

60. Jin S, Ishimoto K, Lory S (1994) Nucleotide sequence of the *rpoN* gene and characterization of two downstream open reading frames in Pseudomonas aeruginosa. J Bacteriol 176: 1316-1322.

61. Morett E, Buck M (1989) In vivo studies on the interaction of RNA polymerase-sigma 54 with the *Klebsiella pneumoniae* and *Rhizobium meliloti* nifH promoters. The role of NifA in the formation of an open promoter complex. J Mol Biol 210: 65-77.

62. Merrick MJ, Coppard JR (1989) Mutations in genes downstream of the *rpoN* gene (encoding sigma 54) of *Klebsiella pneumoniae* affect expression from sigma 54-dependent promoters. Molecular microbiology 3: 1765-1775.

63. Neidhardt FC, Bloch PL, Smith DF (1974) Culture medium for enterobacteria. Journal of bacteriology 119: 736-747.

64. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proceedings of the National Academy of Sciences of the United States of America 97: 6640-6645.

65. Murphy KC, Campellone KG (2003) Lambda Red-mediated recombinogenic engineering of enterohemorrhagic and enteropathogenic *E. coli*. BMC molecular biology 4: 11.

66. Giannella RA, Broitman SA, Zamcheck N (1972) Gastric acid barrier to ingested microorganisms in man: studies in vivo and in vitro. Gut 13: 251-256.

67. Cummings JH (1981) Short chain fatty acids in the human colon. Gut 22: 763-779.

68. Ma Z, Richard H, Tucker DL, Conway T, Foster JW (2002) Collaborative regulation of *Escherichia coli* glutamate-dependent acid resistance by two AraC-like regulators, GadX and GadW (YhiW). J Bacteriol 184: 7001-7012.

69. De Biase D, Tramonti A, Bossa F, Visca P (1999) The response to stationary-phase stress conditions in *Escherichia coli*: role and regulation of the glutamic acid decarboxylase system. Mol Microbiol 32: 1198-1211.

70. Hersh BM, Farooq FT, Barstad DN, Blankenhorn DL, Slonczewski JL (1996) A glutamate-dependent acid resistance gene in *Escherichia coli*. J Bacteriol 178: 3978-3981.

71. Ma Z, Masuda N, Foster JW (2004) Characterization of EvgAS-YdeO-GadE branched regulatory circuit governing glutamate-dependent acid resistance in *Escherichia coli*. J Bacteriol 186: 7378-7389.

72. Sayed AK, Foster JW (2009) A 750 bp sensory integration region directs global control of the *Escherichia coli* GadE acid resistance regulator. Mol Microbiol 71: 1435-1450.

73. Sayed AK, Odom C, Foster JW (2007) The *Escherichia coli* AraC-family regulators GadX and GadW activate gadE, the central activator of glutamate-dependent acid resistance. Microbiology 153: 2584-2592.

74. Hommais F, Krin E, Coppee JY, Lacroix C, Yeramian E, et al. (2004) GadE (YhiE): a novel activator involved in the response to acid environment in *Escherichia coli*. Microbiology 150: 61-72.

75. Gong S, Ma Z, Foster JW (2004) The Era-like GTPase TrmE conditionally activates *gadE* and glutamate-dependent acid resistance in *Escherichia coli*. Mol Microbiol 54: 948-961.

76. Cabedo H, Macian F, Villarroya M, Escudero JC, Martinez-Vicente M, et al. (1999) The *Escherichia coli trmE* (*mnmE*) gene, involved in tRNA modification, codes for an evolutionarily conserved GTPase with unusual biochemical properties. EMBO J 18: 7063-7076.

77. Stim-Herndon KP, Flores TM, Bennett GN (1996) Molecular characterization of *adiY*, a regulatory gene which affects expression of the biodegradative acid-induced arginine decarboxylase gene (*adiA*) of *Escherichia coli*. Microbiology 142 (Pt 5): 1311-1320.

78. Tyrrell R, Verschueren KH, Dodson EJ, Murshudov GN, Addy C, et al. (1997) The structure of the cofactor-binding fragment of the LysR family member, CysB: a familiar fold with a surprising subunit arrangement. Structure 5: 1017-1032.

79. Gong S, Richard H, Foster JW (2003) YjdE (AdiC) is the arginine:agmatine antiporter essential for arginine-dependent acid resistance in *Escherichia coli*. J Bacteriol 185: 4402-4409.

80. Kieboom J, Abee T (2006) Arginine-dependent acid resistance in *Salmonella enterica* serovar *Typhimurium*. J Bacteriol 188: 5650-5653.

81. Bagramyan K, Trchounian A (2003) Structural and functional features of formate hydrogen lyase, an enzyme of mixed-acid fermentation from *Escherichia coli*. Biochemistry (Mosc) 68: 1159-1170.

82. Noguchi K, Riggins DP, Eldahan KC, Kitko RD, Slonczewski JL (2010) Hydrogenase-3 contributes to anaerobic acid resistance of *Escherichia coli*. PloS one 5: e10132.

