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Sigma Factor N: A Novel Regulator of Acid Resistance and Locus of Enterocyte Effacement in Escherichia coli O157:H7

Avishek Mitra
University of South Florida, amitra@mail.usf.edu

Sigma Factor N: A Novel Regulator of Acid Resistance and Locus of Enterocyte Effacement in *Escherichia coli* O157:H7

by

Avishek Mitra

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College of Arts and Sciences
University of South Florida

Major Professor: Dr. James Riordan, Ph.D.
Dr. Lindsey Shaw, Ph.D.
Dr. Katie Scott, Ph.D.
Dr. Stanley Stevens, Ph.D.

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Dedication

I dedicate this work to my parents. Regardless of all the things that we have fought about in the past, they have been the one constant in my life and have been there for me through thick and thin. I wouldn't be who I am or where I am, without their love and support. I want to thank Edin, Mike and Vedad, my three friends who have I have known for over ten years and have become my brothers from other mothers. I feel lucky to have you all in my life and I cannot express enough in words what my parents and my friends mean to me.

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To Pamela, Jason, Nada and Katie, you have all been such great lab mates. I have such great memories with all of you and I wish you all the best in your future. I am definitely going to miss pranking Jason and spoiling movie endings for him. Thank you all for being such great people.

Table of Contents

List of Figures

List of Tables

Abstract

In enterohemorrhagic *E. coli* (EHEC) sigma factor N (σ^N) regulates glutamate-dependent acid resistance (GDAR) and the locus of enterocyte effacement (LEE), discrete genetic systems required for transmission and virulence of this intestinal pathogen. Regulation of these systems requires nitrogen regulatory protein C, NtrC, and is a consequence of NtrC/σ^N-dependent reduction in the activity of sigma factor S (σ^S). This study elucidates pathway components and stimuli for σ^N-directed regulation of GDAR and the LEE in EHEC. Deletion of *fliZ*, the product of which reduces σ^S activity, phenocopies *rpoN* (σ^N) and *ntrC* null strains for GDAR and LEE control, acid resistance and adherence. Upregulation of *fliZ* by NtrC/σ^N is indirect, requiring an intact flagellar regulator *flhDC*. Activation of *flhDC* by NtrC/σ^N and FlhDC-dependent regulation of GDAR and the LEE is dependent on σ^N-promoter $flhD_{P2}$, and a newly described NtrC upstream activator sequence. While the addition of ammonium significantly alters GDAR and LEE expression, acid resistance and adherence, it does so independently of *rpoN*, *ntrC* and the NtrC sensor kinase *ntrB*. Altering the availability of NtrC phosphodonor acetyl phosphate by growth without glucose, with acetate addition, or by deletion of acetate kinase, *ackA*, abrogates NtrC/σ^N-dependent control of *flhDC*, *fliZ*, GDAR and LEE genes.

Chapter One: Regulation of Sigma Factor N and its Contribution to Bacterial Virulence and Pathogenesis

1.1 Bacterial RNA Polymerase

Regulation of gene expression is a vital aspect of survival for organisms in all domains of life. It is the basis for cellular differentiation, adaptation to changing environments, and life itself. Transcription initiation is the first step of gene expression and provides one the most important points of access for differential regulation of genes. In all domains, the multi subunit enzyme called RNA polymerase (RNAP) is the key component involved in transcribing DNA into RNA. The bacterial catalytic core RNAP is intensely conserved consisting of just one form and being composed of the five subunits α_2, β, β' and ω (Fig. 1.1).

The crab claw shape of the RNAP is due to the β (~150kDa) and β' (155kDa) subunits, which form the pincers of the crab claw (443). The α subunits form a dimer (74kDa) and play an important role in assembly of the core RNAP. The dimerized C-terminal domain of the α subunits (α-CTD) primarily interacts with various transcriptional factors dictated by environmental signals and also interacts with promoter DNA sequences signaling the dimerized N-terminal domain (α-NTD) to first recruit and bind the β subunit and then bind the β' subunit for assembly of the RNAP (130, 138, 164, 342). Finally, association of the ω subunit (11kDa) at the interface of the β and β' subunits results in formation of the ~380kDa catalytic core RNA polymerase complex (53).

An immobile domain comprised of α-NTDs, ω and portions of the β and β' subunits contain the active site channel of the core RNAP formed by the cleft between the β and β' subunits. This immobile domain is further bordered by 4 mobile domains. This includes the β' clamp domain,

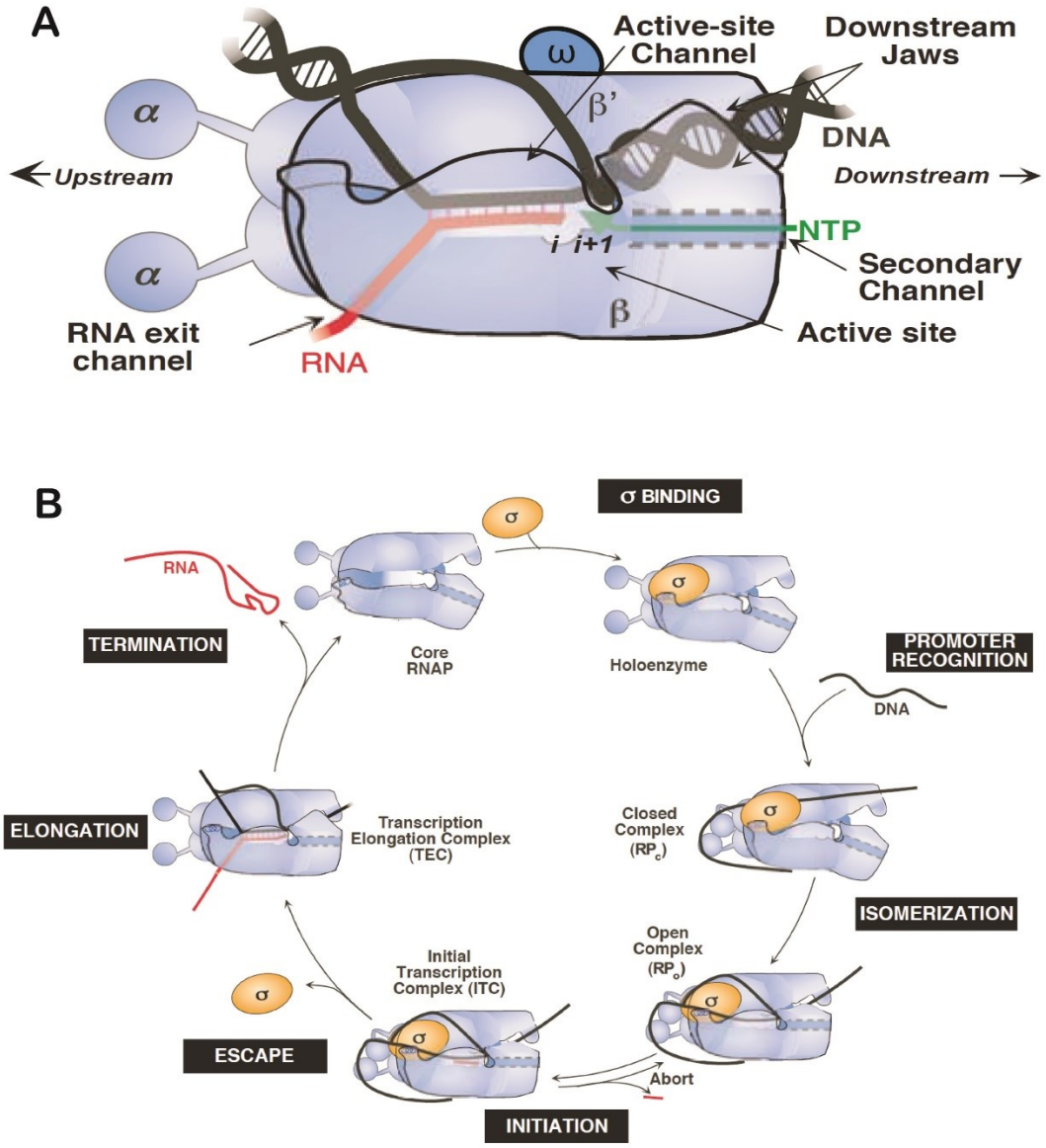

Figure 1.1. Bacterial RNA polymerase (adapted from Geszvain *et al*. 2005). **(A)** RNAP subunits α_2, β, β' and ω form the core RNA polymerase. β and β' subunits form the active site, which is divided into the DNA entry channel and secondary channel for entry of NTPs. **(B)** The transcription and sigma factor cycle. Sigma (orange) associates with core RNAP (blue), targets RNAP to specific promoters and isomerizes closed complex to an open complex to initiate promoter melting and transcription.

which forms a β' pincer that closes on the main channel to hold the template DNA and the RNA:DNA hybrid within the active center. Within the β' pincer are two mobile domains called the lobe and protrusions, which can open and close to make the active site available. The final mobile domain, called the β' flap domain, covers the RNA exit channel regulating the exit of nascent RNA transcript (Fig. 1.1A) (260, 443).

Immediately downstream of the active site, the main channel is separated into a downstream DNA entry channel and a secondary channel. Double stranded DNA enters into the RNAP complex through the DNA entry channel and following DNA melting, the template strand is positioned into the center of the active site. The structure and size of the secondary channel dictates that only NTPs can be positioned and allowed to pass through to enter the active center for addition onto the polymerizing RNA transcript. As nascent transcript elongates, it separates form the RNA:DNA hybrid and is extruded from the RNAP through the RNA exit channel (199, 259).

1.2 Bacterial Sigma Factors and Their Role in Transcription

The core RNAP is completely dependent on another dissociable subunit called a sigma factor, which is crucial for the complete function of the RNAP holoenzyme for promoter recognition and initiation of transcription (53). The key functions of sigma (σ) factors involve: I) binding core RNAP to form the RNAP holoenzyme (Eσ), II) recruiting Eσ to target promoters, III) promoter melting at the transcription start site, and IV) regulating transcription by clearing and releasing the promoter from RNAP (50).

1.2.1 The transcription and sigma factor cycle

The transcription cycle consists of three main steps: initiation, elongation and termination. During initiation, the core RNAP which is in its open complex form binds to a sigma factor to form the RNAP holoenzyme (54). The sigma factor recruits the holoenzyme to promoter DNA, where the holoenzyme forms the closed complex in a process called promoter recognition (55, 200). The closed complex isomerizes to an open complex, during which promoter DNA melting by the sigma factor allows the active site to access the template strand (341). This open complex RNAP can initiate transcription but mostly remains at the promoter in an initial transcription complex creating and releasing very short transcripts in a process called abortive transcription, and then eventually escapes the promoter forming the transcription elongation complex (TEC) (202, 409). After the formation of the TEC, the sigma factor is rapidly released from the holoenzyme back in to the cellular pool of sigma factors for association with core RNAP (254, 316). When RNAP encounters termination sites, structural reorganization within RNAP leads to release of RNA and dissociation of RNAP from template DNA resulting in termination of transcription (Fig. 1.1B).

1.2.2 The σ^{70} family: structure and functions

All bacterial sigma factors can be categorized into two families, σ^{70} and σ^{54}, based on their primary sequence, recognition of consensus promoter sequence and the mode of transcription initiation. Even though there is little sequence similarity between the σ^{70} and σ^{54} families, sigmas of both types bind the same core RNAP to initiate transcription (56, 127, 362). Sigma factors in the σ^{70} family are mostly composed of four helical domains (σ_1, σ_2, σ_3 and σ_4) connected by flexible linkers (Fig. 1.2) (62, 231, 259). The crucial roles of binding core RNAP, promoter recognition

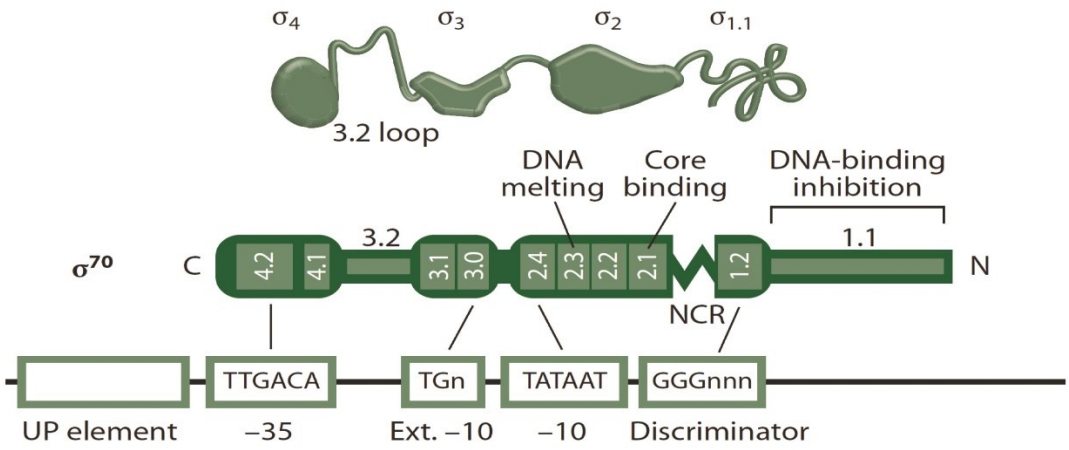

Figure 1.2. Domains and functions of sigma factors in the σ70 family (from Osterberg *et al*. 2011). Globular domains are connected by flexible linkers. σ_1 autoinhibits free sigma from binding promoters and after holoenzyme formations facilitates open complex formation by binding to nonconserved (NCR) discriminator DNA sequence. σ_2 binds core RNAP and initiates promoter melting at -10 (TATAAT) consensus sequence. σ_3 can help recognize extended -10 promoter elements. σ_4 binds -35 (TTGACA) promoter consensus sequence

and promoter melting to initiate transcription are contained within the σ_2 and σ_4 domains of all sigma factors in the σ^{70} family. Within the four domains there are sub regions that play important roles in sigma factor functions. The $\sigma_{1.1}$ region at the N-terminus autoinhibits free sigma from binding promoters and this autoinhibition is repressed when the free sigma binds core RNAP (61, 99). After association with the core RNAP, $\sigma_{1.1}$ can facilitate open complex formation at promoters by binding to the discriminator DNA sequence (411). The $\sigma_{2.2}$ region provides the most important basis of interaction between sigma factors and core RNAP. It first interacts and binds to the β' subunit and then binds the $\sigma_{2.3}$ region, which stimulates $\sigma_{2.3}$ region to initiate promoter melting at the -10 consensus sequence (10, 218, 286). The σ_3 domain helps in recognition of extended -10 promoter elements, if present, and mostly remains positioned within the active site of RNAP (22). The σ_4 domain, containing the highly conserved regions 4.1-4.2, forms a mobile module with the β flap domain of the core RNAP and is essential for binding the -35 consensus promoter sequence (62, 356).

1.3 The σ^{54} Family

In contrast to the sigma factors of the σ^{70} family, alternative sigma factor N (RpoN or σ^{54}) is the only sigma factor in the σ^{54}. Unlike $E\sigma^{70}$, $E\sigma^{54}$ holoenzyme binds at -24 (GG) and -12 (TGC) consensus sequences and remains in an energetically favorable closed complex (50, 423). Open complex formation and transcription initiation by $E\sigma^{54}$ is completely dependent on ATP hydrolysis facilitated by activator proteins called bacterial enhancer binding proteins (bEBPs). These bEBPs are members of the AAA+ (ATPases associated with various cellular activities) protein subfamily and bind enhancer sites upstream of a σ^{54} promoter site, also referred to as upstream activating sequence (UAS). The hydrolysis of ATP by bEBPs results in a conformational change in σ^{54} that

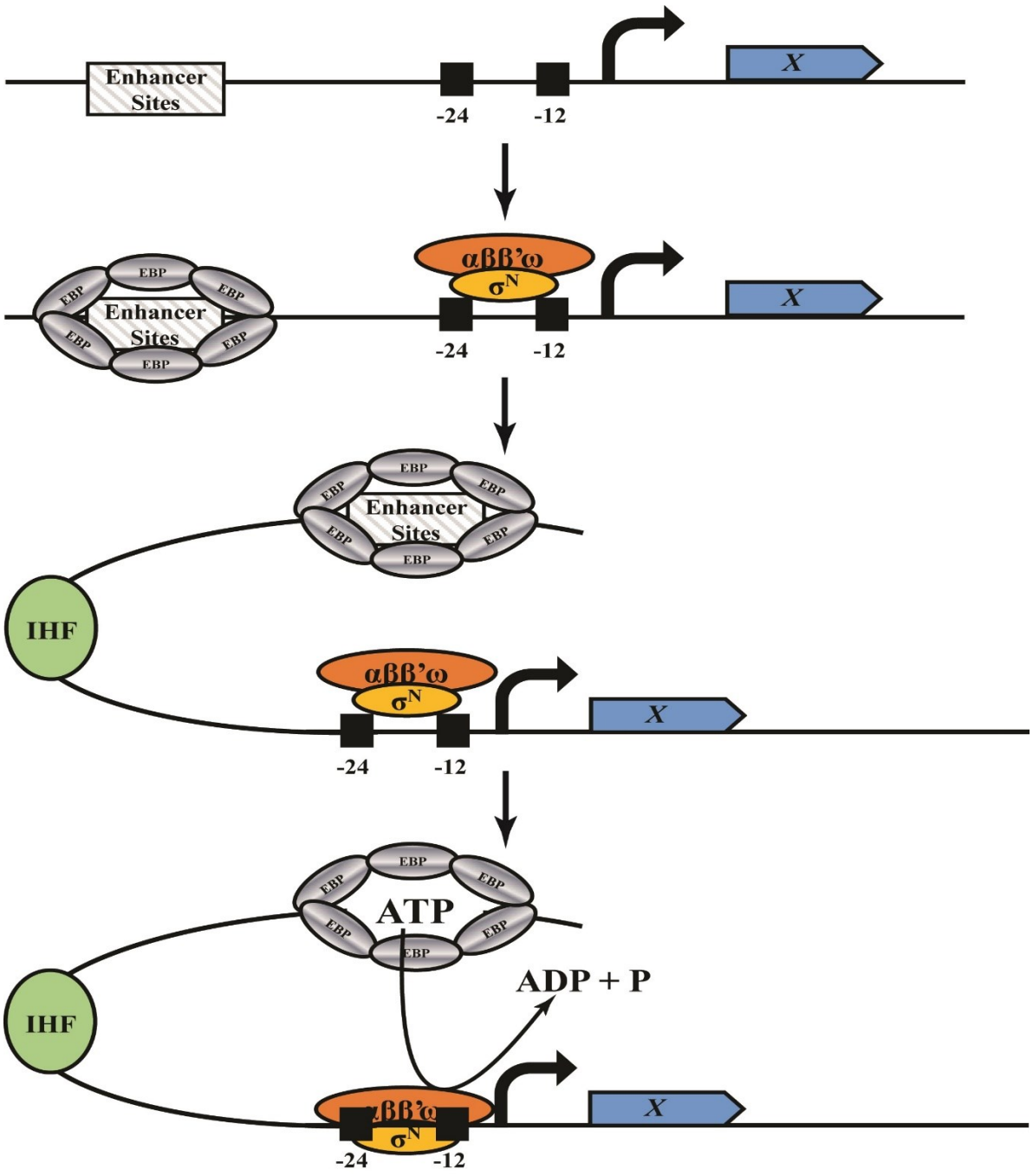

Figure 1.3. Generalized mechanism of σ^{54}-dependent transcription. $E\sigma^{54}$ holoenzyme is unable to form open complex at promoters and needs assistance from bacterial enhancer binding proteins (bEBPs). bEBPs bind to enhancer sites and interaction between bEBP and $E\sigma^{54}$ is mediated through DNA bending by integration host factor. ATP hydrolysis by bEBP initiates open promoter complex formation by σ^{54} and initiation of transcription

initiates promoter melting (306, 346, 417, 418, 434). This $E\sigma^{54}$ activation process very closely resembles that observed for eukaryotic RNA polymerase II, where DNA melting is initiated through ATP hydrolysis by TFIIH (196, 221). Since bEBPs can bind to enhancer sites 100-400 bp upstream of a σ^{54} promoter, DNA between the enhancer sites and promoter must bend to allow interaction between the bEBP and $E\sigma^{54}$ (68). This is facilitated by the DNA bending protein called integration host factor, which binds to specific sequences present between the σ^{54} promoter and the enhancer site (Fig. 1.3) (152, 437).

1.3.1 Key components and structure of the σ^{54} protein

Due to the extreme difference in protein sequence between σ^{70} and alternative sigma factor N (σ^{54}) (18% similarity), the regions of σ^{54} are categorized based on function (Fig. 1.4). Region I (residues 1 to 56) at the N terminus is primarily a glutamine and leucine rich sequence and plays important roles in promoter recognition and promoter melting through interaction with bEBPs (50, 59, 66, 364). Region I has been shown to bind both duplex and premelted -12 consensus (GC) DNA sequence and plays an important role in DNA melting at this site (346, 425). Similar to σ^{70}, after $E\sigma^{54}$ holoenzyme formation, Region I can inhibit spontaneous open complex formation at promoters. Deletion of residues 25-31 in Region I significantly increases $E\sigma^{54(\Delta RegionI)}$ binding to promoters, but transcription from this complex is only favored in solutions that allow transient DNA melting (65, 385, 413). Initiation of melting of duplex -12 (GC) sequence is completely enhancer-dependent and requires direct interaction of bEBPs with Region I. Bacterial enhancer binding proteins directly interact with residues 33-37 in Region I and the requirement of a bEBP for transcription initiation can be bypassed through deletion of Region I but only in the presence of premelted DNA (45, 73).

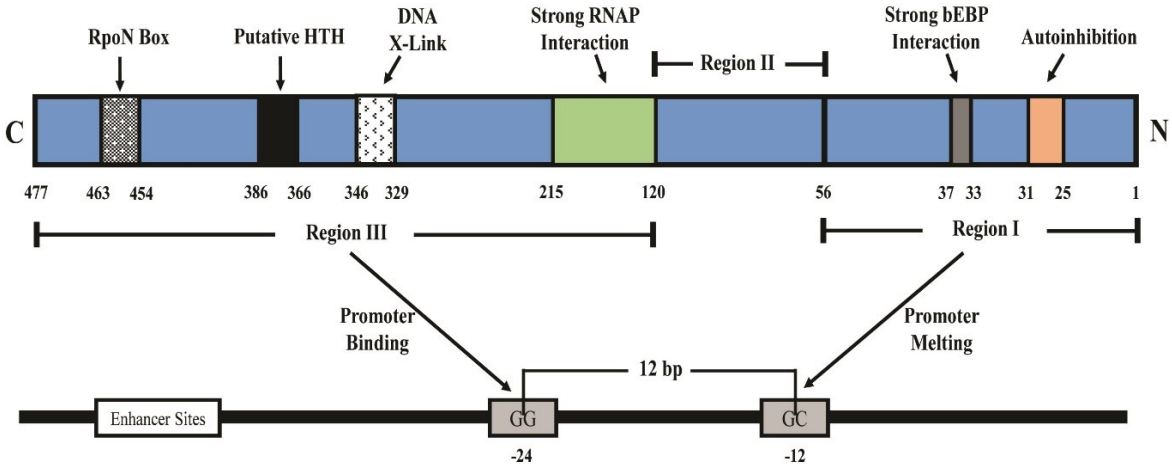

Figure 1.4. Domains and functions of σ^{54}. Regions are described based on functions. Region I (1-56) is essential for interacting with AAA+ domain of bacterial enhancer binding proteins (bEBPs) and initiating promoter melting at -12 (GC) consensus sequence. Region II (56-120) function is still not known. Region III (120-477) is essential for binding core RNAP and -24 (GG) promoter consensus sequence.

Region II is the most variable in length, ranging from 26 residues in *Rhodobacter* to 110 residues in *Bradyrhizobium*, and being completely absent in *Bacillus subtilis* (86, 96, 203) (Fig. 1.4). Sequence analysis has shown that Region II is predominantly comprised of acidic residues and in some organisms these acidic residues occur every third residue and have been termed acid trimer repeats (ATRs) (430). A true generalized function for Region II is still unknown, but the only evidence for a role of Region II has been obtained from studies in *Klebsiella pneumoniae*. Studies have shown that in *Klebsiella pneumoniae* Region II plays a critical role in σ^{54} association to core RNAP and in open complex formation by influencing binding of $E\sigma^{54}$ holoenzyme to DNA (63, 375). In *Klebsiella*, the residues 56 to 106 of Region II are required for binding to core RNAP, whereas residues 56 to 83 are required for complete association of $E\sigma^{54}$ to DNA (375).

Region III (residues 120-477) is extremely conserved and contains the main determinants for binding core RNAP and -24 (GG) consensus sequence (Fig. 1.4). Studies have shown that substitutions within residues 120-215 in Region III impair σ^{54} binding to core RNAP to varied degrees (121). Specifically, deletion of a hydrophobic heptad repeat between residues 158-179 shows a significant impairment in formation of $E\sigma^{54}$ holoenzyme (154). Residues 329 to 463 contain elements that are absolutely essential for binding the -24 (GC) promoter consensus sequence (56, 424). Residues 329-346 contains a domain that results in direct crosslinking with duplex DNA, which allows σ^{54} to bind DNA even when it is not associated with RNAP (64). Near the C-terminal end of σ^{54} is a highly conserved 10 amino acid sequence called the RpoN box (residues 454 to 463) (59, 395). These residues form a HTH motif and specifically an arginine residue (R456) has been shown to be crucial for binding to -24 consensus sequence (104, 105). Deletion or substitution of any residue in the RpoN box leads to complete abrogation of σ^{54} binding to the -24 promoter sequence (395). Another putative HTH motif has been identified between

residues 366-386 and the arginine residue (R383) was experimentally shown to bind the -13 promoter region, but further functions have not been described for these residues (246).

1.3.2 Key components and structure of bacterial enhancer binding proteins

Bacterial enhancer binding proteins (bEBPs) are members of the AAA+ protein family and have two critical functions in σ^{54}-dependent transcription: I) stimulating conformation change in σ^{54} in the holoenzyme through ATP hydrolysis, and II) initiating σ^{54}-directed DNA melting at the -12 promoter site. bEBPs are comprised of three functional domains: an N-terminal regulatory domain (R), a central AAA+ domain (C) and a C-terminal DNA binding domain (D). The core AAA+ domain, which drives ATP hydrolysis and conformational change in σ^{54}, is absolutely essential for a functioning bEBP and as such the R and D domains are sometimes absent in some bEBPs as observed in the case of PspF (*E. coli*), FlgR (Campylobacter), HrpR and HrpS (Pseudomonas) (48, 108, 158). The central AAA+ domain is comprised of seven highly conserved regions (C1-C7), which contain elements essential to the function of a bEBP (Fig. 1.5). The Walker A (region C1) and Walker B (region C4) motifs play roles in nucleotide binding and hydrolysis, respectively (136, 412). The Walker A motif has a consensus sequence GxxxxGK(T/S) and is assisted by residues in Sensor II (region C7) to bind ATP, which triggers bEBP oligomerization into higher order rings (24, 345, 351). bEBP monomers are usually ordered into hexameric rings, where the cleft between two adjacent protomers form the catalytic center for ATP hydrolysis (319). The Walker B motif has a consensus sequence hhhhDE (h – hydrophobic residue), which binds Mg^{2+} required for ATP hydrolysis (338, 352). The arginine residues (R fingers) in region C6 stabilize the ring structure by reaching into the catalytic core of an adjacent

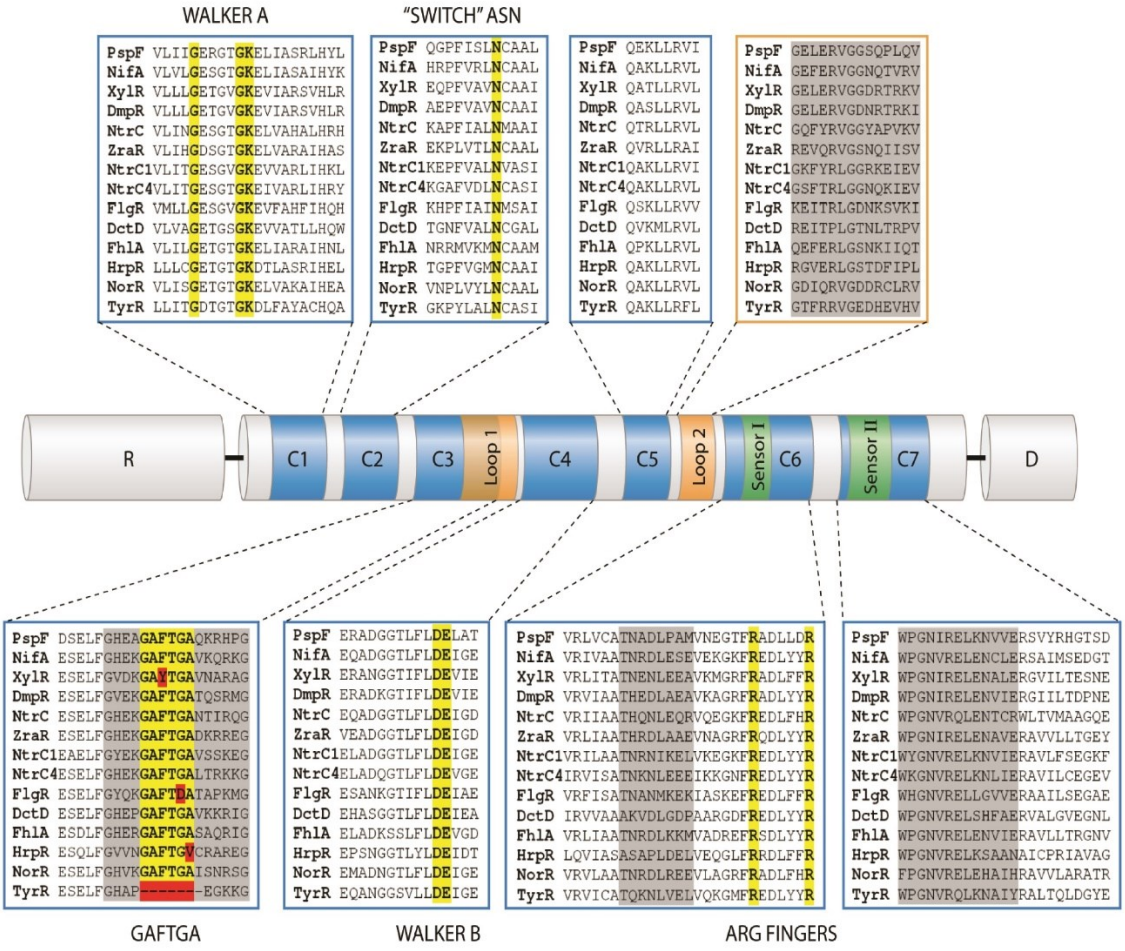

Figure 1.5. σ^{54} bacterial enhancer binding proteins (bEBPs) (from Bush *et al*. 2012). Sequence alignment showing all the conserved regions in the central AAA+ domain of all bEBPs. Walker A is assisted by Sensor II to bind ATP, triggering bEBP oligomerization into higher order rings. Cleft between two adjacent bEBP promoters form the catalytic center for ATP hydrolysis. Walker B binds Mg^{2+} and performs ATP hydrolysis. The R fingers reach into the catalytic core of an adjacent subunit, function in nucleotide sensing and work in conjunction with Walker B in intersubunit catalysis. When ATP is bound to the Walker motifs, the glutamate (E) in Walker B forms a bond with a switch asparagine (N) and upon conversion of ATP to ADP this bond is broken. Change in nucleotide state is communicated by the switch asparagine and the residues of Sensor I to the L1 and L2 loops. The GAFTGA motif in L1 loop with assistance from the L2 loop directly cause conformational change in σ^{54}, which leads to open complex formation.

subunit, function in nucleotide sensing and work in conjunction with Walker B in intersubunit catalysis (136, 224, 273, 351). When ATP is bound to the Walker motifs, the glutamate (E) in Walker B forms a bond with a switch asparagine (N) in region C2 and upon conversion of ATP to ADP this bond is broken. This change in nucleotide state is communicated by the switch asparagine and the residues of Sensor I (region C6) to the L1 and L2 loops (183, 185, 320, 350). The tip of the loop 1 (L1) in region C3 holds the GAFTGA motif and with the assistance from the residues in loop 2 (L2) directly causes conformational change in σ^{54}, which leads to Eσ^{54} closed complex formation (Fig. 1.5) (319, 444, 445).

The N-terminal regulatory domain (R) of bEBPs are involved in sensing various intra- and extra-cellular cues, which are directly coupled to activation or repression of bEBPs. The R domain has a diverse set of motifs for sensing various signals which can be categorized into three classes: V4R motifs, response regulator motifs, and GAF domains (Fig. 1.6) (362, 364). bEBPs with V4R motifs sense and bind small ligands, usually aromatic compounds that are substrates of enzymatic pathways that they control (363). For example, the bEBP, DmpR, in *Pseudomonas* binds various aromatic compounds that are involved in phenol catabolism, which then directly derepresses the inhibition of C domain (266, 368, 427). bEBPs with response regulator domains are parts of two-component systems, where a conserved residue in the R domain of the bEBP is directly phosphorylated by a cognate membrane bound sensor kinase (67). One of the most well studied bEBPs in this category is the *E. coli* nitrogen regulatory protein NtrC. During nitrogen limited conditions, the membrane bound sensor kinase NtrB autophosphorylates itself and then transfers the phosphate to a specific aspartate residue in the R domain of NtrC. This signals inactive NtrC dimers to oligomerize into active hexameric rings, allowing for the formation of a core catalytic complex for ATP hydrolysis required for open complex formation in σ^{54}-dependent transcription

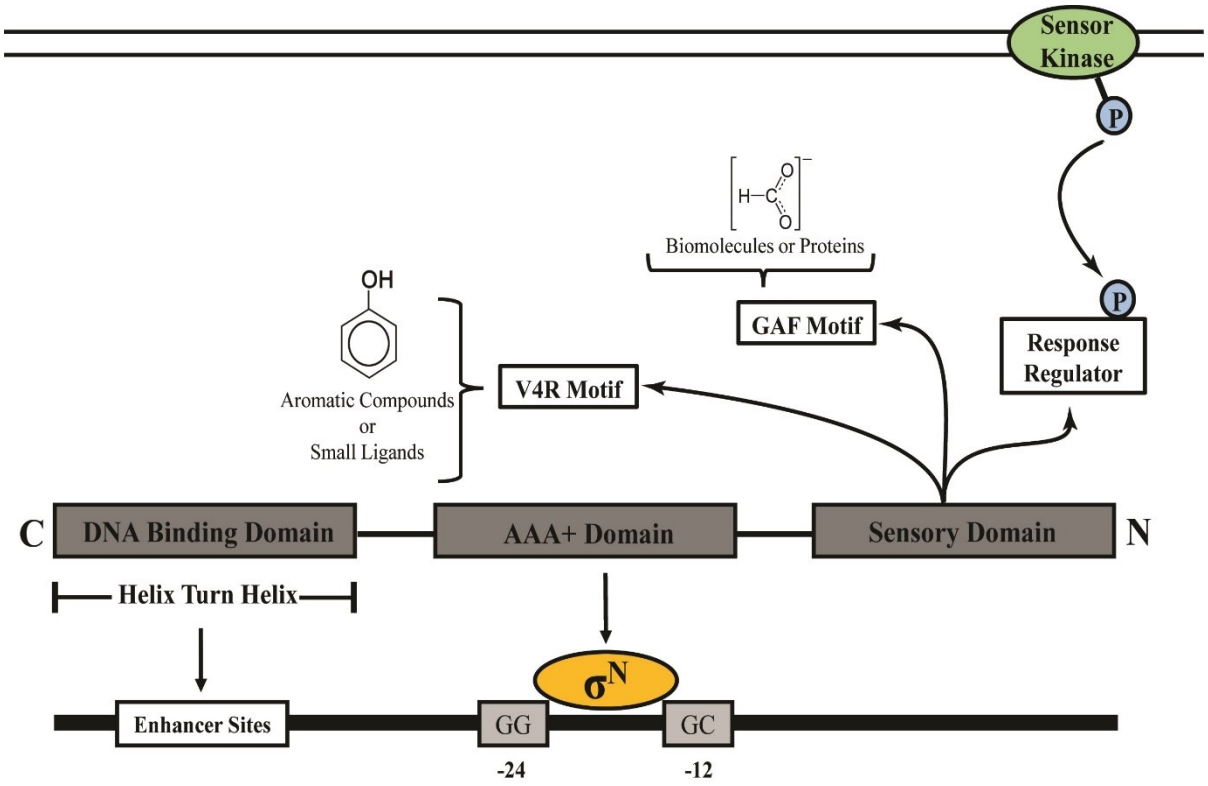

Figure 1.6. Schematic illustration of different N-terminal sensory domains and C-terminal helix-turn-helix (HTH) DNA-binding domain. The variable N-terminal sensory domains can contain V4R or GAF or response regulator motifs. V4R motifs can recognize and bind aromatic compounds (such as phenolic compounds) or small ligands. GAF domains can bind small biomolecules (such as formate) or other proteins. Response regulators are parts of two component systems and are phosphorylated by their cognate membrane bound sensor kinase.

(329, 364, 383). bEBPs with GAF motifs in the R domain respond to a wide range of inputs such as ligands, biomolecules and proteins. The *E. coli* bEBP NorR, which is involved in expression of nitric oxide reductase, directly binds nitric oxide (NO) at the GAF domain and derepresses activity of NorR (87, 159). Similarly, another bEBP, FhlA, binds formate to activate transcription of formate hydrogen lyase (153). The GAF domain in the bEBP NifA responds to different inputs in different organisms for the same response to nitrogen limitation. For example, in *Azotobacter vinelandii*, NifA is repressed by direct binding of NifL under nitrogen excess and activated by binding of 2-oxoglutarate to the GAF domain under nitrogen limitation (23, 372). In *Bradyrhizobium japonicum*, NifA production can be directly affected at the level of transcription in response to oxygen limitation and in *Herbaspirillum seropedicae* NifA can respond to nitrogen limitation by direct binding of the PII signal transduction protein to the GAF domain (114, 275, 376).

The C-terminal DNA binding domain contains a helix-turn-helix motif that is vital for binding of bEBPs to enhancer sequences (Fig. 1.6) (382, 434). Most enhancer sites exhibit dyad symmetry and as a result, bEBPs initially bind as inactive dimers as has been shown in the cases of NtrC, NorR and XylR (14, 291, 337, 406). Different studies have suggested that the D domain may also have functions in bEBP oligomerization, stabilization and fidelity. It has been proposed that the binding of bEBPs as dimers at the enhancer sites increases the local concentration of bEBPs, which may facilitate oligomerization and formation of the higher order ring structure (59). Evidence from EM reconstruction of bEBP bound to various nucleotides suggests that during transition of ATP to ADP conformational changes within the D domain may stabilize bEBP ring arrangement or facilitate σ^{54} interaction with bEBP oligomer (59, 94).

In *Escherichia coli*, it has been proven that under varying conditions response regulators of two component systems can be cross phosphorylated by non-cognate sensor kinases (408). bEBP response regulators such as NtrC can also be directly phosphorylated by high energy phosphoryl donors as acetyl phosphate (197). In *Campylobacter jejuni*, deletion of the D domain of the bEBP FlgR results in constitutive activation of σ^{54}-dependent transcription and thus has been proposed that the D domain may prevent cross phosphorylation by non-cognate sensor kinases or high energy phosphoryl donors as acetyl phosphate (186).

1.3.3 bEBPs lacking regulatory and DNA binding domains

Beyond the described architecture for the bEBPs, there are variants of bEBPs that can lack a regulatory input (R) domain or C-terminal DNA binding (D) domain and in some extreme cases contain highly variant versions of the Walker and GAFTA domains. In *Pseudomonas syringae*, HrpR and HrpS activate σ^{54}-dependent transcription of *hrpL*, which is involved in regulation of the hrp-hrc pathogenicity island (158). Both HrpR and HrpS lack any recognizable regulatory input domain and are constitutively active, but their activity in the absence of any signal is repressed by direct binding of the regulatory protein HrpV. Environmental cues lead to dissociation of HrpV from the complex, which allows HrpR/HrpS to activate σ^{54}-dependent transcription of *hrpL* (189, 364). Another example of a bEBP lacking a regulatory domain is that of PspF in *E. coli*, which is involved in activation of the phage shock operon (89). Even though the exact physiological context for the function of the phage shock operon is not known, it is predicted that the phage shock operon is activated in response to membrane stress (184, 419). Similar to *P. syringae*, under uninduced conditions, PspF is bound and inactivated by PspA, encoded by the first gene (*pspA*) of the *psp* operon, and during membrane stress dissociation of

PspA allows PspF to initiate σ^{54}-dependent transcription of the *psp* operon (49, 106, 108, 160, 187). In bEBPs that lack a C-terminal binding domain, they overcome this obstacle by activating σ^{54}-dependent transcription directly from solution. Studies have shown that bEBPs lacking a DNA binding domain have to reach a higher concentration relative to DNA bound bEBPs to initiate transcription (25). A well-studied example of such a bEBP is FlgR, which is a crucial activator of flagellar genes in *Helicobacter pylori* (27, 378). Bramhachary *et al*. has shown that FlgR lacks the HTH motif common in the C-terminal domain of other bEBPs and initiates σ^{54}-dependent transcription independent of any enhancer site. It was also shown that FlgR has the ATPase capability to activate transcription of $E\sigma^{54}$ even in *E. coli* (48).

1.4 Factors That Influence σ^{54}-Dependent Transcription

All genes required for growth and housekeeping are expressed by the housekeeping sigma factor (σ^{70}), whereas alternative sigma factors are primarily involved in regulation of genes under duress and also for bacterial virulence (193, 276). σ^{54} was first identified as a sigma factor in *E. coli* during analysis of the *gln* operon, which is involved in glutamine synthetase production and nitrogen assimilation (150, 157). Further analysis identified σ^{54} as an alternative sigma factor with crucial functions in activating genes required during nitrogen regulated (Ntr) response, flagellar biosynthesis, and bacterial pathogenesis (325, 327). Besides σ^{54}, in *E. coli* there are other alternative sigma factors that are tightly regulated through various direct active and indirect passive mechanisms programmed within the cell. Active mechanisms involve directly regulating expression of the sigmas, harnessing the sigma factors through antisigma factors, keeping them in an inactive form or maintaining high proteolytic turnover of sigma factors (26, 143, 144, 147, 193, 263). Passive mechanisms involve direct competition between sigmas for core RNAP based on

their intrinsic affinity for RNAP, sequestration of σ^{70} from holoenzyme allowing alternative sigmas to bind core RNAP and influencing activity of alternative sigmas which increase affinity for core RNAP (151, 229, 295, 296). Even though the functions of σ^{54} have been well studied and more are being discovered, there are still significant gaps in knowledge about how σ^{54} itself is regulated. One specific area requiring further research is identifying what factors stimulate or regulate association of σ^{54} to core RNAP and their exact mode of action. The following sections introduce elements that are known to influence σ^{54}-dependent transcription and review their mechanisms.

1.4.1 The effects of ppGpp and its cofactor DksA on σ^{54}-dependent transcription

When bacteria encounter nutrient limitation, ppGpp triggers a change in global transcription called stringent response, which downregulates synthesis of stable RNAs (rRNA, tRNA), ribosomal proteins and increases production of factors required for stress response (307). ppGpp can directly bind RNAP and alter activity of RNAP resulting in inhibition and stimulation of target promoters (3, 85, 277, 289, 364, 410). The effect of ppGpp at promoters is facilitated by another protein called DksA, which binds the active site of RNAP through the secondary channel and amplifies the impact of ppGpp (289). DksA causes structural changes within RNAP, which alters interaction with the -6 to +6 region of σ^{70} promoters (39, 217, 340). ppGpp and DksA also indirectly influence transcription at promoters through sigma factor competition. It is hypothesized that when ppGpp and DksA inhibit RNAP binding to σ^{70} promoters, its causes RNAP to dissociate from σ^{70} allowing alternative sigma factors to form holoenzyme and direct transcription from their respective target promoters (276). These functions of ppGpp eventually identified their vital role in activation of σ^{54}-dependent transcription (31, 32, 181, 210, 386, 388).

The most concrete evidence of a direct effect of ppGpp and DksA on σ^{54} promoters has been obtained from investigation of the *dmp* operon in *P. putida* and *E. coli*. The *dmp* operon in *Pseudomonas sp.* contains genes required for growth on phenol and phenolic derivatives as sole carbon sources (366). The structural genes are transcribed from a σ^{54}-dependent promoter termed Po (369). The bEBP required for transcription of Po is DmpR (encoded by *dmpR*), which is divergently transcribed from a σ^{70}-dependent promoter termed Pr (290, 365, 367). When ppGpp levels are increased, transcription of Po concomitantly increases and reducing ppGpp or DksA levels significantly reduces expression of Po. Po expression can be completely abrogated by removing both ppGpp and DksA, showing a synergistic effect (32, 388). In vitro transcription assays shows that ppGpp and DksA do not have any direct stimulatory effect on the Po promoter suggesting some indirect stimulatory effect on Po (32). This gap in knowledge was finally resolved after it was determined that ppGpp and DksA influence expression of DmpR through the Pr promoter. Deletion of *dksA* or reducing ppGpp production substantially decreases DmpR levels and in vitro transcription assays validated that ppGpp-DksA directly stimulate σ^{70}-dependent transcription of the Pr promoter (32, 181). Therefore, ppGpp-DksA directly activate *dmpR* expression from Pr, which leads to DmpR-σ^{54}-dependent expression from the Po promoter. Besides the effect on the Po promoter, ppGpp-DksA has also been shown to have similar stimulatory effect on other σ^{54} promoters. The Po promoter was replaced with σ^{54} promoters of *Pu* (*P. putida*), *nifh* (*K. pneumoniae*), *glnA* (*E. coli*) and *pspA* (*E. coli*) and their response to ppGpp and DksA was determined in vitro. All promoters exhibited significantly decreased activation in the absence of either ppGpp or DksA, showing a strong dependence on both and an indirect role for ppGpp and DksA in activation of σ^{54} promoters (31, 32).

As mentioned previously, the expression of the distinct sigma regulons is in part dependent on the intrinsic ability of sigmas to compete with other sigma factors for binding RNAP. At any given point the amount of any sigma factor in the cell exceeds that of available core RNAP, which makes the amount of functional RNAP an important rate limiting step (132, 300). Studies have shown that in *E. coli* the levels of σ^{54} and σ^{F} always remain constant at about 10% and 50% of that of σ^{70}, respectively, and during transition into stationary phase σ^{S} reach 30% of that of σ^{70} (173, 178). Also, in stationary phase the levels of free RNAP decreases to approximately 65% of that of log phase levels (192). Even though out of all the alternative sigma factors σ^{54} has the second highest affinity for core RNAP, the constant low levels of σ^{54} and competition from other sigmas make it extremely difficult to form holoenzyme (229). Unlike the other sigma factors which are regulated with their antisigma factors, there are no known antisigma factors for σ^{54}. As such, sigma factor competition plays a crucial role in σ^{54}-dependent transcription. This idea has been further validated through both in vivo and in vitro experimentation with the DmpR-Po system. As explained previously, in the absence of ppGpp, DmpR-σ^{54}-dependent expression of the Po promoter significantly decreases (32, 388). Studies have shown that creating mutations within *rpoD* (encoding σ^{70}) or the β and β' subunits which allow the formation of Eσ^{54} can restore Po expression in the absence of ppGpp and DksA (210, 386). Similarly, artificially manipulating levels of σ^{54} and σ^{70} through overexpression of σ^{70} and reduction of σ^{70} also significantly increases Po expression in the absence of ppGpp and DksA (31, 32). The role for sigma factor competition was even further validated through deletion of *rpoS* (encoding σ^{S}), which lead to increased expression of Po (210). Altogether, a complete indirect mechanism of influencing σ^{54}-dependent transcription has been proposed and is referred to as the passive model for ppGpp and DksA regulation of σ^{54} (269, 364).

In this passive model for ppGpp and DksA, it is hypothesized that the increased σ^{54}-dependent transcription is partly due to increased availability of free RNAP. Studies suggest that ppGpp can destabilize $E\sigma^{70}$ promoter open complexes, which causes RNAP to fall off promoters (21, 448). During exponential growth in rich media it has been shown that about 60-70% of $E\sigma^{70}$ is occupied at promoters of rRNA and tRNA operons and ppGpp levels are very low or barely detectable (262). As such, there are very low amounts of free RNAP available to form $E\sigma^{54}$. It is hypothesized that during stringent response when ppGpp levels significantly increase, it binds and destabilizes $E\sigma^{70}$ occupied at the rRNA operons, which increases levels of free RNAP and allows σ^{54} to form holoenzyme leading to increased σ^{54}-dependent transcription (269, 276, 364). It has yet to be experimentally proven if there is total increase in σ^{54}-holoenzyme in the presence of ppGpp.

1.4.2 Regulation of σ^{54}-dependent transcription by integration host factor

As mentioned previously, σ^{54}-dependent transcription is absolutely dependent on activation by enhancer binding proteins and in some cases on integration host factor (IHF), which bends DNA between the enhancer site and promoter allowing interaction of bEBPs with $E\sigma^{54}$. Apart from bending DNA and positioning enhancer and promoter sites for efficient activation, additional functions for control of σ^{54}-dependent transcription have been identified for IHF from different studies in *Pseudomonas* and *E. coli*. Investigation of the Po promoter region has identified that IHF plays a role in the activation of Po, independent of DmpR and the enhancer sites (387). Upstream of the Po promoter there are two IHF binding sites: IHF1 overlaps with the enhancer sites (UAS) and IHF2 is located between the UAS and Po promoter. The IHF2 site has been shown to provide sufficient binding of IHF and robust DmpR-σ^{54}-dependent transcription of

Po. Removal of the UAS showed that IHF can stimulate Po expression with assistance from DmpR in solution and that it is not involved in recruitment of DmpR to UAS. Increasing concentration of $E\sigma^{54}$ does not affect IHF-mediated Po expression, which also proves that IHF does not stimulate Po expression through recruitment of $E\sigma^{54}$ to the promoter. Analysis of the holoenzyme at the promoter revealed that IHF binding causes a structural change in the AT rich region around IHF2, which stabilizes $E\sigma^{54}$ open promoter complexes at Po (387).

The second example of a distinct role for IHF in σ^{54}-dependent transcription is derived from analysis of the Pu promoter in *P. putida* (35). For maximal expression from the Pu promoter, the α-CTD of RNAP has to make contacts with a specific DNA sequence just upstream of the IHF binding site. Removal of the DNA sequence or absence of IHF significantly decreases expression from the Pu promoter, which showed that IHF may play a role in association of the α-CTD of RNAP with the upstream DNA element (35). Subsequently, it was determined that in the absence of IHF only one of the α-CTD of RNAP makes contact with the DNA sequence and in the presence of IHF both α-CTD of RNAP make contact with the DNA sequence forming a stable closed complex at the Pu promoter (228).

Another example of a role for IHF in σ^{54}-transcription has been derived from experimentation with the *psp* operon in *E. coli*. The *psp* operon is transcribed from a σ^{54} promoter with assistance from the activator PspF and interaction of PspF with $E\sigma^{54}$ is mediated by IHF induced DNA bending. PspF is divergently expressed from the *psp* operon from a σ^{70} promoter and autoregulates its own expression by binding and repressing the σ^{70} promoter (89, 187). Gel shift assays have shown that PspF and IHF individually bind their respective sites at the promoter, but when both are present the shift in promoter DNA is increased approximately 48-fold, which is significantly higher than the sum of their individual shifts. It has been predicted that PspF binding

to enhancer sites results in an energetically favorable configuration which leads to significantly higher recruitment and binding of IHF. Alternatively, it has been shown that IHF binding also leads to increased recruitment of PspF to enhancer sites. This is a unique example of co-operative binding of IHF and an activator for σ^{54}-dependent system (188).

1.4.3 Other factors that affect σ^{54} association with RNAP

Rsd was initially identified from *E. coli* cell extracts of stationary phase cultures, where an additional protein was found in complex with σ^{70}-holoenzyme (175). Further experimentation identified that Rsd binds σ^{70} at a 1:1 ratio and not with any other sigma factors or components of core RNAP. Rsd was shown to inhibit transcription from σ^{70} promoter by replacing σ^{70} in the holoenzyme (175). Studies have also shown that Rsd sequesters σ^{70} and inhibits σ^{70} from binding core RNAP and -35 promoter DNA (161). Rsd has since been designated as the σ^{70} antisigma factor, which sequesters σ^{70} allowing association of alternative sigma factors during stationary phase. Observing expression of the Po promoter in an *rsd* null strain in *E. coli* and a *pfrA* (*rsd* homolog) null strain in *P. putida* revealed that deletion of these genes had no significant effect on DmpR-σ^{54}-dependent expression of Po, but overexpression of *rsd* increased Po expression in the absence of ppGpp (31, 210). Even though the physiological levels of Rsd did not produce any effect, the possible role of Rsd for other low affinity promoters or under other growth conditions have still yet to be determined. Some studies have also shown that different potassium salts can also affect sigma factor selectivity in core RNAP. Kundu *et al*. demonstrated that increasing concentration of potassium glutamate and potassium acetate significantly impedes σ^{70}-dependent transcription and increases σ^{F}-dependent transcription in a dose dependent manner (204). Lee *et al*. also demonstrated using potassium glutamate and potassium acetate that potassium levels affect

interaction between core RNAP and σ^{70} and σ^{S} (213). Therefore, it is possible that potassium levels in the cell may similarly affect σ^{54} association with core RNAP, but these effects have yet to be determined.

1.5 Role of σ^{54} in Bacterial Pathogenesis and Stress Fitness

σ^{54} was initially discovered during the investigation of the response of *E. coli* to nutrient limitation (150). When *E. coli* is cultivated in media without ammonia or with a single organic nitrogen source (nitrogen limiting conditions), it results in expression of about 100 genes, collectively called the nitrogen regulated (Ntr) response. Growth in nitrogen limited environment results in low intracellular levels of glutamine, culminating in the phosphorylation and activation of the bEBP NtrC by the sensor kinase NtrB and NtrC-σ^{54} dependent activation of Ntr genes. The primary function of Ntr genes is to assimilate nitrogen through induction of transport/scavenging systems and nitrogen degradation pathways when nitrogen is limiting (327). Under these conditions, glutamine synthetase (GS) catalyzes the synthesis of L-glutamine from ammonia and L-glutamate. However, roles of σ^{54} extend beyond just assimilation of nitrogen during nitrogen limitation response and have broad implications on cell metabolism. In commensal *E. coli* σ^{54} has been directly implicated in catabolism of arginine and agamatine, transport of amino acids such as arginine, histidine, glutamate and aspartate. σ^{54} also plays a key role in formate metabolism by directly activating the FhlA regulon, which encodes formate catabolic genes. Studies have shown that σ^{54} is directly responsible for activating expression of the *prp* operon and the *zra* operon, whose genes encode components crucial for propionate metabolism and maintaining zinc homeostasis, respectively. Recent studies have also shown that σ^{54} activates the *rtc* operon whose gene products are the only RNA cyclase and RNA ligase found in *E. coli* suggesting an important

role for σ^{54} in RNA metabolism, but the actual physiological context has yet to be determined (for detailed review see (327)). Other than playing an important role in metabolism many studies have also implicated σ^{54} as a crucial determinant of bacterial pathogenesis in many organisms. The following sections present a review of the different organisms in which σ^{54} plays an important role in virulence and pathogenesis.

1.5.1 Borrelia burgdorferi

Borrelia burgdorferi, a spirochetal bacterium, is the causative agent of Lyme disease and the most common vector-borne disease in the US (15). It maintains a natural reservoir in *Ixodes scapularis* ticks and is transmitted to mammalian (rodent or human) hosts through bite of an infected tick (52, 379). Following a localized skin lesion the pathogen can disseminate to the heart, joints and nervous system causing carditis, arthritis and neurological complications (281). First, *B. burgdorferi* has to adapt to conditions and colonize in the tick host and then subsequently adapt and colonize within the mammalian host. Adaptation and colonization within different hosts is primarily driven through differential regulation of outer surface lipoproteins (Osp) (353). Outer surface lipoprotein A (OspA) and OspB are absolutely essential for colonization of the tick midgut and highly expressed when the bacteria are ingested by the tick through a blood meal (95, 279, 284, 354, 440). Outer surface lipoprotein C (OspC) is essential for colonization of the mammalian host and highly expressed when the bacteria are transferred from the tick into mammal through the bite (133, 285, 353, 398). In conjunction to OspC, other surface proteins like decorin binding protein A (DbpA) and Mlp8 also function in colonization of the mammalian host (278). The alternative sigma factor S (RpoS) plays a crucial role in the switching of outer surface lipoproteins and the complex natural cycle of *B. burgdorferi*, by indirectly repressing OspA and OspB and

directly activating OspC, DbpA and Mlp-8 during mammalian infection (156, 398, 440). Expression of *rpoS* (encoding RpoS) is directed from a σ^{54} promoter, and deletion of *rpoN* completely abrogates expression of *rpoS, opsC, dbpA* and derepresses expression of *ospA* (57, 115, 374). As with any σ^{54}-dependent promoter, activation of *rpoS* is completely dependent on the unique bEBP Rrp2, which does not bind to any specific DNA sequence and activates *rpoS* transcription independent of any enhancer sequences (41, 43, 134, 438). Initially it was hypothesized that Rrp2 phosphorylation and its activation was dependent on it cognate sensor kinase HK2. Subsequent experimentation identified that phosphorylation and activation of Rrp2 was dependent specifically on production of acetyl phosphate, a high energy phosphoryl donor (435). Another unique feature for *rpoS* transcription in *Borrelia* is that σ^{54} is also completely dependent on another DNA binding protein called BosR, which very closely resembles a Fur homolog (280). This dependence on both a bEBP (Rrp2) and a non bEBP (BosR) for transcriptional activation by σ^{54} represents a unique case that has never been observed for σ^{54} in other bacteria. Inactivation of any component in the Rrp2-BosR-RpoN pathway for activation of *rpoS* significantly mitigates virulence and recovery of the pathogen from mice models proving their necessity for full pathogenic potential in *Borrelia* for mammalian infection (280).

1.5.2 Escherichia coli O157:H7

Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 is a serious food and water borne pathogen, responsible for outbreaks of bloody diarrhea (hemorrhagic colitis), severe acute anemia and the life threatening illness hemolytic uremic syndrome (HUS) (232, 245, 422). To cause disease, EHEC must pass through the harsh acidic milieu of the upper gastrointestinal tract, and then colonize the lower gastrointestinal tract (4, 384, 422). After entry into the stomach, EHEC

ceases growth, enters into stationary phase and activates expression of genes for acid resistance response, which allow survival of EHEC during passage through the gastric acid barrier of the stomach (pH 1-3) and determines a low oral infectious dose (<100 cells/ml) (76, 397). After entering the lower gastrointestinal tract, increased availability of nutrients, neutral pH and physiologic temperature signal EHEC to grow exponentially and colonize. Colonization is dependent on the pathogenicity island called the locus of enterocyte effacement (LEE), which encodes a type III secretion system and several effector proteins, absolutely crucial for intimate interaction of EHEC with intestinal cells (102, 293). Therefore, both acid resistance and type III secretion mechanisms are vital for EHEC transmission and pathogenesis. In *E. coli* O157:H7, alternative sigma factor S (σ^S) is a common regulator of both acid resistance and the LEE (169, 313). When the extracellular pH drops to pH 1-3, σ^S activates expression of the central transcriptional activator gene, *gadE*, whose protein product GadE activates glutamate-dependent acid resistance for maintaining cytoplasmic pH (70, 117, 219). Posttranslational regulation of σ^S plays an important role in regulation of LEE encoded genes through two major LEE regulators, Ler and GrlR. In one mechanism TTS genes are de-repressed through proteolytic activity of ClpXP on σ^S (169). In another mechanism stabilization of *rpoS* mRNA increases expression of TTS genes (206).

Recent study demonstrated that deletion of *rpoN* impacts expression of GDAR and the LEE in EHEC during exponential growth (332). Inactivating σ^{54} (RpoN) significantly increases expression of GDAR genes correlating with increased fitness in acid after a two hour acid challenge. Whereas, the WT strain exhibits no acid resistance during exponential phase, the *rpoN* mutant exhibits almost a 1000 fold increase in acid survival after a two hour challenge, demonstrating that σ^{54} is involved in negative regulation of GDAR. Deletion of σ^{54} also

significantly decreases expression of genes encoding the central LEE regulator, *ler*, and receptor for A/E lesions, *tir*, demonstrating that σ^{54} plays a positive regulatory role in regulation of the LEE. The influence of σ^{54} on GDAR and LEE was shown to be dependent on σ^{S}, because inactivation of *rpoS* (encoding σ^{S}) in the *rpoN* mutant strain abolishes σ^{54}-dependent control of GDAR and the LEE (332). This is another example of where σ^{54} influences σ^{S} for control of mechanisms that influence pathogenesis, but unlike *Borrelia burgdorferi* in EHEC σ^{54} influences σ^{S} indirectly in a negative manner. In EHEC, σ^{54} negatively influences both stability and activity of σ^{S} to regulate GDAR and LEE expression and is dependent on the bEBP NtrC for this control. Deletion of *ntrC*, encoding NtrC, phenocopies the *rpoN* mutant for control of GDAR and the LEE (251). Negative regulation of σ^{S} activity by NtrC-σ^{54} is mediated through FliZ, an antagonist of σ^{S} activity, and upregulation of *fliZ* by NtrC-σ^{54} has been shown to be indirect, requiring an intact flagellar regulator FlhDC. Phosphorylation and activation of NtrC for control of GDAR and the LEE was shown to be independent of nitrogen availability within the cell, and like *Borrelia* is dependent on acetyl phosphate. Manipulation of acetyl phosphate levels through deletion of acetate kinase (*ackA*) and substitution of carbon source abrogates NtrC-σ^{54}-dependent control of *flhDC*, *fliZ*, GDAR and LEE genes (Mitra *et al*. unpublished, in review). Altogether, NtrC-σ^{54} play an important role in regulation of key pathogenic mechanisms in EHEC by sensing extra- and intracellular availability of carbon.

1.5.3 Pseudomonas aeruginosa

Pseudomonas aeruginosa is one of the most common causes of infections in burn injuries and are commonly associated with infections in patients with cystic fibrosis (CF) (123). The large amounts of viscous bronchial secretions, high osmolarity and low nitrogen and phosphate

conditions within CF lungs, stimulate production of alginate, allowing *P. aeruginosa* to proliferate and cause severe infections in the respiratory tract (34, 97, 238). Alginate, an exopolysaccharide, provides an important protective barrier against antibiotics and host immunity in the host lungs (208, 238). Its production is dependent on two genes, *algD* and *algC*, which are under direct transcriptional control of σ^{54}. Studies have shown that alginate production is influenced by nitrogen availability within the cell, which is cued through σ^{54} to activate or repress expression of *algD* and *algC*. Removal of *rpoN*, encoding σ^{54}, significantly reduces *algDC* expression and production of alginate making σ^{54} an important determinant for successful colonization within the host lungs (46, 449). Deletion of *rpoN* also severely abrogates production of pili and flagella by directly affecting expression of pilin and flagella biosynthesis genes, leading to loss of adhesion to various cell types and motility and making the pathogen extremely avirulent (78, 84, 91, 190, 403). *P. aeruginosa* also depends on flagella and pili production for successful colonization within the respiratory tract in CF patients. Absence of either flagella or pili significantly abrogates virulence in *P. aeruginosa*. Studies from burned mice model shows that non-flagellated or pili negative strains have a ten times higher LD_{50} and significantly reduced recovery from skin relative to WT, making them highly susceptible to host defense mechanisms (253, 347). Altogether, the positive regulatory roles of σ^{54} in alginate, flagella and pili production make it an important determinant of *P. aeruginosa* pathogenesis.

1.5.4 Pseudomonas syringae

Pseudomonas syringae is an opportunistic pathogen and the causative agent of leaf blights and many related diseases in plants (149). During pathogenesis, *P. syringae* invades the plant through the stomata or wounds and colonizes the surfaces of cells (194). The range of plants that

P. syringae can invade and colonize is strain specific, and when introduced into a non-compatible host, the host's defense system responds by eliciting a rapid localized programmed cell death termed hypersensitive response (HR) to prevent further colonization (142, 194, 432). *P. syringae* strains contain avirulence (*avr*) genes which interact with non-compatible hosts and ensure interaction with compatible hosts and successful infection and colonization (357). Within *P. syringae* is a pathogenicity island (PAI) called the *hrp*/*hrc* gene cluster, which encodes components of a type III secretion apparatus and effector proteins (*avr* genes), crucial for effective identification and colonization of the host (129, 170, 267, 301, 357). In conjunction to the *hrp*/*hrc* PAI, some *P. syringae* pathovars also produce a phytotoxin called coronatine, which induces chlorosis in the host (6, 29). Expression of the majority of *hrp*, *hrc* and *avr* genes in the pathogenicity island are under the direct transcriptional control of an extracytoplasmic sigma factor called HrpL (145, 432). It was eventually identified that transcription of *hrpL* is under direct control of σ^{54} and is dependent on two separate bEBPs, HrpR and HrpS (158, 193). Subsequent studies showed that strains lacking *rpoN* are extremely impaired at colonizing host cells and in production of coronatine, which significantly reduces the ability of *P. syringae* to cause disease (6, 146). The extreme defective phenotype of *rpoN* mutants is not just due to decreased expression of PAI, since complementation with *hrpL* only marginally reproduces wild type phenotype but not coronatine production, proving that σ^{N} contributes to *P. syringae* virulence through both *hrpL*-dependent and -independent mechanisms (193).

1.5.5 Salmonella typhi

Salmonella typhi is the causative agent of typhoid fever and poses a serious problem in many developing nations (287). An important determinant of virulence in *S. typhi* is the

lipopolysaccharide (LPS) layer, a major component in the outer membrane of all gram negative bacteria. LPS provides crucial protection to the pathogen from lytic action of serum complement and also functions in adherence to epithelial cells during initial stages of infection (182, 225, 299). LPS biosynthesis is dependent on nutrient availability, pH, osmolarity and temperature (5, 162, 335). Expression of operons and genes encoding components of LPS core and O antigen are positively regulated by the RfaH elongation factor (17, 110, 234, 310, 416). Most of these operons and genes contain a conserved 8 bp motif called *ops* (operon polarity suppressor) at the 5'-UTR, which induces transcriptional pausing both *in vivo* and *in vitro* (11, 216). RfaH recognizes these *ops* sequences and stimulates expression of these operons and genes by repressing transcriptional pausing (12). It has been proven that RfaH is absolutely necessary for production of LPS in *S. typhi* (336). RfaH dependent expression of LPS is growth phase dependent with expression of *rfaH* and production of LPS being lowest during exponential phase and maximal during stationary phase (38, 336). It was eventually shown that expression of *rfaH* is activated from a σ^{54} promoter and that deletion of *rpoN* significantly abrogates the growth phase dependent expression of *rfaH* and production of LPS. Involvement of σ^{54} also led to the finding that both *rfaH* expression and LPS production was similarly influenced by nitrogen availability, where nitrogen limitation significantly increased RfaH levels and LPS production (38).

1.5.6 Other species

Besides the organisms reviewed in the preceding sections, σ^{54} has also been implicated to be important for virulence in other pathogens. In *Vibrio cholerae* deletion of *rpoN* leads to loss of motility and inability to colonize in an infant mouse model of cholera. The inability to colonize in mouse was shown to be independent of motility suggesting a more significant role for σ^{54} in

regulation of *V. cholerae* virulence (198). Similarly, σ^{54} was also shown to be necessary for the fish pathogen *Vibrio anguillarum* in penetration of the fish epithelium, but it is not required for virulence following penetration of the epithelium (270). In *Legionella pneumophila*, mutation of *rpoN* significantly impairs the ability of the pathogen to compete with the wild type in an infection model, proving that σ^{54} is required for full *in vivo* virulence and fitness (349). Studies in the plant pathogen *Erwinia amylovora* show that σ^{54} is absolutely necessary for virulence, which was linked to the direct regulatory effect of σ^{54} in expression of the type III secretion system (9, 77).

Concluding Remarks

During various nutrient limitation and upon encountering stressful conditions bacteria have to rapidly adapt to survive. Integration of environmental signals by sigma factors and coupling them to differential gene regulation provides the basis for adaption and survival in extreme environments. σ^{54} is a major player in nitrogen limitation response, amino acid synthesis and transport, carbon metabolism and various other vital cellular processes. However, in recent years, studies have shown that functions of σ^{54} extend beyond just maintaining cellular metabolism. σ^{54} is absolutely crucial for expression of virulence factors in *Borrelia*, *Pseudomonas* and *Vibrio* species and for complete *in vivo* disease progression. In EHEC σ^{54} is responsible for activation of the locus of enterocyte effacement, which is crucial for immune subversion and colonization in the human intestine. For full activity σ^{54} is completely dependent on enhancer binding proteins (bEBPs), which provide the bridge between environmental signals and activation of σ^{54}-dependent transcription. However, in many pathogens the environmental factors and the molecular basis that influence bEBP-σ^{54}-directed regulation of pathogenesis are poorly understood. Defining the molecular basis of σ^{54}-directed regulation and understanding the environmental factors that

influence these pathways are crucial for development of chemotherapy to prevent disease progression in many of these pathogens.

Chapter Two: Sigma Factor N, Liaison to an NtrC and RpoS Dependent Regulatory Pathway Controlling Acid Resistance and the LEE in Enterohemorrhagic *Escherichia coli*

Note to Reader

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2.1 Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) is dependent on acid resistance for gastric passage and low oral infectious dose, and the locus of enterocyte effacement (LEE) for intestinal colonization. Mutation of *rpoN*, encoding sigma factor N (σ^N), dramatically alters the growth-phase dependent regulation of both acid resistance and the LEE. This study reports on the determinants of σ^N-directed acid resistance and LEE expression, and the underlying mechanism attributable to this phenotype. Glutamate-dependent acid resistance (GDAR) in TW14359Δ*rpoN* correlated with increased expression of the *gadX-gadW* regulatory circuit during exponential growth, whereas upregulation of arginine-dependent acid resistance (ADAR) genes *adiA* and *adiC* in TW14359Δ*rpoN* did not confer acid resistance by the ADAR mechanism. LEE regulatory (*ler*), structural (*espA* and *cesT*) and effector (*tir*) genes were downregulated in TW14359Δ*rpoN*, and mutation of *rpoS* encoding sigma factor 38 (σ^S) in TW14359Δ*rpoN* restored acid resistance and LEE genes to WT levels. Stability, but not the absolute level, of σ^S was increased in

TW14359Δ*rpoN*; however, increased stability was not solely attributable to the GDAR and LEE expression phenotype. Complementation of TW14359Δ*rpoN* with a σ^N allele that binds RNA polymerase (RNAP) but not DNA, did not restore WT levels of σ^S stability, *gadE*, *ler* or GDAR, indicating a dependence on transcription from a σ^N promoter(s) and not RNAP competition for the phenotype. Among a library of σ^N enhancer binding protein mutants, only TW14359Δ*ntrC*, inactivated for nitrogen regulatory protein NtrC, phenocopied TW14359Δ*rpoN* for σ^S stability, GDAR and *ler* expression. The results of this study suggest that during exponential growth, NtrC-σ^N regulate GDAR and LEE expression through downregulation of σ^S at the post-translational level; likely by altering σ^S stability or activity. The regulatory interplay between NtrC, other EBPs, and σ^N-σ^S, represents a mechanism by which EHEC can coordinate GDAR, LEE expression and other cellular functions, with nitrogen availability and physiologic stimuli.

2.2 Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is an enteric pathogen commonly implicated in food-borne outbreaks of hemorrhagic colitis, and in the life-threatening illness hemolytic uremic syndrome (18, 243, 244). To cause disease in humans, EHEC must overcome two formidable innate barriers to infection: the acidity of the stomach, and competition for intestinal colonization sites. For the former, EHEC (and other *E. coli*) has evolved multiple discrete acid resistance mechanisms (reviewed in (116)), which allow for survival in highly acidic environments such as the stomach, and which determine a low oral infectious dose (75, 396). For competitive gut colonization, EHEC utilize a type III secretion system (T3SS) encoded on the locus of enterocyte effacement (LEE) pathogenicity island (109, 137, 241, 292). This T3SS translocates EHEC

effector proteins into host intestinal cells that mediate intimate attachment to the gut and subvert host cellular processes (reviewed in (429)).

The expression of acid resistance and the LEE is influenced by various environmental and intracellular signals, including nutrient availability, stress, and growth phase (69, 128, 226, 227, 373), (1, 2, 30, 191, 195, 441). During exponential growth acid resistance is largely repressed, but is activated as cultures transition into stationary phase (69); for the LEE, the inverse is true (30). This pattern of expression may reflect the importance of colonization and replication when resources are abundant, and that of stress durability when they are scarce. Many auxiliary regulators communicate these changes in growth conditions to regulatory components of both acid resistance and the LEE (93, 201, 207, 211, 227, 343, 360, 399). Alternative sigma factor 38 (σ^S) is a global regulator that plays an important role in coordinating acid resistance and LEE expression with growth phase. σ^S is a protein of low abundance during exponential growth, but accumulates during transition into stationary phase (209). The acid resistance phenotype of stationary phase cultures is largely attributed to σ^S and expectedly, strains mutated for *rpoS* (encoding σ^S) are sensitive to acid (69, 373), whereas LEE expression is both decreased and increased in response to *rpoS* mutation, depending on growth conditions (168, 207, 377, 401). Not surprisingly, *rpoS* mutants are impaired in their ability to survive passage in both murine and bovine models of infection (312). σ^S is tightly regulated at multiple levels of control (reviewed in (147)), and the factors that dictate *rpoS*/σ^S expression indirectly influence acid resistance, the LEE, and EHEC pathogenesis.

Recently, another alternative sigma factor, sigma N (σ^N), has been shown to control structural and regulatory genes of both acid resistance and the LEE in EHEC serotype O157:H7 (332). When bound to RNA polymerase (RNAP), the RNAP-σ^N holoenzyme ($E\sigma^N$) directs

transcription from an estimated twenty-one promoters in *E. coli* which specify the transcription of over sixty genes involved in nitrogen and carbon metabolism, and stress resistance (51, 308, 327, 420). EHEC strains null for *rpoN* (encoding σ^N) express elevated levels of acid resistance genes belonging to the glutamate-dependent acid resistance (GDAR) system, and reduced levels of expression for genes encoded on all five operons of the LEE (332). This altered expression of GDAR and LEE genes is restricted to exponential phase cultures. Furthermore, GDAR upregulation in *rpoN* mutants is correlated with increased survival in acidic environments, and is dependent on an intact *rpoS* gene, suggesting that GDAR is controlled by an as yet uncharacterized σ^N-σ^S regulatory pathway in *E. coli* (332).

There is precedent for such a pathway in *Borrelia burgdorferi*, in which a σ^N-σ^S regulatory pathway controls the expression of membrane lipoproteins essential for transmission and pathogenesis (42, 155, 439). In the *B. burgdorferi* model, σ^N has been shown to directly activate *rpoS* transcription, which is contrary to *E. coli* in which *rpoS* inactivation abrogates the GDAR phenotype of an *rpoN* null mutant, suggesting that σ^N downregulates *rpoS*/σ^S by some unknown mechanism. There is evidence that this negative regulation is at the post-transcriptional level, as *rpoN* mutation does not alter *rpoS* mRNA levels (332). In addition, a recent study reported increased levels and stability of σ^S in an *rpoN* mutant of the nonpathogenic *E. coli* strain K-12 MG1655 (101). This study further explores the regulatory interplay of σ^N and σ^S, and uncovers mechanistic details about σ^N-σ^S directed control of acid resistance and the LEE, and other genetic factors which contribute to the expression of this regulatory pathway.

2.1 Results

2.2.1 σ^N-σ^S directed regulation of glutamate-dependent acid resistance and the locus of enterocyte effacement

Independent regulatory pathways control glutamate-dependent acid resistance (GDAR) genes in response to discrete environmental stimuli through transcriptional modulation of the central regulator *gadE*. These include pathways that stimulate *gadE* during exponential growth in minimal, acidified media (EvgAB-YdeO) (226, 236), or during stationary phase growth in rich media (σ^S-GadX-GadW) (227) , or rich media containing glucose (TrmE) (128). The growth conditions under which *rpoN*-dependent acid resistance is expressed do not conform precisely to any of these stimulating environments. And yet, mutation of *rpoS* in an *rpoN* null background suppresses GDAR, suggesting that in the WT background σ^N negatively regulates GDAR through a σ^S-dependent pathway; namely, σ^S-GadX-GadW. To explore this further, transcript levels of GDAR regulatory genes from these activating circuits were measured in WT and mutant backgrounds of TW14359 during exponential growth.

As anticipated, *gadE* transcript levels were significantly higher in TW14359Δ*rpoN* compared to TW14359 ($p=0.001$), as well as TW14359Δ*rpoS* ($p=0.007$), and TW14359Δ*rpoN*Δ*rpoS* ($p=0.005$) (Fig. 2.1A). Adding to this, both *gadX* and *gadW* transcripts were upregulated in TW14359Δ*rpoN* ($p<0.05$), but not in TW14359Δ*rpoS* for *gadX*, or TW14359Δ*rpoN*Δ*rpoS* for either *gadX* or *gadW*. Transcript levels for *trmE* and *ydeO*, key regulators of alternative pathways for *gadE* activation, were in low abundance, and did not differ significantly between strains (Fig 2.1A); the presence of amplicons for *trmE* and *ydeO* was validated by gel electrophoresis. Thus, a *rpoN* null mutation leads to increased expression of the GDAR-activating GadX-GadW pathway, agreeing with the *rpoS*-dependency of the phenotype.

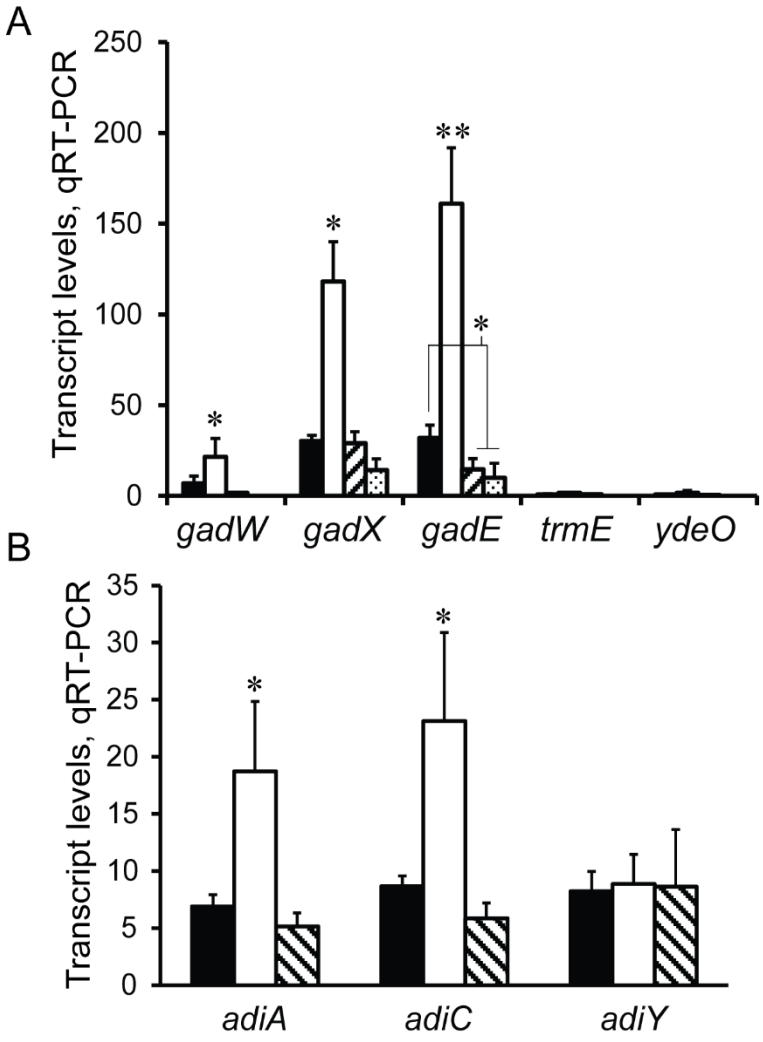

Figure 2.1. Transcript levels for acid resistance genes. Gene transcript levels as determined by qRT-PCR are plotted for genes of the GDAR system **(A)** and genes of the ADAR system **(B)**. Mean transcript levels are normalized to the 16S rRNA gene *rrsH*. Transcript levels are plotted against WT TW14359 (filled), TW14359Δ*rpoN* (empty), TW14359Δ*rpoN* Δ*rpoS* (hatched), and TW14359Δ*rpoS* (stippled, *gadX* and *gadE* only) for **(A)**. Asterisks denote significant differences by Tukey's HSD following a significant F-test ($n \geq 3$, $p<0.05$ [*]; $p<0.01$ [**]). Error bars indicate standard error of the mean.

In addition to GDAR, σ^S regulates at least two more acid resistance systems in *E. coli*: the arginine-dependent acid resistance (ADAR) system (220), and the oxidative-dependent acid resistance (ODAR) system (312). Both GDAR and ADAR systems protect the cell from acid by a proton scavenging mechanism that is facilitated by the conversion of glutamate to γ-aminobutyric acid (GDAR) or arginine to agmatine (ADAR), and catalyzed by amino acid decarboxylases. ODAR on the other hand does not require glutamate or arginine, and is repressed by glucose (reviewed in (116)). Except for *rpoS*, the regulatory and structural determinants of ODAR are not well understood, and thus were not investigated in this study. For ADAR, the structural genes *adiA* (arginine decarboxylase) and *adiC* (arginine-agmatine exchanger) were slightly but significantly upregulated in TW14359Δ*rpoN* relative to TW14359 and TW14359Δ*rpoN*Δ*rpoS* ($p<0.05$) (Fig. 2.1B). However, *adiY*, encoding a putative regulator of *adiA* and *adiC* (380), was not altered in expression in either of the mutant backgrounds. Despite the increase in *adiA* and *adiC* expression in TW14359Δ*rpoN*, there was no corresponding increase in acid resistance by the ADAR mechanism (Table 2.1), and exclusion of either glutamate or arginine from acidified EG media resulted in no growth for any strains (data not shown). Therefore the only known requirements for *rpoN*-dependent acid resistance are *rpoS*, *gadE*, and glutamate.