83. Ma Z, Gong SM, Richard H, Tucker DL, Conway T, et al. (2003) GadE (YhiE) activates glutamate decarboxylase-dependent acid resistance in *Escherichia coli* K-12. Molecular Microbiology 49: 1309-1320.

84. Mates AK, Sayed AK, Foster JW (2007) Products of the *Escherichia coli* acid fitness island attenuate metabolite stress at extremely low pH and mediate a cell density-dependent acid resistance. J Bacteriol 189: 2759-2768.

85. Salmon KA, Hung SP, Steffen NR, Krupp R, Baldi P, et al. (2005) Global gene expression profiling in *Escherichia coli* K12: effects of oxygen availability and ArcA. J Biol Chem 280: 15084-15096.

86. Belik AS, Tarasova NN, Khmel IA (2008) [Regulation of biofilm formation in *Escherichia coli* K12: effect of mutations in HNS, StpA, lon, and *rpoN* genes]. Mol Gen Mikrobiol Virusol: 3-5.

87. Gardner AM, Gessner CR, Gardner PR (2003) Regulation of the nitric oxide reduction operon (norRVW) in *Escherichia coli*. Role of NorR and sigma54 in the nitric oxide stress response. J Biol Chem 278: 10081-10086.

88. Hunt TP, Magasanik B (1985) Transcription of glnA by purified *Escherichia coli* components: core RNA polymerase and the products of *glnF*, *glnG*, and *glnL*. Proc Natl Acad Sci U S A 82: 8453-8457.

89. Ronson CW, Nixon BT, Albright LM, Ausubel FM (1987) Rhizobium meliloti *ntrA* (*rpoN*) gene is required for diverse metabolic functions. J Bacteriol 169: 2424-2431.

90. Totten PA, Lara JC, Lory S (1990) The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J Bacteriol 172: 389-396.

91. Ninfa AJ, Reitzer LJ, Magasanik B (1987) Initiation of transcription at the bacterial glnAp2 promoter by purified *E. coli* components is facilitated by enhancers. Cell 50: 1039-1046.

92. Ma Z, Richard H, Foster JW (2003) pH-Dependent modulation of cyclic AMP levels and GadW-dependent repression of RpoS affect synthesis of the GadX regulator and *Escherichia coli* acid resistance. J Bacteriol 185: 6852-6859.

93. Chen G, Schellhorn HE (2003) Controlled induction of the RpoS regulon in *Escherichia coli*, using an RpoS-expressing plasmid. Can J Microbiol 49: 733-740.

94. Goh K, Chua D, Beck B, McKee ML, Bhagwat AA (2011) Arginine-dependent acid-resistance pathway in *Shigella boydii.* Arch Microbiol 193: 179-185.

95. Cui S, Meng J, Bhagwat AA (2001) Availability of glutamate and arginine during acid challenge determines cell density-dependent survival phenotype of *Escherichia coli* strains. Appl Environ Microbiol 67: 4914-4918.

96. Eboigbodin KE, Newton JR, Routh AF, Biggs CA (2006) Bacterial quorum sensing and cell surface electrokinetic properties. Appl Microbiol Biotechnol 73: 669-675.

97. Schneider BL, Kiupakis AK, Reitzer LJ (1998) Arginine catabolism and the arginine succinyltransferase pathway in *Escherichia coli*. J Bacteriol 180: 4278-4286.

98. Shaibe E, Metzer E, Halpern YS (1985) Metabolic pathway for the utilization of L-arginine, L-ornithine, agmatine, and putrescine as nitrogen sources in *Escherichia coli* K-12. J Bacteriol 163: 933-937.

99. Wedel A, Kustu S (1995) The bacterial enhancer-binding protein NTRC is a molecular machine: ATP hydrolysis is coupled to transcriptional activation. Genes Dev 9: 2042-2052.

100. Jones DH, Franklin FC, Thomas CM (1994) Molecular analysis of the operon which encodes the RNA polymerase sigma factor sigma 54 of *Escherichia coli*. Microbiology 140 (Pt 5): 1035-1043.

101. Powell BS, Court DL, Inada T, Nakamura Y, Michotey V, et al. (1995) Novel Proteins of the Phosphotransferase System Encoded within the Rpon Operon of *Escherichia-Coli* - Enzyme Iia(Ntr) Affects Growth on Organic Nitrogen and the Conditional Lethality of an Era(Ts) Mutant. Journal of Biological Chemistry 270: 4822-4839.

102. Model P, Jovanovic G, Dworkin J (1997) The *Escherichia coli* phage-shock-protein (psp) operon. Molecular microbiology 24: 255-261.

103. Pfluger-Grau K, Gorke B (2010) Regulatory roles of the bacterial nitrogen-related phosphotransferase system. Trends Microbiol 18: 205-214.

104. Merrick M, Gibbins J, Toukdarian A (1987) The nucleotide sequence of the sigma factor gene ntrA (*rpoN*) of *Azotobacter vinelandii*: analysis of conserved sequences in NtrA proteins. Molecular & general genetics : MGG 210: 323-330.