σ^S has also been shown to upregulate and downregulate transcription of LEE genes in EHEC. For upregulation, σ^S is hypothesized to enhance expression of the central regulator of the LEE, *ler* (encoded on operon *LEE1*), in a manner dependent on the non-coding RNA DsrA (207). It has also been reported that both the *LEE3* and *LEE5* operons possess σ^S-responsive promoters (377). For downregulation, σ^S is proposed to stimulate an unknown repressor of PchA, which is a positive regulator of *ler* (167, 168, 401). The mutation of *rpoN* leads to the downregulation of LEE genes during exponential growth (332). Since σ^N controls GDAR through a σ^S-dependent

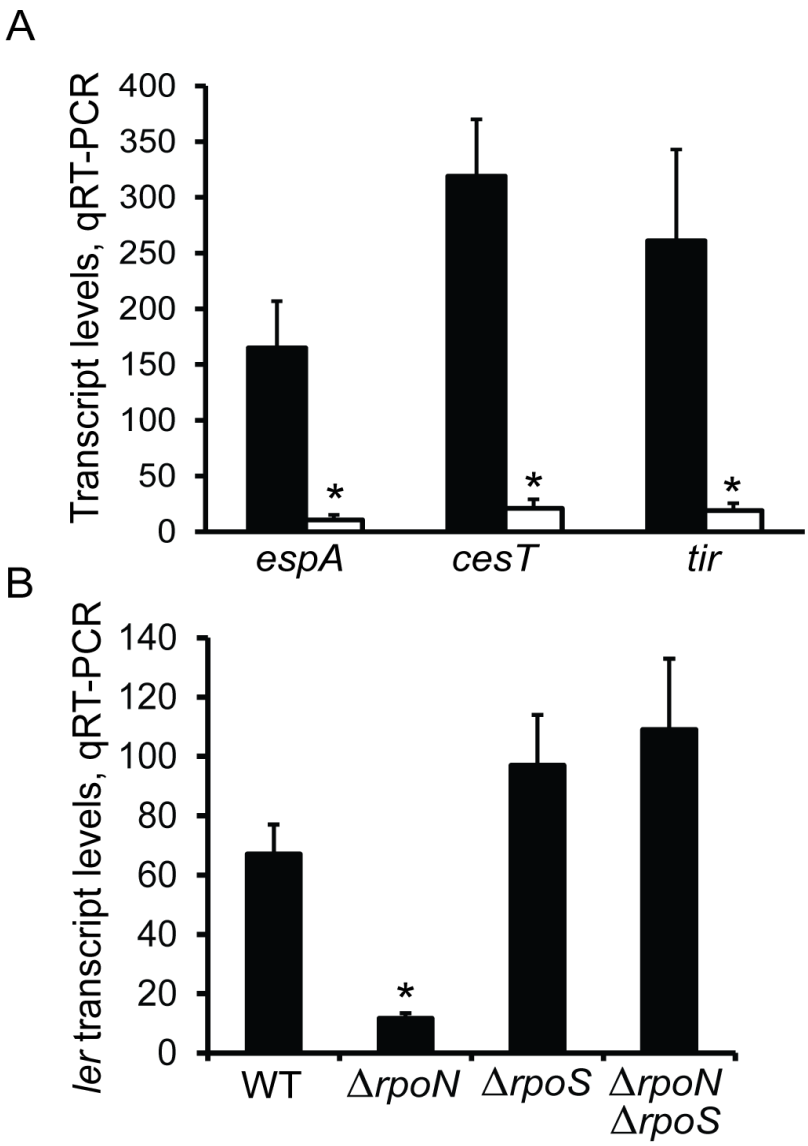

Figure 2.2. Transcript levels for LEE genes. (A) gene transcript levels as determined by qRT-PCR are plotted for representative LEE genes in WT TW14359 (filled) and TW14359Δ*rpoN* (empty). **(B)** *ler* transcript levels by qRT-PCR are plotted against TW14359 and various mutant derivative strains of TW14359. Mean transcript levels are normalized to the 16S rRNA gene *rrsH*. For panel A, an asterisk denotes a significant difference between TW14359 and TW14359Δ*rpoN* for each gene by Welch's t-test ($n \geq 3$, $p < 0.05$). For panel B, the asterisk denotes a significant difference between TW14359Δ*rpoN* and the remaining strains by Tukey's HSD following a significant F-test ($n \geq 3$, $p < 0.05$). Error bars indicate standard error of the mean.

pathway, it was predicted that σ^N-directed regulation of the LEE may be similarly dependent on *rpoS*. As expected, transcript levels for LEE genes encoding the T3SS translocon component *espA* (encoded on *LEE4*), the effector chaperone *cesT* (on *LEE5*), and the translocated intimin receptor *tir* (on *LEE5*) were downregulated during exponential growth of TW14359Δ*rpoN* relative to TW14359 ($p<0.05$) (Fig. 2.2A). In addition, transcript levels of *ler* (on *LEE1*) were reduced in TW14359Δ*rpoN* compared to TW14359 ($p=0.015$) and TW14359Δ*rpoS* ($p=0.011$) (Fig. 2.2B). Importantly, mutation of *rpoS* in TW14359Δ*rpoN* restored *ler* expression to levels consistent with TW14359Δ*rpoS*; *ler* expression was increased in *rpoS* null backgrounds relative to WT, but not significantly increased. These results indicate that σ^N positively regulates the LEE during exponential growth in an *rpoS*-dependent manner, and is consistent with the role of σ^S as a negative regulator of LEE expression via the PchA-Ler pathway (167, 168, 401).

2.2.2 Effect of rpoN mutation on rpoS mRNA and σ^S stability in EHEC

There is evidence that the mutation of *rpoN* in EHEC does not alter *rpoS* mRNA levels, but instead leads to post-transcriptional alternations in *rpoS*/σ^S (332). The mutation of *rpoN* in *E. coli* strain K-12 MG1655 was recently shown to lead to increased σ^S levels and stability (101). However, there are substantial differences at the genomic level between K-12 and EHEC O157:H7 strains (139). As an important example, the TW14359 genome (and the genomes of many other EHEC strains), does not contain two of the thirteen σ^N enhancer-binding proteins found in K-12 and most other *E. coli*. This study thus aimed to validate the effect of *rpoN* mutation on σ^S levels and stability in the EHEC background and under the growth conditions that promote σ^N-dependent control of GDAR and the LEE.

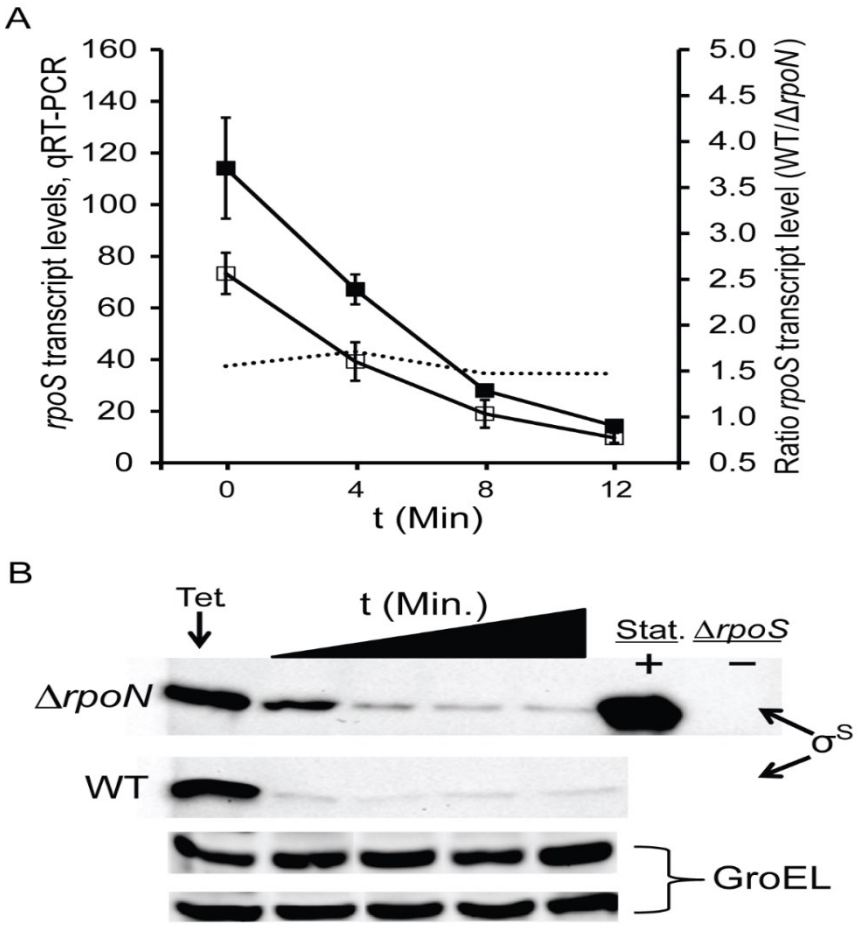

Figure 2.3. Stability of *rpoS* mRNA and σ^S. (A) Mean *rpoS* transcript levels (1st ordinate) and ratio of *rpoS* transcript (2nd ordinate) plotted against time following addition of rifampin at t=0 min for WT TW14359 (filled) and TW14359Δ*rpoN* (empty); ratio is indicated by the dotted line. Error bars denote standard error of the mean (n≥3). **(B)** Representative western immunoblots for σ^S as a function to time following addition of tetracycline at t=0 min for TW14359 (WT) and TW14359Δ*rpoN* (Δ*rpoN*); blots are in increments of 4 min. Stationary phase (Stat.) protein extracts were used as a positive control for σ^S, and TW14359Δ*rpoS* (Δ*rpoS*) as a negative control. Equal loading was controlled for by westerns for GroEL (top row is Δ*rpoN*, bottom row is WT).

As anticipated, no difference was observed in the stability of *rpoS* mRNA between TW14359 and TW14359Δ*rpoN* (Fig. 2.3A). After 12 min of rifampin addition, *rpoS* transcript was barely detectable in both backgrounds and the mean half-life for *rpoS* transcript was estimated at 2.43 min (TW14359) and 2.51 min (TW14359Δ*rpoN*), which agrees with previous estimates (283, 442). Before addition of rifampin, however, levels of *rpoS* transcript were higher (1.5-fold) in TW14359Δ*rpoN* compared to TW14359, but not significantly higher. In agreement with experiments using strain MG1655, σ^S was more stable in TW14359Δ*rpoN* compared to TW14359, however absolute levels were not observed to be higher in TW14359Δ*rpoN* (Fig. 2.3B) as described for MG1655 (101). In TW14359, σ^S was barely detectable after 4 min of tetracycline addition, but was detected for up to 12 min in TW14359Δ*rpoN*. The mean half-life for σ^S was estimated at 2.4 min for TW14359 and 5.5 min for TW14359Δ*rpoN*, increasing by 2.3-fold in the *rpoN* null background. The half-life for σ^S has been estimated at 1.4-6.5 min in exponential cultures of *E. coli* (209, 258, 355), and 10.5-30 min in stationary phase cultures (209, 355). These results reveal that in TW14359Δ*rpoN*, *rpoS*-dependency and control of GDAR and the LEE is correlated with an increase in exponential phase stability, but not absolute levels, of σ^S.

2.2.3 Role for core RNA polymerase and σ^N–dependent transcription in the σ^S stability, GDAR and LEE expression phenotype of TW14359ΔrpoN

The ability of *E. coli* sigma factors to successfully compete for core RNA polymerase (RNAP) differs substantially. For example, the RNAP binding affinity of σ^N is second only to the primary sigma factor, σ^{70}, whereas σ^S binding affinity lies at the bottom of this rank order (83, 230). In addition, the relative cellular abundance of each sigma factor influences gene expression through competition for RNAP (111). During exponential growth, σ^N levels have been estimated to be at

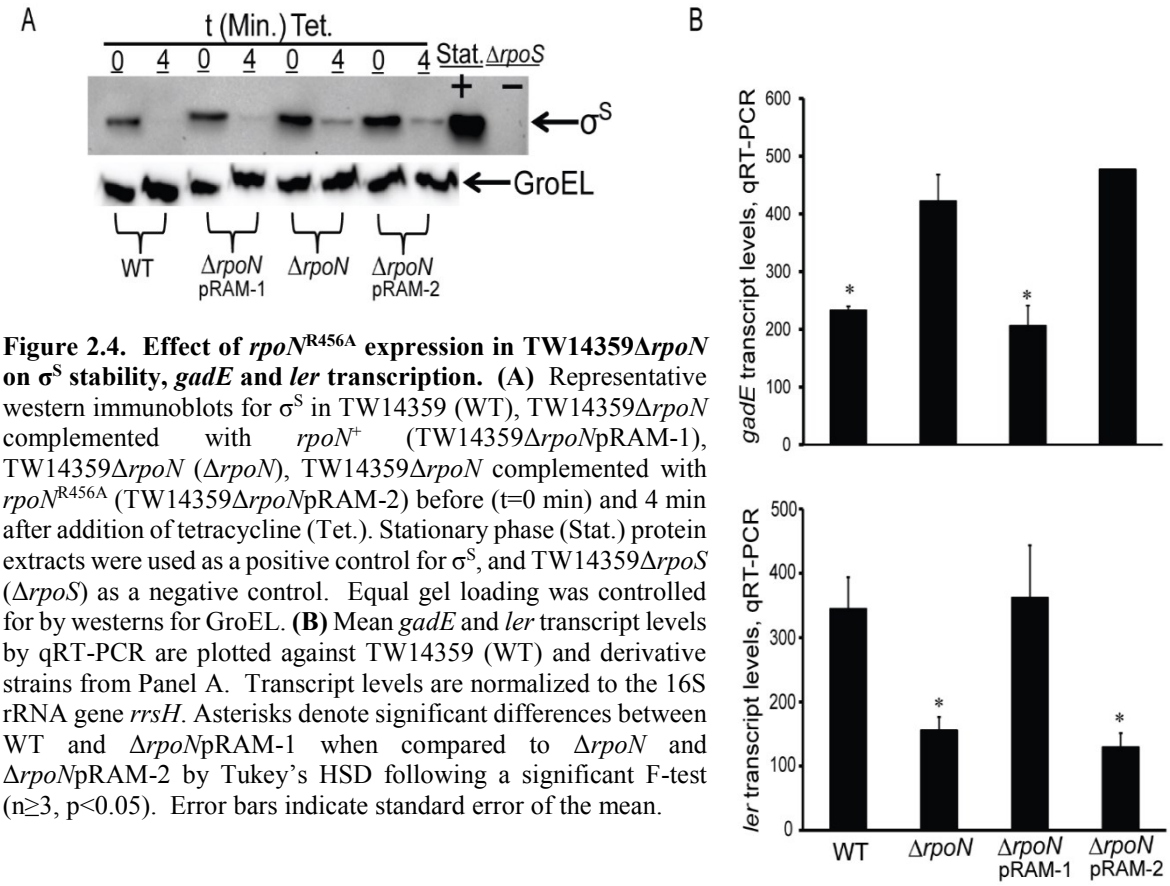

Figure 2.4. Effect of *rpoN*^{R456A} expression in TW14359Δ*rpoN* on σ^S stability, *gadE* and *ler* transcription. (A) Representative western immunoblots for σ^S in TW14359 (WT), TW14359Δ*rpoN* complemented with *rpoN*$^+$ (TW14359Δ*rpoN*pRAM-1), TW14359Δ*rpoN* (Δ*rpoN*), TW14359Δ*rpoN* complemented with *rpoN*^{R456A} (TW14359Δ*rpoN*pRAM-2) before (t=0 min) and 4 min after addition of tetracycline (Tet.). Stationary phase (Stat.) protein extracts were used as a positive control for σ^S, and TW14359Δ*rpoS* (Δ*rpoS*) as a negative control. Equal gel loading was controlled for by westerns for GroEL. **(B)** Mean *gadE* and *ler* transcript levels by qRT-PCR are plotted against TW14359 (WT) and derivative strains from Panel A. Transcript levels are normalized to the 16S rRNA gene *rrsH*. Asterisks denote significant differences between WT and Δ*rpoN*pRAM-1 when compared to Δ*rpoN* and Δ*rpoN*pRAM-2 by Tukey's HSD following a significant F-test ($n \geq 3$, $p < 0.05$). Error bars indicate standard error of the mean.

10-16% of σ^{70}, whereas σ^S is barely detectable (163, 174, 179). Together, this suggests that σ^S is at a substantial disadvantage for competitive RNAP binding during exponential growth. However, in an *rpoN* null background, the absence of competing σ^N may allow for an increase in σ^S RNAP binding sufficient enough to protect σ^S from ClpXP degradation, leading to increased transcription from σ^S promoters. This hypothesis might explain the σ^S stability, GDAR and LEE expression phenotype of TW14359Δ*rpoN*.

To examine this possibility, a mutant version of the *rpoN* gene (*rpoN*^{R456A}) was constructed, the product of which can efficiently form Eσ^N holoenzyme but cannot bind DNA to direct transcription from σ^N promoters [91, 92]. If the increased stability of σ^S in TW14359Δ*rpoN* is solely the result of increased RNAP binding by σ^S, the expression of *rpoN*^{R456A} in TW14359Δ*rpoN* should reproduce WT levels of σ^S stability. This was not determined to be the case however, as the stability of σ^S in TW14359Δ*rpoN*pRAM-2 did not differ from that of TW14359Δ*rpoN*, and both were increased in comparison to TW14359 and TW14359Δ*rpoN*pRAM-1 (Fig. 2.4A). The effect of *rpoN*^{R456A} expression on the GDAR and LEE expression phenotype of TW14359Δ*rpoN* was also examined. Transcript levels for the GDAR regulator *gadE*, and the LEE regulator *ler* in TW14359Δ*rpoN* and TW14359Δ*rpoN*pRAM-2 did not differ, and were significantly higher or lower than TW14359 and TW14359Δ*rpoN*pRAM-1, respectively ($p<0.05$) (Fig. 2.4B). Interestingly, survival by GDAR for TW14359Δ*rpoN*pRAM-2 was partially reduced compared to TW14359Δ*rpoN*, but remained substantially higher than TW14359 and TW14359Δ*rpoN*pRAM-1 (Table 2.1).

2.2.4 Sensitivity of σ^N-dependent GDAR and LEE expression to protease inhibition

The low abundance of σ^S during exponential growth is due to rapid proteolytic turnover by the serine protease complex ClpXP (209, 355). In strains mutated for *clpP* (the protease of ClpXP), σ^S is completely stable in exponential phase (355), however in exponential phase cultures of TW14359Δ*rpoN*, σ^S is still largely unstable (Fig. 2.3B), suggesting that there remains a sufficient amount of σ^S proteolysis. To reproduce the level of increased σ^S stability characteristic of TW14359Δ*rpoN* in the WT background, subinhibitory concentrations of the serine protease inhibitor 3, 4-dichloroisocoumarin (3, 4-DCI) (97) were titrated into growing exponential cultures and σ^S stability was measured.

The addition of 5 μM 3, 4-DCI (or 1/12X MIC) increased σ^S stability levels in TW14359 similar to σ^S stability levels observed in TW14359Δ*rpoN* without the addition of 3, 4-DCI (Fig. 2.5A). Addition of 3, 4-DCI further increased σ^S levels in TW14359Δ*rpoN* as well, revealing that σ^S stability is sensitive to serine protease inhibition in both backgrounds. It was predicted that if the GDAR and LEE expression phenotype of TW14359Δ*rpoN* was simply a result of decreased σ^S proteolysis, then experimentally increasing σ^S stability with 3,4-DCI should reconstitute a similar phenotype in TW14359. For GDAR this was not shown to be true, as 3, 4-DCI had no impact on survival of TW14359 in acid, and only marginally increased percent survival in TW14359Δ*rpoN* (Table 2.1). Thus increased stability of σ^S alone cannot account for GDAR in TW14359Δ*rpoN*. The expression of LEE genes is known to be positively influenced by ClpP through its proteolytic effect on σ^S (168, 401). Consistent with this, 3, 4-DCI addition reduced expression from ler_{P430}-*lacZ* in TW14359 as indicated by a decrease in percent β-galactosidase activity relative to untreated controls (Fig. 2.5B). Since addition of 3,4-DCI further increased σ^S stability in TW14359Δ*rpoN*, it was expected that this increase would correspond with a further

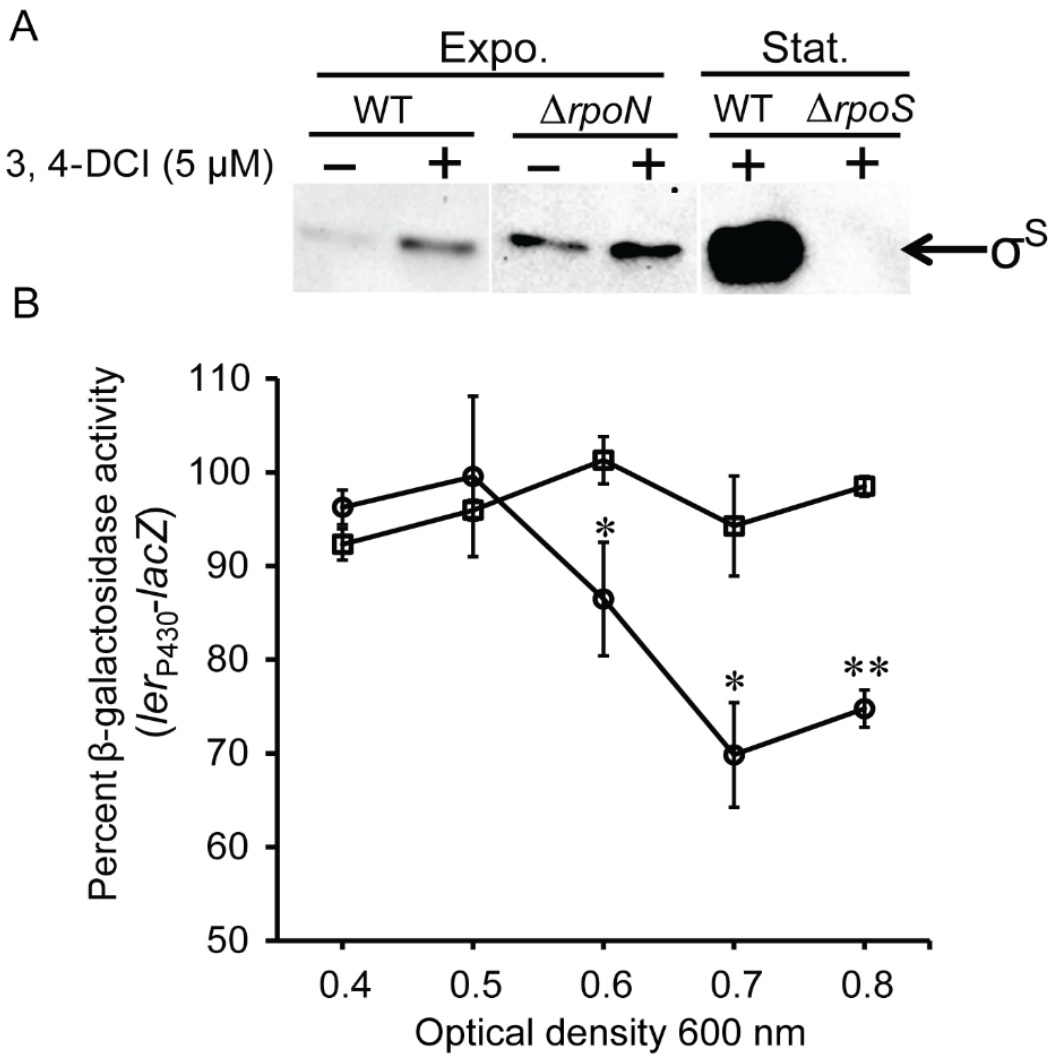

Figure 2.5. Effect of the serine protease inhibitor 3,4-DCI on σ^S stability and ler expression. (A) Representative western immunoblots for σ^S stability in TW14359 (WT) and TW14359Δ*rpoN* (Δ*rpoN*) during exponential phase (Expo.) 4 min after the addition of tetracycline, and with or without 3,4-DCI, as well as in WT and TW14359Δ*rpoS* (Δ*rpoS*) during stationary phase (Stat.) with 3,4-DCI. Equal gel loading was controlled for by westerns for GroEL. **(B)** Expression from *ler*$_{P430}$-*lacZ* as measured by mean percent β-galactosidase activity following addition of 3,4-DCI and relative to untreated controls during exponential growth for TW14359 (circles) and TW14359Δ*rpoN* (squares). Asterisks denote significant differences between TW14359 and TW14359Δ*rpoN* at each OD_{600} by Welch's t-test ($n \geq 3$, $p<0.05$ [*]; $p<0.01$ [**]).

decrease in *ler* expression. On the contrary, *ler*$_{P430}$-*lacZ* expression did not differ in 3,4-DCI-treated TW14359Δ*rpoN* cultures compared to untreated controls, and β-galactosidase activity was unchanged throughout growth compared to significantly reduced activity in TW14359 ($p<0.05$) (Fig. 2.5B). These results reveal that although σ^S stability is sensitive to protease inhibition using 3, 4-DCI in TW14359Δ*rpoN*, GDAR and *ler* expression is not and indicates that the underlying mechanism responsible for these phenotypes are at least partially distinct. The addition of 1/2X MIC of 3, 4-DCI did not significantly alter the outcome for GDAR or *ler* expression in either strain (data not shown).

2.2.5 Identification of the enhancer-binding protein required for σ^N-directed regulation of GDAR and the LEE

σ^N is a unique sigma factor in its requirement for enhancer-binding proteins (EBP) to initiate transcription (reviewed in (361)). If σ^S stability, GDAR and LEE expression in the *rpoN* mutant is dependent on σ^N-directed transcription, at least one of these EBPs is required for this control. To examine this, a library of EBP isogenic deletion mutants in TW14359 was constructed and screened for GDAR during exponential growth. Of the eleven mutants, only TW14359Δ*glnG* and TW14359Δ*fhlA* expressed GDAR comparable to levels observed for TW14359Δ*rpoN* (Table 2.1). *fhlA* encodes a regulator of formate metabolism (344), and *ntrC* (also *glnG*) encodes NtrC, a major regulator of nitrogen assimilation (330, 450). The impact of *fhlA* or *ntrC* mutation on LEE expression was then determined by transforming pRJM-1 containing *ler*$_{P430}$-*lacZ* into both EBP isogenic backgrounds, TW14359Δ*rpoN* and TW14359, and β- galactosidase activity was measured during exponential growth. Expression from *ler*$_{P430}$-*lacZ* increased in TW14359 to mid-exponential phase ($OD_{600}=0.5$), then tapered off as cells entered late exponential phase

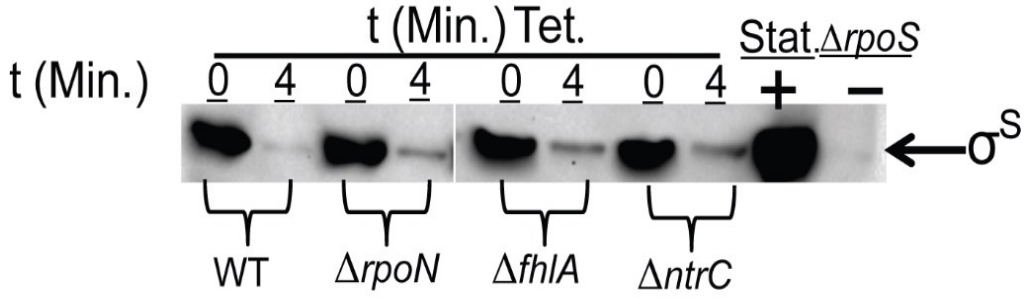

Figure 2.6. Stability of σ^S in σ^N enhancer binding protein mutants. Representative western immunoblots for σ^S in TW14359 (WT), TW14359Δ*rpoN* (Δ*rpoN*), TW14359Δ*fhlA* (Δ*fhlA*), and TW14359Δ*ntrC* (Δ*ntrC*) before (t=0 min) and 4 min after addition of tetracycline (Tet.). Stationary phase (Stat.) protein extracts were used as a positive control for σ^S, and TW14359Δ*rpoS* (Δ*rpoS*) as a negative control. Equal loading was controlled for by westerns for GroEL.

(OD_{600}=1.0) (Fig. 2.6). For TW14359Δ*rpoN*, *ler*$_{P430}$-*lacZ* expression only slightly increased during growth, and was significantly reduced to 56% of WT levels at OD_{600}=0.5, concordant with qRT-PCR data (p=0.008) (Figs. 2.2 and 2.6). Mutation of *fhlA* had no apparent effect on *ler*$_{P430}$-*lacZ* expression, yet *ntrC* mutation reduced *ler*$_{P430}$-*lacZ* expression to 50% of WT at OD_{600}=0.5 (p=0.006) to levels comparable with TW14359Δ*rpoN* (Fig. 2.6). Thus the mutation of *ntrC* faithfully reproduces the GDAR and LEE expression phenotype of TW14359Δ*rpoN*. Interestingly, σ^S stability was increased in both EBP mutant backgrounds to the level of stability observed in TW14359Δ*rpoN* (Fig. 2.6). These results reveal that mutation of *fhlA* and *ntrC* similarly influence σ^S stability, yet only *ntrC* mutation phenocopies GDAR and LEE expression observed in TW14359Δ*rpoN*. A strain deleted for both *rpoN* and *ntrC* was constructed to validate the dependence on *rpoN* for NtrC-directed GDAR and LEE expression, but the mutant was too growth-impaired in DMEM to be phenotypically informative.

2.3 Discussion

The importance of σ^N in *E. coli* metabolism, particularly nitrogen metabolism, is undisputed. Strains mutated for *rpoN* are growth-impaired under nitrogen-limiting conditions due to an inability to activate nitrogen regulatory response promoters. Mutation of *rpoN* also clearly affects many genes in *E. coli* that are not directly tied to metabolism, but which are perhaps cued to the metabolic status of the cell through σ^N, such as those involved in the regulation of motility (82, 447), NO detoxification (122), and biofilm formation (28). In the present study, the phenotype of acid resistance and LEE expression previously described for *rpoN* mutants in EHEC (332), represents a case in which σ^N-dependent regulation is indirectly communicated through the downregulation of another sigma factor, σ^S. The antagonistic interplay of σ^N and σ^S in the control

of these discrete systems resembles that described on a genomic scale by Dong et al. (101), in which it was estimated that as many as 60% of σ^N regulated genes are counter-regulated by σ^S.

For control of acid resistance, σ^N negatively regulates the σ^S-directed GadX-GadW pathway of glutamate-dependent acid resistance (GDAR) activation. This agrees with the dependence on *rpoS* and *gadE* for acid resistance formerly described for *rpoN* mutants (332), and with research showing that *rpoS* expression in a Δ*gadXW* background cannot induce the GDAR central regulator *gadE* (348). In this regulatory circuit, σ^S drives the transcription of *gadX*, the product of which then activates *gadE* transcription. GadX also downregulates GadW, which is a negative regulator of σ^S (227). As observed for GDAR, σ^N is clearly dependent on *rpoS* for upregulation of the LEE, conforming to the role of σ^S as a negative regulator of LEE expression (168, 401). This σ^N-σ^S regulatory pathway is predicted to converge on the LEE central regulator, *ler*. The fact that *ler* expression was not observed to be significantly decreased in previous microarray studies of *rpoN* mutated EHEC (332) but is in the current study, may be explained by the increased sensitivity of qRT-PCR.

The GDAR and LEE expression phenotype of TW14359Δ*rpoN* correlates with an increase in σ^S stability similar to that described for K-12 (101), however no increase in σ^S levels was observed as was for K-12. This disparity in results could reflect genetic differences between K-12 and TW14359, or differences in experimental growth conditions. For the latter, the M9 glucose media used by Dong et al. (101) should be strongly growth restrictive for *rpoN* mutants, which are auxotrophic for glutamine in minimal media containing glucose (330). As the production of σ^S is sensitive to reduced growth (209), increased σ^S levels during growth of *rpoN* mutants in M9 glucose may be attributed to metabolic stress, and not specific to σ^N. The growth of *rpoN* mutants is impaired in DMEM, but not prohibitively, as it contains glutamine.

This study further scrutinized the genetic basis for and significance of increased σ^S stability in the GDAR and LEE expression phenotype of *rpoN*. The expression of a transcriptionally silent allele of σ^N (*rpoN*^{R456A}) in TW14359Δ*rpoN* did not reconstitute WT levels of σ^S stability, *gadE* or *ler* expression, suggesting that competition for core RNAP is unlikely to be the primary underlying mechanism for this phenotype, and that transcription from a σ^N promoter(s) is a requirement. The RNAP competition hypothesis implies that the simple removal of a competing sigma factor may allow for increased competition of the remaining sigma factors for RNAP core. However, due to the low intrinsic affinity of σ^S for RNAP (230), all else being equal, it is more likely that σ^{70}, or other sigma factors present during exponential phase (ex. σ^F) will out-compete σ^S for extant core. Naturally, this competition dynamic changes in stationary phase cultures, as small molecules and proteins modulate the ability of specific sigma subunits to interact with RNAP.

Addition of the serine protease inhibitor 3,4-DCI was shown to result in increased σ^S stability in TW14359, and further increased σ^S stability in TW14359Δ*rpoN*. This cumulative increase in σ^S stability in TW14359Δ*rpoN* could reflect the sum of effects of 3,4-DCI and *rpoN* mutation on a common pathway (i.e. ClpP), or independent pathways. There is no direct evidence however, that 3, 4-DCI is increasing σ^S stability by inhibiting ClpP. Regardless of which is true, increasing σ^S stability alone by interfering with proteolysis did not alter GDAR and LEE expression in TW14359Δ*rpoN*, suggesting that the mechanistic basis of these phenotypes is distinct. Mutation of *rpoN* could lead to increased σ^S activity at promoters, or modulate its affinity for RNAP. For the former, both FliZ and 6S RNA have been reported to reduce σ^S activity at selective promoters (294, 405). Interestingly, transcript levels of *fliZ* were markedly upregulated in *rpoN* null K-12 (101), but not in EHEC (332). For the latter, various proteins and small molecules are known to facilitate Eσ^S holoenzyme formation, including Crl (100), Rsd (176), and

ppGpp (180). Currently, the involvement of any of these regulators in σ^N-σ^S control of GDAR and the LEE is unknown.

This study revealed that a strain mutated for *ntrC*, encoding nitrogen regulatory protein NtrC, is phenotypically similar to an *rpoN* mutant in regards to σ^S stability, GDAR and LEE expression. NtrC is a canonical σ^N EBP, activating transcription from at least 16 promoters in *E. coli* by binding as a hexameric ring to an upstream activator sequence (UAS) distal to the σ^N -24/-12 binding site (98, 421, 450). The transcription of *ntrC* dramatically increases when *E. coli* is grown in media that does not contain ammonia (i.e. DMEM), and plays an integral role in controlling nitrogen utilization pathways. This finding suggests that the product(s) of an NtrC/σ^N driven promoter directly or indirectly downregulates σ^S, which in-turn affects GDAR and LEE expression. Currently however, there is no experimental evidence to support a role for any of the known NtrC/σ^N regulated genes in this. Alternatively, NtrC could activate σ^N promoters independent of DNA binding, which may relax the site selectivity of NtrC/σ^N dependent transcription initiation. Examples of this have been described for Rrp2 of *B. burgdorferi*, and FlgR of *Campylobacter jejuni*, that activate σ^N promoters in the absence of known UAS sites for these EBPs by some unknown mechanism (40, 58, 186). There is also a precedent for NtrC regulating transcription independent of σ^N. NtrC binds to the core promoters of $glnA_{P1}$ and $glnA_{P3}$, repressing *glnLG*/*glnALG* (glutamine synthetase operon) transcription by interfering with σ^{70}-dependent initiation (330). Other *E. coli* promoters that are directly downregulated by NtrC have not however been described.

This study further identified FhlA as a putative EBP involved in the control of σ^S and GDAR, but not the LEE. FhlA activates transcription from multiple operons involved in formate metabolism, including structural components of the formate hydrogen lyase hydrogenase-3 (Hyd-

3) complex. Interestingly, the Hyd-3 complex has been reported to confer acid resistance by a unique mechanism that involves the consumption of protons during the conversion of formic acid to CO_2 and H_2 (268). However, the fact that *fhlA* mutation leads to acid resistance is inconsistent with its role as a positive regulator of the Hyd-3 acid resistance mechanism. Adding to this, Hyd-3 has only been shown to be protective under anaerobic growth conditions (268), together suggesting that the acid resistance conferred by *fhlA* mutation is independent of this mechanism. Alternatively, mutation of *fhlA* may lead to the accumulation of formic acid during growth on glucose (DMEM contains 4 g/l glucose) leading to acid-adaptation. Volatile fatty acid (VFAs, including acetic, formic and butyric acid) production during growth on glucose has been attributed to inorganic acid resistance in *Salmonella* and *E. coli* (16, 205). The broader significance of this finding is that multiple σ^N EBPs regulate GDAR and the LEE by discrete pathways, some of which may be independent of *rpoS*. In further support of this hypothesis, the EBP QseF has been independently shown to be important for attaching and effacing lesion formation, and for the control of T3SS effectors in response to autoinducer 3 (AI-3) and norepinephrine/epinephrine (321, 323, 324). The mutation of *qseF* did not however affect GDAR in this study (data not shown).

Given the essential roles of NtrC and σ^N in nitrogen metabolism, the results of this study infer that these proteins coordinate the expression of GDAR and the LEE with nitrogen (i.e. NH_3) availability through σ^S. This proposed regulatory pathway shares many similarities with that described for *rfaH* expression and O-antigen production in *Salmonella enterica*. Specifically, σ^N has been observed to activate *rfaH* transcription in an *rpoS*-dependent manner (36). However, the mutation of *rpoN* was epistatic for *rfaH* control by σ^S, indicating a regulatory relationship in which σ^S is positively controlling σ^N; there is no evidence that σ^S influences *rpoN*/σ^N expression or

activity in *E. coli* (101, 332). Remarkably however, *rfaH* transcription was further determined to be stimulated under nitrogen-limiting conditions (37), which suggests the potential for involvement of NtrC in σ^N-σ^S dependent control of O-antigen production in *S. enterica*.

This study concludes that σ^N exerts its regulatory influence on GDAR and the LEE through negative post-translational control of σ^S. Thus the inactivation of *rpoN* relaxes the requirement for stationary phase-induced mechanisms of σ^S accumulation during exponential growth. Furthermore, the results suggest that σ^N-σ^S dependent GDAR and LEE expression is at least partially controlled by NtrC, an EBP that activates transcription from σ^N promoters specifying genes for nitrogen utilization. The regulatory interplay of NtrC and other EBPs with σ^N and σ^S is likely to play a significant role in coordinating transcription with the various nutritional and physiological stimuli EHEC is exposed to during transmission, and in the course of infection.

2.4 Materials and Methods

2.4.1 Bacterial strains and culture conditions

The strains and plasmids used in this study are listed in Table 2.2. Strains were stocked at -80°C in glycerol (15% v/v final) diluted in Lysogeny Broth (LB) and were maintained in LB or on LB with 1.5% agar (LBA). Unless otherwise noted, overnight (18-20 h) cultures grown in MOPS (50 mM)-buffered Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, cat. #D2902, St. Louis, MO) (257) containing 4 g/l glucose and 4 mM glutamine (pH 7.4) were used to inoculate fresh DMEM to a final OD_{600}=0.05 and cultured at 37°C on a rotary shaker (200 RPM) using a 1:10 ratio of media-to-flask volume as described (332). The growth of strains in DMEM was monitored by taking OD_{600} readings at 1 h intervals over 12 h (Fig. S1). Antibiotics (Sigma-

Aldrich) were added to cultures when required. The *rpoS*$^{+}$ status of strains was confirmed by catalase activity and glycogen storage following previous protocols (44, 148).

2.4.2 Directed gene deletion and site-specific mutation

Gene deletion mutants were constructed using the λ Red recombinase-assisted approach (92, 261) and as described (332). Primers used for the deletion of σ^{N} EBPs, as well as *rpoN* and *rpoS* are provided in Table 2.3. For site-specific mutation, a 1,518 bp *Cla*I/*Hind*III-digested PCR fragment containing the *rpoN* gene from strain TW14359 nucleotide positions 4,144,833-4,146,311 was generated using primers rpoN-45/*Cla*I and rpoN+1455/*Hin*dIII (Table 2.3). This fragment was ligated into *Cla*I/*Hin*dIII-digested pACYC177 to produce pRAM-1 (Table 2.2). Point mutations C1366G and G1367C were introduced into the *rpoN* gene present on the pRAM-1 template plasmid by PCR using mutagenic primers rpoNR456A-F and rpoNR456A-R (Table 2.3) and *Pfu Ultra*™ high fidelity DNA polymerase (Agilent, Santa Clara, CA) to produce pRAM-2 (Table 1). The resultant σ^{N} allele has a R456A mutation (*rpoN*^{R456A}) in the DNA binding domain which interferes with the ability of the protein to bind DNA, but does not affect its capacity for RNAP association and holoenzyme formation (394, 415). pRAM-1, in addition to pRAM-2 purified from *E. coli* XL10-Gold® (Agilent) transformants, were transformed into strain TW14359Δ*rpoN* as described (332). Genetic constructs were validated by PCR, and restriction mapping, or by DNA sequencing and qRT-PCR.

2.4.3 Tests for acid resistance

Acid resistance by the glutamate- and arginine-dependent systems was measured as described (332) with slight adaptations. For the glutamate-dependent acid resistance mechanism,

mid-exponential (OD_{600}=0.5) DMEM cultures were inoculated to 10^6 CFU/ml final cell density into E minimal glucose (EG) media with or without 5.7 mM L-glutamate at pH 7 (control) or acidified with HCl (pH 2). To test for arginine-dependent acid resistance, exponential phase DMEM cultures were inoculated into EG media as above but with or without 0.6 mM L-arginine at pH 7 and pH 2.5. EG media acid resistance test environments were incubated at 37°C (200 RPM) for 1 h before sampling. For cell counts (CFU/ml) and percent survival determinations, samples were serially-diluted in PBS (pH 7), plated to LBA and incubated overnight at 37°C.

2.4.4 Quantitative real-time PCR (qRT-PCR)

Primers for qRT-PCR are provided in Table 2.3. RNA purification, cDNA synthesis, qRT-PCR cycling conditions and data analysis followed previously described protocols (265, 332).

2.4.5 Protein extraction, SDS-PAGE and western immunoblots

To extract total cellular protein, 10 ml culture samples were centrifuged at 10,000 x *g* for 2 min and the cell pellet was washed twice with sterile water with centrifugation as above. Washed cell pellets were resuspended in 0.7 ml 0.5 M triethyl ammonium bicarbonate buffer (TEAB) (Sigma-Aldrich) and sonicated with a Sonic Dismembrator 120 (Fisher, Waltham, MA) at 50% amplitude for 30 sec intervals totaling 5 min, followed by incubation at 95°C in 4X Laemmli Buffer for 5 min. Total cell protein was collected from lysed cells by centrifugation at 10,000 x *g* for 5 min, and supernatant was removed by aspiration. For western immunoblots, 10-30 µg extracted protein was resolved using 10% SDS-PAGE at 13 V/cm for 80 min before transfer at 15 V for 20 min to polyvinylidene fluoride (PVDF) membranes using a Trans-Blot semi-dry transfer cell (Bio-Rad, Hercules, CA). For detection of σ^S, PVDF membranes were blocked in Tris-

buffered saline (1X Tris, pH 7.4) with 0.1% (v/v) Tween-20 (TBST) containing 5% skim milk for 2 h at room temperature before incubation with anti-σ^S mAbs (Neoclone, Madison, WI) diluted 1:5000 in TBST containing 2% skim milk overnight on a Veri Mix platform rocker (Fisher) at 4°C. Membranes were then incubated for 1 h at room temperature with HRP-conjugated goat anti-mouse pAbs (Bio-Rad) diluted 1:10,000 in TBST with 2% skim milk. Protein was detected using an enhanced chemiluminescence (ECL) Plus detection system (Amersham-Pharmacia, Piscataway, NJ) following the manufacturer's instructions. Protein levels were measured and analyzed using a ChemiDoc XRS and Image Lab Software (Bio-Rad). The amount of protein loaded was measured using a Bradford protein assay standard curve. Equal loading was validated by western blots for GroEL using anti-GroEL mAbs (Bio-Rad) diluted 1:40,000 in TBST with 2% skim milk. Western blots were repeated a minimum of three times in independent trials.

2.4.6 σ^S and rpoS mRNA stability

Cultures were grown to mid-exponential phase (OD_{600}=0.5) before the addition of a subinhibitory concentration of the transcription inhibitor rifampin (300 μg/ml final) or the translation inhibitor tetracycline (60 μg/ml final). Sampling was performed immediately before addition of antibiotics, and at 4 min intervals thereafter for 12 min (*rpoS* mRNA stability) or 16 min (σ^S protein stability). RNA was purified and validated as described (265). For *rpoS* mRNA stability, gene transcript levels were measured using qRT-PCR and primers rpoS+356 and rpoS+466 (Table 2.3). Protein was extracted, and σ^S levels measured by western immunoblots. The half-life in minutes for *rpoS* mRNA and σ^S was extrapolated from gene transcript or protein levels, respectively, using linear regression analysis and as described (33). The strength of linearity was estimated by the correlation coefficient (r^2), and exceeded 0.85 (85%) for all analyses.

2.4.7 lacZ transcriptional fusions and β-galactosidase assay

A 429-bp *Bam*HI/*Eco*RI digested PCR fragment generated using primers ler-1/BamHI and ler-430/EcoRI (Table 2.3) and corresponding to nucleotide positions 4,679,303-4,679,731 in strain TW14359 was cloned into the similarly digested vector pRS551 (371) using T4-DNA ligase (Fisher) to create pRJM-1 (Table 2.2). This cloned fragment included 429-bp upstream of the translation initiation codon for *ler* (ECSP_4703) and both *ler* P1 and P2 promoters transcriptionally fused to *lacZ* (*ler*$_{P430}$-*lacZ*). pRJM-1 purified from DH5α transformants was used for transformation into various WT and mutant backgrounds. The *ler*$_{P430}$-*lacZ* fusion was confirmed by PCR and sequencing. To measure β-galactosidase activity from *ler*$_{P430}$-*lacZ*, 50 µl culture samples taken at OD_{600}=0.25 (early exponential), OD_{600}=0.5 (mid-exponential) and OD_{600}=1.0 (late exponential) were immediately added to 950 µl Z-buffer (1M KCl, 1 mM MgSO4, 0.05 M β-mercaptoethanol, 0.06 M Na_2HPO_4, 0.04 M $NaH_2PO_4 \bullet H_2O$, pH 7) with 0.1 ml chloroform and 50 µl 0.1% (v/v) SDS) and mixed vigorously for 30 sec. Samples were then incubated static at 28°C for 5 min before addition of 0.2 ml ortho-nitrophenyl β-D-galactopyranoside (ONPG, 4 mg/ml in 0.1 M phosphate buffer, pH 7) at 28°C for 20 min. Following development of the yellow cleavage product orthonitrophenol, the reaction was terminated by the addition of 0.5 ml Stop Solution (1M Na_2CO_3) and samples were mixed and then centrifuged at 21,000 x *g* for 5 min before measuring β-galactosidase activity. β-galactosidase activity was converted to Miller Units as described (248).

2.4.8 Serine protease inhibition

Selective inhibition of serine protease activity was performed using subinhibitory concentrations (i.e. 1/12X minimum inhibitory concentration (MIC) or 5 μM) of 3, 4-dichloroisocoumarin (3,4-DCI) (Sigma-Aldrich) (309). The MIC for 3,4-DCI was at 60 μM for both WT and *rpoN* null backgrounds. The effect of 3,4-DCI addition to growing cultures on σ^S stability, GDAR and LEE expression was determined as described above. For σ^S stability, 3, 4-DCI was added to cultures at mid-exponential phase (OD_{600}=0.4) and incubated to OD_{600}=0.5 before addition of 60 μg/ml tetracycline. Sampling was performed immediately before tetracycline addition and 4 min after addition. For GDAR and LEE expression, 3,4-DCI was added at OD_{600}=0.4 as for σ^S stability, and then GDAR tested, or β-galactosidase activity measured from *ler*$_{P430}$-*lacZ* as described above. Control cultures did not contain 3, 4-DCI for all experiments.

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Table 2.1 Acid resistance by the GDAR and ADAR mechanisms.

		Percent survival (SD)[a]	
Growth condition	**Strain/genotype**	**GDAR**	**ADAR**
DMEM	TW14359	<0.01[b]	<0.01
	TW14359Δ*rpoN*	24.2 (0.24)	<0.01
	TW14359Δ*fhlA*	21.2 (0.31)	ND[c]
	TW14359Δ*glnG*	15.7 (1.88)	ND
	TW14359Δ*rpoN*Δ*rpoS*	<0.01	<0.01
	TW14359Δ*rpoN* pRAM-1	0.141 (0.11)	0.125 (0.79)
	TW14359Δ*rpoN* pRAM-2	10.61 (1.22)	ND
DMEM + 3, 4-DCI[d]	TW14359	<0.01	ND
	TW14359Δ*rpoN*	29.1 (9.3)	ND

[a] Percent survival by the glutamate-dependent (GDAR) and arginine-dependent (ADAR) acid resistance system; standard deviation (SD).

[b] Less than 10 CFU/ml remains following 1 h exposure to acidified GDAR or ADAR test environment.

[c] Not determined (ND).

[d] DMEM growth media with addition of 5 μM 3,4-dichloroisocoumarin (3,4-DCI).

Table 2.2 Strain and plasmids used in this study.

Strain/plasmid	Relevant characteristics	Source/reference
Strain name:		
DH5α	Vector propagation, *recA1 endA1*	
XL10-Gold®	Competent cells	Agilent, Santa Clara, CA
TW14359	WT 2006 outbreak, western U.S.	(232)
EcRPF-6	TW14359Δ*rpoN*	This study
EcRPF-9	TW14359Δ*rpoN*Δ*rpoS*	This study
EcRPF-7	TW14359Δ*rpoS*	This study
EcRAM-26	TW14359Δ*glnG*	This study
EcRAM-25	TW14359Δ*fhlA*	This study
EcRAM-28	TW14359Δ*qseF*	This study
EcRAM-27	TW14359Δ*pspF*	This study
EcRAM-29	TW14359Δ*ygeV*	This study
EcRAM-4	TW14359*norR*::*kan* Kan^R	This study
EcRAM-7	TW14359*rtcR*::*kan* Kan^R	This study
EcRAM-3	TW14359*hyfR*::*kan* Kan^R	This study
EcRAM-11	TW14359*zraR*::*kan* Kan^R	This study
EcRAM-8	TW14359*tyrR*::*kan* Kan^R	This study
EcRAM-5	TW14359*prpR*::*kan* Kan^R	This study
Plasmid name:		
pACYC177	Low copy cloning vector, Amp^R Kan^R P15A	(74)
pRAM1	*rpoN*::pACYC177, Amp^R Kan^S	This study
pRAM2	$rpoN^{R456A}$::pACYC177 Amp^R Kan^S	This study
pRS551	*lac* fusion vector, Amp^R Kan^R $lacZ^+$ ColE1	(371)
pRJM-1	pRS551 containing ler_{P430}-*lacZ*fusion	This study

Table 2.3 Primers used in this study.

Primer Name	Type	Sequence (5'à3')	Source/reference
adiA+1463	Real-Time	CACAAACCGGCAAAACCTAT	This study
adiA+1542	Real-Time	ATGCATTACCCAGCAGTCCT	This study
adiC+1076	Real-Time	ATGACCGACACGGAAGAAAC	This study
adiC+987	Real-Time	CGTCGGTATTTTGATGACCA	This study
adiY+276	Real-Time	CCTGACACCAGACGTCTTTC	This study
adiY+357	Real-Time	GCGTGTTCGTTCCTTTTCTG	This study
cesT+296	Real-Time	TCCCTCTCGATGATGCTACC	343
cesT+445	Real-Time	TGTCGCTTGAACTGATTTCCT	343
crl+103	Real-Time	TCGATTGTCTGGCTGTATGC	This study
crl+244	Real-Time	AGTCGCCTGCTTTATCGAAC	This study
espA+128	Real-Time	AGGCTGCGATTCTCATGTTT	343
espA+310	Real-Time	GAAGTTTGGCTTTCGCATTC	343
gadE+309	Real-Time	TGGTAAACACTTGCCCCATA	343
gadE+419	Real-Time	AGCGTCGACGTGATATTGCT	343
gadW+445	Real-Time	ATCGCCAAACGTTGGTATCT	This study
gadW+536	Real-Time	CAGGTGTTTTCATCCTGCAA	This study
gadX+319	Real-Time	CGCTATGCAGAAATGCTACG	This study
gadX+413	Real-Time	ACGTTCAGAAGCAGCGGTAT	This study
ler+109	Real-Time	CGAGAGCAGGAAGTTCAA	343
ler+214	Real-Time	GTCCATCATCAGGCACAT	343
rpoS+356	Real-Time	TATCGAAGAGGGCAACCTGG	343
rpoS+466	Real-Time	GTTCAATCGTCTGGCGAATC	343
tir+664	Real-Time	ACTTCCAGCCTTCGTTCAGA	343
tir+869	Real-Time	TTCTGGAACGCTTCTTTCGT	343
trmE+1083	Real-Time	AGTCTTTGCCGAGAGACGAA	This study
trmE+991	Real-Time	ACCGTGGTACGCAATAAAGC	This study
ydeO+548	Real-Time	TAGATGCCAGAATGCAGCAC	This study
ydeO+631	Real-Time	TGGCATAACCACATTGTTCG	This study
ler-1/BamHI	Cloning	CGGGATCCAATAAATAATCTCCGCATGC	This study
ler-430/EcoRI	Cloning	CGGAATTCGGATTCACTCGCTTGCCGCC	This study
rpoN+1455/HindIII	Cloning	AACACAAGCTTGTGTCTTCCTTATCGGTTGG	This study
rpoN-45/ClaI	Cloning	ATTATCGATGGGTAGAAGTTTGCGACGTT	This study
fhlA+2106/P2	Mutation	CAGGCAGATCTGTCCGGCAATTTGCAGTTAAATCAATGCCcatatgaatatcctccttag	This study
fhlA-66/P1	Mutation	CTAAATCTCCTATAGTTAGTCAATGACCTTTTGCACCGCTgtgtaggctggagctgcttc	This study
glnG+1503/P2	Mutation	CCGGGCAAGATCATACTGAACTTATCGGAACAGTAAAGCGcatatgaatatcctccttag	This study
glnG-50/P1	Mutation	CATACCGAGTTCTCGGTTTACCTGCCTATCAGGAAATAAAgtgtaggctggagctgcttc	This study
hyfR+2004/P2	Mutation	TGCAAAAGCAGATTACAACACCTCGCGAACCGAGATCCCCcatatgaatatcctccttag	This study
hyfR-43/P1	Mutation	TTCTCATTAATAAGGACTGTTGATGGCTATGTCAGACGAGgtgtaggctggagctgcttc	This study
norR+1510/P2	Mutation	AGTTGTGATGATTTTGTGCCAGTGCCTGACGAATAGTTTCcatatgaatatcctccttag	This study
norR-107/P1	Mutation	ACCTCAATTTATTCAGCGTGTTCTAAAAAGATGTCTTGCTgtgtaggctggagctgcttc	This study
prpR+1676/P2	Mutation	CCTATGTAAACATCCCCGATGCGTAAGTTTATCGGTGATCcatatgaatatcctccttag	This study
prpR-117/P1	Mutation	TAATCCGCAAATATGCGTTTCAGTTAACGTTTCAGGCAATgtgtaggctggagctgcttc	This study
pspF+1029/P2	Mutation	CACGCCGCATCCGGCAAGTTGTATTGCCCAACTTCGCTAAcatatgaatatcctccttag	This study
pspF-41/P1	Mutation	GCAACATGCCAGGATGAATTAGCTAATTACACTAACAAGTgtgtaggctggagctgcttc	This study
qseF+1333/P2	Mutation	ATTCCTTGAAATCGTTTGCATCCAGCTCGTGTCGGGAAACATATGAATATCCTCCTTAG	This study
qseF+20/P1	Mutation	ATTTATTATTGGTCGATGACGATCCGGGATTGCTGAAACTGTGTAGGCTGGAGCTGCTTC	This study
rpoN+1430/P2	Mutation	ACGAGCTGTTTACGCTGGTTTGACGGCGGAATGGATAAAGcatatgaatatcctccttag	343
rpoN-12/P1	Mutation	ACGATTCTGAACATGAAGCAAGGTTTGCAACTCAGGCTTAgtgtaggctggagctgcttc	343
rpoNR456-F	Mutation	GGAACAAGGTATCATGGTGGCACGCGCCACTGTTGCGAAGTACCGAGAGTCT	This study
rpoNR456-R	Mutation	AGACTCTCGGTACTTCGCAACAGTGGCGCGTGCCACCATGATACCTTGTTCC	This study
rpoS+1104/P2	Mutation	TTGCCGGGTAGGACGCTGACGTGTCTTATCCAGGCGACAAcatatgaatatcctccttag	343
rpoS-56/P1	Mutation	GGAACCAGGCTTTTGCTTGAATGTTCCGTCAAGGGATCACgtgtaggctggagctgcttc	343
tyrR+1523/P2	Mutation	TGGCTTAAGCCATATTCCCGCAACTTATTGGCAATCGCGGcatatgaatatcctccttag	This study
tyrR-94/P1	Mutation	TCTTTGTGTCAATGATTGTTGACAGAAACCTTCCTGCTATgtgtaggctggagctgcttc	This study
yfhA+1333/P2	Mutation	ATTCCTTGAAATCGTTTGCATCCAGCTCGTGTCGGGAAAGcatatgaatatcctccttag	This study
yfhA+20/P1	Mutation	ATTTATTATTGGTCGATGACGATCCGGGATTGCTGAAACTgtgtaggctggagctgcttc	This study
ygeV+1806/P2	Mutation	CCTGAATTCAGGCCGGATTCACTGATGTTATGTGTTTAACcatatgaatatcctccttag	This study
ygeV-77/P1	Mutation	GAGTTAATATGATCATGATCTGTGAACCATCAACGTCTTCgtgtaggctggagctgcttc	This study
zraR+1312/P2	Mutation	TTGCCAACAGCGTTTTGCGCGTGATCCCTAACTGACGGGCcatatgaatatcctccttag	This study
zraR-14/P1	Mutation	TATCGATATTCTGGTGGTGGATGATGACATTAGCCACTGCgtgtaggctggagctgcttc	This study

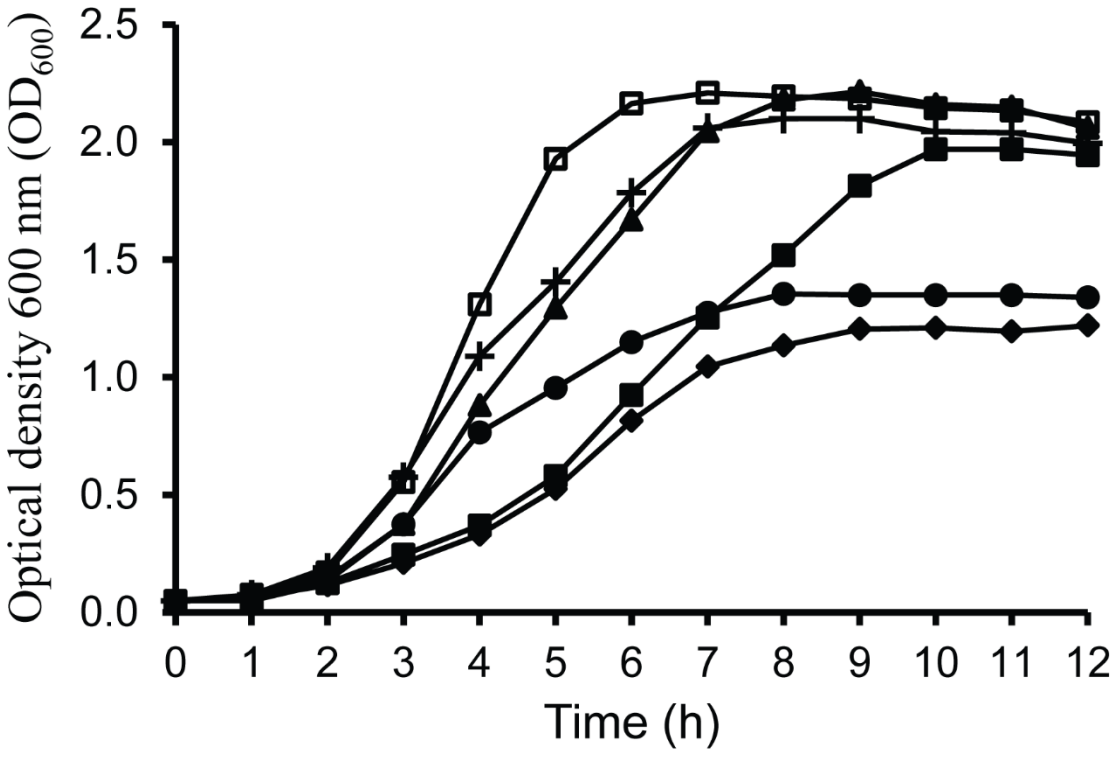

Figure S2.1. Growth of strains in Dulbecco's Modified Eagle's Medium (DMEM). Mean (n=2) optical density 600 nm (OD_{600}) plotted for TW14359 (empty squares), TW14359Δ*rpoN* (filled squares), TW14359Δ*rpoS* (circles), TW14359Δ*fhlA* (plus signs), TW14359Δ*ntrC* (triangles), and TW14359Δ*rpoN*Δ*rpoS* (diamonds). Individual OD_{600} measurements for each strain varied by less than 5%. For ler_{P430}-*lacZ* expression (Fig. 6), sampling was done for all strains except for TW14359Δ*rpoS* and TW14359Δ*rpoN*Δ*rpoS* at OD_{600}=0.25, OD_{600}=0.5, and OD_{600}=1.0 approximately corresponding to early-, mid- and late-exponential phase, respectively. For all remaining experiments, sampling was done at OD_{600}=0.5.

Chapter Three: Essential Pathway Components and Stimuli for NtrC/σ^N-Dependent Regulation of Acid Resistance and the Locus of Enterocyte Effacement (LEE) in Enterohemorrhagic *Escherichia coli* (EHEC)

3.1 Abstract

In enterohemorrhagic *E. coli* (EHEC) sigma factor N (σ^N) regulates glutamate-dependent acid resistance (GDAR) and the locus of enterocyte effacement (LEE), discrete genetic systems required for transmission and virulence of this intestinal pathogen. Regulation of these systems requires nitrogen regulatory protein C, NtrC, and is a consequence of NtrC/σ^N-dependent reduction in the activity of sigma factor S (σ^S). This study elucidates pathway components and stimuli for σ^N-directed regulation of GDAR and the LEE in EHEC. Deletion of *fliZ*, the product of which reduces σ^S activity, phenocopies *rpoN* (σ^N) and *ntrC* null strains for GDAR and LEE control, acid resistance and adherence. Upregulation of *fliZ* by NtrC/σ^N is indirect, requiring an intact flagellar regulator *flhDC*. Activation of *flhDC* by NtrC/σ^N and FlhDC-dependent regulation of GDAR and the LEE is dependent on σ^N-promoter $flhD_{P2}$, and a newly described NtrC upstream activator sequence. While the addition of ammonium significantly alters GDAR and LEE expression, acid resistance and adherence, it does so independently of *rpoN*, *ntrC* and the NtrC sensor kinase *ntrB*. Altering the availability of NtrC phosphodonor acetyl phosphate by growth without glucose, with acetate addition, or by deletion of acetate kinase, *ackA*, abrogates NtrC/σ^N-dependent control of *flhDC*, *fliZ*, GDAR and LEE genes.

3.2 Introduction

Alternative sigma factor N (σ^N) when bound to RNA polymerase directs the transcription of genes for carbon and nitrogen metabolism, stress fitness, and regulation (327). In an increasing number of bacterial pathogens, σ^N has been shown to also regulate genes for virulence and transmission, and to be required for complete *in vivo* disease progression (7, 20, 88, 165, 249, 274, 358, 414). For most species, the mechanism underlying σ^N-dependent regulation of pathogenesis remains unknown. The exception lies in the model of *Borrelia burgdorferi*, and to a lesser extent, enterohemorrhagic *E. coli* (EHEC). In *B. burgdorferi*, the causative agent of Lyme borreliosis, σ^N activates outer surface lipoproteins (OspA and OspC) essential for transmission from the tick vector to a mammalian host, and for establishment of infection (133, 155, 284). This Osp activation pathway requires another sigma factor (σ^S), the transcription of which is directly activated from a σ^N-promoter in what has been dubbed a σ^N-σ^S regulatory cascade (141, 374). In EHEC serotype O157:H7, a food-borne pathogen attributed to outbreaks and sporadic cases of bloody diarrhea (hemorrhagic colitis) (318), σ^N (encoded by *rpoN*) represses transcription of glutamate-dependent acid resistance (GDAR) genes, while activating the locus of enterocyte effacement (LEE) pathogenicity island (332). The GDAR system allows for low oral infectious dose during gastric passage (75, 396), while the LEE encodes a type III secretion (T3S) apparatus that translocates virulence factors into host intestinal cells mediating intimate adherence and immune subversion (109, 241, 292). Thus, σ^N in EHEC regulates major determinants of fecal-oral transmission and colonization.

Like *B. burgdorferi*, a σ^N-σ^S regulatory pathway has been described for EHEC, and has been further implicated in the control of GDAR and LEE genes in this pathogen (332). However for EHEC, the underlying mechanism by which σ^S is regulated is not completely understood. σ^S

controls the expression of hundreds of genes in *E. coli*; it is an activator of GDAR system genes (*gad* genes) (227), and can both activate and repress the LEE (168, 207, 401). Strains null for *rpoN* are characterized by a phenotype of increased GDAR and decreased LEE expression that is dependent on an intact *rpoS* (encoding σ^S), and while deletion of *rpoN* in either EHEC, or laboratory *E. coli* strain K-12 MG1655, has no impact on *rpoS* transcription, both the stability and activity of σ^S have been shown to increase (101, 252, 332). Mitra et al. (252) demonstrated that this effect of σ^N on σ^S stability/activity is indirect and dependent on transcription from a σ^N-promoter, and not competition of these sigma factors for core RNA polymerase (RNAP). What additional regulatory component(s) is required downstream of σ^N for control of σ^S, GDAR and the LEE is not yet known. Unlike other *E. coli* sigma factors, the initiation of transcription by σ^N requires activation by enhancer binging proteins (EBP) that communicate various environmental signals to the RNAP-σ^N holoenzyme complex ($E\sigma^N$) (361). Of the eleven EBPs encoded within the EHEC O157:H7 background, only deletion of *ntrC* (encoding NtrC) phenotypically reproduces the *rpoN* null background for control of σ^S, GDAR and the LEE (252). Nitrogen regulatory protein C, NtrC (also NRI), is the response regulator of a two-component system that activates σ^N-dependent transcription of genes for the assimilation and utilization of nitrogen, relieving slowed growth under nitrogen-limiting conditions (450). It is thus plausible that nitrogen availability plays a fundamental role in activation of the σ^N-σ^S regulatory pathway in EHEC. In this study, essential components of the mechanism for σ^N-dependent control of GDAR and the LEE are identified, and their genetic interactions tested. The significance of nitrogen metabolism and basis of pathway activation are also examined.

3.3 Results

3.3.1 FliZ phenocopies NtrC and σ^N for control of GDAR and the LEE

Previous studies have revealed NtrC and σ^N to negatively regulate GDAR and positively regulate the LEE by reducing the activity of alternative sigma factor S (σ^S) (252, 332). For this to occur, NtrC-σ^N must increase or decrease the expression of a gene(s) whose product, in-turn, alters σ^S-dependent transcription. One of two proteins were predicted to fulfill this role: Crl or FliZ. Crl enhances RNAP-σ^S holoenzyme formation, thus increasing transcription from σ^S promoters (311, 407), whereas FliZ interferes with σ^S promoter binding and transcription initiation, thus reducing σ^S-dependent transcription (294, 297). During growth in DMEM (OD_{600}=0.5), both *crl* and *fliZ* expression were shown to be reduced in TW14359Δ*ntrC* and TW14359Δ*rpoN* when compared to TW14359 ($p<0.05$) (Fig. 1A), however, only TW14359Δ*fliZ* phenocopied TW14359Δ*ntrC* and TW14359Δ*rpoN* for the control of GDAR and LEE genes (Fig. 3.1B). In TW14359Δ*crl*, both *gadE* and *gadB* were increased in expression compared to TW14359 ($p<0.05$), but less than for TW14359Δ*rpoN*, TW14359Δ*ntrC* and TW14359Δ*fliZ*, in which expression levels for all genes were nearly identical ($p<0.01$) (Fig. 3.1B). The expression of LEE genes *ler*, *tir*, *espA* and *cesT* did not differ between TW14359 and TW14359Δ*crl*, but were uniformly reduced in TW14359Δ*ntrC*, TW14359Δ*rpoN* and TW14359Δ*fliZ* backgrounds ($p<0.05$) (Fig. 3.1B). Both *gadE* and *ler* expression was restored to near WT levels in *fliZ* complement strain TW14359Δ*fliZ*pRAM-8 or by the deletion of *rpoS* in TW14359Δ*fliZ* (Fig. 3.1B). Consistent with the effect of *fliZ* deletion on *gadE* and *gadB* expression, counts (CFU/ml) of TW14359Δ*fliZ* recovered following exposure to acidified (pH 2) EG media for 1 h increased by 10- to 100-fold compared to TW14359, TW14359Δ*fliZ*pRAM-8 and TW14359Δ*fliZ*Δ*rpoS*, yet remained ~10-fold less than that observed for TW14359Δ*ntrC* and TW14359Δ*rpoN* (Fig. 3.1C). Furthermore, the

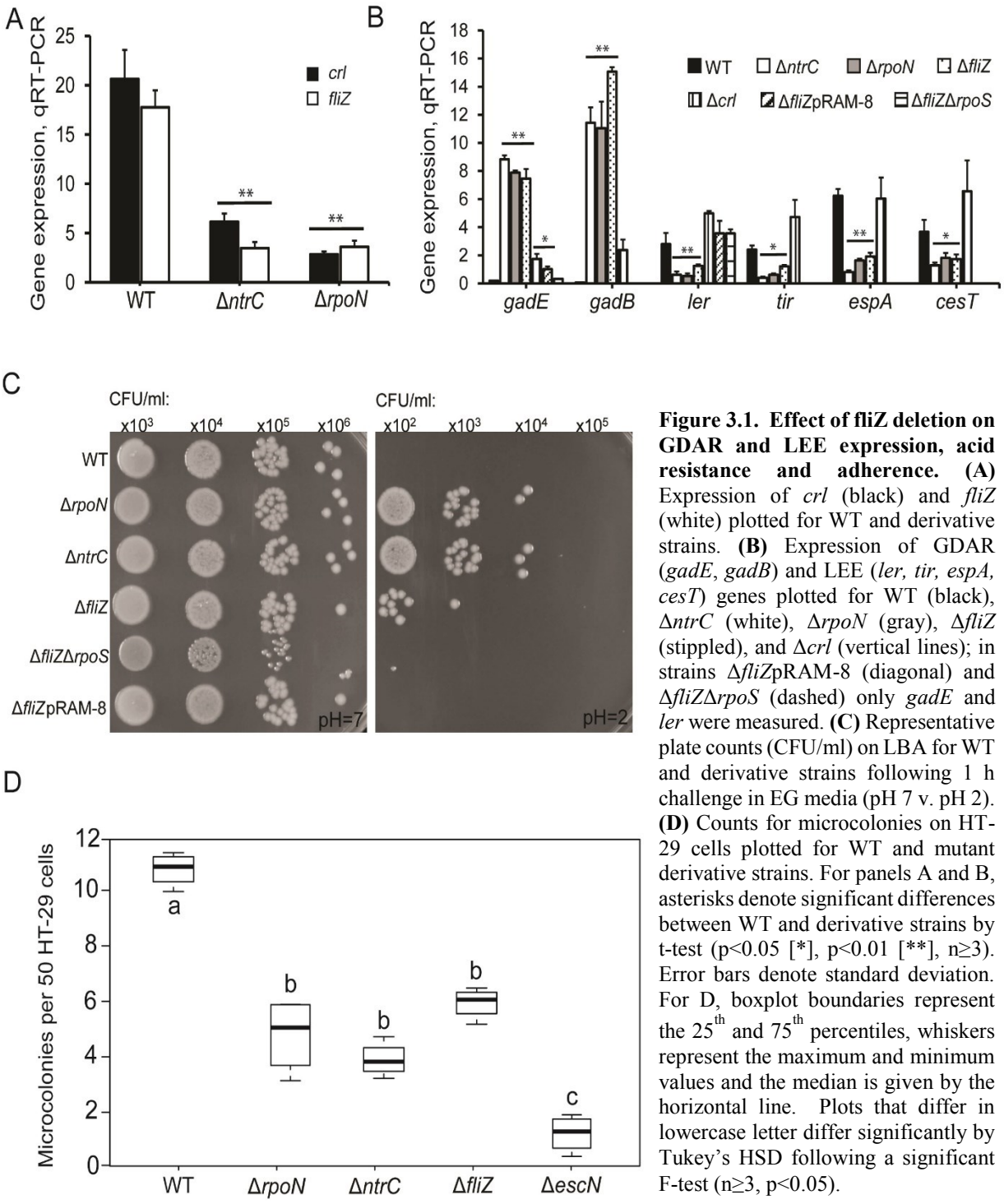

Figure 3.1. Effect of fliZ deletion on GDAR and LEE expression, acid resistance and adherence. (A) Expression of *crl* (black) and *fliZ* (white) plotted for WT and derivative strains. **(B)** Expression of GDAR (*gadE*, *gadB*) and LEE (*ler, tir, espA, cesT*) genes plotted for WT (black), Δ*ntrC* (white), Δ*rpoN* (gray), Δ*fliZ* (stippled), and Δ*crl* (vertical lines); in strains Δ*fliZ*pRAM-8 (diagonal) and Δ*fliZ*Δ*rpoS* (dashed) only *gadE* and *ler* were measured. **(C)** Representative plate counts (CFU/ml) on LBA for WT and derivative strains following 1 h challenge in EG media (pH 7 v. pH 2). **(D)** Counts for microcolonies on HT-29 cells plotted for WT and mutant derivative strains. For panels A and B, asterisks denote significant differences between WT and derivative strains by t-test ($p<0.05$ [*], $p<0.01$ [**], $n\geq3$). Error bars denote standard deviation. For D, boxplot boundaries represent the 25th and 75th percentiles, whiskers represent the maximum and minimum values and the median is given by the horizontal line. Plots that differ in lowercase letter differ significantly by Tukey's HSD following a significant F-test ($n\geq3$, $p<0.05$).

ability to form microcolonies on HT-29 intestinal cells was decreased in TW14359Δ*fliZ* compared to TW14359 (p=002), and matched that observed for TW14359Δ*ntrC* and TW14359Δ*rpoN* (Fig. 3.1D).

3.3.2 σ^N utilizes FlhDC for activation of fliZ transcription

fliZ is encoded as the second gene of a three gene operon (*fliAZY*), the transcription of which is directed from as least two promoters, $fliA_{P1}$ and $fliA_{P2}$. Neither of these promoters are σ^N-dependent, however $fliA_{P1}$ is activated by the regulator of flagellar biosynthesis and motility FlhDC, for which there is a predicted σ^N-dependent promoter, $flhD_{P2}$ (447). In addition, a putative activator sequence (UAS) for NtrC is present ~1-kb upstream of $flhD_{P2}$. It was thus hypothesized that the control of *fliZ* by NtrC-σ^N is a consequence of direct activation of *flhDC* transcription from this promoter.

In agreement with this, *flhDC* expression was similarly reduced in both TW14359Δ*ntrC* and TW14359Δ*rpoN* backgrounds compared to TW14359 during growth in DMEM (OD_{600}=0.5) ($p<0.05$) (Fig. 3.2A). Also, *flhDC* significantly decreased *gadE* levels and increased *ler* levels when overexpressed in TW14359Δ*ntrC* and TW14359Δ*rpoN* ($p<0.05$), but not in TW14359Δ*fliZ* (Fig. 3.2B). To define *cis*-elements of the *flhDC* promoter region important for NtrC-σ^N dependent regulation, *flhDC* mRNA copy number was measured from three promoter fragments (Fig. 3.2C) cloned into arabinose inducible vector pBAD22 and transformed into TW14359Δ*flhDC*. As anticipated, *flhDC* copy number was reduced when expressed from a fragment in which the putative NtrC UAS was removed (Frag. II) compared to the WT *flhDC* promoter fragment (Frag. I) (p=0.004) (Fig. 3.2C). *flhDC* copy number was further reduced when expressed from a fragment in which both the NtrC UAS and putative σ^N promoter $flhDC_{P2}$ were removed (Frag. III), but not

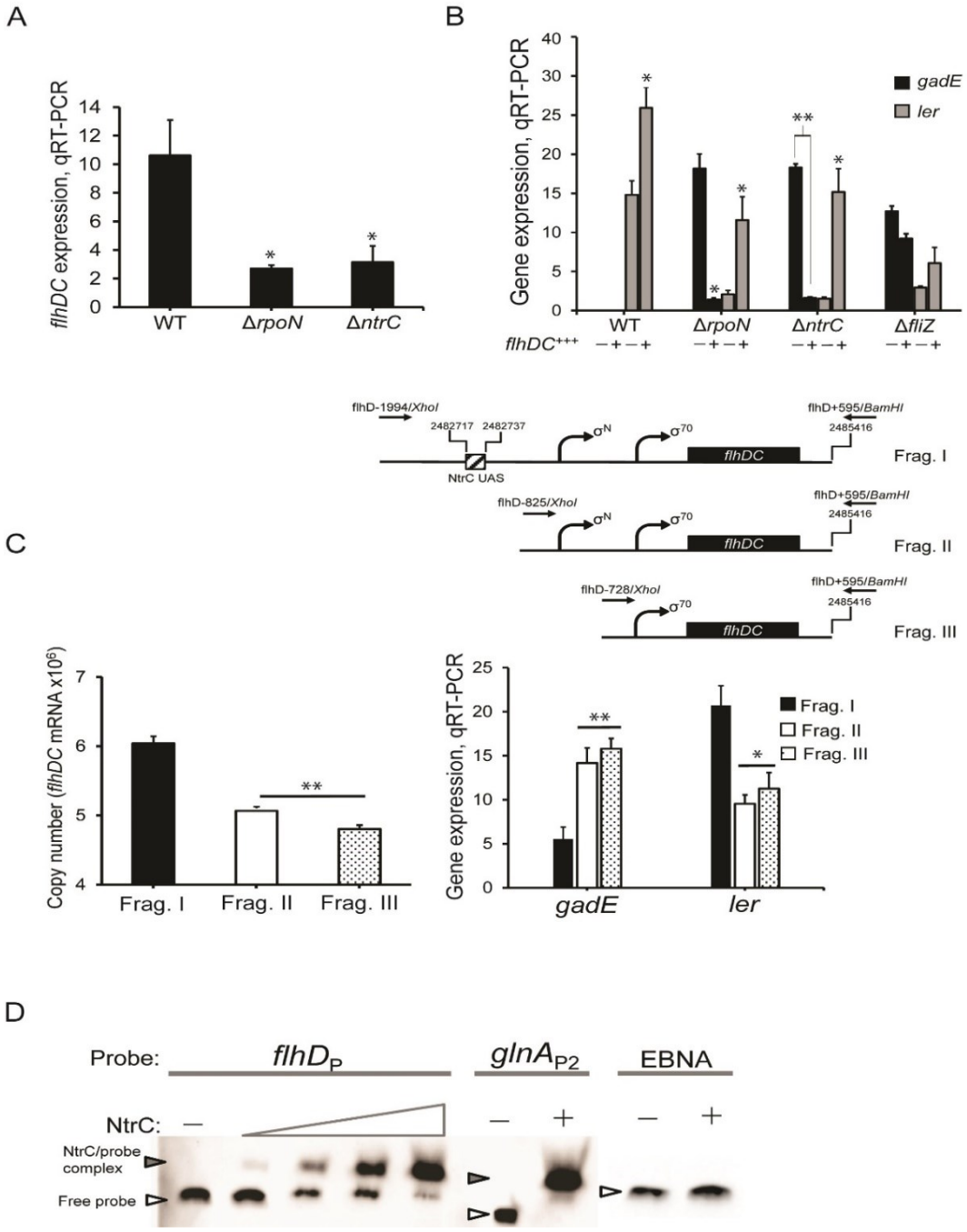

Figure 3.2. Regulation of flhDC by NtrC-σ^N and effect on *gadE* and *ler* expression. **(A)** Expression levels for *flhDC* plotted for WT and derivative strains. The asterisk denotes a significant difference between WT and mutated strains by t-test ($p<0.05$, $n\geq3$). **(B)** Expression levels for *gadE* (black) and *ler* (gray) plotted for WT and derivative strains containing pRAM-3 (*flhDC*::pBAD22); expression of pRAM-3 is either uninduced (−) or induced (+) with arabinose. Asterisks indicate significant differences between uninduced and induced treatments by t-test ($p<0.05$ [*], $p<0.01$ [**], $n\geq3$). **(C)** Absolute *flhDC* mRNA copy number and expression levels for *gadE* and *ler* measured in the Δ*flhDC* background expressing cloned *flhDC* fragments, Frag. I (black), Frag. II (white), and Frag. III (stippled); topology of *flhDC* promoter fragments is included, top right panel C. See text for details. **(D)** EMSA for NtrC binding to the $flhDC_P$ promoter and $glnA_{P2}$ promoter; EBNA is Epstein Barr nuclear antigen DNA. Inset arrows indicate the location of the NtrC/probe complex (filled arrow) or free probe (empty arrow). See text for details. Error bars denote standard deviation for all panels.

significantly less than for Frag. II. Correspondingly, *gadE* expression increased ($p<0.01$) and *ler* expression decreased ($p<0.05$) in TW14359Δ*flhDC* expressing either Frag. II or Frag. III when compared to Frag. I (Fig. 2C). Thus, the putative NtrC UAS site and σ^N promoter $flhDC_{P2}$ are required for full expression of *flhDC* and for regulation of *gadE* and *ler*. Purified 6xHis-NtrC was observed to retard the mobility by EMSA of a 200-bp *flhD* promoter probe containing the putative NtrC UAS in a manner similar to the NtrC–dependent glutamine synthetase promoter, $glnA_{P2}$ (Fig. 3.2D). No shift was observed for *flhD* or *glnA* promoter probes in the absence of 6xHis-NtrC, or for the negative control Epstein Barr nuclear antigen (EBNA) DNA probe (Fig. 3.2D).