105. Debarbouille M, Martin-Verstraete I, Kunst F, Rapoport G (1991) The *Bacillus subtilis sigL* gene encodes an equivalent of sigma 54 from gram-negative bacteria. Proc Natl Acad Sci U S A 88: 9092-9096.

106. Geerse RH, van der Pluijm J, Postma PW (1989) The repressor of the PEP:fructose phosphotransferase system is required for the transcription of the *pps* gene of *Escherichia coli*. Molecular & general genetics : MGG 218: 348-352.

107. Lee CA, Saier MH, Jr. (1983) Mannitol-specific enzyme II of the bacterial phosphotransferase system. III. The nucleotide sequence of the permease gene. J Biol Chem 258: 10761-10767.

108. Zimmer B, Hillmann A, Gorke B (2008) Requirements for the phosphorylation of the *Escherichia coli* EIIANtr protein in vivo. FEMS Microbiol Lett 286: 96-102.

109. Kalamorz F, Reichenbach B, Marz W, Rak B, Gorke B (2007) Feedback control of glucosamine-6-phosphate synthase GlmS expression depends on the small RNA GlmZ and involves the novel protein YhbJ in *Escherichia coli*. Molecular Microbiology 65: 1518-1533.

110. Milewski S (2002) Glucosamine-6-phosphate synthase-the multi-facets enzyme. Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology 1597: 173-192.

111. Reitzer LJ, Magasanik B (1986) Transcription of *glnA* in E. coli is stimulated by activator bound to sites far from the promoter. Cell 45: 785-792.

112. Campbell EA, Tupy JL, Gruber TM, Wang S, Sharp MM, et al. (2003) Crystal structure of *Escherichia coli* sigmaE with the cytoplasmic domain of its anti-sigma RseA. Mol Cell 11: 1067-1078.

113. Jishage M, Iwata A, Ueda S, Ishihama A (1996) Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: intracellular levels of four species of sigma subunit under various growth conditions. J Bacteriol 178: 5447-5451.

114. Pahel G, Tyler B (1979) A new *glnA*-linked regulatory gene for glutamine synthetase in *Escherichia coli*. Proc Natl Acad Sci U S A 76: 4544-4548.

115. Reitzer LJ, Magasanik B (1985) Expression of glnA in *Escherichia coli* is regulated at tandem promoters. Proc Natl Acad Sci U S A 82: 1979-1983.

116. Wolfe AJ, Millikan DS, Campbell JM, Visick KL (2004) *Vibrio fischeri* sigma54 controls motility, biofilm formation, luminescence, and colonization. Appl Environ Microbiol 70: 2520-2524.

117. Janakiraman RS, Brun YV (1997) Transcriptional and mutational analyses of the *rpoN* operon in *Caulobacter crescentus*. J Bacteriol 179: 5138-5147.

118. Keseler IM, Kaiser D (1997) sigma54, a vital protein for *Myxococcus xanthus*. Proc Natl Acad Sci U S A 94: 1979-1984.

119. Doucleff M, Malak LT, Pelton JG, Wemmer DE (2005) The C-terminal RpoN domain of sigma54 forms an unpredicted helix-turn-helix motif similar to domains of sigma70. J Biol Chem 280: 41530-41536.

120. Homerova D, Rezuchova B, Skovierova H, Kormanec J (2010) The expression of the *rpoE* operon is fine-tuned by the internal *rseA*p promoter in *Salmonella enterica* serovar *Typhimurium*. Biologia 65: 932-938.

121. Lee CR, Cho SH, Kim HJ, Kim M, Peterkofsky A, et al. (2010) Potassium mediates *Escherichia coli* enzyme IIA(Ntr)-dependent regulation of sigma factor selectivity. Mol Microbiol 78: 1468-1483.

122. Ueta M, Ohniwa RL, Yoshida H, Maki Y, Wada C, et al. (2008) Role of HPF (hibernation promoting factor) in translational activity in *Escherichia coli*. J Biochem 143: 425-433.

123. Jurado P, Fernandez LA, de Lorenzo V (2003) Sigma 54 levels and physiological control of the Pseudomonas putida Pu promoter. J Bacteriol 185: 3379-3383.

124. Overbeeke N, Lugtenberg B (1980) Expression of outer membrane protein e of *Escherichia coli* K12 by phosphate limitation. FEBS Lett 112: 229-232.

125. Korteland J, Lugtenberg B (1984) Increased efficiency of the outer membrane PhoE protein pore in *Escherichia coli* K-12 mutants with heptose-deficient lipopolysaccharide. Biochim Biophys Acta 774: 119-126.

126. Taschner NP, Yagil E, Spira B (2004) A differential effect of sigmaS on the expression of the PHO regulon genes of *Escherichia coli*. Microbiology 150: 2985-2992.

127. Moreau PL (2007) The lysine decarboxylase CadA protects *Escherichia coli* starved of phosphate against fermentation acids. J Bacteriol 189: 2249-2261.