3.3.3 Auxotrophy for glutamine enhances glutamate-dependent acid resistance

NtrC-σ^N direct the transcription of nitrogen regulated (Ntr) response genes, the primary function of which is to assimilate nitrogen through induction of transport/scavenging systems and nitrogen degradation pathways when nitrogen is limiting (reviewed in (327)). Under these conditions, glutamine synthetase (GS) catalyzes the synthesis of L-glutamine from ammonia and L-glutamate. The gene for GS (*glnA*) is maximally expressed from the σ^N promoter $glnA_{P2}$ in a manner dependent on NtrC. Thus, strains that are null for *rpoN* or *ntrC* cannot initiate transcription from $glnA_{P2}$ and are auxotrophic for glutamine when nitrogen is limiting. Since the acid resistance of TW14359Δ*rpoN* is entirely dependent on glutamate availability (332), the significance of glutamine auxotrophy and *glnA* to the expression of this phenotype was determined. Growth of TW14359Δ*rpoN* in MOPS media containing 0.2% glucose and 0.1% L-histidine (i.e. high energy but nitrogen-limiting) is impaired due to auxotrophy for glutamine (Gln−) (Fig. 3.3A). However, after 48 h the outgrowth of a prototrophic (Gln+) suppressor mutant (TW14359Δ*rpoN*Gln+) was repeatedly observed in which WT growth in MOPS media was restored (Fig. 3.3A), and in which

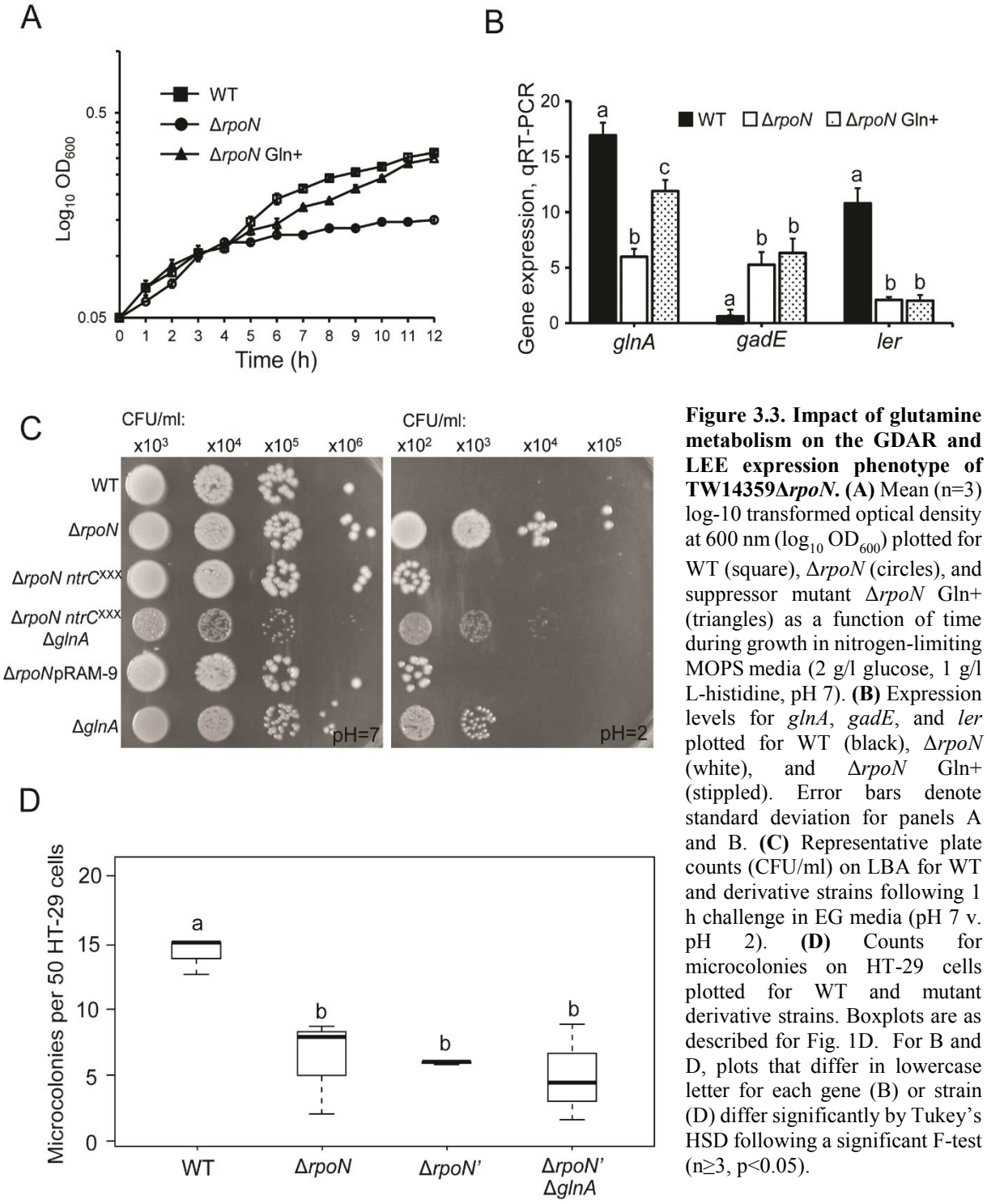

Figure 3.3. Impact of glutamine metabolism on the GDAR and LEE expression phenotype of TW14359Δ*rpoN*. (A) Mean (n=3) log-10 transformed optical density at 600 nm ($\log_{10}$ OD_{600}) plotted for WT (square), Δ*rpoN* (circles), and suppressor mutant Δ*rpoN* Gln+ (triangles) as a function of time during growth in nitrogen-limiting MOPS media (2 g/l glucose, 1 g/l L-histidine, pH 7). **(B)** Expression levels for *glnA*, *gadE*, and *ler* plotted for WT (black), Δ*rpoN* (white), and Δ*rpoN* Gln+ (stippled). Error bars denote standard deviation for panels A and B. **(C)** Representative plate counts (CFU/ml) on LBA for WT and derivative strains following 1 h challenge in EG media (pH 7 v. pH 2). **(D)** Counts for microcolonies on HT-29 cells plotted for WT and mutant derivative strains. Boxplots are as described for Fig. 1D. For B and D, plots that differ in lowercase letter for each gene (B) or strain (D) differ significantly by Tukey's HSD following a significant F-test ($n \geq 3$, $p < 0.05$).

the expression of *glnA* was significantly increased compared to TW14359Δ*rpoN* during growth in DMEM (OD_{600}=0.5) (p=0.013) (Fig. 3.3B); *glnA* expression was still however slightly but significantly lower in TW14359Δ*rpoN*Gln+ when compared to TW14359 (p=0.02). Mutations which suppress Gln− in *E. coli* have been mapped to *ntrC*, and to *cis*-elements controlling *glnA* transcription. *glnA* can be transcribed from three promoters: $glnA_{P1}$ and $glnA_{P3}$ are σ^{70} promoters that are repressed by NtrC during nitrogen-limitation, whereas $glnA_{P2}$ is a σ^{N} promoter that is activated by NtrC under the same conditions. Mutations in the DNA-binding domain of NtrC (amino acid residues 400-470) at the C-terminus result in the de-repression of $glnA_{P1}$ and/or $glnA_{P3}$, while mutations in the promoter(s) enhance transcription from $glnA_{P1}$ or result in formation of a *de novo* σ^{70} consensus at $glnA_{P2}$ (328). Partial DNA sequencing of *ntrC* and the *glnA* promoter region did not reveal any of these described mutations in TW14359Δ*rpoN*Gln+. Sequencing of the TW14359Δ*rpoN*Gln+ genome however, revealed a single adenine deletion in the *ntrC* ORF at nucleotide position 4,910,080 (accession NC_013008, NCBI), resulting in a frame-shift mutation. This mutation occurs early in the ORF at +285 relative to the start codon and results in a premature stop codon or opal (UGA) mutation at amino acid position 106. It was thus suspected that increased expression of *glnA*, and growth in the absence of glutamine for TW14359Δ*rpoN* Gln+ (Fig. 3.3B), reflects de-repression at the $glnA_{P1}$ and $glnA_{P3}$ promoters due to NtrC inactivation.

Expression levels for *gadE* and *ler* did not differ between TW14359Δ*rpoN* and TW14359Δ*rpoN* Gln+ during growth in DMEM (OD_{600}=0.5), indicating that the Gln− status of TW14359Δ*rpoN* has no impact on GDAR and LEE gene regulation. Interestingly however, counts (CFU/ml) recovered from acidified EG media were decreased by ~1000-fold for TW14359Δ*rpoN*Gln+ and for TW14359Δ*rpoN* overexpressing *glnA* (strain TW14359Δ*rpoN*pRAM-9) when compared to TW14359Δ*rpoN* (Fig. 3.3C), clearly indicating a

role for *glnA* in the complete acid resistance phenotype of TW14359Δ*rpoN*. Adding to this, CFU/ml recovered from acidified EG increased by ≥100-fold in both TW14359Δ*glnA* and TW14359Δ*rpoN*Δ*glnA*Gln+ backgrounds. The CFU/ml recovered from acidified EG media for all strains was at least 10-fold greater than that observed for TW14359. Consistent with qRT-PCR data on *ler* (Fig. 3A), the number of microcolonies formed on HT-29 cells in TW14359Δ*rpoN*Gln+ was significantly reduced when compared to TW14359 ($p<0.05$), but did not differ from TW14359Δ*rpoN* or TW14359Δ*rpoN*Δ*glnA*Gln+ (Fig. 3.3D).

3.3.4 NtrC-σ^N is not activated by a canonical signal for regulation of GDAR and the LEE

When *E. coli* is cultivated in media without ammonia, intracellular levels of glutamine are low, culminating in the phosphorylation and activation of NtrC by sensor kinase NtrB and NtrC-σ^N dependent transcription. It was thus suspected that the absence of ammonia in DMEM prompts NtrC-σ^N dependent transcription of *flhDC*, activating the pathway for GDAR and LEE regulation, and that supplementation of DMEM with ammonia would offset this effect. If so, ammonia would be expected to stimulate *gad* gene expression and repress the LEE in TW14359, but to have no effect in the TW14359Δ*rpoN* and TW14359Δ*ntrC* backgrounds. While the addition of ammonium chloride (2 g/l NH_4Cl_2) was observed to slightly but insignificantly increase GDAR gene (*gadE* and *gadB*) expression in TW14359, expression in TW14359Δ*ntrC* and TW14359Δ*rpoN* uniformly decreased ($p<0.05$) (Fig. 3.4A). Correspondingly, ammonium addition reduced CFU/ml recovered for TW14359Δ*ntrC* and TW14359Δ*rpoN* by ~100- to 1000-fold but had no observable effect on CFU/ml recovered for TW14359 (Fig. 3.4B). For the LEE, ammonium addition increased *ler*, *tir*, *espA*, and *cesT* expression in all backgrounds (Fig. 3.4C) and correspondingly increased the

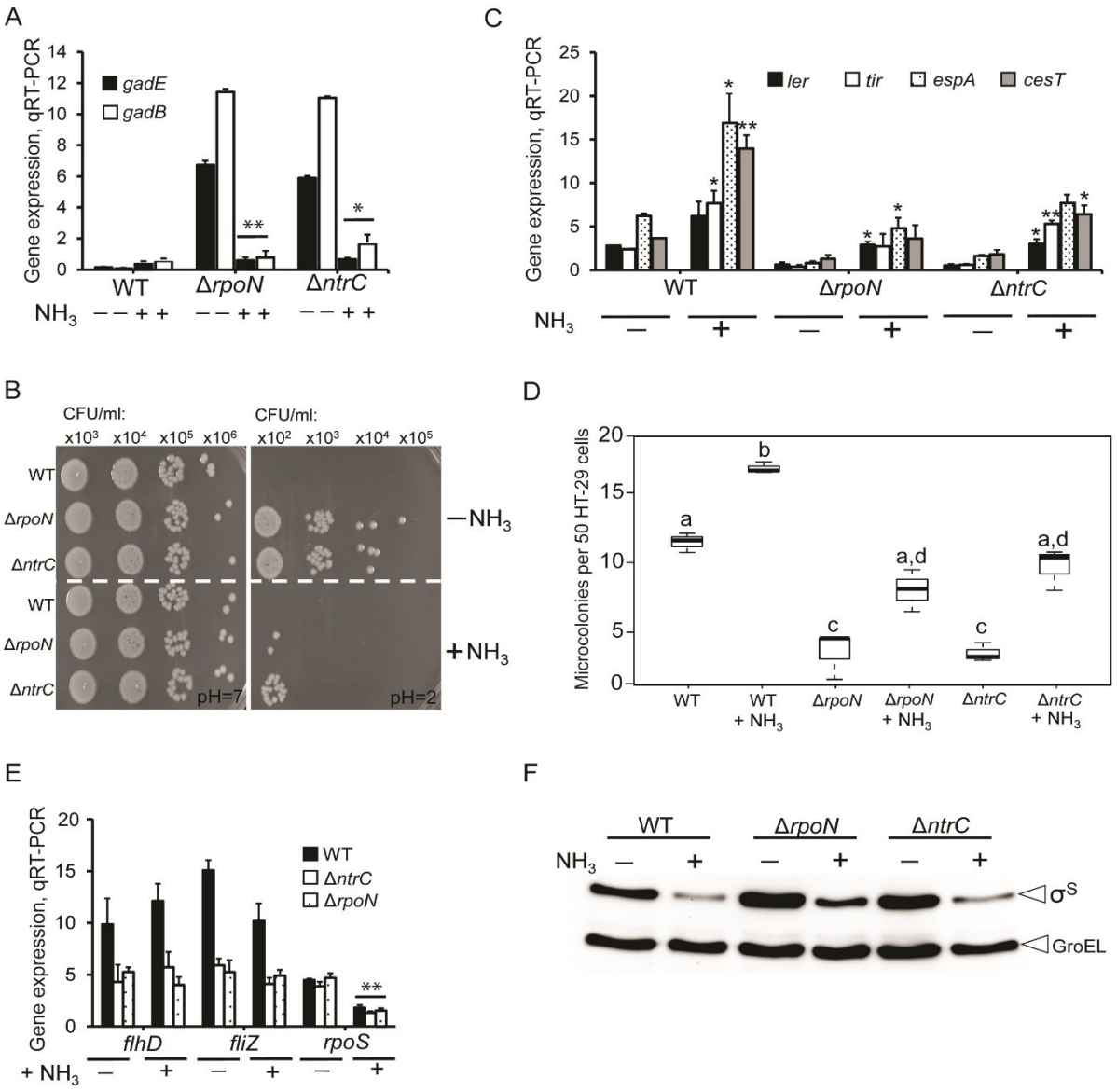

Figure 3.4. Role for ammonium in the NtrC-σ^N dependent pathway controlling GDAR and the LEE. (A) Expression levels for *gadE* (filled) and *gadB* (empty) without (−) and with (+) the addition of NH_4Cl_2 plotted for WT and derivative strains; asterisks denote significant difference between treatments by t-test ($p<0.05$ [*], $p<0.01$ [**], $n\geq3$). **(B)** Representative plate counts (CFU/ml) on LBA for WT and derivative strains grown without ($-NH_4$) or with ($+NH_4$) NH_4Cl_2 added to DMEM and following 1 h challenge in EG media (pH 7 v. pH 2). **(C)** Expression levels for *ler* (black), *tir* (white), *espA* (stippled), and *cesT* (gray) for WT and derivative strains grown without ($-NH_3$) or with ($+NH_3$) NH_4Cl_2 added to DMEM. **(D)** Counts for microcolonies on HT-29 cells plotted for WT and mutant derivative strains grown without ($-NH_4$) or with ($+NH_4$) NH_4Cl_2. Boxplots are as described for Fig. 1D. **(E)** Expression levels of *flhDC*, *fliZ* and *rpoS* plotted for WT (black), Δ*ntrC* (white), and Δ*rpoN* (stippled). **(F)** Representative western blot for σ^S and GroEL (control) in WT, Δ*rpoN* and Δ*ntrC* grown without (−) or with (+) NH_4Cl_2 added to DMEM. For A, C and E, asterisks denote significant differences between treatments by t-test ($p<0.05$ [*], $p<0.01$ [**], $n\geq3$). For D, plots that differ in lowercase letter differ significantly by Tukey's HSD following a significant F-test ($n\geq3$, $p<0.05$). Error bars denote standard deviation.

number of microcolonies formed on HT-29 cells for all strains ($p<0.05$). The same observations were made when substituting equimolar ammonium sulfate for ammonium chloride (data not shown). These results reveal that ammonium does in fact influence GDAR and LEE gene expression, but by a mechanism that is independent of *ntrC* and *rpoN*. In support of this data, the expression of pathway components (*gadE*, *ler*, *flhDC* and *fliZ*) for control of GDAR and the LEE were not altered in a strain deleted for the NtrC cognate sensor kinase, *ntrB*. Interestingly, growth in DMEM containing ammonium was observed to significantly reduce *rpoS* expression in TW14359, TW14359Δ*ntrC* and TW14359Δ*rpoN* ($p<0.01$), while having no impact on *flhDC* or *fliZ* expression in these backgrounds (Fig. 3.4E). This reduction in *rpoS* transcript levels correlated with a reduction in σ^S levels in all backgrounds with ammonium, however σ^S levels were not as strongly reduced in TW14359Δ*rpoN* when compared to TW14359 or TW14359Δ*ntrC* (Fig. 3.4F).

Feng et al (112) demonstrated phosphotransfer to, and activation of, NtrC in *E. coli* by the small molecule phosphodonor acetyl phosphate (acetyl~P). Acetyl~P readily accumulates during growth on glucose or in the presence of excess acetate, but not during growth on glycerol (239, Wolfe 2005). It was thus of interest to determine the effect of glucose and acetyl~P availability on σ^N-dependent control of pathway components for the regulation of GDAR and the LEE. During growth in MOPS media containing glucose (2 g/l) and NH_4Cl_2 (1 g/l) (OD_{600}=0.5), the expression of *flhDC*, *fliZ*, and *ler* was decreased and *gadB* increased in TW14359Δ*ntrC* and TW14359Δ*rpoN* when compared to TW14359 ($p<0.05$) (Fig. 3.5A), similar to that observed during growth in DMEM media (Fig. 3.1A, B and Fig. 3.2A). Substituting 0.2% (v/v) glycerol for glucose as the sole carbon source reduced *flhDC*, *fliZ* and *ler* expression in TW14359 and *rpoN* complement strain TW14359Δ*rpoN*pRAM-1 ($p<0.05$), but not in TW14359Δ*ntrC* and TW14359Δ*rpoN* (Fig. 3.5A).

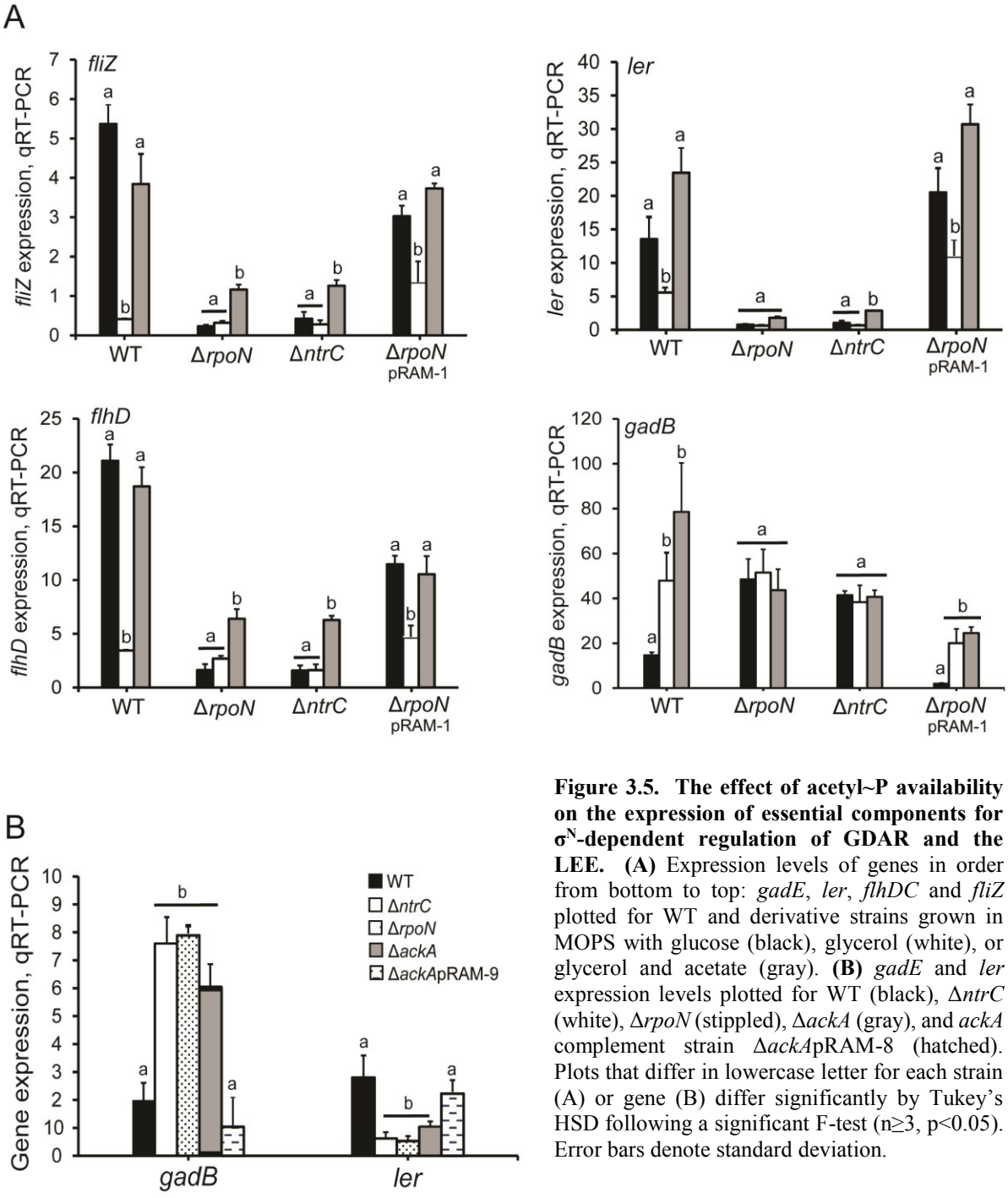

Figure 3.5. The effect of acetyl~P availability on the expression of essential components for σ^N-dependent regulation of GDAR and the LEE. (A) Expression levels of genes in order from bottom to top: *gadE*, *ler*, *flhDC* and *fliZ* plotted for WT and derivative strains grown in MOPS with glucose (black), glycerol (white), or glycerol and acetate (gray). **(B)** *gadE* and *ler* expression levels plotted for WT (black), Δ*ntrC* (white), Δ*rpoN* (stippled), Δ*ackA* (gray), and *ackA* complement strain Δ*ackA*pRAM-8 (hatched). Plots that differ in lowercase letter for each strain (A) or gene (B) differ significantly by Tukey's HSD following a significant F-test ($n \geq 3$, $p < 0.05$). Error bars denote standard deviation.

Likewise, glycerol substitution increased *gadB* expression in TW14359 and TW14359Δ*rpoN*pRAM-1 ($p<0.05$), but not in TW14359Δ*ntrC* and TW14359Δ*rpoN*. The addition of sodium acetate (2 g/l) to glycerol treatments restored *flhDC*, *fliZ* and *ler* expression to levels observed for glucose in TW14359, however *gadB* expression was slightly but insignificantly increased when compared to glycerol treatments (Fig. 3.5A). In TW14359Δ*ntrC* and TW14359Δ*rpoN*, acetate was still observed to generally increase *fliZ*, *flhDC* and *ler* expression, yet had no impact on *gadB* expression in these backgrounds, which may reflect a more generalized, *ntrC*- and *rpoN*-independent effect of acetate on the expression of these genes. To further examine the effect of acetate and acetyl~P availability on this regulatory pathway, *gadB* and *ler* expression was measured in a strain null for acetate kinase (*ackA*), the product of which catalyzes the interconversion of acetate to acetyl~P (339). In TW14359Δ*ntrC*, TW14359Δ*rpoN* and TW14359Δ*ackA*, *gadB* expression was significantly and uniformly increased when compared to TW14359 ($p<0.01$) (Fig. 3.5B). Complementation with *ackA* (strain TW14359Δ*ackA*pRAM-8) restored *gadB* expression to WT levels. For *ler*, expression was similarly reduced in TW14359Δ*ackA*, TW14359Δ*ntrC*, and TW14359Δ*rpoN* when compared to WT and TW14359Δ*ackA*pRAM-8 ($p<0.05$).

3.4 Discussion

In the present study NtrC and σ^N have been shown to positively regulate the expression of *crl* and *fliZ*, the products of which control the activity of σ^S. It is predicted that of the two, only FliZ is a required component of the σ^N pathway controlling σ^S, GDAR and the LEE. What impact *crl* upregulation in TW14359Δ*rpoN* has on σ^S, if any, is as yet unclear. Crl and FliZ play antagonistic roles in the regulation of σ^S. Crl directly binds σ^S facilitating interaction with RNA polymerase

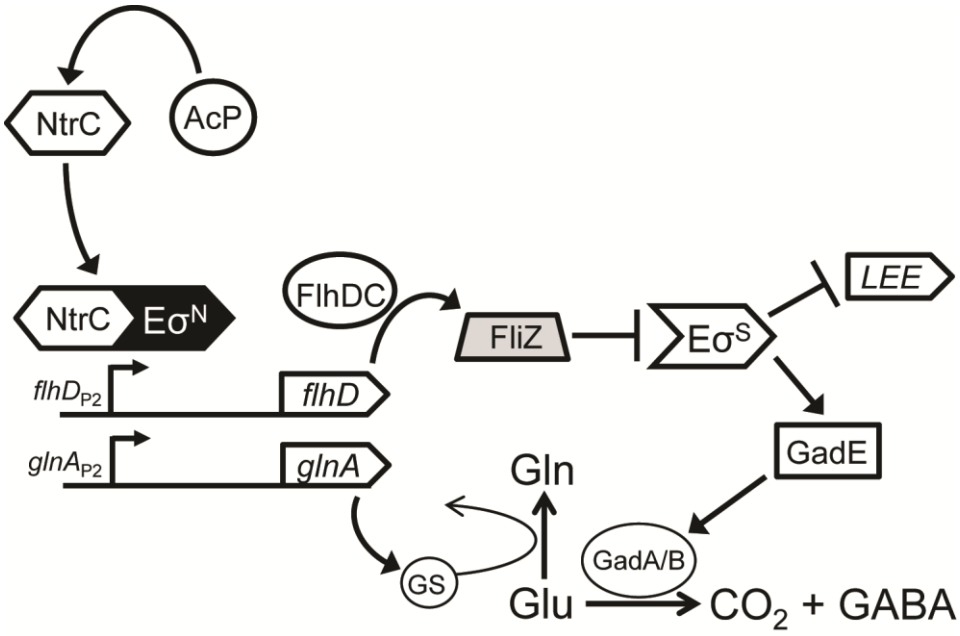

Figure 3.6: Model predicting NtrC-σ^N dependent regulation of GDAR and the LEE. During exponential growth in DMEM (a nitrogen-limiting media), NtrC activates transcription from σ^N-dependent promoters for *flhD* and *glnA*. FlhDC (regulator of flagellar biosynthesis) directly activates *fliZ*, the product of which reduces the activity of σ^S-RNAP ($E\sigma^S$) holoenzyme. σ^S indirectly downregulates LEE expression by repressing the LEE activator *pchA* (not shown) by an unknown mechanism, while upregulating the GDAR activator *gadE* through increased transcription of *gadX* (not shown). The upregulation of *glnA* (encoding glutamine synthetase, GS) increases the conversion of extant glutamate (Glu) to glutamine (Gln), thus depleting the substrate for GDAR system decarboxylases (GadA/GadB) and the potential for proton scavenging and acid detoxification. Acetyl~P (AcP) is a non-cognate phosphodonor that can activate NtrC-dependent transcription from σ^N promoters for *flhD* and *glnA*. The model is an amalgam of experimental observations inferred from this and previous studies (112, 215, 227, 296, 331, 401, 447). See the text for further details.

and holoenzyme ($E\sigma^S$) formation (47), whereas FliZ acts downstream of $E\sigma^S$ formation, binding to the -10 box of σ^S promoters (297) precluding promoter recognition by $E\sigma^S$. Thus, FliZ may be dominant to Crl in σ^N-directed control of σ^S activity. Alternatively, Crl has been shown to reduce σ^S stability in an RssB-dependent manner during all stages of growth (407). It is therefore plausible that the increased stability of σ^S in *rpoN* null backgrounds (101, 252) results from reduced *crl* expression. This is consistent with the observation that in TW14359Δ*rpoN* the GDAR and LEE expression phenotype cannot be reproduced by increasing σ^S stability alone (252).

The transcription of *fliZ* is largely determined by FlhDC, a global regulator of motility genes (119). FlhD forms a heterodimer with FlhC, directly activating transcription of the *fliAZY* operon from the σ^{70}-dependent promoter $fliA_P$. This study determined that *flhDC* was required for σ^N-directed regulation of GDAR and LEE genes in a manner that was dependent on an intact *fliZ*. Based on our results, it is predicted that NtrC and σ^N directly activate transcription of *flhDC* during exponential growth in DMEM (4 g/l glucose, with no NH_4) requiring the putative σ^N-promoter $flhD_{P2}$, and a newly identified NtrC box at position 2481732-2487152 (Fig. 3.6). This NtrC box is nearly identical to the predicted NtrC consensus (113), differing by a single nucleotide in the dyad repeat region. Upregulation of FlhDC leads to increased transcription of *fliZ* (119), the product of which decreases the activity of σ^S (297) (Fig. 3.6). This suggests that during exponential growth NtrC-σ^N keep the activity of extant σ^S in check by increasing FlhDC-dependent transcription of *fliZ*. One consequence of this reduced σ^S activity in EHEC is an increase in LEE expression (332) and correspondingly, increased *in vitro* microcolony formation. While FlhDC has been formerly shown to effect adherence in *E. coli*, until now, the association has been negative. Leathem et al. (212) reported that the deletion of *flhDC* in *E. coli* K-12 increased colonization of a mouse, while constitutive expression of *flhDC* in another study, reduced

adherence of EHEC to HeLa cells (166). Since the former study is in the K-12 MG1655 background, the effect of *flhDC* on colonization is clearly LEE-independent. For EHEC however, *flhDC* and the LEE are known to be inversely regulated; expression of LEE-encoded GrlA downregulates *flhDC* and motility in a manner dependent on RcsB, a response regulator of the Rcs phosphorelay system (166, 256). Perhaps FlhDC is used by σ^N to initiate transcription of the LEE, and then is repressed as GrlA accumulates as part of a GrlA-RcsB feedback loop initiating intimate adherence. This would be consistent with the transience and growth-phase dependence of σ^N dependent regulation of the LEE (252).

By reducing the activity of σ^S, σ^N also helps to maintain a low level of GDAR gene expression during exponential growth (Fig. 3.6). However, unlike σ^N-dependent LEE regulation, it is predicted that full expression of the GDAR phenotype in *rpoN* null strains is a consequence of two discrete but concurrent mechanisms. One requires σ^S for the activation of GDAR system genes (*gad* genes), the products of which confer acid resistance by a proton-scavenging mechanism involving the decarboxylation (GadA/GadB decarboxylases) and subsequent protonation of glutamate to yield γ-amino butyric acid (GABA) (Fig. 3.6). In the absence of glutamate, GDAR is defective in protecting *E. coli* from acid stress (reviewed in (116)). It is this cellular glutamate that is the source of the corresponding mechanism. Specifically, under nitrogen-limiting conditions (ex. growth in DMEM), NtrC-σ^N activate transcription of glutamine synthetase (*glnA*), which catalyzes the conversion of glutamate (Glu) to glutamine (Gln) (Fig 3.6). Strains null for *rpoN* or *ntrC* are therefore unable to activate *glnA* in response to reduced nitrogen availability, leading to glutamate accumulation and auxotrophy for glutamine. These strains are thus characterized by elevated levels of both the components (i.e. *gadE*, *gadA/B*, *gadC*) and substrate (glutamate) for GDAR. This mechanistic duality is reflected in the observation that neither *fliZ*

nor *glnA* deletion can fully recapitulate the GDAR phenotype of an *rpoN* null background. Since as many as 60% of σ^N-regulated genes have been shown to be antagonistically controlled by σ^S in *E. coli* (101), the interplay of these sigma factors likely has a more global impact on virulence, fitness and metabolism than simply control of GDAR and the LEE.

The precise activating signal for NtrC-σ^N dependent regulation of GDAR and the LEE is as yet unknown. Phosphorylation and activation of NtrC is sensitive to changes in the intracellular levels of glutamine. When *E. coli* is grown in the absence of ammonium, glutamine levels are low, signaling the phosphorylation of NtrC by its cognate sensor kinase NtrB, and NtrC-dependent activation of σ^N promoters for nitrogen assimilation (326). Although the addition of ammonium to DMEM did have a significant impact on GDAR and LEE expression, it did so independently of *ntrC* and *rpoN*. This effect of ammonium on the expression of *E. coli* colonizing factors has been formerly observed in EPEC, as well as for enterotoxigenic *E. coli* (ETEC). In EPEC, ammonium has been shown to reduce expression of the bundle-forming pilus genes *bfpA* and *bfpT*, and to reduce T3S-secretion of the EspA, EspB and EspC translocon proteins (195, 235, 315). For ETEC, ammonium increased expression of the 987P fimbria genes *fasH* and *fasA* (107). Idiosyncrasies between EPEC and ETEC colonizing factor expression in response to ammonium correlate with differences in tissue tropism and reflect the availability of ammonium in the intestine; its concentration gradually increasing towards the distal small intestine (107, 235, 402). This natural gradient of intestinal ammonium may have a significant influence on the decision for colonization in all *E. coli*. However for EPEC, repression of *bfp* was shown to require a *trans*-acting factor that was absent, or present but not functional in *E. coli* K-12 (235). How the ammonium signal is communicated to GDAR in EHEC and to the LEE in EHEC and EPEC requires further study.

Based on the findings of this study, it is proposed that NtrC is autophosphorylated by a non-cognate phosphodonor in the σ^N pathway controlling GDAR and the LEE. Acetyl~P is a plausible candidate (Fig. 3.6), as it is a known NtrC phosphodonor (13, 112), and experimental alteration of acetyl~P levels by substituting either glycerol or glycerol and acetate for glucose, or by the deletion of acetate kinase (*ackA*), alters the expression of pathway components for regulation of GDAR and the LEE in a manner dependent on *rpoN* and *ntrC*. Requirement for acetyl~P is consistent with the growth-phase dependency of σ^N for GDAR and LEE regulation. The cellular pool of acetyl~P during growth with glucose peaks during exponential phase, and drops off precipitously during transition into stationary phase (314, 389). Correspondingly, control of *gad* and LEE genes by NtrC and σ^N is restricted to the mid-exponential phase of growth (252, 332). Remarkably, acetyl~P also serves as a phosphodonor for Rrp2, a σ^N EBP found in *B. burgdorferi* and required for activation of the σ^N-σ^S pathway regulating virulence expression in this pathogen (433). Thus, the use of acetyl~P for autophosphorylation of σ^N EBPs may be a phenomenon that is conserved across different species of bacteria. Why acetyl~P would be used in place of the cognate sensor kinase NtrB in *E. coli* is not yet known. It has been formerly proposed that the phosphorylation of NtrC by acetyl~P may be used to initiate transcription of Ntr genes during transition to a nitrogen poor environment, as cellular NtrB levels are very low when nitrogen is abundant (112). Yet, in this study *ntrB* was clearly dispensable for GDAR and LEE regulation when grown in nitrogen-limiting media containing glucose, suggesting that acetyl~P alone is sufficient to activate this pathway. It remains to be determined if *ntrB* is required for GDAR and LEE regulation by NtrC-σ^N in nitrogen-limiting media lacking glucose. The broader significance of this finding is that acetyl~P levels in *E. coli* are sensitive to many factors including,

nutrients, temperature, anaerobiosis and pH (428), suggesting that it may be used by NtrC to communicate various environmental cues to σ^N.

3.5 Experimental procedures

3.5.1 Bacterial strains and culture conditions

All strains and plasmids used in this study are listed in Table 3.1. Luria Bertani (LB) starter cultures were inoculated with a single colony of each strain and grown at 37°C with shaking (200 RPM) to an optical density at 600 nm (OD_{600}) of 0.5. Unless otherwise indicated, these cultures were used to inoculate either Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO) buffered with 50 mM MOPS and containing 0.4% (w/v) glucose, or MOPS minimal medium. MOPS medium was prepared as described (264), and contained 0.4% (w/v) glucose, 0.1% (w/v) NH_4Cl, and 0.1% (w/v) L-glutamine. Cultures were grown for 18-20 h before inoculating into fresh DMEM or MOPS to a final OD_{600}=0.05, respectively, using a 1:10 ratio of media-to-flask volume and grown at 37°C, 200 RPM. Appropriate antibiotics were added to cultures as required.

3.5.2 Procedures for genetic manipulation

Nonpolar gene deletion mutants were constructed using the λ Red recombinase-assisted approach (92, 261) and as described (332). Primers used for the construction of deletion mutants are listed in Table 3.2. For overexpression of *flhDC*, a 932-bp PCR fragment containing *flhDC* of strain TW14359 (nucleotide positions 2485400-2484469) was generated using primers flhDC-F/EcoRI and flhDC-R/XbaI. An *Eco*RI/*Xba*I digested fragment of the product was cloned into

similarly digested arabinose-inducible expression vector pBAD22 (135) to produce pRAM-3. pRAM-3 purified from DH5α transformants was then used to transform TW14359 and derivative strains producing EcRAM-51 through EcRAM-53. For *flhDC* promoter expression studies, a 2,942-bp *Xho*I/*Bam*HI digested PCR fragment (nucleotide positions 2487394-2484453) was generated using primers flhD-1994/XhoI and flhC+595/BamHI. This fragment contained the *flhDC* ORFs, and 1994-bp of DNA upstream of the *flhD* start codon including a σ^N promoter (2486152-2486138), a σ^{70} promoter (2485633-2485604), and a predicted NtrC box (2487152-2487132). This was ligated into *Xho*I/*Bam*HI digested pACYC177 to produce pRAM-4. The same approach was used for pRAM-5 and pRAM-6 construction, however the cloned fragment in pRAM-5, generated using primers flhD-825/XhoI and flhD+595/BamHI (positions 2486228-2484453), did not include the predicted NtrC box. For the fragment in pRAM-6, generated using primers flhD-728/XhoI and flhD+595/BamHI (positions 2486128-2484453), both the NtrC box and the σ^N promoter were excluded. Plasmids were purified from DH5α transformants and used to transform TW14359Δ*flhDC* producing strains EcRAM-59 to EcRAM-61. For *fliZ* complementation, a 552-bp PCR fragment containing the *fliZ* ORF was created using primers fliZ-Clone/F and fliZ-Clone/R and cloned into the arabinose inducible pBAD-TA vector (Invitrogen) to yield pRAM-8, which was then used to transform EcRAM-49 to produce EcRAM-66. For *ackA* complementation, the *ackA* ORF was amplified using primers ackA-Clone/F and ackA-Clone/R and cloned into the high copy pSC-B vector (Agilent) to create pRAM-9, which was then used to transform EcRAM-63 to produce EcRAM-68. The *rpoN* complement strain EcRAM-36 was constructed previously (252). All genetic constructs were validated using a combination of restriction mapping, DNA sequencing and qRT-PCR.

3.5.3 Quantitate Real-Time PCR (qRT-PCR)

RNA purification, cDNA sysnthesis, qRT-PCR cycling conditions and data analysis for relative quantitation of gene expression followed previously described protocols (252, 256, 332). Analysis was performed using a Realplex2 Mastercycler (Eppendorf). Cycle threshold (C_t) data were normalized to *rrsA* (16S rRNA gene) and normalized C_t values (ΔC_t) were transformed to arbitrary gene expression units using $2^{-\Delta Ct}/10^{-6}$ as described (222). A previous method was used for the quantitation of *flhD* mRNA copy number (60). Briefly, a 154-bp PCR product containing *flhD* was generated using flhD+63 and flhD+216, column purified (Qiagen) and serially-diluted in molecular grade water. C_t was measured for each dilution to generate a standard curve plotting C_t as a linear function of DNA concentration (ng/µl). The strength of linearity was estimated by the correlation coefficient (r^2), which exceeded 0.90 for all curves. DNA concentration was extrapolated from a standard curve using experimental C_t values and then converted to *flhD* copy number based on the estimated weight of a single 154-bp *flhD* dsDNA fragment of 47-kDa. Gene expression levels and *flhD* copy number were compared between samples using the appropriate *t*-test or by ANOVA and Tukey's HSD ($n \geq 3$, $a=0.05$) using R v. 2.13.0.

3.5.4 Protein extraction, SDS-PAGE and western blots

Protein extraction, purification and procedures for western blots followed a previously described protocol (252, 256). Monoclonal antibodies for σ^S and GroEL were acquired from Neoclone (Madison, WI) and Bio-Rad (Carlsbad, CA), respectively. Densitometry was used to estimate differences in protein levels using a ChemiDoc XRS+ Imaging System and Image Lab 3.0 (Bio-Rad). Western blots were repeated a minimum of three times in independent trials.

3.5.5 Purification of NtrC

A 1425-bp *Nco*I/*Xho*I-digested PCR fragment generated using primers ntrC+F/NcoI and ntrC-R/XhoI was cloned into similarly digested pET-24d producing pRAM7 and replacing the *ntrC* stop codon with a C-terminal 6x His tag. pRAM7 was transformed into propagating *E. coli* strain BL-21, which was grown in LB containing ampicillin (100 µg/ml) to OD_{600}=0.4 before induction of 6xHis-tagged *ntrC* with 1 mM IPTG for 16 h at 20°C (200 RPM). Cultures were harvested by centrifugation (5000 x g, 20 min) and 6xHis-NtrC was purified using a nickel Ni-NTA Protein Purification Kit (Qiagen) according to the manufacturer's instruction.

3.5.6 Electrophoretic mobility shift assay, EMSA

EMSA was performed using the LightShift Chemiluminescence EMSA Kit (Pierce, Rockford, IL) according to the manufacturer's instruction. Biotin end-labeled DNA probes were generated by PCR using flhD-1842/Biotin and flhD-1634/Biotin for the $flhD_P$ promoter probe, and glnA-311/Biotin and glnA-112/Biotin for the $glnA_{P2}$ promoter probe; biotin end-labeled Epstein-Barr nuclear antigen (EBNA) DNA was supplied with the kit. The $flhD_P$ promoter probe (strain TW14359 nucleotide position 2487034-2487242) contained a putative NtrC binding site flanked by 0.1-kb. For the $glnA_{P2}$ promoter probe, a confirmed NtrC box (nucleotide position 4913213-4913228) was flanked by 0.1-kb. Binding reactions (20 µl per reaction) contained 20 fmol of biotin end-labeled DNA probe, 50 mM KCl, 5 mM MgCl2, 1% (v/v) glycerol, 0.05% (v/v) NP-40, 50 ng/µl poly(dI-dC) copolymer competitor, 10X molar excess BSA (10 mg/ml), and 0 µM, 2 µM, 4 µM, 8 µM or 16 µM purified C-terminally labeled 6xHis-NtrC. Reactions were incubated for 40 min at room temperature, and were then separated by electrophoresis using 8% non-denaturing acrylamide gels prepared in 0.5X TBE buffer at 4°C for 80 min at 160V, and DNA/protein

complexes transferred to a nylon membrane (Biodyne). Membranes were UV cross-linked at 120,000 mJ/cm^2 for 1 min and detected by chemiluminescence using the Biotin Detection System (Pierce) and a ChemiDoc XRS+ Imaging System including Image Lab 3.0 (Bio-Rad).

3.5.7 Selection of suppressor mutants for glutamine auxotrophy

Spontaneous suppressor mutants for glutamine auxotrophy were selected in the TW14359Δ*rpoN* background by growth in MOPS minimal media without the addition of glutamine. Briefly, overnight cultures of TW14359Δ*rpoN* grown in MOPS media were inoculated into fresh MOPS containing 0.4% glucose and 0.1% NH_4Cl and grown at 37°C (200 RPM). The outgrowth of suppressor mutants (TW14359Δ*rpoN* Gln+) consistently occurred following 48 h incubation. Single colonies of suppressor mutants were obtained by subculture from MOPS media to LB with 1.5% agar, and confirmed by growth in MOPS containing 0.2% glucose and 0.1% (w/v) L-histidine as described (328), and by qRT-PCR analysis of glutamine synthetase *glnA* expression. Three independent suppressor mutants were selected and validated by this approach. The mutation leading to suppression was determined using a combination of PCR and Sanger sequencing of amplified DNA fragments (MWG Operon, Huntsville, AL), and next generation whole genome sequencing.

3.5.8 Whole genome next generation DNA sequencing and analysis

Genomic DNA was extracted from TW14359Δ*rpoN* and a single suppressor mutant of TW14359Δ*rpoN* (TW14359Δ*rpoN* Gln+) using Puregene® Kits (Gentra, Minneapolis, MN). One microgram of DNA from each strain was enzymatically sheared into libraries of ~200-bp fragments using the Ion Xpress™ Plus Fragment Library Kit (Life Technologies, NY). Each DNA

library was purified using the E-Gel® SizeSelect™ 2% Agarose system (Invitrogen, NY), and the integrity and quantity of each was determined using a Bioanalyzer high-sensitivity DNA chip (Agilent, CA). Libraries were diluted and template-positive Ion Sphere Particles (ISPs) prepared using the Ion OneTouch 200 Template Kit (Life Technologies). ISPs were sequenced using an IonTorrent™ Personal Genome Machine and the Ion PGM 200 Sequencing Kit (Life Technologies) following the manufacturer's instructions. Whole genome sequencing data was exported from the Ion Torrent Server and analyzed using the Genomics Suite software package (CLC Bio, Denmark). Genomes were assembled using the TW14359 genome (NC_013008, NCBI) as a reference, followed by quality-based variant detection to identify polymorphisms with a minimum coverage of 10X and 100% detection frequency. Polymorphisms common to both strains (relative to the reference TW14359 genome), and those in homopolymeric nucleotide tracts, were excluded resulting in the identification of specific genetic variations between TW14359Δ*rpoN* and TW14359Δ*rpoN* Gln+.

3.5.9 Adherence assay

Adherence to epithelial cells was determined following a previously described protocol (256). Briefly, human HT-29 colonic epithelial cells were grown to confluence on polylysine-treated glass coverslips placed within the wells of 24 well culture plates at 37°C with 5% CO_2. Overnight DMEM cultures were diluted 1:40 (v/v) in fresh DMEM and 0.05 ml of this dilution was used to inoculate each well which already contained 0.45 ml of sterile DMEM. After 3 h, plate wells were washed five times with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7) to remove non-adherent bacteria from the coverslips, and fresh DMEM was then added before incubating for an additional 3 h. Plate wells were subsequently washed three times in PBS, and

then fixed with ice cold (−20°C) 100% methanol for 10 min before staining with Giemsa diluted in PBS 1:20 (v/v) for 20 min. Giemsa-stained coverslips were examined at 1000X magnification by oil immersion, and microcolonies were scored as discrete clusters of five or more bacterial cells as previously defined (2, 167, 242). For each sample, a minimum of ten viewing frames were observed and the average number of microcolonies was reported per 50 HT-29 cells. Microcolony counts were compared between strains by Tukey's HSD following a significant F-test ($n \geq 3$, $\alpha = 0.05$) (R v. 2.13.0).

3.5.10 Tests for acid resistance

Acid resistance by the glutamate-dependent system was measured for exponential phase cultures grown in DMEM as previously described (252, 332) with slight adaptations. Strains were grown in DMEM to OD_{600}=0.5 before inoculating (10^6 CFU/ml final cell density) into E minimal glucose (EG) media containing 5.7 mM L-glutamate adjusted with HCl to pH 7 (control) or pH 2. Cultures were sampled for counts (CFU/ml) after 1 h incubation at 37°C (200 RPM) by plating serial dilutions to LB with 1.5% agar and incubating overnight. Experiments were repeated a minimum of three times in independent trials.

Conflict of Interest

The authors state that there are no conflicts of interest.

Table 3.1 Strains and plasmids used in this study

Strain/Plasmid	Relevant characteristics	Source/reference
Strain name:		
DH5α	Vector propagation, *recA1 endA1*	
BL-21		(250)
TW14359	WT 2006 outbreak, western US	(232)
EcRPF-6	TW14359Δ*rpoN*	(252)
EcRAM-26	TW14359Δ*ntrC*	(252)
EcRAM-43	TW14359Δ*rpoN*Gln+, suppressor mutant for Gln auxotrophy	This study
EcRAM-45	EcRAM-43Δ*glnA*	This study
EcRAM-47	TW14359*crl*::*kan* Kan^R	This study
EcRAM-49	TW14359Δ*fliZ*	This study
EcRAM-51	EcRFP-6 pRAM-3 Amp^R	This study
EcRAM-52	EcRAM 26 pRAM-3 Amp^R	This study
EcRAM-53	EcRAM 49 pRAM-3 Amp^R	This study
EcRAM-58	TW14359Δ*flhDC*	This study
EcRAM-59	EcRAM 58 pRAM-4 Amp^R	This study
EcRAM-60	EcRAM 58 pRAM-5 Amp^R	This study
EcRAM-61	EcRAM 58 pRAM-6 AmpR	This study
EcRAM-63	TW14359Δ*ackA*	This study
EcRAM-66	TW14359Δ*fliZ*pRAM-8	This study
EcRAM-68	TW14359Δ*ackA*pRAM-9	This study
Plasmid name:		
pACYC177	Low copy cloning vector, Amp^R Kan^R P15A	(74)
pET-24d	IPTG inducible His-tagging vector, Kan^R	Novagen
pBAD22	Mid copy arabinose inducible cloning vector, Amp^R	(135)
pSC-B	High copy cloning vector, Amp^R Kan^R	StrataClone
pBAD-TA	Mid-copy arabinose inducible cloning vector, Amp^R	Inivitrogen
pRAM-1	*rpoN*::pACYC177, Amp^R Kan^S	(252)
pRAM-3	*flhDC*::pBAD22, Amp^R	This study
pRAM-4	*flhDC*::pACYC177 positions +948 to -1994 relative to start codon	This study
pRAM-5	*flhDC*::pACYC177 positions +948 to -825 relative to start codon	This study
pRAM-6	*flhDC*::pACYC177 positions +948 to -728 relative to start codon	This study
pRAM-7	*ntrC*::pET-24d containing ORF, Kan^R	This study
pRAM-8	*fliZ*::pBAD, Amp^R	This study
pRAM-9	*ackA*::pSC-B, Amp^R Kan^R	This study

Table 3.2 Primers used in this study

Primer Name	Type	Sequence (5'→3')	Source/reference
cesT+296	Real-Time	TCCCTCTCGATGATGCTACC	343
cesT+445	Real-Time	TGTCGCTTGAACTGATTTCCT	343
crl+103	Real-Time	TCGATTGTCTGGCTGTATGC	This study
crl+244	Real-Time	AGTCGCCTGCTTTATCGAAC	This study
espA+128	Real-Time	AGGCTGCGATTCTCATGTTT	343
espA+310	Real-Time	GAAGTTTGGCTTTCGCATTC	343
flhC+191	Real-Time	AACCAGTCGGTTGAGAATGG	This study
flhC+32	Real-Time	GGATATTCAGCTGGCAATGG	This study
flhD+216	Real-Time	GTGGCTGTCAAAACGGAAGT	This study
flhD+63	Real-Time	CAGCGTCTGATTGTTCAGGA	This study
fliZ-187	Real-Time	CGGAACAAAAATCATGGGCG	This study
fliZ-371	Real-Time	TGCTCGTGTAGATGATTCCC	This study
gadE+309	Real-Time	TGGTAAACACTTGCCCCATA	343
gadE+419	Real-Time	AGCGTCGACGTGATATTGCT	343
glnA+1064	Real-Time	CGTGCTTTCGGAGAAGAAAC	This study
glnA+890	Real-Time	ACTACATTGGCGGCGTAATC	This study
katE+734	Real-Time	TGCAACCTGAAACTCTGCAC	This study
katE+897	Real-Time	TTTACCTGCCAGTGGTTTCC	This study
ler+109	Real-Time	CGAGAGCAGGAAGTTCAA	343
ler+214	Real-Time	GTCCATCATCAGGCACAT	343
otsA+130	Real-Time	GGTGAAACAGGGAATGAGGA	This study
otsA+287	Real-Time	ACCAGATCGAGGCGATAATG	This study
rpoH+110	Real-Time	GGCTGAAAAGCTGCATTACC	This study
rpoH+261	Real-Time	CATCAGGCCGATGTTACCTT	This study
rpoS+356	Real-Time	TATCGAAGAGGGCAACCTGG	343
rpoS+466	Real-Time	GTTCAATCGTCTGGCGAATC	343
rtcA+518	Real-Time	GGTGGCATCGTTTAACACCT	This study
rtcA+684	Real-Time	ATATTCATAACCTGCCGCGC	This study
rtcB+128	Real-Time	TGCGGTAATGCCTGATGTAC	This study
rtcB+249	Real-Time	TAGTGCGTTCATTCCACAGC	This study
tir+664	Real-Time	ACTTCCAGCCTTCGTTCAGA	343
tir+869	Real-Time	TTCTGGAACGCTTCTTTCGT	343
flhD-1842/Biotin	EMSA	BIOTIN-ATAATAAAGCGGCGTCCAGC	This study
flhD-1634/Biotin	EMSA	BIOTIN-ATAATAAAGCGGCGTCCAGC	This study
glnA-311/Biotin	EMSA	BIOTIN-CAGGATCACAAACATCCTCC	This study
glnA-112/Biotin	EMSA	BIOTIN-CCCTAAAAGGCGTTATCATGC	This study
ackA-Clone/F	Cloning	AATGTCGGTGTCATCATGCG	This study
ackA-Clone/R	Cloning	GGGACACGGTTTATCCTCTT	This study
flhD+948/BamH1	Cloning	GCCTCGAGCGCCACACCGTATCAGTTAA	This study
flhD-1994/XhoI	Cloning	CGGGATCCGGCAAAGCGTTTCGAACAGA	This study
flhD-728/XhoI	Cloning	CGGGATCCATGGAGAAACGACGCAATCC	This study
flhD-825/XhoI	Cloning	CGGGATCCTTTCCGGTGTAACCGCAACA	This study
flhDC-F/EcoRI	Cloning	CGGAATTCAtgcatacctccgagttgct	This study
flhDC-R/XbaI	Cloning	GCTCTAGATTAAACAGCCTGTACTCTCT	This study
fliZ-Clone/F	Cloning	CGGAATTCATGATGGTGCAGCACCTGAA	This study
fliZ-Clone/R	Cloning	GCTCTAGATTAATATATATCAGAAGCAG	This study
ntrC+F/NcoI	Cloning	CATGCCATGGGCCAACGAGGGATAGTCTGGGT	This study
ntrC-R/XhoI	Cloning	CCGCTCGAGCTCCATCCCCAGCTCTTTTA	This study
crl+449/P2	Mutation	cagttttaatgattattgccggatgtgatgcatccggcacCATATGAATATCCTCCTTAG	This study
crl-41/P1	Mutation	gccaatttggtaaaacagttgcatcacaacaggagatagcaGTGTAGGCTGGAGCTGCTTC	This study
flhD+985/P2	Mutation	AAAGCAGCGGTACGTCGTTACCGCTGCTGGAATGTTGCGCCATATGAATATCCTCCTTAG	This study
flhD-60/P1	Mutation	gacatcacggggtgcggtgaaaccgcataaaaataaagttGTGTAGGCTGGAGCTGCTTC	This study
fliZ+637/P2	Mutation	tcaccccgaatgttgttatgtctgtttgcagtgtagagccCATATGAATATCCTCCTTAG	This study
fliZ-45/P1	Mutation	cgtcagtaaatgccgcactttaactttgactaccaggagtGTGTAGGCTGGAGCTGCTTC	This study
glnA+1371/P2	Mutation	AGTCATACGCACGCGGTCATCTTCTTCGCGACGCAGAGCGCATATGAATATCCTCCTTAG	This study
glnA-72/P1	Mutation	acggcgacacggccaaaataattgcagatttcgttaccacGTGTAGGCTGGAGCTGCTTC	This study

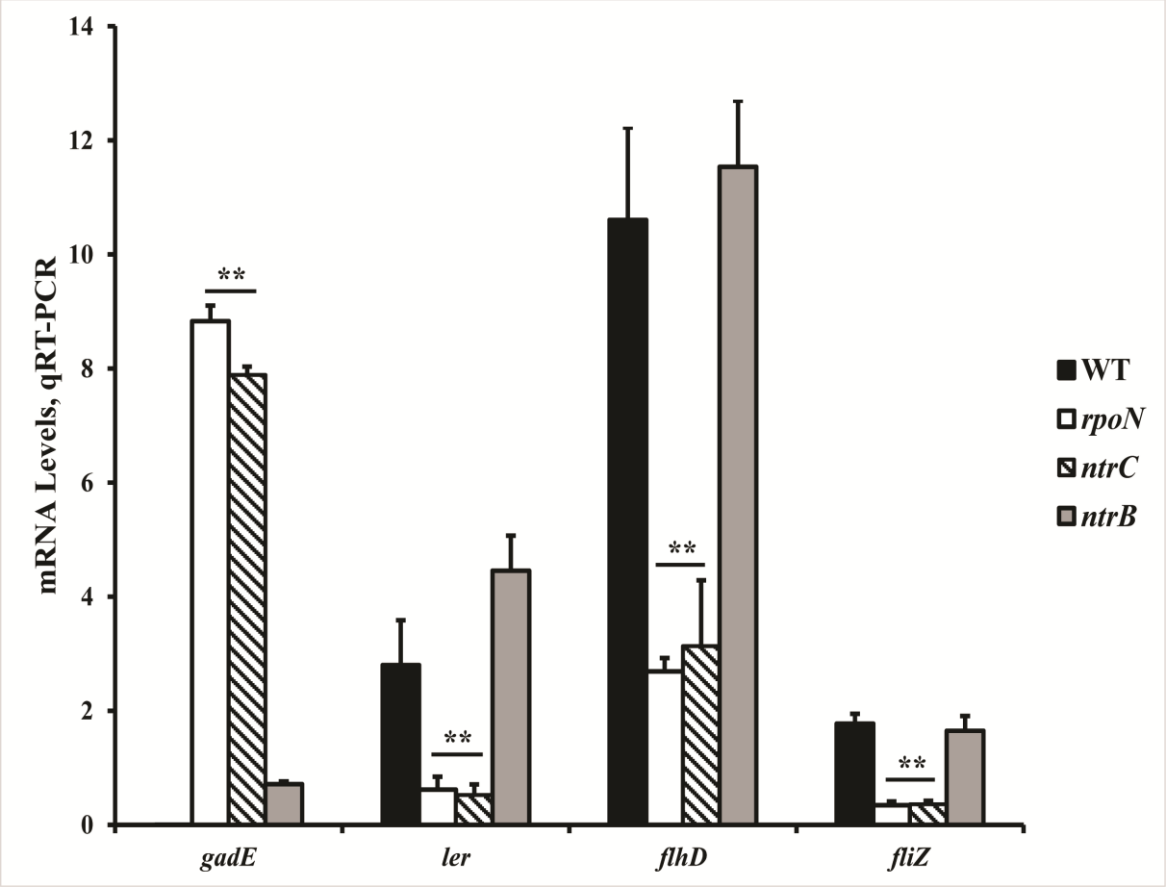

Figure S3.1: Effect of ntrB deletion on the expression of genes for GDAR and LEE control. Gene expression levels plotted for WT (black), Δ*rpoN* (white), Δ*ntrC* (hatched), and *ntrB* (gray). Asterisks denote significant difference between WT and respective isogenic mutants by t-test ($p<0.05$ [*], $p<0.01$ [**], $n\geq3$). Error bars denote standard deviation.

Chapter Four: RtcBA and RtcR are Important Determinants of Enterohemorrhagic *Escherichia coli* (EHEC) Stress Response and Contribute to EHEC Pathogenesis

4.1 Abstract

In recent years, alternative sigma factor N (σ^N) has increasingly been identified to be a vital determinant for pathogenesis in many organisms such as *Borrelia*, *Pseudomonas*, *Salmonella* and *Vibrio* species. Enterohemorrhagic *E. coli* (EHEC), a food borne pathogen, depends on glutamate dependent acid resistance (GDAR) for efficient passage through the gastric acid barrier of the stomach and on the locus of enterocyte effacement (LEE) for immune subversion and colonization in the intestine. In EHEC σ^N and its cognate enhancer binding proteins (bEBPs) NtrC and QseF have been identified as important regulators of GDAR and the LEE. The bEBPs play a crucial role by sensing environmental cues and coupling them to activation of σ^N-dependent transcription for regulation of GDAR and the LEE. Besides NtrC and QseF there are 9 other bEBPs that function in conjunction with σ^N for activation of promoters, but their contribution to EHEC stress fitness and pathogenesis have not been explored. This study explores the contribution of σ^N EBPs in EHEC stress fitness and pathogenesis. Whereas, deletion of *norR*, *prpR*, *tyrR* significantly reduces the ability of EHEC to survive in acid, deletion of *rtcR* negatively impacts EHEC stress fitness in acid, peroxide, salt and heat. Deletion of *rtcBA*, whose expression is activated by RtcR, phenocopied the *rtcR* mutant for EHEC stress fitness. Reduced fitness was correlated with decreased expression of key regulatory genes for stress responses in both the *rtcR* and *rtcBA*

mutants. Deletion of *rtcR* and *rtcBA* also significantly reduced EHEC adherence to HT-29 epithelial cells, killing of *Galleria mellonella* and survival within macrophages indicating the mutants attenuate EHEC virulence. Decreased adherence to HT-29 cells correlated with reduced expression of LEE genes. Transcriptome analysis using RNAseq identified differential regulation of 166 ORFs, of which 109 were downregulated and 57 were upregulated. For the downregulated ORFs, 68% perform crucial functions in the transport and metabolism of various sugars and in the transport of quorum sensing molecules, 9% function in stress response, 11% encode structural components of curli, fimbriae and flagella, with the remaining having unknown functions.

4.2 Introduction

Transcription initiation is the first step of gene expression and provides one the most important points of access for differential regulation of genes. During initiation, the core RNAP enzyme which is in its open complex form binds to a dissociable subunit called a sigma factor to form the RNAP holoenzyme (Eσ) (54). Sigma factors recruit the holoenzyme (Eσ) to promoter DNA and forms a closed complex in a process called promoter recognition. This is then followed by promoter melting by the sigma factor to initiate transcription of genes (55, 200). Whereas, all genes required for growth and housekeeping are expressed by the housekeeping sigma factor (σ^{70}), bacteria employ alternative sigma factors to express genes required for adapting to changing environments and also for virulence and pathogenesis (193, 276). In *Escherichia coli*, σ^{N} has been directly implicated in catabolism of arginine and agamatine, transport of amino acids such as arginine, histidine, glutamine, glutamate and aspartate. Studies have shown that σ^{N} is directly responsible for activating expression of the *fhlA*, *prp* and *zra* operon, whose genes encode components crucial for formate and propionate metabolism and in maintaining zinc homeostasis,

respectively. Altogether, σ^N has broad functions in maintaining general cell metabolism (for detailed review see (327)). Unlike all other bacterial sigma factors that can readily form open complex and initiate transcription, σ^N remains in an energetically favorable closed complex after forming holoenzyme ($E\sigma^N$) (50, 423). Open complex formation and transcription initiation by $E\sigma^N$ is completely dependent on activator proteins called bacterial enhancer binding proteins (bEBPs). The bEBPs are members of the AAA+ (ATPases associated with various cellular activities) protein subfamily and bind upstream activating sequences (UAS) called enhancer sequences. After binding, the bEBPs hydrolyze ATP and interact with σ^N, resulting in a conformation change within σ^N to initiate promoter melting and transcription (306, 346, 417, 418, 434).

σ^N bEBPs are comprised of three functional domains: an N-terminal regulatory domain (R), a central AAA+ domain (C) and a C-terminal DNA binding domain (D). The highly conserved core AAA+ domain (C), which drives ATP hydrolysis and conformation change in σ^N, is absolutely essential for a functioning bEBP and as such the R and D domains are sometimes absent in some bEBPs, as observed in the case of PspF (*E. coli*), FlgR (Campylobacter), HrpR and HrpS (Pseudomonas) (48, 108, 158). The N-terminal regulatory domain (R) is highly variable and contains different motifs like V4R motifs, response regulator motifs and GAF domains. These motifs play a crucial role in sensing intra- and extra-cellular cues and coupling them to σ^N-dependent activation of promoters (362, 364). The C-terminal DNA binding domain contains a helix-turn-helix motif that allows bEBPs to bind and oligomerize at UAS and then initiate ATP hydrolysis (382, 434). Since the bEBPs can bind to enhancer sites 100-400 bp upstream of a σ^N promoter, DNA between the enhancer sites and promoter must bend to allow interaction between the bEBP and $E\sigma^N$ (68). This is facilitated by the DNA bending protein called integration host

factor, which binds to specific sequences present between the σ^N promoter and the enhancer site (152, 437).

In an increasing number of bacterial pathogens, σ^N and its activator proteins (bEBPs) have been shown to regulate genes for virulence and transmission, and to be required for complete *in vivo* disease progression (7, 20, 88, 165, 249, 274, 358, 414). In *B. burgdorferi*, σ^N and Rrp2 (bEBP) are essential for transmission from the tick vector to a mammalian host, and for establishment of infection (133, 155, 284). In *Pseudomonas aeruginosa*, σ^N and AlgB (bEBP) control alginate production, which is an important determinant for survival within lungs of cystic fibrosis patients (46, 431, 449). *Escherichia coli* serotype O157:H7 (EHEC), is a food-borne pathogen responsible for outbreaks and sporadic cases of bloody diarrhea (hemorrhagic colitis) and the life threatening hemolytic uremic syndrome (HUS) (318). EHEC is dependent on the glutamate-dependent acid resistance (GDAR) system for efficient passage through the harsh acidic milieu of the stomach and to determine a low oral infectious dose (75, 396). It is also completely dependent on the locus of enterocyte effacement (LEE) for expression of a type III secretion (T3S) apparatus that translocates virulence factors into host intestinal cells mediating intimate adherence and immune subversion (109, 241, 292). Studies have shown that σ^N (encoded by *rpoN*) and the bEBPs NtrC (encoded by *ntrC*), FhlA (encoded by *fhlA*) and QseF (encoded by *qseF*) are important regulators of GDAR and the LEE (324, 332). QseF is the response regulator of the QseEF two-component system and responds to autoinducer-3 (AI-3), and host hormones norepinephrine and epinephrine to activate expression of key LEE genes required for intimate adherence to intestinal epithelia (322, 324). NtrC and σ^N were also recently identified to be key activators for LEE genes and repressors of GDAR genes during exponential EHEC growth, but, the specific environmental signals influencing the NtrC-σ^N directed regulation of GDAR and the LEE are not fully understood

yet. It was also demonstrated that the FhlA acts a repressor of GDAR during exponential growth (251). These studies show that there is precedent for σ^N and its bEBPs to be important determinants in EHEC transmission and colonization. In EHEC, there are total of eleven σ^N bEBPs and besides QseF, FhlA and NtrC their role(s) in EHEC fitness and pathogenesis have not been previously explored. This study explores the roles of all the enhancer binding proteins in EHEC fitness and pathogenesis. In this study the *rtc* operon, encoding RtcB (RNA ligase) and RtcA (RNA cyclase), and the σ^N enhancer binding protein RtcR, which activates expression of the *rtc* operon are shown to play an important role in EHEC survival under different environmental conditions and contribute to EHEC virulence and pathogenesis.

4.3 Results

4.3.1 Role of σ^N bEBP in EHEC stress fitness

To determine the contribution of σ^N bacterial enhancer binding proteins (bEBPs) to EHEC fitness, the survival of O157:H7 strain TW14359 was compared to isogenic mutant strains representing each of the 11 known bEBPs in response to acid, oxidative, salt and heat stress. Since many of the σ^N bEBPs have very low basal expression (Fig. S4.2), they were overexpressed *in trans* in the isogenic mutants to determine their effects on EHEC stress fitness. The bEBPs whose deletion and overexpression had no effect on stress fitness are reported in Table 4.1. Missing values in Table 4.1 are for bEBPs that produced significant effects and are discussed here. Significant reductions in percent survival were observed for TW14359Δ*norR* (67%) TW14359Δ*prpR* (49%), TW14359Δ*rtcR* (52%), and TW14359Δ*tyrR* (57%) compared to TW14359 (85%) following 1 h incubation in acidified (pH 2) EG media ($p<0.05$) (Fig. 4.1A). For

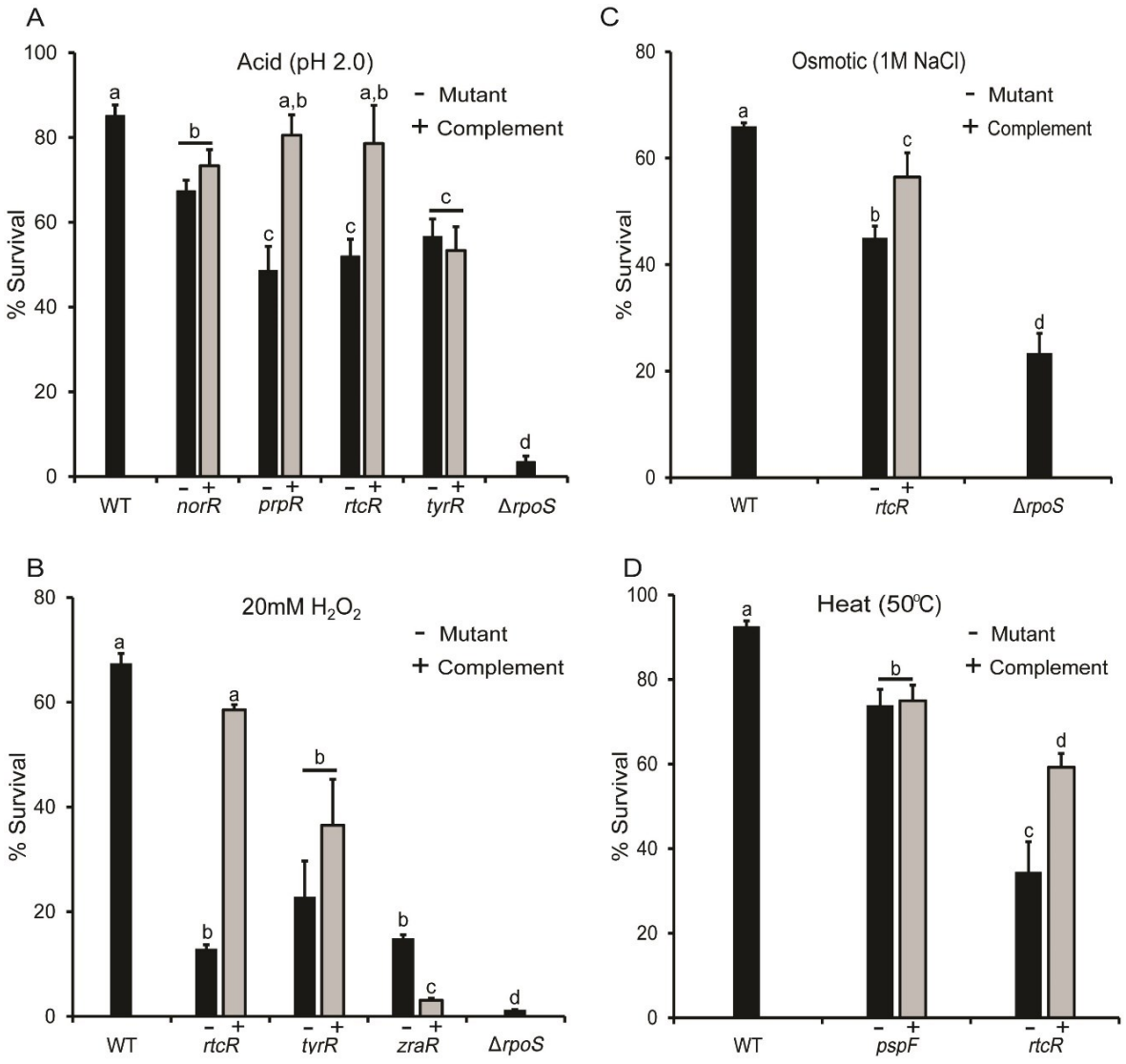

Figure 4.1. Effect of bEBP deletion on EHEC fitness in stressors. Percent survival in **(A)** Acid (pH 2.0), **(B)** 20 mM H_2O_2, **(C)** 1M NaCl, and **(D)** Heat (50ºC) resistance for TW14359 (WT), bEBP mutants (-) and complement strains (+). Plots that differ in lowercase letter differ significantly by Tukey's HSD following a significant F-test ($n = 6$, $p < 0.05$). Error bars denote standard deviation.

oxidative stress, percent survival was significantly reduced in response to 20 mM hydrogen peroxide for strains TW14359Δ*rtcR* (13%), TW14359Δ*tyrR* (23%), and TW14359Δ*zraR* (15%) compared to TW14359 (67%) ($p<0.01$) (Fig. 4.1B). For salt (1 M NaCl) and heat (50C) stress, only the deletion of *rtcR* (salt and heat) and *pspF* (heat) significantly reduced survival compared to wild-type. For salt, survival was reduced to 44% for TW14359Δ*rtcR* compared to 66% for TW14359 ($p=0.02$) (Fig. 4.1C), whereas for heat, TW14359Δ*rtcR* was reduced to 35% survival and TW14359Δ*pspF* to 74% survival compared to 92% for TW14359 ($p<0.05$) (Fig. 4.1D). Thus of all EBPs, only the deletion of *rtcR* had a negative impact on survival in response to all stressors during stationary phase growth. Complementation restored near wild-type levels of resistance to stressors for TW14359Δ*prpR*pRAM-16, and TW14359Δ*rtcR*pRAM-19, but not for *norR*+, *tyrR*+, and *pspF*+. In fact, for TW14359Δ*zraR*pRAM-22 (*zraR*+), sensitivity to peroxide increased with survival (dropping to 3%), and was comparable to that of a strain deleted for stationary phase stress resistance factor, *rpoS* (strain TW14359Δ*rpoS*) (Fig. 4.1C). Altogether, the results identify new distinct functions for NorR, TyrR and PrpR in EHEC stress fitness which have not been described before and show that RtcR is a major contributor to general stress response in EHEC.

4.3.2 RtcR and genes of the **rtc** *operon contribute to EHEC stress fitness*

The salient observation from the initial screening of all the bEBP mutants was that deletion of *rtcR* negatively impacted stress fitness for EHEC. RtcR was originally implicated in σ^N-dependent regulation of the *rtc* operon containing the genes *rtcB* and *rtcA* (124, 317). Subsequent in vitro studies identified that RtcB and RtcA are the only discovered RNA ligase and an RNA cyclase, respectively, in *E. coli* and are involved in RNA repair mechanisms (71, 90, 391-393). Since deletion of *rtcR* reduces stress fitness, it prompted further investigation into the possible

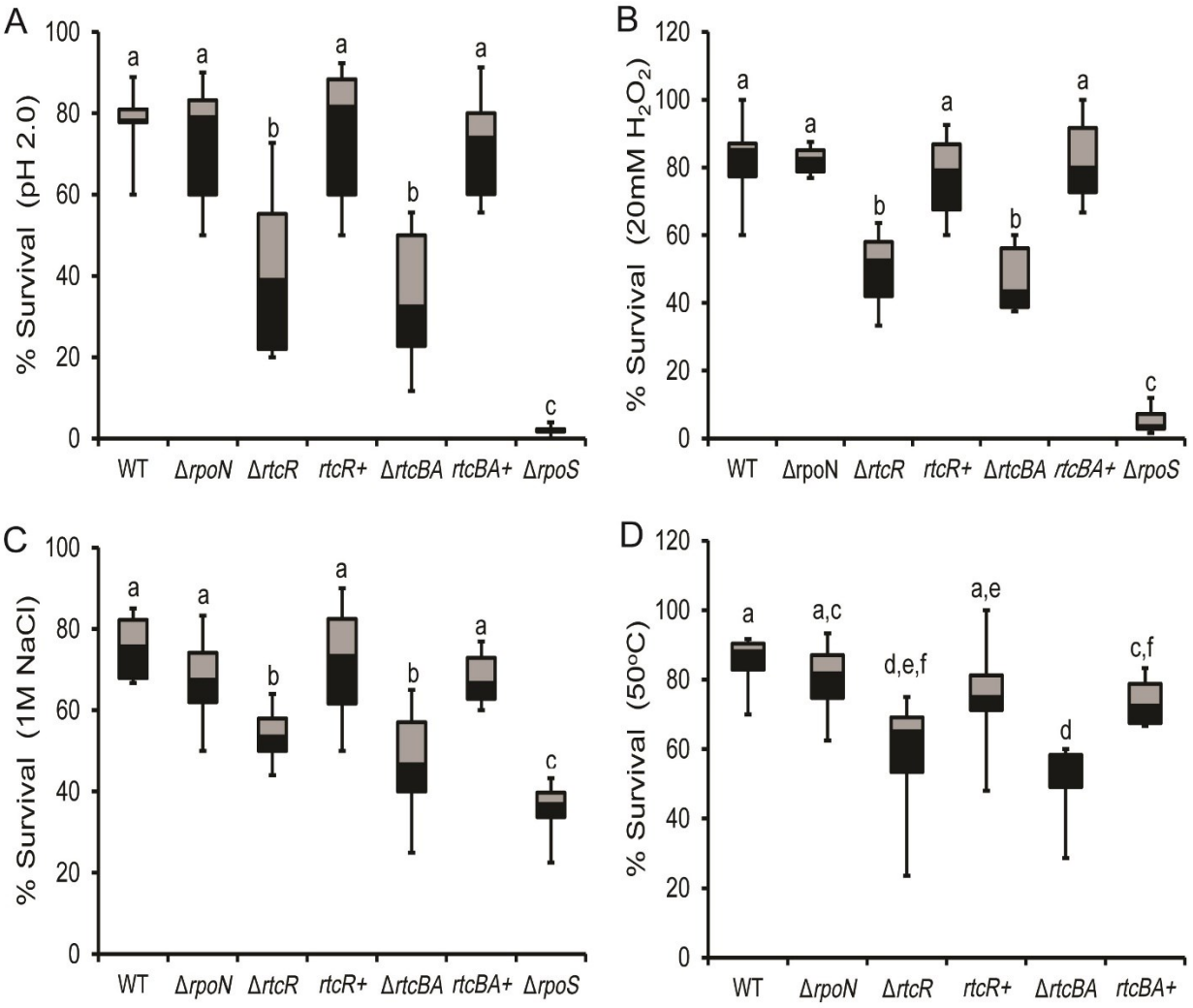

Figure 4.2. Effect of *rpoN*, *rtcR* and *rtcBA* deletion on EHEC fitness in stressors. Percent survival of TW14359, TW14359Δ*rpoN*, TW14359Δ*rtcR*, TW14359Δ*rtcBA*, Δ*rtcR*pRAM-19 (*rtcR*+) and Δ*rtcBA*pRAM-23 (*rtcBA*+) in acid **(A)**, peroxide **(B)**, salt **(C)** and heat **(D)**. For boxplot, boundaries represent the 25th and 75th percentiles, whiskers represent the maximum and minimum values and the median is given by the horizontal line. Plots that differ in lowercase letter differ significantly by Tukey's HSD following a significant F-test ($n = 6$, $p < 0.05$).

contribution of RtcB and RtcA in this phenotype. To determine if the effect of *rtcR* deletion is being mediated through *rtcB* and *rtcA*, a strain deleted for both genes (TW14359Δ*rtcBA*) was screened for stress fitness in parallel with TW14359Δ*rtcR*. Since RtcR is dependent on σ^N for the expression of *rtcBA*, survival was also compared to TW14359Δ*rpoN*. As observed previously, TW14359Δ*rtcR* exhibited generalized reduction in stress fitness when compared to TW14359 (Fig. 4.2). While deletion of *rtcBA* further reduced recovery from acid (pH 2.0) to 35% compared to 41% for TW14359Δ*rtcR*, the reduction was not significant (Fig. 4.2A). A similar observation was made for TW14359Δ*rtcR* and TW14359Δ*rtcBA* in response to peroxide and salt stress (Fig. 4.2 B & C). Percent survival in response to heat was only marginally reduced in TW14359Δ*rtcBA* (52%) when compared to TW14359ΔrtcR (58%) (Fig. 4.2D). Complementation with *rtcR* (strain TW14359Δ*rtcR*pRAM-19) only partially restored survival to WT levels (Fig. 4.2D). Recovery of TW14359Δ*rtcBA* increased to 73% ($p = 0.003$) after complementation with *rtcBA*, however it was never restored to WT levels and the difference from WT remained statistically significant ($p = 0.03$) (Fig. 4.2D). Interestingly, for all stressors, survival of the *rtcR* and *rtcBA* mutants was significantly different from TW14359Δ*rpoN* ($p < 0.05$) (Fig. 4.2). Deletion of *rpoN* exhibited no influence on survival of EHEC and resembled WT levels in all the stressors except during heat shock. TW14359Δ*rpoN* showed a slight reduction in recovery to 80% compared to the 85% survival for TW14359, however this was not statistically significant ($p = 0.40$) (Fig. 4.2D). Since the fitness pattern of TW14359Δ*rpoN* did not resemble that of the *rtcR* and *rtcBA* mutants, it appears that the mechanism through which RtcB, RtcA and RtcR influence general stress resistance does not involve σ^N.

4.3.3 The RtcR regulon

Whole genome RNA sequencing was performed to examine the impact of *rtcR* inactivation on the transcriptome of *E. coli* O157:H7 TW14359 grown in DMEM. Inactivation of *rtcR* resulted in the differential expression of 166 ORFs, of which, 109 ORFs were downregulated and 57 were upregulated (Table 4.2). For the ORFs that were downregulated in TW14359Δ*rtcR*, 74 ORFs (~68%) perform crucial functions in transport and metabolism of sugars and amino acids. Examples of some downregulated operons and genes include: *ast* (arginine biosynthesis/ transport), *lysA* (lysine biosynthesis), *fad* (fatty acid metabolism), *fru* (fructose transport), *mal* (maltose transport), *mgl* (galactose transport), *prpR* (propionate metabolism), *lsr* (autoinducer-2) and several porins and predicted inner membrane proteins (Table 4.2). Moreover, 10 ORFs (9%) functioning in stress response, 12 (11%) encoding structural components of curli (*csgA*), fimbriae (*fimA*) and flagella (*fliL*), with the remaining having unknown functions were also downregulated (Table 4.2). For the ORFs that were upregulated, the only specific categories of genes that were apparent were genes encoding structural components of the translation apparatus (*rpm* and *rps* genes) and cold shock response (*csp*) (Table 4.2). Examples of other upregulated genes include: *stx2B* (Shiga toxin), *grlR* (repressor of the LEE), *kil1* (host killing protein) and *nleE* (non-LEE encoded effector protein). These results demonstrate that a significant number of genes in the RtcR regulon are involved in biosynthesis and transport of arginine, fructose, maltose and galactose. Most of these sugars become a vital source of carbon in the absence of glucose. Downregulation of these genes in the *rtcR* mutant suggests that the mutant may be defective in scavenging different carbon sources in the absence of glucose and RtcR has an important role in utilization of these secondary carbon sources.

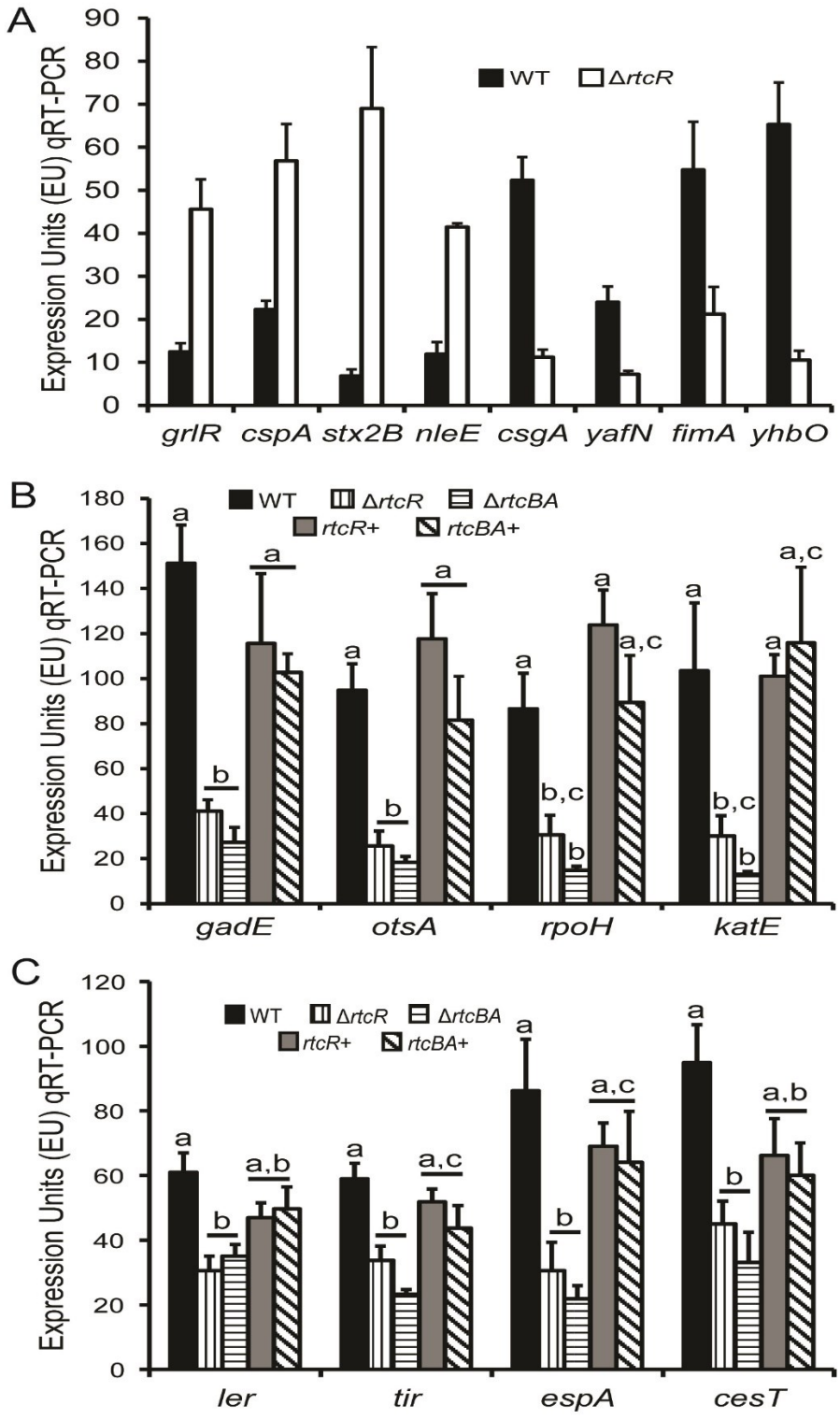

Figure 4.3. Effect of *rtcR* and *rtcBA* deletion on stress and LEE genes mRNA levels and EHEC adherence. (A) Validation of RNA sequencing analysis by qRT-PCR. mRNA levels of stress **(B)** and LEE **(C)** genes in WT (black), Δ*rtcR* (vertical bars), Δ*rtcBA* (horizontal bars), Δ*rtcR*pRAM-19 (*rtcR*+) (gray), Δ*rtcR*pRAM-23 (rtc*BA*+) (dashed). Error bars denote standard deviation. Plots that differ in lowercase letter differ significantly by Tukey's HSD following a significant F-test ($n \geq 3$, $p < 0.05$).

4.3.4 Contribution of rtc genes in EHEC fitness and virulence

Quantitative real-time PCR (qRT-PCR) was used to validate expression of eight genes, of which four were upregulated (*grlR*, *cspA*, *stx2B* and *nleE*) and four were downregulated (*csgA*, *yafN*, *fimA* and *yhbO*) in TW14359Δ*rtcR* by RNAseq analysis. The expression of both up- and down-regulated gene sets by qRT-PCR was shown to be consistent with RNAseq (Fig. 4.3), and the magnitude of altered expression was highly correlated (r^2=0.96) (Fig. 4.3A). Since the *rtcR* mutant exhibited reduced survival in response to acid, peroxide, salt and heat stress, it stood to reason that the expression of genes involved in the protective response to the stressors may be correspondingly altered. This included the genes *gadE* (acid), *otsA* (salt), *rpoH* (heat) and *katE* (hydrogen peroxide). Even though, these key regulatory genes for stress responses were observed to be downregulated by RNAseq, they were below the critical cutoff for significance (~3-fold change). However, by qRT-PCR analysis of TW14359Δ*rtcR* compared to wild-type, these genes demonstrated a much larger and significant fold reduction in expression ($p<0.03$) (Fig. 4.3B). Complementation of both the *rtcR* and *rtcBA* mutants restored *gadE*, *otsA*, *rpoH* and *katE* expression to near WT levels, indicating that the *rtc* structural and enzymatic genes are involved in control of stress genes (Fig. 4.3B). One of the genes that was significantly upregulated in TW14359Δ*rtcR* was *grlR*, an important repressor of the locus of enterocyte effacement (LEE) (Table 4.2) (166). The LEE genes encode structural and effector proteins crucial for the EHEC T3SS and colonization of the human and animal host. Since *grlR* was upregulated in the *rtcR* mutant, it was predicted that LEE genes may be concomitantly downregulated in the *rtcR* background. As expected, the mRNA levels of LEE genes *ler*, *tir*, *espA* and *cesT* were uniformly reduced in TW14359Δ*rtcR* and TW14359Δ*rtcBA* backgrounds compared to TW14359 ($p<0.05$) (Fig. 4.3C). For all LEE genes, except *cesT*, expression was restored to near WT levels with

complementation (Fig. 4.3B). Altogether, this data shows that RtcR and RtcBA play an important role in the positive regulation of EHEC stress fitness and virulence genes.

4.3.5 RtcB/RtcA and RtcR influence EHEC virulence

To cause disease, EHEC must pass through the harsh acidic milieu of the upper gastrointestinal tract, and then colonize the lower gastrointestinal tract (4, 384, 422). After entry into the stomach, EHEC ceases growth, enters into stationary phase and activates expression of stress response genes. This allows survival of EHEC during passage through the gastric acid barrier of the stomach (pH 1-3) and determines a low oral infectious dose (<100 cells/ml) (76, 397). After entering the lower gastrointestinal tract, increased availability of nutrients, neutral pH and physiologic temperature signal EHEC to grow exponentially and colonize. Colonization is dependent on the LEE, which encodes a type III secretion system and several effector proteins, absolutely crucial for intimate interaction of EHEC with intestinal cells (102, 293). Therefore, both acid resistance and type III secretion mechanisms are vital for EHEC transmission and pathogenesis. Since deletion of *rtcR* and *rtcBA* negatively impacts both the stress response and LEE expression, it stands to reason that it may impact transmission and virulence of EHEC. To test this hypothesis, the ability of TW14359Δ*rtcR* and TW14359Δ*rtcBA* to adhere to HT-29 human intestinal cells, to kill *Galleria mellonella* (invertebrate wax worm model) and to survive inside macrophages was compared to wild-type TW14359.

First, the effect of *rtcR* and *rtcBA* deletion on adherence to human intestinal HT-29 epithelial cells was observed. Consistent with the pattern of LEE expression in these backgrounds, HT-29 adherence was significantly decreased in TW14359Δ*rtcR* and TW14359Δ*rtcBA* compared to TW14359 ($p < 0.05$) (Fig. 4.4C). Interestingly, even though the *rtcR* and *rtcBA* complement

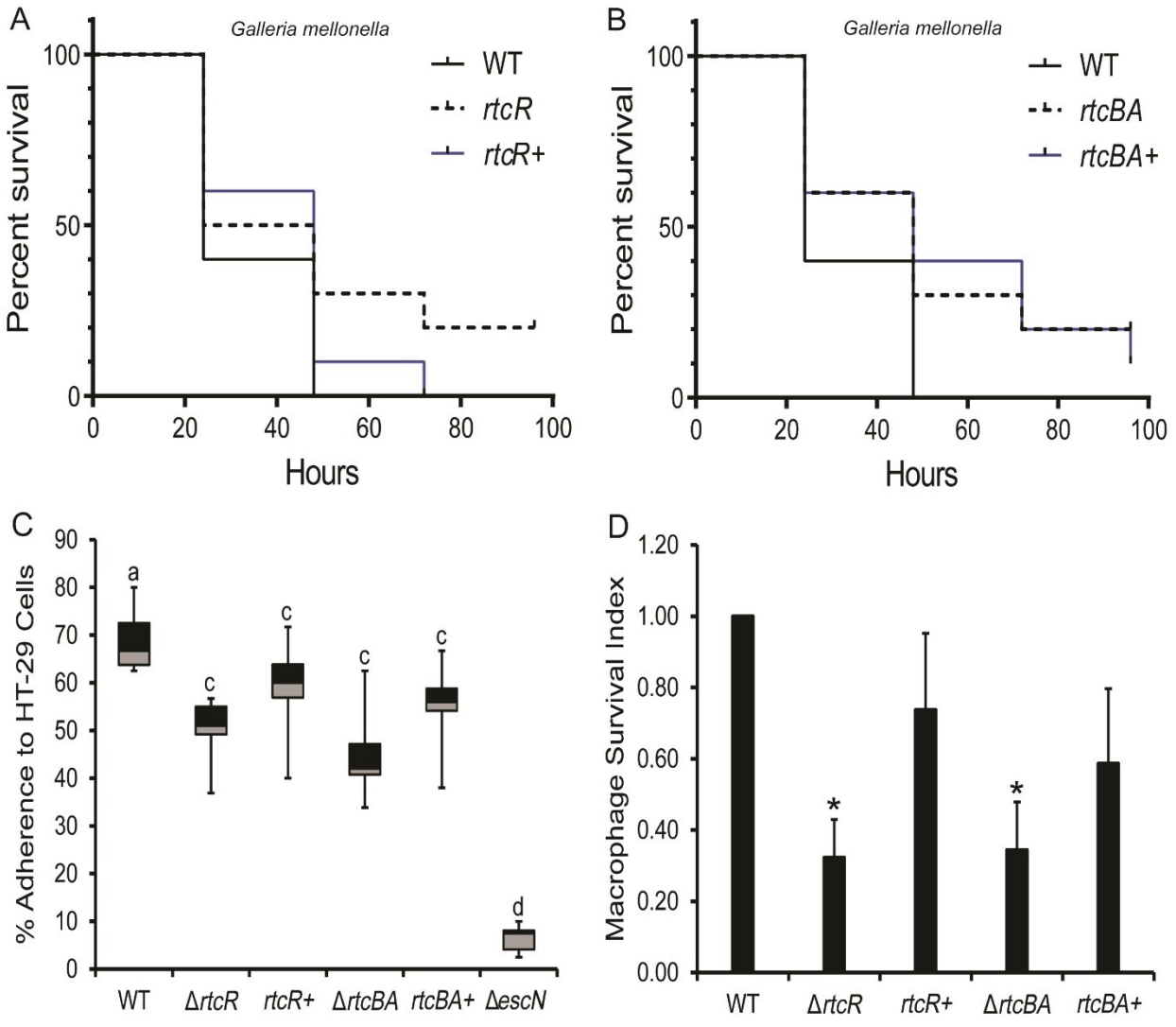

Figure 4.4. Effect of *rtcR* and *rtcBA* deletion on EHEC virulence. (A) Percent survival of *Galleria mellonella* (insects) after infection with WT (black), Δ*rtcR* (spotted) and Δ*rtcR*pRAM-19 (*rtcR*+) (blue). **(B)** Percent survival of *Galleria mellonella* (insects) after infection with WT (black), Δ*rtcBA* (spotted) and Δ*rtcBA*pRAM-23 (*rtcBA*+) (blue). **(C)** Percent adherence of WT, Δ*rtcR*, Δ*rtcBA*, Δ*rtcR*pRAM-19 (*rtcR*+), Δ*rtcR*pRAM-23 (rtc*BA*+) to HT-29 epithelial cells. **(D)** Survival of Δ*rtcR*, Δ*rtcBA*, Δ*rtcR*pRAM-19 (*rtcR*+) and Δ*rtcBA*pRAM-23 (*rtcBA*+) relative to WT within macrophage. For **(A)** and **(B)** percent survival of worm was plotted using Prism GraphPad (v6) and significant difference was determined using Mann-Whitney U-test ($n = 30$, $p < 0.05$). For boxplots **(C)**, boundaries represent the 25th and 75th percentiles, whiskers represent the maximum and minimum values and the median is given by the horizontal line. For **(D)** error bars denote standard deviation and asterisks denote significance by student's t-test ($n = 6$, $p<0.05$).

strains showed increased adherence to HT-29 cells, it was never restored completely to WT levels (Fig. 4.4C). Second, *G. mellonella* were inoculated with WT and mutant EHEC strains to determine their virulence. *G. mellonella* serves as a powerful animal infection model for many bacterial pathogens due to its advanced antimicrobial defenses (317). Therefore, it can provide important information regarding the ability of TW14359Δ*rtcR* and TW14359Δ*rtcBA* to colonize and infect. Each *G. mellonella* was infected with 10^5 CFU and their survival was monitored at 24 hour intervals. After 48 h, all *G. mellonella* infected with TW14359 were dead, whereas 30% of those infected with TW14359Δ*rtcR* survived, with 10% surviving the duration of experiment (96 h) (Fig. 4.4A). A nearly identical pattern of survival was observed for TW14359Δ*rtcBA* (Fig. 4.4B). While complementation with *rtcR* (strain TW14359Δ*rtcR*pRAM-19) partially restored virulence, with no *G. mellonella* surviving by 72 h (Fig. 4.4A), complementation with *rtcBA* (strain TW14359Δ*rtcBA*pRAM-23) did not (Fig. 4.4B).

To further determine the effect of rtcR/*rtcBA* deletion on virulence, a macrophage survival assay was performed. When EHEC colonizes the intestine, it is exposed to the underlying human macrophages and can be rapidly killed by them (359). Studies have shown that EHEC can survive within macrophages for up to 24 hours and during this time expression of stress genes (*soxRS*, *katE*, *gadA*) is significantly upregulated (304). Therefore, it was hypothesized that RtcR and RtcBA may play an important role for survival in macrophages due to their contribution to generalized stress fitness. As predicted deletion of both *rtcR* and *rtcBA* negatively impacted recovery of EHEC from macrophage. TW14359Δ*rtcR* and TW14359Δ*rtcBA* both exhibited a 3-fold reduction in recovery compared to TW14359 ($p < 0.03$) (Fig. 4.4D). Complementation with *rtcR* and *rtcBA* partially increased recovery of EHEC from macrophages (Fig. 4.4D).

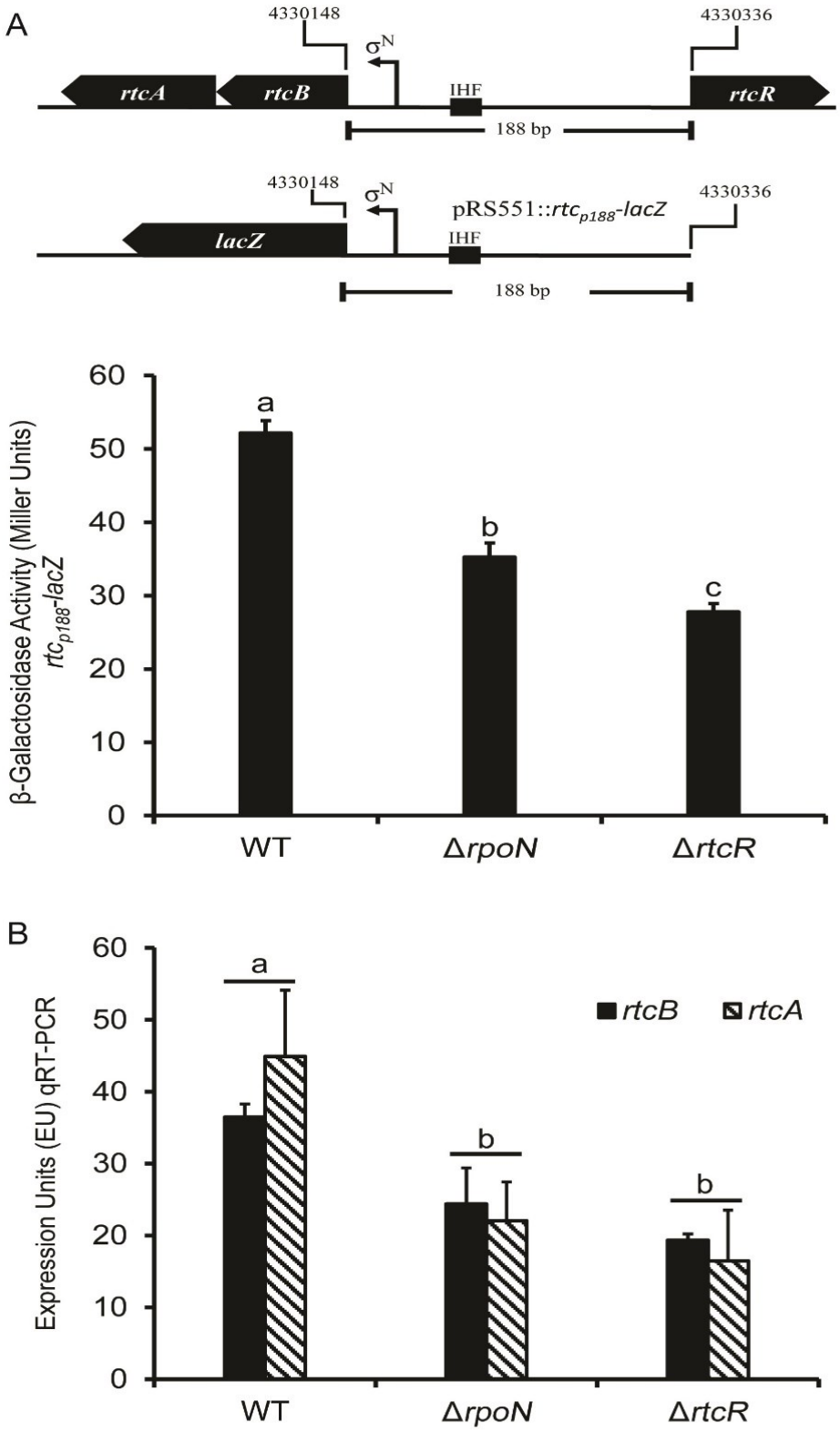

Figure 4.5. Analysis of *rtcB* and *rtcA* mRNA levels. (A) Mean expression from rtc_{P188}-*lacZ* represented as β-galactosidase activity from TW14359, TW14359Δ*rpoN* and TW14359Δ*rtcR*. **(B)** mRNA levels of *rtcB* (black) and *rtcA* (gray) in TW14359, TW14359Δ*rpoN* and TW14359Δ*rtcR*. Plots that differ in lowercase letter differ significantly by Tukey's HSD following a significant F-test ($n = 6$, $p < 0.05$). Error bars denote standard deviation.

4.3.6 RtcR phenocopies RpoN for expression of the rtc operon

The different stress fitness profile for TW14359Δ*rpoN* and TW14359Δ*rtcR* suggested the possibility of other σ^N-independent promoters upstream of the *rtc* operon controlling *rtcBA* expression. The impact of *rpoN* or *rtcR* mutation on *rtc* operon expression was determined by transforming pRAM-24 containing rtc_{p188}-*lacZ* (Fig. 4.5A top panel) into TW14359Δ*rpoN* and TW14359Δ*rtcR* and measuring β-galactosidase activity. Expression from rtc_{p188}-*lacZ* was significantly reduced in the *rpoN* mutant to ~73% ($p = 0.02$) of WT levels (Fig. 4.5A). Interestingly, rtc_{p188}-*lacZ* expression was further reduced in the *rtcR* mutant and even though it was not a marked difference, it was still statistically significant from the *rpoN* mutant ($p = 0.03$). As a result, it was hypothesized that this difference of promoter expression may also affect the expression of the *rtc* operon. However, expression of both *rtcB* and *rtcA* were unchanged in the TW14359Δ*rpoN* and TW14359Δ*rtcR* showing that the difference of survival in stressors for TW14359Δ*rpoN* and TW14359Δ*rtcR* is not due to differential expression of the *rtc* operon (Fig. 4.5B).

4.4 Discussion

The goal of this study was to explore the contribution of σ^N bEBPs to *E. coli* O157:H7 stress fitness and pathogenesis. It has been determined that the bEBPs NorR, PrpR, TyrR and RtcR are required for the full expression of stress fitness in EHEC. PrpR is responsible for σ^N-dependent activation of the *prp* operon which encodes genes necessary for propionate metabolism (214). Short term exposure to propionate and its metabolism has been shown to increase expression of many genes in the σ^S regulon and confer some sort of resistance to moderately acidic conditions (pH 5-6), but several orders higher than was tested in this study (305). In fact, metabolism of short chain fatty

acids like butyrate and acetate has been shown to induce acid resistance in EHEC, suggesting that propionate metabolism and PrpR may have some role in the acid resistance observed in this study (Fig. 4.1A) (120). NorR, which also positively affected acid resistance (Fig. 4.1A), has only previously been associated with *E. coli* response to nitric oxide stress (87). Whether there is any link between nitric oxide stress and acid resistance still remains unclear. This study also shows that deletion of *tyrR* reduced survival of EHEC in both acid and peroxide environment (Fig. 4.1A and B). TyrR is a very interesting protein in that it shares very little sequence similarity with any σ^N bEBPs. The absence of the key GAFTGA domain, which prevents it from associating with σ^N-holoenzyme, and its role in stimulation of σ^{70} promoters classifies TyrR as an atypical EBP (303). TyrR has so far only been identified in activation and repression of genes involved in aromatic amino acid synthesis and transport (302). It is well know that amino acids like glutamate, glutamine and arginine play key roles in *E. coli* acid resistance (70, 116, 118), but as to how aromatic amino acids or TyrR could affect these acid resistance mechanisms needs to be further explored. On the other hand, the basis for why *tyrR* deletion and overexpression affects peroxide resistance may be clearer. Genes of the TyrR regulon are involved in production of chorismate and enterobactin (126). Chorismate is required for synthesis of ubiquinone, which is a crucial component of the aerobic respiratory chain involved in reduction of reactive oxygen species. Therefore, deletion of *tyrR* could be influencing levels of ubiquinone resulting in a reduced recovery of TW14359Δ*tyrR* from peroxide. Alternatively, overexpression of *tyrR* may be causing increased production of enterobactin, a siderophore involved in iron acquisition. High intracellular levels of enterobactin could: I) lead to sequestration of iron from key enzymes and proteins within the cell rendering crucial enzymes inactive, or II) increase iron levels to toxic levels within the cell, producing more ROS through the Fenton reaction. In both cases, it could be detrimental to

the cell and explain why even if overexpression of *tyrR* increases chorismate production, it does not recover fitness to WT levels in peroxide (Fig. 1B).

This study demonstrates that RtcB, RtcA and RtcR positively influence stress fitness and virulence genes and phenotypes of EHEC. The deletion of *rtcR* and *rtcBA* negatively impact survival of *E. coli* O157:H7 during acid, peroxide, salt and heat stress. Decreased survival of the *rtcR* and *rtcBA* mutants in response to stress challenge was directly correlated to reduced mRNA levels of key regulatory genes of the *E. coli* stress response (Fig. 4.3B). Studies have shown that during environmental duress bacteria can respond to various stressors by inducing expression of ribonuclease or ribotoxin encoding genes belonging to toxin-antitoxin (TA) systems. Under normal conditions, these ribotoxins are mostly inactivated through direct binding of cognate antitoxins (125). During stress response, it has been suggested that the activated toxins can initiate programmed cell death or arrest cell growth through translation inhibition by direct cleaving of mRNA and tRNA (8, 79, 223, 272, 446). The toxins encoded from type II TA systems are all mRNA interfases that have been shown to directly inhibit translation through mRNA cleavage (79, 125, 140, 446). In *E. coli*, the extensively studied type II TA system RelEB is activated under various stress and starvation conditions which eventually causes a global reduction in translation by mRNA cleavage (80, 81, 282). An important fact about these mRNA interfases and ribonucleases is that their cleaving mechanism eventually results in generation of RNA 2'3'-cyclic phosphate and 5'-OH ends which later become crucial substrates for RNA repair mechanisms (8, 223, 272, 400). Following RNA cleavage, RNA cyclases create RNA 2'3'-cyclic phosphate ends, which serve as important substrates for RNA ligases to link to RNA 5'-OH ends to regenerate the 3'5'-phosphodisester RNA backbone.

RtcA (encoded by *rtcA*) is a RNA 3'-terminal phosphate cyclase that catalyzes a three step process of generating RNA 2',3'-cyclic phosphate ends important for RNA metabolism. In the first step, RtcA uses a divalent cation (Mn^{2+} or Mg^{2+}) and ATP to form the covalent RtcA-Amp intermediate. In the second step the adenylate from Rtc-Amp is transferred to the RNA 3'-phosphate terminus forming an activated phosphoanhydride intermediate. In the third step, the RNA 2'-OH attacks the 3'-phosphate of the intermediate to generate the RNA 2'3'-cyclic phosphate product (71, 124). This RNA 2'3'-cyclic phosphate product serves as important substrates for RNA ligases during tRNA/rRNA restriction repair and splicing. Interestingly, RtcB (encoded by *rtcB*) is a newly discovered family of RNA ligase that has a very unique mechanism of RNA ligation. After RtcA catalyzes formation of the RNA 2'3'-cyclic phosphate ends, RtcB uses its 2',3'-cyclic phosphodiesterase activity to break the cyclic phosphate end and creates a 3'-monophosphate end. RtcB then uses Mn^{2+} and GTP to form the covalent RtcB-guanylate intermediate where the GTP provides the energy for ligating the 3'-monophosphate end to a RNA 5'-OH end to restore the 3'5'-phosphodiester backbone (391-393). The crucial RNA repair functions of RtcB and RtcA have only been shown in vitro and the actual physiological context and contributions of RtcB and RtcA in *E. coli* had not been described. Many groups have proposed that the *rtc* operon may have an important role during stress response which produces RNA damage within the cell (391-393). Knowledge of the only known bacterial RNA repair system comes from the different bacteria that contain the *pnkP-hen1* operon encoding the PnkP ligase and Hen1 cyclase (72, 171). Since the PnkP-Hen1 operon is absent in *E. coli*, it strongly supports the proposed role for the *rtc* operon in *E. coli* RNA repair during stress response.

This study clearly shows that deletion of *rtcR* and *rtcBA* have a significant impact on survival of *E. coli* O157:H7 in response to various stressors. Decreased resistance to low pH from

rtcR and *rtcBA* deletion would make EHEC more vulnerable to the low pH within the host stomach, and reduce its chances of determining a low infectious dose. Similarly, the significantly reduced recovery from macrophages can be correlated to decreased resistance to peroxide in the *rtcR* and *rtcBA* mutants, which would suggest that the *rtcR* mutant would be more vulnerable to oxygen-dependent killing mechanisms within macrophages (Fig. 4.4D). Deletion of *rtcR* and *rtcBA* also negatively impact adherence to HT-29 intestinal epithelial cells and it may be partially due to reduced mRNA levels of LEE genes (Fig. 4.3C). Adherence in EHEC is also dependent on other factors like fimbriae, curli and flagella production. Interestingly, qRT-PCR for the major subunit of type I fimbriae, *fimA*, and the major subunit of curlin, *csgA*, has shown that they were both downregulated ~4-fold in TW14359Δ*rtcR* (Fig 4.3A). Therefore, the reduced adherence observed in the *rtcR* and *rtcBA* mutants could be more due to the combined effect of reduced curli and fimbriae genes expression than the LEE. The contribution of RtcR and RtcBA to EHEC virulence is further supported by observing killing of *Galleria mellonella*. Whereas, wild-type EHEC killed all worms by 48 h, approximately 10% of *G. mellonella* survived after being inoculated with the *rtcR* and *rtcBA* mutants (Fig. 4.4A and B), showing that RtcR and RtcBA is required for virulence potential in EHEC.

Another interesting observation is that TW14359Δ*rpoN* did not produce a similar phenotype as TW14359Δ*rtcR* for EHEC fitness in the stressors. In silico analysis has shown that RtcR has the functional features of σ^N bEBPs: a N-terminal regulatory domain, a conserved central AAA+ domain for ATP hydrolysis, and a C-terminal DNA binding domain. Moreover, in lab strain *E. coli*, RtcR has been shown to be necessary for σ^N-dependent transcription of the *rtc* operon (124). Thus, it would make sense that deletion of *rpoN* should at least produce a similar pattern of survival as the *rtcR* mutant in the stressors. However, the stress profile for TW14359Δ*rtcR*

mutant suggests that RtcR influences EHEC fitness in a manner independent of σ^N. This suggested that there could be a promoter in close proximity to the σ^N promoter, which may be repressed through σ^N binding. Therefore, absence of σ^N in TW14359Δ*rpoN* could derepress this promoter and allow expression of the *rtc* operon. Whereas in the *rtcR* mutant, the presence of σ^N would still repress this alternate promoter resulting in an overall reduced expression of the *rtc* operon. Analysis of the promoter region upstream of the *rtc* operon reveals that there is no difference in activation of the *rtc* operon between TW14359Δ*rpoN* and TW14359Δ*rtcR* (Fig. 5.5). Another possibility for this discrepancy could be from the effect of sigma factor competition. It is well known that alternative sigma factors have to compete with each other for association with RNAP because at any given point the amount of any sigma factor in the cell exceeds that of available core RNAP making the amount of functional RNAP an important rate limiting step (132, 300). Moreover, in stationary phase, the levels of free RNAP decreases to approximately 65% of that of log phase levels, making holoenzyme formation even more challenging for alternative sigma factors (192). In *E. coli*, σ^N has the highest affinity for core RNAP, second to the housekeeping sigma factor (σ^{70}), and its constant protein levels throughout all phases of growth make it much more challenging for other alternative sigma factors to form holoenzyme (173, 177, 229). Therefore, there are two factors that could explain why TW14359Δ*rtcR* does not perform as well as the TW14359Δ*rpoN*: 1) presence of σ^N and binding to core RNAP inhibits holoenzyme formation by other alternative sigma factors to activate expression of their regulons during stress, and 2) reduced levels of RtcBA results in an overall reduction in RNA repair following environmental stress. In the absence of σ^N in TW14359Δ*rpoN* would shift holoenzyme formation in favor of other alternative sigma factors and stimulate expression of stress response genes. However, following the same logic of sigma factor competition would also suggest that the *rpoN*

mutant should at least perform better than WT in response to all the stressors, which was not observed in the stress screen (Fig. 5.2). The functional independence of RtcR from σ^N presents an interesting paradox in σ^N-EBP regulation which needs to be further explored. Overall, the findings of this study supports the previously stated ideas that genes of the *rtc* operon are involved in *E. coli* stress response and play an important role in maintenance of cellular fitness through RNA repair.

4.5 Experiment Procedures

4.5.1 Bacterial strains and culture conditions

All strains and plasmids used in this study are listed in Table 4.3. Luria Bertani (LB) starter cultures were inoculated with a single colony of each strain and grown at 37°C with shaking (200 RPM). Unless otherwise indicated, these cultures were used to inoculate either LB or Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, St. Louis, MO) buffered with 50 mM MOPS and containing 0.4% (w/v) glucose. Cultures were grown for 18-20 h before inoculating into fresh LB or DMEM to a final OD_{600}=0.05, respectively, using a 1:10 ratio of media-to-flask volume and grown at 37°C, 200 RPM. Appropriate antibiotics were added to cultures as required.

4.5.2 Procedures for genetic manipulation

Nonpolar gene deletion mutants were constructed using the λ Red recombinase-assisted approach (92, 261) and as described (332). Primers used for the construction of deletion mutants are listed in Table 4.4. For complementation of isogenic mutants, fragment containing gene ORFs were created using primers listed in Table 4.4, and then cloned into the arabinose inducible vector

pBAD-TA (Invitrogen) to create pRAM-11 through pRAM-23. The constructs were then transformed into respective isogenic mutant strains to create the complement strains. All mutants and complements were validated through PCR and restriction mapping.

4.5.3 lacZ transcriptional fusions and β-galactosidase assay

A 188-bp *BamHI*/*EcoR*I digested PCR fragment, generated using primers Rtc_p-188/EcoRI and Rtc_p-1/BamHI (Table 4.4) and corresponding to nucleotide positions 43330148-4330336 in strain TW14359 (Fig. 4.5A top panel), was cloned into similarly digested vector pRS551 using T4-DNA ligase (Fisher) to create pRAM-24 (Table 4.3). This cloned fragment included 188-bp upstream of the translation initiation codon for *rtcB* (ECSP_4373) and the σ^N promoter transcriptionally fused to *lacZ* (rtc_{P188}-*lacZ*). pRAM-24 purified from DH5α transformants was used for transformation into TW14359, TW14359Δ*rpoN* and TW14359Δ*rtcBA* backgrounds. The rtc_{P188}-*lacZ* fusion was confirmed by PCR and sequencing. β-galactosidase activity from rtc_{P188}-*lacZ* was measured as previously described (251). Statistical rigor was determined by Tukey's HSD following a significant F-test ($n = 6$, $p < 0.05$).

4.5.4 Quantitate Real-Time PCR (qRT-PCR)

RNA purification, cDNA sysnthesis, qRT-PCR cycling conditions and data analysis for relative quantitation of gene expression followed previously described protocols (252, 256, 332). Analysis was performed using a Realplex2 Mastercycler (Eppendorf). Briefly, cycle threshold (C_t) data were normalized to *rrsH* (16S rRNA gene) and normalized C_t values (ΔC_t) were transformed to arbitrary gene expression units using $2^{-\Delta Ct}/10^{-6}$ as described (222). All primers for

qRT-PCR is listed in Table 4.4. Gene expression units were compared statistically between strains by Tukey's HSD following a significant F-test ($n = 3$, $p < 0.05$).

4.5.5 Stress fitness assays

Overnight LB cultures of all strains were inoculated into 10 ml LB at a 1:200 dilution and then grown to stationary phase ($OD_{600} = 3.0$) (Fig. S4.1). For complement strains, arabinose was added to a final concentration of 0.5% (w/v) and expression of bEBPs was induced for 10 min. Following induction, cultures were inoculated into stress test environments or just PBS (for controls) and then incubated at 37ºC with shaking (200 RPM). Test cultures were sampled for cell counts (CFU/ml) at specified times, and were serially diluted in PBS (pH 7.0) before plating to LBA for overnight incubation at 37ºC. For oxidative stress, strains were inoculated into PBS containing 20 mM hydrogen peroxide for 15 min before sampling. For salt and heat stress, strains were inoculated into PBS containing 1 M NaCl, or PBS preheated to 50C, respectively, and incubated for 1 h before sampling. For acid resistance, the glutamate-dependent system was tested as described previously (252, 332). For all experiments, CFU/ml data was transformed to mean percent survival and statistically compared using Tukey's HSD following a significant F-test ($n = 6$, $p < 0.05$).

4.5.6 Adherence assay

Adherence to epithelial cells was determined following a previously described protocol (256) with slight adaptations. Briefly, human HT-29 colonic epithelial cells were grown to confluence in 6 well culture plates at 37°C with 5% CO_2. Overnight DMEM cultures of strains were inoculated into fresh 10 ml DMEM and grown to exponential phase to $OD_{600} = 0.4$.

Expression of bEBPs was induced in complement strains as described above. Following induction, 0.05 ml of culture was used to inoculate each well containing 0.45 ml of sterile DMEM. After 4 h of static incubation, plate wells were washed five times with PBS to remove non-adherent bacteria and HT-29 cells in each well were lysed with 0.5 ml of 0.1% Triton X-100. Lysate was then serially diluted and plated on LBA for determining cell counts (CFU/ml). For all experiments, CFU/ml data was transformed to mean percent adherence and compared statistically by Tukey's HSD following a significant F-test ($n = 6$, $p < 0.05$).

4.5.7 Macrophage survival assay

The human monocyte cell line THP-1 (ATCC® TIB-202™) was used for determining survival of all bacterial strains within macrophage using the protocol of Poirier et al. (304). THP-1 cells were maintained in RPMI 1640 media containing 10% FBS, penicillin (100 µg/ml), and streptomycin (100 µg/ml) and subcultured every 2-3 days when cell counts reached 8 x 10^5 cells/ml. Macrophages were seeded at 10^5 cells/well in a 24 well tissue culture plate and a mature macrophage-like state was induced with 0.1 µM PMA (phorbol 12-myristate 13 acetate) for 48 h. Stationary phase cultures were added to each well at a multiplicity of infection of 10. After brief centrifugation ($t = 0$) the plate was incubated for 20 min for phagocytosis at 37ºC with 5% CO_2. Following phagocytosis, the medium was twice replaced with fresh RPMI containing gentamycin to kill all extracellular bacteria and incubated at 37ºC with 5% CO_2. The first gentamycin treatment was at 100 µg/ml for 2 h, and the second at 12 µg/ml for 1 h. All infected monolayers were then lysed with 0.1% Triton X-100 and the lysate was serially diluted and plated on LBA for determining cell counts (CFU/ml). For all experiments, CFU/ml data was transformed to mean

percent recovery. Initial (t=0) and final (CFU/ml) were converted to percent survival and significance was determined by Tukey's HSD following a significant F-test ($n = 6$, $p < 0.05$).

4.5.8 Virulence assays using Galleria mellonella

Virulence assays in *G. mellonella* were performed using fifth instar larvae purchased from Georgia Crickets (Winder, GA). Upon receipt, larvae were stored on wood chips at 4°C and only larvae free of melanization and injury were selected for use within 2 weeks. Overnight cultures were grown in DMEM to $OD_{600} = 0.5$, and where appropriate, expression of EBP genes was induced with arabinose as described above. Samples (1 ml) were pelleted by centrifugation (5,000 x g) and washed twice with 10 mM $MgSO_4$ with repeated centrifugation between washes, and finally re-suspended in 1 ml of 10 mM $MgSO_4$. After the wash, 5 µl of each re-suspension containing 10^5 CFU (previously determined through serial dilution plating) was inoculated in *G. mellonella* larva ($n = 30$) by hemolymph injection via the 4th posterior proleg using a 26s gauge, 10 µl Hamilton syringe (Hamilton Company, cat. 80366). Uninjected controls ($n = 10$) were also included in each trial. Inoculated larvae were incubated at 37°C in the dark for 96 h and the number of dead worms were recorded for each trial every 24 hours. At each assay time point, larvae were scored as dead if they did not respond to touch. Survival curves were created using the method of Kaplan and Meier in Prism GraphPad (version 6) and significant differences in survival between strains was determined using the Mann-Whitney U-test ($n = 30$, $p < 0.05$).

4.5.9 Transcriptomic analysis of TW14359ΔrtcR via RNAseq.

RNA was isolated from WT and mutant strains as previously described (251) from three independent cultures for both strains. Genomic DNA was removed from RNA samples with

DNAse I (Turbo DNA-free, Ambion) and concentration and purity of RNA was determined using an Agilent 2100 Bioanalyzer. Equimolar amounts of RNA, from each of the three replicate preparations, were pooled and ribosomal RNA was removed using Ribo-Zero (Epicentre) and MicrobExpress (Invitrogen) Kits and removal of ribosomal RNA was again confirmed using the bioanalyzer. The pooled RNA samples were then used for RNA-seq analysis using IonTorrent Total RNAseq kit v2 according to the manufacturer's instructions (Life Technologies). Templated Ion Sphere Particles were generated using the Ion OneTouch 200 Template kit v2 and sequencing was performed on an IonTorrent 318 chip using an Ion PGM 200 Sequencing kit. Data generated was exported to the CLC Genomics Workbench software package for analysis. Reads were aligned to the TW14359 genome (NC_013008, NCBI) and expression values for each gene determined as RPKM (Reads Per Kilobase of exon model per Million mapped reads) values. An RPKM threshold of detection value of 10 was imposed as a lower level cut off, and data were normalized using the quantile normalization approach (240). Genes demonstrating changes in expression lower than 2-fold were excluded from further analysis.

Conflict of Interest

The authors state that there are no conflicts of interest.

Table 4.1. Effect of bEBP deletion and overexpression on EHEC stress fitness.

	Percent Survival			
Strain	**Acid**	**Peroxide**	**Salt**	**Heat**
WT	85.0	67.4	66.0	92.5
Δ*fhlA*	81.0	70.0	68.0	90.0
fhlA+	87.0	67.0	68.0	88.0
Δ*hyfR*	80.0	65.0	60.0	94.0
hyfR+	82.0	62.0	60.0	90.0
Δ*norR*	*	67.0	70.0	88.0
norR+	*	68.0	68.0	96.0
Δ*ntrC*	88.0	60.0	60.0	85.2
ntrC+	80.0	60.0	65.0	96.0
Δ*prpR*	*	68.0	70.0	90.0
prpR+	*	72.0	70.0	90.0
Δ*pspF*	80.0	75.0	70.0	*
pspF+	90.0	72.0	73.0	*
Δ*tyrR*	*	*	59.8	85.8
tyrR+	*	*	64.3	89.6
Δ*qseF*	89.0	68.0	67.0	90.0
qseF+	87.0	63.0	60.0	91.0
Δ*ygeV*	88.0	66.0	73.0	90.0
ygeV+	89.0	67.0	69.0	87.0
Δ*zraR*	86.0	*	68.0	88.0
zraR+	80.0	*	67.0	90.0

[+] Isogenic bEBP mutants overexpressing bEBP *in trans* from inducible vector pBAD-TA.

* Missing data points are for strains that show significant effect on stress fitness and discussed in results (Fig. 4.1).

Table 4.2 Genes differentially regulated in TW14359Δ*rtcR*.

Gene	Function	Downregulated (Δ*rtcR*/WT)
Metabolism & Transport:		
abgA	Predicted glutamate metabolism protein	-4.26
aceA	Isocitrate lyase	-4.19
aceB	Malate synthase	-9.57
acs	Acetyl-CoA synthetase	-3.07
actP	Acetate/glycolate permease	-3.01
aldA	Aldehyde dehydrogenase, major metabolism protein	-3.44
argI	Arginine biosynthesis	-3.09
astA	Arginine biosynthesis	-4.99
astB	Arginine biosynthesis	-4.4
astC	Arginine biosynthesis	-3.4
astD	Arginine biosynthesis	-4.52
astE	Arginine biosynthesis	-3.57
cpsB	Colanic acid biosynthesis	-3.4
dctA	Dicarboxylate transporter	-3.21
ddpB	RpoN-dependent predicted component of ABC transporter	-14.3
ddpC	RpoN-dependent predicted component of ABC transporter	-3.94
ddpD	RpoN-dependent predicted component of ABC transporter	-5.79
fadA	Fatty acid catabolism	-4.57
fadB	Fatty acid catabolism	-5.25
fadE	Acyl-CoA dehydrogenase	-3.65
fadH	Fatty acid metabolism	-3.88
fadI	Fatty acid metabolism	-6.25
frdB	Fumurate reductase iron-sulfur protein	-3.52
fruA	Fructose PTS permease	-3.57
fruB	Fructose PTS permease	-16.94
fruK	Phosphofrucktokinase	-3.32
gabP	Amino acid, polyamine transporter	-3.15
galS	Galactose transport and catabolism repressor	-7.01
hcaR	Phenylpropionate catabolism	-5.7
hybG	Chaperon protein for active hydrogenase 2	-4.26
lacI	Repressor of *lac* operon for lactose utilization	-6.86
lamB	Sugar porin for diffusion of maltose and maltodextrins	-4.57
lldP	Inner membrane permease for lactate and glycolate	-4.72
lldR	Transcriptional repressor of lactate transport and catabolism	-3.03
lsrA	ATP binding subunit of AI-2 ABC transporter	-3.5
lsrB	Periplasmic binding protein of AI-2 ABC transporter	-3.01
lsrC	Innermembrane subunit of AI-2 ABC transporter	-13.66
lsrF	Predicted class I aldolase	-3.14
lsrG	AI-2 degrading protein	-8.44
lsrK	AI-2 kinase	-3.63
lysA	Lysine biosynthesis	-4.92
malE	Periplasmic binding protein of maltose ABC transporter	-3.94
malF	Membrane subunit of maltose ABC transporter	-4.24

Table 4.2 (continued) Genes differentially regulated in TW14359Δ*rtcR*.

Gene	Function	Downregulated (Δ*rtcR*/WT)
Metabolism & Transport:		
malG	Membrane subunit of maltose ABC transporter	-5.2
malK	ATP binding subunit of maltose ABC transporter	-6.76
malM	Maltose regulon periplasmic binding protein	-3.69
malX	Maltose/glucose PTS permease	-3.61
manX	Subunit of mannose PTS permease	-4.15
manY	Subunit of mannose PTS permease	-4.8
manZ	Subunit of mannose PTS permease	-3.43
melA	Utilization of α-galactosides as nutrients	-3.52
mglA	ATP binding subunit of galactose ABC transporter	-3.94
mglB	Periplasmic binding subunit of galactose ABC transporter	-6.02
mglC	Membrane subunit of galactose ABC transporter	-3.98
prpR	Propionate metabolism genes transcriptional activator	-4.99
nrdG	Anaerobic growth	-4.91
rihA	Cytidine and uridine ribonuclease hydrolase	-3.23
sfsB	Regulator of genes of maltose metabolism	-5.02
ugpB	Component of glycerol-3-phosphate ABC transporter	-4.47
ulaB	L-ascorbate PTS permease	-6.44
uxuB	D-glucouronate catabolism	-5.41
yahN	Predicted neutral amino acid efflux pump	-5.19
ybdK	Carboxylate-amine ligase	-3.43
ydcS	Periplasmic binding protein of spermidine/putrescine ABC transporter	-3.65
ydcT	Subunit of spermidine/putrescine ABC transporter	-3.31
ydeN	Putative sulfatase	-5.89
yedL	Predicted acyltransferase	-4.97
yfdZ	Glutamate-pyruvate aminotransferase	-3.78
ygaT	Unknown carbon starvation induced gene	-3.98
yhhY	Predicted acetyltransferase	-46.7
yidE	Predicted transporter	-3.11
ykgE	Predicted oxidoreductase	-5.48
yqeF	Predicted acyltransferase	-3.51
ytfR	ATP binding subunit of galactofuranose/glactopyranose ABC transporter	-3.55
Stress Response:		
csiE	RpoS-dependent stationary phase inducible protein	-3.11
gamW	Host nuclease inhibitor protein	-3.41
priC	Primosome required for restart of stalled replication form	-3.06
uspF	Class II universal stress protein and fimbrial adhesion	-3.7
yafN	Antitoxin for mRNA interfase toxin YafO	-5.36
yehZ	Periplasmic binding protein of ABC transporter for osmoprotection	-3.22
yfiL	Promotes stress-induced mutagenesis repair	-3.42
yghZ	L-glyceraldehyde 3-phosphate reductase for methlyglyoxal detoxification	-5.73
yhbO	NaCl induced stress response protein	-7.14
yncN	HicA toxin of the HicA-HicB toxin-antitoxin system	-32.27

Table 4.2 (continued) Genes differentially regulated in TW14359Δ*rtcR*.

Gene	Function	Downregulated (Δ*rtcR*/WT)
Adherence:		
afuA	Periplasmic ferric iron-binding protein	-3.76
cedA	Cell division	-8.32
csgA	Major curlin subunit	-4.22
fimA	Major type I fimbriae subunit	-3.99
fliL	Inner membrane associated flagellar biosynthesis component	-5.52
putA	Transcriptional repressor flavoprotein	-3.04
ybgD	Fimbrial-like adhesin protein	-4.64
Lipoproteins:		
blc	Outermembrane lipoprotein	-3.08
yaiY	Inner membrane protein	-3.19
yaiZ	Predicted inner membrane protein	-79.55
ybhQ	Predicted inner membrane protein	-3.36
yfdY	Biofilm associated protein	-3.11
Unknown/Hypothetical:		
phnB	Unknown	-3.32
ydcJ	Unknown	-4.96
yeaH	Unknown	-3.28
yeaL	Unknown	-4.12
yegP	Unknown	-3.27
yghA	Unknown	-3.13
yhaL	Unknown	-3.69
yibH	Unknown	-4.1
yibT	Unknown	-3.06
yicS	Unknown	-5.12
yneG	Unknown	-3.52
ynfD	Unknown	-3.88
yraR	Unknown	-4.03

Table 4.2 (continued) Genes differentially regulated in TW14359Δ*rtcR*.

Gene	Function	Upregulated (Δ*rtcR*/WT)
borW	Bor protein precursor	4.3
creA	Unknown	3
cspA	Major cold shock protein	5.06
cspE	Transcription antiterminator, regulator of RNA stability	3.87
cspG	Cold shock protein	5.87
dinI	Positive modulator of RecA	3.24
dsrB	Unknown	3
ecpR	(MatA) regulator of planktonic/sessile lifestyle	3.84
escI	Predicted type III secretion component	3
grlR	Negative regulator of LEE	3.02
hha	Catabolite repression	3.19
holE	Theta subunit of DNA polymerase	4.28
ilvC	Isoleucine biosynthesis	6.68
kil1	Host killing protein	5.47
ndh	Aerobic respiration	7.25
nleE	Non-LEE encoded effector protein	3.88
nsrR	Nitric oxide stress	3.15
orf16	Predicted type III secretion component	15.25
orf4	Predicted type III secretion component	4.38
pabA	Glutamine amidotransferase activity	4.03
pdhR	Pyruvate dehydrogenase complex regulator	9.75
ppdB	Unknown	3.09
rplV	L22 protein, component of 50S ribosome subunit	3.17
rpmD	L30 protein, component of 50S ribosome subunit	6.17
rpmG	L33 protein, component of 50S ribosome subunit	7.66
rpmH	L34 protein, component of 50S ribosome subunit	6.04
rpmJ	L36 protein, component of 50S ribosome subunit	4.27
rpsL	S12 protein, component of 30S ribosome subunit	3.19
sepD	LEE-encoded type III secretion component	15.38
stx2B	Shiga toxin II subunit B	8.88
tatA	Export of folded protein across inner membrane	3.29
waaL	O antigen ligase	4.39
wbdN	Glycosyl transferase	3.4
wzy	O antigen polymerase	3.26
yacG	DNA gyrase inhibitor	5.79
ybfA	Isochorismate synthase	4.15
ybhT	Predicted membrane transport, cell envelope stress	6.8
ybiJ	Unknown	5.8
ydiE	Unknown	3.64
ydiH	Unknown	8.62
yeaS	Leucine exporter	3.18
yecJ	Unknown	3
yedR	Predicted inner membrane protein	3
yeeN	Unknown	3.05

Table 4.2 (continued) Genes differentially regulated in TW14359Δ*rtcR*.

Gene	Function	Upregulated (Δ*rtcR*/WT)
yfgG	Unknown	4.23
ygjN	mRNA interfase, cell growth inhibitor, stress response	8.52
yhbY	Predicted RNA binding protein	3.08
yhdL	Ribosome rescue factor	5.55
yheV	Unknown	6.29
yiiF	Unknown	6.94
yjbJ	Predicted inner membrane protein	5.24
yjcB	Predicted inner membrane protein	13.59
yjeT	Conserved inner membrane protein	5.62
yliE	c-di-GMP-specific phosphodiesterase	3.06
ynjI	Predicted inner membrane protein	5.89
yqgC	Unknown	3.67
yrdB	Unknown	4.85

Table 4.3 Strains and plasmids used in this study

Strain/Plasmid	Relevant characteristics	Source/reference
Strain name:		
DH5α	Vector propagation, *recA1 endA1*	Mitra *et al*. 2012
TW14359	WT 2006 outbreak, western US	This study
EcRPF-6	TW14359Δ*rpoN*, Glutamine auxotroph	Mitra *et al*. 2012
EcRAM-25	TW14359Δ*fhlA*	Mitra *et al*. 2012
EcRAM 86	TW14359Δ*hyfR*	This study
EcRAM 87	TW14359Δ*norR*	This study
EcRAM-26	TW14359Δ*ntrC*	Mitra *et al*. 2012
EcRAM 88	TW14359Δ*prpR*	This study
EcRAM 27	TW14359Δ*pspF*	This study
EcRAM 28	TW14359Δ*qseF*	This study
EcRAM 89	TW14359Δ*rtcR*	This study
EcRAM 90	TW14359Δ*tyrR*	This study
EcRAM 29	TW14359Δ*ygeV*	This study
EcRAM 91	TW14359Δ*zraR*	This study
EcRAM 85	TW14359Δ*rtcBA*	This study
EcRAM 92	EcRPF-6 pRAM-11 Amp^R	This study
EcRAM 93	EcRAM-25 pRAM-12 Amp^R	This study
EcRAM 94	EcRAM-86 pRAM-13 Amp^R	This study
EcRAM 95	EcRAM-87 pRAM-14 Amp^R	This study
EcRAM 96	EcRAM-26 pRAM-15 Amp^R	This study
EcRAM 97	EcRAM-88 pRAM-16 Amp^R	This study
EcRAM 98	EcRAM-27 pRAM-17 Amp^R	This study
EcRAM 99	EcRAM-28 pRAM-18 Amp^R	This study
EcRAM 100	EcRAM-89 pRAM-19 Amp^R	This study
EcRAM 101	EcRAM-90 pRAM-20 Amp^R	This study
EcRAM 102	EcRAM-29 pRAM-21 Amp^R	This study
EcRAM 103	EcRAM-91 pRAM-22 Amp^R	This study
EcRAM 104	EcRAM-85 pRAM-23 Amp^R	This study
EcRAM 105	EcRPF-6 pRAM-24 Amp^R	This study
EcRAM 106	EcRAM-89 pRAM-24 Amp^R	This study
Plasmid name:		
pRS551	*lacZ* fusion vector, AmpR KanR *lacZ*+ ColE1	
pBAD-TA	Mid copy arabinose inducible cloning vector, AmpR	Inivitrogen
pRAM-11	*rpoN*::pBAD Amp^R	This study
pRAM-12	*fhlA*::pBAD Amp^R	This study
pRAM-13	*hyfR*::pBAD Amp^R	This study
pRAM-14	*norR*::pBAD Amp^R	This study
pRAM-15	*ntrC*::pBAD Amp^R	This study
pRAM-16	*prpR*::pBAD Amp^R	This study
pRAM-17	*pspF*::pBAD Amp^R	This study
pRAM-18	*qseF*::pBAD Amp^R	This study
pRAM-19	*rtcR*::pBAD Amp^R	This study
pRAM-20	*tyrR*::pBAD Amp^R	This study
pRAM-21	*ygeV*::pBAD Amp^R	This study

Table 4.3 (continued) Strains and plasmids used in this study

Strain/Plasmid	Relevant characteristics	Source/reference
Plasmid name:		
pRAM-22	*zraR*::pBAD Amp^R	This study
pRAM-23	*rtcBA*::pBAD Amp^R	This study
pRAM-24	pRS551 containing rtc_{p188}-*lacZ* fusion	This study

Table 4.4 Primers used in this study

Primer Name	Sequence (5'→3')	Source/reference
qRT-PCR:		
gadE+309	TGGTAAACACTTGCCCCATA	Mitra *et al*. 2012
gadE+419	AGCGTCGACGTGATATTGCT	Mitra *et al*. 2012
ler+109	CGAGAGCAGGAAGTTCAA	Mitra *et al*. 2012
ler+214	GTCCATCATCAGGCACAT	Mitra *et al*. 2012
tir+664	ACTTCCAGCCTTCGTTCAGA	Mitra *et al*. 2012
tir+869	TTCTGGAACGCTTCTTTCGT	Mitra *et al*. 2012
cesT+296	TCCCTCTCGATGATGCTACC	Mitra *et al*. 2012
cesT+445	TGTCGCTTGAACTGATTTCCT	Mitra *et al*. 2012
espA+128	AGGCTGCGATTCTCATGTTT	Mitra *et al*. 2012
espA+310	GAAGTTTGGCTTTCGCATTC	Mitra *et al*. 2012
rtcB+128	TGCGGTAATGCCTGATGTAC	This study
rtcB+249	TAGTGCGTTCATTCCACAGC	This study
rtcA+518	GGTGGCATCGTTTAACACCT	This study
rtcA+684	ATATTCATAACCTGCCGCGC	This study
katE+734	TGCAACCTGAAACTCTGCAC	This study
katE+897	TTTACCTGCCAGTGGTTTCC	This study
otsA+130	GGTGAAACAGGGAATGAGGA	This study
otsA+287	ACCAGATCGAGGCGATAATG	This study
rpoH+110	GGCTGAAAAGCTGCATTACC	This study
rpoH+261	CATCAGGCCGATGTTACCTT	This study
grlR-RT/F	AAGACTCCTGTGGGGAAGGT	This study
grlR-RT/R	GGACATGAAGTATGATGTCC	This study
stx2b-RT/F	GGCGGATTGTGCTAAAGGTA	This study
stx2b-RT/R	GCACTTTGCAGTAACGGTTG	This study
cspA-RT/F	TTCATCACTCCTGACGATGG	This study
cspA-RT/R	TTACAGGCTGGTTACGTTGC	This study
nleE-RT/F	CTAGGAGAACAACGGGCAAA	This study
nleE-RT/R	TATTTCCCCAGGCATGTAGC	This study
cspA-RT/F	TGCAAGCTGATGCTCGTAAC	This study
cspA-RT/R	GCTGTTACCAAAGCCACGTT	This study
fimA-RT/F	ATCGTTGTTCTGTCGGCTCT	This study
fimA-RT/R	GTACGAACCTGTCCTAACTG	This study
yafN-RT/F	CTCTTAAGTGCCAGCGCATT	This study

Table 4.4 (continued) Primers used in this study

Primer Name	Sequence (5'→3')	Source/reference
qRT-PCR:		
yafN-RT/R	CTCGGCGTGTAATTTCCTCT	This study
yhbO-RT/F	ACCTGTTTTCGCCATCTGTC	This study
yhbO-RT/R	CCACGACTTCCTGATCGTAA	This study
Cloning:		
rpoN–F-TA	GAGGAATAATAAATGAAGCAAGGTTTGCAACT	This study
rpoN–R-TA	TCATCAAACGAGCTGTTTACGCT	This study
fhlA-F-TA	GAGGAATAATAAATGTCATATACACCGATGAG	This study
fhlA-R-TA	TCATTAAATCAATGCCGATTTAT	This study
hyfR–F-TA	GAGGAATAATAAATGGCTATGTCAGACGAGGC	This study
hyfR–R-TA	TCATTACAACACCTCGCGAACCG	This study
norR–F-TA	GAGGAATAATAAATGAGTTTTTCCGTTGATGT	This study
norR–R-TA	TCATTAATCCTTCAATCCCAGAC	This study
ntrC–F-TA	GAGGAATAATAAATGCAACGAGGGATAGTCTG	This study
ntrC–R-TA	TCATCACTCCATCCCCAGCTCTT	This study
prpR–F-TA	GAGGAATAATAAATGGCACATCCACCACGGCT	This study
prpR–R-TA	TCATCAGCTTTTCAGCCGCCGCC	This study
pspF–F-TA	GAGGAATAATAAATGGCAGAATACAAAGATAA	This study
pspF–R-TA	TCACTAAATCTGGTGCTTTTTCA	This study
qseF–F-TA	GAGGAATAATAAAGCCATAAACCTGCGCATTT	This study
qseF–R-TA	TCATCATTCCTTGAAATCGTTTG	This study
rtcR–F-TA	GAGGAATAATAAATGCGTAAAACAGTGGCTTT	This study
rtcR–R-TA	TCATCAACTGGAGCTGTGCTGAT	This study
tyrR–F-TA	GAGGAATAATAAATGCGTCTGGAAGTCTTTTG	This study
tyrR–R-TA	TCATTACTCTTCGTTCTTCTTCT	This study
ygeV–F-TA	GAGGAATAATAAATGGAGCTTGCTACTACGCA	This study
ygeV–R-TA	TCATTATGTGTTTAACAACTCAT	This study
zraR–F-TA	GAGGAATAATAAATGACGCACGATAATATCGA	This study
zraR–R-TA	TCACTAACGCGACAATTTTGCCA	This study
RtcBA-F-TA	GAGGAATAATAAATGAATTACGAATTACTGAC	This study
RtcBA-R-TA	TCATCAATCAGTGAGTTTGGTTA	This study
rtcB-187/EcoRI	gccGAATTCcttagatatccttataaaag	This study
rtcB-1/BamH1	gcGGATCCtttgtttttctcttttcgtt	This study
Deletion:		
hyfR-43/P1	TTCTCATTAATAAGGACTGTTGATGGCTATGTCAGACGAGGTGTAGGCTGGAGCTGCTTC	This study
hyfR+2004/P2	TGCAAAAGCAGATTACAACACCTCGCGAACCGAGATCCCCCATATGAATATCCTCCTTAG	This study
norR-107/P1	ACCTCAATTTATTCAGCGTGTTCTAAAAAGATGTCTTGCTGTGTAGGCTGGAGCTGCTTC	This study
norR+1510/P2	AGTTGTGATGATTTTGTGCCAGTGCCTGACGAATAGTTTCCATATGAATATCCTCCTTAG	This study
prpR-117/P1	TAATCCGCAAATATGCGTTTCAGTTAACGTTTCAGGCAATGTGTAGGCTGGAGCTGCTTC	This study
prpR+1676/P2	CCTATGTAAACATCCCCGATGCGTAAGTTTATCGGTGATCCATATGAATATCCTCCTTAG	This study
pspF-41/P1	GCAACATGCCAGGATGAATTAGCTAATTACACTAACAAGTGTGTAGGCTGGAGCTGCTTC	This study

Table 4.4 (continued) Primers used in this study

Primer Name	Sequence (5'→3')	Source/reference
Deletion:		
pspF+1029/P2	CACGCCGCATCCGGCAAGTTGTATTGCCCAACTTCGCTAACATATGAATATCCTCCTTAG	This study
rtcR-83/P1	TTATATCTTTACGTCCGTAAACGGAGATTTCCCGCAAAGCGTGTAGGCTGGAGCTGCTTC	This study
rtcR+1564/P2	ACGTCAGACCAAAACGCGCCAGGTATTTGCGTAGCCGATCCATATGAATATCCTCCTTAG	This study
tyrR-94/P1	TCTTTGTGTCAATGATTGTTGACAGAAACCTTCCTGCTATGTGTAGGCTGGAGCTGCTTC	This study
tyrR+1523/P2	TGGCTTAAGCCATATTCCCGCAACTTATTGGCAATCGCGGCATATGAATATCCTCCTTAG	This study
qseF+20/P1	ATTTATTATTGGTCGATGACGATCCGGGATTGCTGAAACTGTGTAGGCTGGAGCTGCTTC	This study
qseF+1333/P2	ATTCCTTGAAATCGTTTGCATCCAGCTCGTGTCGGGAAACATATGAATATCCTCCTTAG	This study
ygeV-77/P1	GAGTTAATATGATCATGATCTGTGAACCATCAACGTCTTCGTGTAGGCTGGAGCTGCTTC	This study
ygeV+1806/P2	CCTGAATTCAGGCCGGATTCACTGATGTTATGTGTTTAACCATATGAATATCCTCCTTAG	This study
zraR-14/P1	TATCGATATTCTGGTGGTGGATGATGACATTAGCCACTGCGTGTAGGCTGGAGCTGCTTC	This study
zraR+1312/P2	TTGCCAACAGCGTTTTGCGCGTGATCCCTAACTGACGGGCCATATGAATATCCTCCTTAG	This study
rtcB-57/P1	TTCTGGCACGACGGTTGCAATTATCAGGACCGCAAACAACGTGTAGGCTGGAGCTGCTTC	This study
rtcA+1077/P2	TTACCTCTACCGGATAGTCACACTGATGAGTCTGATCCGGCATATGAATATCCTCCTTAG	This study

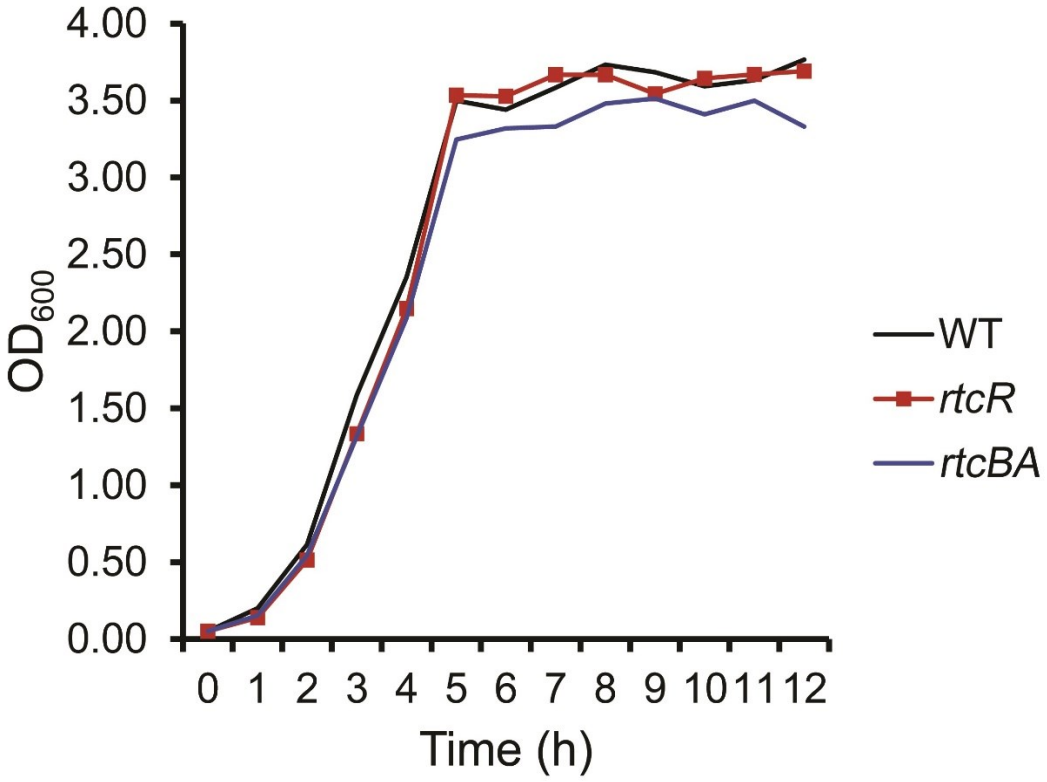

Figure S4.1. Growth of strains in LB. Mean (n=2) optical density 600 nm (OD_{600}) plotted for TW14359 (black), TW14359Δ*rtcR* (squares), TW14359Δ*rtcBA* (blue). Individual OD_{600} measurements for each strain varied by less than 5%. For stress fitness assays, sampling was done for all strains at OD_{600} = 3.00 approximately corresponding to early stationary phase. For all remaining experiments, sampling was done at OD_{600}=0.5.

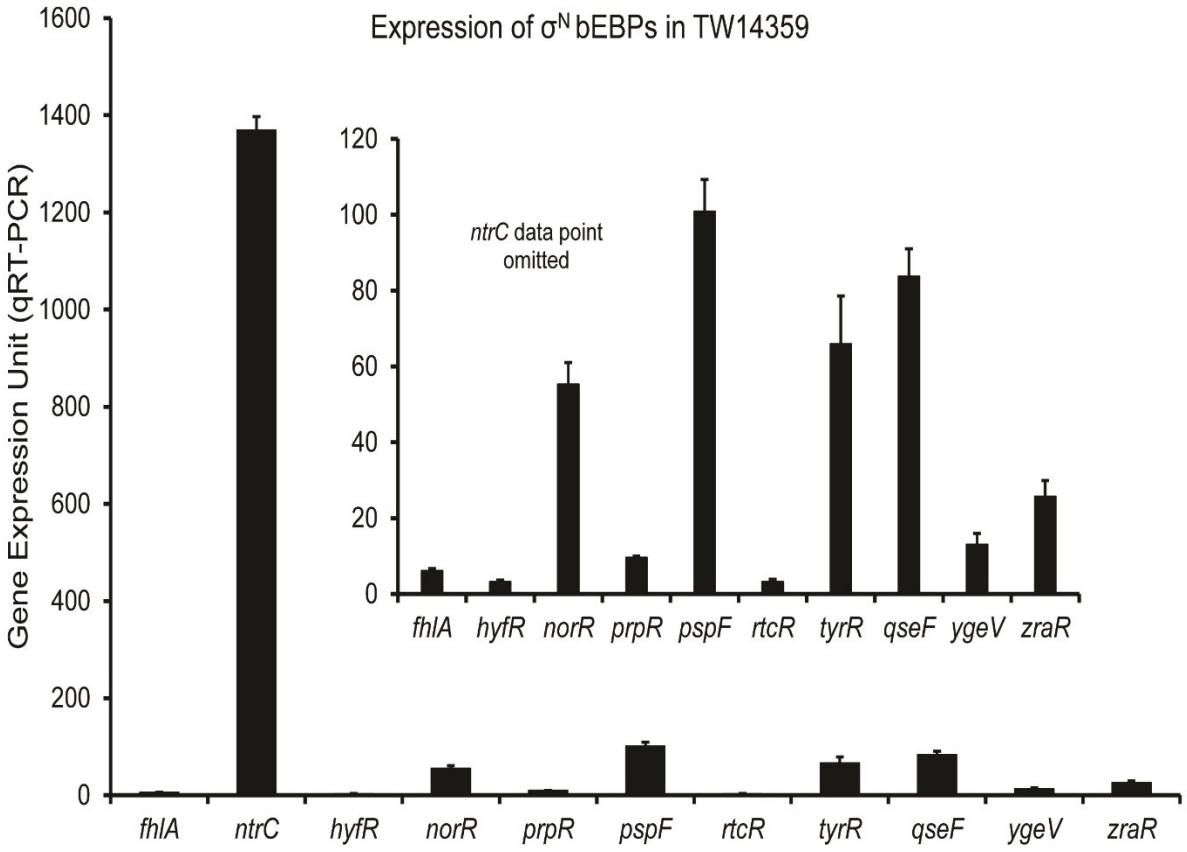

Figure S4.2. Expression of σ^N bEBPs in TW14359. Expression of bEBPs observed during mid-exponential phase in LB. Most bEBPs have low basal expression under normal growth. Inset graph shows bEBP expression with *ntrC* datapoint removed.

Chapter Five: Purpose, Conclusions, Implications and Recommendations

5.1 Purpose of This Research Study

Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 is a serious food and water borne pathogen, responsible for outbreaks of bloody diarrhea (hemorrhagic colitis), severe acute anemia and the life threatening illness hemolytic uremic syndrome (HUS) (232, 245, 422). To cause disease in humans, EHEC is completely dependent on extreme acid resistance (XAR) mechanisms to pass through the harsh acidic milieu of the upper gastrointestinal tract, and the locus of enterocyte effacement (LEE) to colonize the lower gastrointestinal tract (4, 384, 422). As such, XAR and the LEE systems are vital for EHEC virulence and pathogenesis. Riordan *et al*. identified that the gene *rpoN* (encoding σ^{54}) modulates XAR and the LEE in a negative and positive manner, respectively, during exponential growth (333). It was also demonstrated that deletion of *rpoN* (encoding σ^{54}) increases survival of EHEC in acid, but significantly affects colonization revealing the important contributions of σ^{54} in EHEC virulence and transmission. There is precedent for this because in recent years σ^{54} has also been implicated to be an important determinant of virulence and transmission, and to be required for complete *in vivo* disease progression in many other pathogens like *Borrelia*, *Pseudomonas*, *Vibrio*, *Salmonella* and *Erwinia* (7, 20, 88, 165, 249, 274, 358, 414). While the molecular basis of σ^{54}-directed regulation of virulence mechanisms are well understood in these pathogens, σ^{54}-directed regulation of EHEC pathogenesis was not known. This study sought to delineate the molecular basis of σ^{54}-directed regulation of XAR and the LEE and the environmental factors that influence this regulation in EHEC.

5.2 Conclusions of This Research Study

This study has conclusively shown that σ^{54} is an important determinant of EHEC virulence and transmission. Even though deletion of *rpoN* significantly increases survival of EHEC in extreme acid environments, it also at the same time significantly impairs the ability of EHEC to attach to human intestinal epithelial cells. Thus, it was hypothesized that in WT EHEC during exponential phase, σ^{54} acts as a repressor of XAR and activator of the LEE. It has been clearly proven that σ^{54} is dependent on an intact *rpoS* gene, encoding the alternative sigma factor S (σ^S), and the interplay of sigma factors between σ^{54} and σ^S provide the basis of σ^{54}-directed control of XAR and the LEE. Increased stability of σ^S upon deletion of *rpoN* suggested that σ^{54} regulates σ^S at the posttranslational level. Modulation of σ^S stability by σ^{54} could happen in two possible ways: I) either through a passive competition for core RNAP, where absence of σ^{54} allows σ^S to bind core RNAP, protecting it from proteolytic cleavage, or II) through an active mechanism, where σ^{54} controls some factor that influences σ^S stability. Complementation of TW14359Δ*rpoN* with a mutant σ^{54} allele that binds core RNAP and is defective in activating transcription demonstrated that the increased σ^S stability is not due to competition for core RNAP. Whether the stability of σ^S has actual relevance to σ^{54}-dependent control of XAR and the LEE is still inconclusive because the two systems could not be modulated just by influencing σ^S stability (251). Therefore, it was hypothesized that σ^{54} was probably influencing σ^S activity towards XAR and LEE promoters through some other factor. This proved to be true as deletion of *fliZ*, encoding an antagonist of σ^S activity, reproduced a similar phenotype as the *rpoN* mutant, which could again be restored to WT levels through complementation with *fliZ*. It was further demonstrated that σ^{54} requires the master

regulator protein FlhDC to regulate production of FliZ, which led to the discovery of the σ^{54}-FlhDC-FliZ-σ^{S} cascade that controls XAR and the LEE in EHEC.

To determine the environmental signals that influence σ^{54}/σ^{S} dependent regulation of XAR and the LEE, it was important to define the activator protein that works in conjunction with σ^{54} for regulation of this pathway. σ^{54} is an unique sigma factor in that it is completely dependent on activator proteins called enhancer binding proteins (bEBPs) for open complex formation at promoters and transcription initiation. These bEBPs bind upstream activating sequences (UAS) and through ATP hydrolysis provide σ^{54} the energy required for open complex formation and transcription initiation (381, 423, 426). The bEBPs have a variable N-terminal sensory domain containing different motifs that recognize extra- and intracellular cues to activate σ^{54} transcription. In EHEC there are 11 bEBPs that work in tandem with σ^{54} for regulation of the σ^{54} regulon. Thus, logic dictated that at least one of these bEBPs must be required for σ^{54}-directed regulation of XAR and the LEE. Moreover, identification of the bEBPs involved in the σ^{54}/σ^{S} pathway would also provide insight into the activating signals for the pathway. As hypothesized, deletion of the bEBP NtrC, modulated XAR and the LEE in a manner which was a phenocopy of the *rpoN* mutant. This was extremely insightful as σ^{54} and NtrC are two well-known crucial regulators of genes required for nitrogen assimilation in many gram negative bacteria. When *E. coli* is grown in the absence of ammonium, glutamine levels are low, signaling the phosphorylation of NtrC by its cognate sensor kinase NtrB, and NtrC-dependent activation of σ^{54} promoters for nitrogen assimilation (326). Deletion of either *rpoN* or *ntrC* (encoding NtrC) results in glutamine auxotrophy, which severely mitigates the ability of *E. coli* to assimilate nitrogen during nitrogen limitation (327). As such, it was hypothesized that NtrC may bridge the gap between nitrogen availability and σ^{54} resulting in modulation of σ^{S}, XAR and the LEE. Experimentation with an *rpoN* mutant strain

that was prototroph for glutamine production demonstrated that while glutamine prototrophy had no influence on expression of XAR and LEE genes, it significantly affected survival of the *rpoN* mutant in acid. This was hypothesized to be a result of direct competition for available glutamate molecules between glutamine synthetase (for nitrogen assimilation) and the decarboxylase (for XAR) enzymes. This demonstrated that while nitrogen assimilation may not directly affect expression of XAR genes, it directly affects XAR at a biochemical level. Alternatively, it was also hypothesized that increasing nitrogen levels in the medium by adding ammonium chloride would downregulate NtrC- σ^{54}-dependent expression in WT EHEC, which should result in a XAR and LEE phenotype very similar to the *rpoN* and *ntrC* mutant strains. Whereas the increased nitrogen levels would not have any effect on XAR and LEE in the *rpoN* and *ntrC* mutants. Interestingly, this hypothesis was completely disproven as increasing nitrogen availability modulated XAR and the LEE in all strains completely independent of NtrC-σ^{54}. Altogether, these results definitively proved that nitrogen availability was not the environmental signal influencing NtrC-σ^{54} directed regulation of σ^{S}, XAR and the LEE.

The effect of ammonium independent of NtrC-σ^{54} led to formulating a new hypothesis for the activating signal of the σ^{54}-σ^{S} pathway. It was proposed that NtrC is autophosphorylated by a non-cognate phosphodonor in the σ^{54} pathway controlling XAR and the LEE and a plausible candidate could be acetyl~P, which is a known NtrC phosphodonor (13, 112). This was proven to be the case as alteration of acetyl~P levels by substituting either glycerol or glycerol and acetate for glucose, or by the deletion of acetate kinase (*ackA*), alters the expression of pathway components for regulation of GDAR and the LEE in a manner dependent on *rpoN* and *ntrC*. There is precedent for this as acetyl~P also serves as a phosphodonor for Rrp2, a σ^{N} EBP found in *B. burgdorferi* and required for activation of the σ^{N}-σ^{S} pathway regulating virulence expression in

this pathogen (433). Thus, the use of acetyl~P for autophosphorylation of σ^{54} EBPs may be a phenomenon that is conserved across different species of bacteria. Since, acetyl~P is common by product of carbon metabolism, the results suggested that the NtrC-σ^{54} regulates σ^{S}, XAR and the LEE in response to carbon availability within the cell. Altogether, this research study has shown that NtrC-σ^{54} are important determinants of virulence and transmission in EHEC and that they do not response to their canonical activating signals for regulation of σ^{S}, XAR and the LEE.

This study also explored the contribution of σ^{54} bEBPs in EHEC fitness and pathogenesis and demonstrated that the bEBPs NorR, PrpR, TyrR and RtcR contribute to EHEC stress fitness in different stressors. The salient observation after screening of the bEBP mutants was the effect deletion of *rtcR* resulted on EHEC fitness and virulence. RtcR was originally implicated in σ^{54}-dependent regulation of the *rtc* operon containing the genes *rtcB* and *rtcA* (124, 317). Subsequent in vitro studies demonstrated that RtcB and RtcA are the only RNA ligase and RNA cyclase, respectively, in *E. coli* and are involved in RNA repair mechanisms (71, 90, 391-393). Removal of RtcR and RtcBA negatively impacts EHEC fitness in acid, peroxide, salt and heat environments and also attenuates EHEC virulence. The decreased resistance to low pH would make EHEC more vulnerable to the low pH within the host stomach, and reduce its chances of determining a low infectious dose. Similarly, the significantly reduced recovery from macrophages of the *rtcR* and *rtcBA* mutants can be correlated to decreased resistance to peroxide suggesting that the mutants are more sensitive to oxygen-dependent killing mechanisms within macrophages. Deletion of *rtcR* and *rtcBA* also negatively impacts adherence to HT-29 intestinal epithelial cells, which may be partially due to a combined effect of reduced LEE, fimbriae and curli genes expression.

Previous studies had hypothesized that genes of the *rtc* operon may play a role during bacterial stress response, although it had never been experimentally shown (71, 90, 393). During

stress response, it has been has been shown that mRNA interfases of type II toxin-antitoxin systems inhibit translation by cleaving mRNA to initiate programmed cell death or arrest cell growth (79, 125, 140, 446). In *E. coli*, the extensively studied type II TA system RelEB is activated under various stress and starvation conditions which eventually causes a global reduction in translation by mRNA cleavage (80, 81, 282). Cleaving by mRNA interfases result in generation of RNA 2'3'-cyclic phosphate and 5'-OH ends which later become crucial substrates for RNA repair mechanisms (8, 223, 272, 400). Interestingly, RtcA (encoded by *rtcA*) is a RNA 3'-terminal phosphate cyclase that catalyzes a three step process of generating RNA 2',3'-cyclic phosphate ends (71, 124). RtcB (encoded by *rtcB*) is a newly discovered family of RNA ligase that breaks the cyclic phosphate end and then ligates the 3'-monophosphate end to a RNA 5'-OH end to restore the 3'5'-phosphodiester backbone (391-393). So far, the functions of RtcBA had only been shown *in vitro* and this is the first time an actual physiological context has been identified where RtcR/RtcBA play an important role in EHEC. The combined effects of low stress and adherence genes expression impact EHEC fitness and pathogenesis. In conclusion, the findings support the previously proposed hypothesis that genes of the *rtc* operon are involved in EHEC stress response and may maintain cellular fitness through RNA repair.

5.3 Implications of This Research Study

EHEC infections present with hemorrhagic colitis in 90% of cases, which progress to HUS in 3-15% of cases (19, 131, 334, 370). Even though individuals of all age can develop HUS, it is more common in children and the elderly (384). In the US alone, there are more than 75,000 cases of infection every year, resulting in 325,000 hospitalization and 5,200 deaths (245). Currently there are no established procedures to prevent or control EHEC disease progression. Research in

prevention efforts have focused primarily on three main mechanisms: I) immunization with Shiga toxin components; II) use of toxin sequestration mechanism in the gastrointestinal tract; and III) use of probiotic strains to prevent toxin uptake (103, 233, 237, 271, 288, 390, 404, 436). So far, these models have been largely ineffective. The lack of any therapy or prevention measures to control EHEC infection reveals the necessity for research which may lead to the elucidation of new drug targets or prevention strategies.

This study extends the functions of RtcR/RtcBA, NtrC and σ^{54} beyond cellular metabolism and demonstrates that they are important for EHEC transmission and colonization. The effects of *rtcR*/*rtcBA* deletion and *ntrC*/*rpoN* deletion were observed during different growth phases in EHEC. Deletion of *rtcR* and *rtcBA* specifically impacted EHEC fitness in acid during stationary phase. Whereas, deletion of *rpoN* and *ntrC* deletion specifically impacted EHEC adherence during exponential growth. This is very important because when EHEC enters the stomach it enters into stationary phase to induce XAR and after passing through the stomach and reaching the intestines it enters exponential growth and induces LEE for colonization. Supposedly, if molecular target based drugs were developed for RtcR/RtcBA and NtrC/σ^{54}, the administration of this combined chemotherapy could be a new approach to EHEC treatment by attacking two separate components of EHEC pathogenesis. First, targeting the RtcR/RtcBA component would potentially reduce fitness and increase sensitivity of EHEC to acid in the stomach, significantly reducing the bacterial load entering the intestines. Second, targeting the NtrC/σ^{54} component would potentially inhibit EHEC from colonizing the gastrointestinal tract. If EHEC could be prevented from colonizing that would significantly reduce the chances of developing hemorrhagic colitis and potentially HUS. Moreover, RtcR/RtcBA and NtrC/σ^{54} have been shown to influence curli/fimbriae and flagella, respectively. As such, targeting them RtcR/RtcBA and NtrC/σ^{54} would also negatively

impact curli, fimbriae and flagella production, which are important determinants of EHEC virulence. Altogether, the findings of this research study show that NtrC/σ^{54} and RtcR/RtcBA have the potential to become drug targets for treatment in EHEC infections.

5.4 Future Studies and Direction

Unlike other alternative sigma factors in *E. coli*, knowledge of the regulation of σ^{54} itself is severely lacking and not understood. The *rpoN* operon is a five gene polycistronic operon, with *rpoN* being the first gene followed by *hpf*, *ptsN*, *yhbJ* and *npr* (Fig. 5.1). Similar to the glucose EIIBA phosphotransferase system, the protein products of *ptsN* and *npr* form the nitrogen phosphotransferase system. However, it has been shown that their functions do not involve transport of molecules, but are rather regulatory (298). $EIIA^{Ntr}$ (encoded by *ptsN*) has been shown to play an important role in sigma factor competition between σ^{70} and σ^{S} in response to potassium

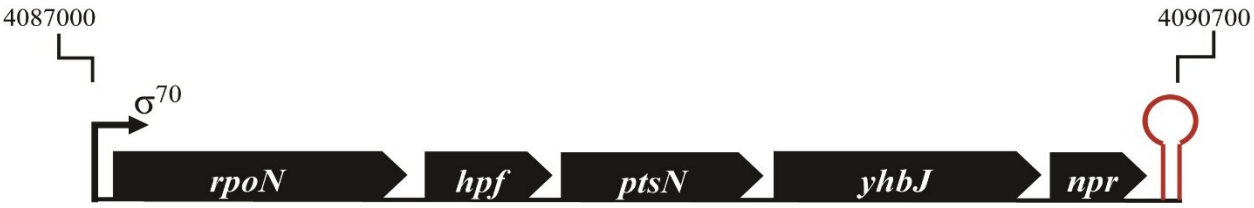

Figure 5.1. RpoN operon in *E. coli* O157:H7 strain TW14359. Organization of genes in the *rpoN* operon.

levels (213). Hpf and $EIIA^{Ntr}$ have been shown to negatively regulate genes of the σ^{54} regulon in *Klebsiella pneumoniae*, and in *P. aeruginosa* $EIIA^{Ntr}$ functions in conjunction with σ^{54} to regulate genes for nitrogen metabolism (172, 247, 255). The organization of these genes in an operon with *rpoN* strongly suggests that they may perform functions that affect σ^{54} itself or at least in regulation of σ^{54}-dependent genes. One of the future studies will be to investigate the roles of the genes in the *rpoN* operon in regulation of σ^{54} and σ^{54}-dependent genes.

As mentioned previously, in *E. coli* K-12 it had been shown that activation of the *rtcBA* is dependent on σ^{54} and its cognate bEBP RtcR. Therefore, logically the inactivation of *rpoN* should phenotypically reproduce the same effect as observed for *rtcR* and *rtcBA* mutants for EHEC fitness. However, the *rpoN* mutant did not phenotypically match the *rtcR* and *rtcBA* mutants suggesting that regulatory effect of RtcR/RtcBA on EHEC fitness may not be fully dependent on σ^{54}. Analysis of the promoter region upstream of *rtcBA* revealed no difference in activation between the *rpoN* and *rtcR* mutant. However, promoter expression analysis was only tested under normal growth and it is possible that under different stress conditions there is differential expression of *rtcBA* in the *rpoN* and *rtcR* mutants. Therefore, another future study will focus on defining the molecular basis for regulation of the *rtc* operon. This will include: I) identify how *rtcR* is regulated, II) identify the upstream activating sequences where RtcR binds to initiate σ^{54}-dependent transcription, and III) identify if there are any σ^{54}-independent promoters for activating *rtcBA* expression.

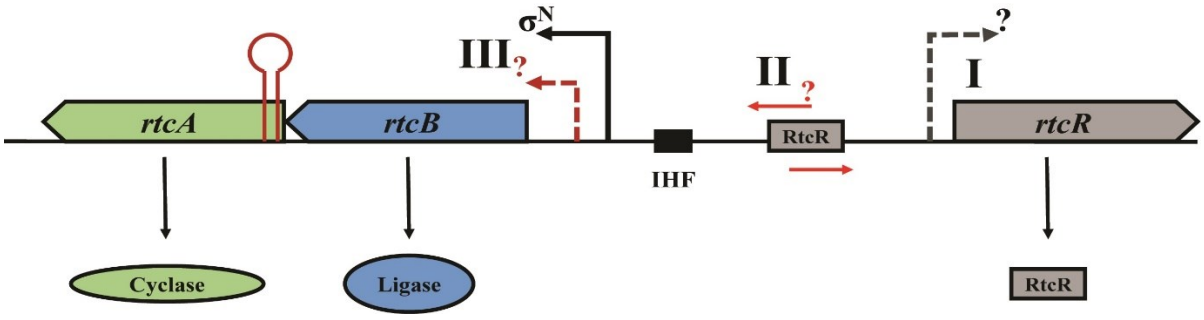

Figure 5.2. Future studies for defining the regulation of the *rtc* operon. I) identify how *rtcR* is regulated, II) identify the upstream activating sequences where RtcR binds to initiate σ^{54}-dependent transcription, and III) identify if there are any σ^{54}-independent promoters for activating *rtcBA* expression.

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Appendix A: PlosOne Permission for Inclusion of Chapter Two in Dissertation

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Sigma factor N, liaison to an ntrC and rpoS dependent regulatory pathway controlling acid resistance and the LEE in enterohemorrhagic Escherichia coli.

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