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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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 (σN), dramatically alters the growth-phase dependent regulation of both acid resistance and the LEE. This study reports on the determinants of σN-directed acid resistance and LEE expression, and the underlying mechanism attributable to this phenotype. Glutamate-dependent acid resistance (GDAR) in TW14359ΔrpoN 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 TW14359ΔrpoN did not confer acid resistance by the ADAR mechanism. LEE regulatory (ler), structural (espA and ces7) and effector (tir) genes were downregulated in TW14359ΔrpoN, and mutation of rpoS encoding sigma factor 38 (σS) in TW14359ΔrpoN restored acid resistance and LEE genes to WT levels. Stability, but not the absolute level, of σS was increased in TW14359ΔrpoN; however, increased stability was not solely attributable to the GDAR and LEE expression phenotype. Complementation of TW14359ΔrpoN with a σN allele that binds RNA polymerase (RNAP) but not DNA, did not restore WT levels of σS stability, gadE, ler or GDAR, indicating a dependence on transcription from a σN promoter(s) and not RNAP competition for the phenotype. Among a library of σN enhancer binding protein mutants, only TW14359ΔntrC, inactivated for nitrogen regulatory protein NtrC, phenocopied TW14359ΔrpoN for σS stability, GDAR and ler expression. The results of this study suggest that during exponential growth, NtrC-σN regulate GDAR and LEE expression through downregulation of σS at the post-translational level; likely by altering σS stability or activity. The regulatory interplay between NtrC, other EBPs, and σN-σS, represents a mechanism by which EHEC can coordinate GDAR, LEE expression and other cellular functions, with nitrogen availability and physiologic stimuli.

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 [1–3]. 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 [4], which allow for survival in highly acidic environments such as the stomach, and which determine a low oral infectious dose [5,6]. For competitive gut colonization, EHEC utilize a type III secretion system (T3SS) encoded on the locus of enterocyte effacement (LEE) pathogenicity island [7–10]. This T3SS translocates EHEC effector proteins into host intestinal cells that mediate intimate attachment to the gut and subvert host cellular processes [11].

The expression of acid resistance and the LEE is influenced by various environmental and intracellular signals, including nutrient availability, stress, and growth phase [12–21]. During exponential growth acid resistance is largely repressed, but is activated as cultures transition into stationary phase [13]; for the LEE, the inverse is true [18]. 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 [12,22–28]. Alternative sigma factor 38 (σS) is a global regulator that plays an important role in coordinating acid resistance and LEE expression with growth phase. σS is a protein of low abundance during exponential growth, but accumulates during transition into stationary phase [29]. The acid resistance phenotype of stationary phase cultures is largely attributed to σS and expectedly, strains mutated for rpoS (encoding σS) are sensitive to acid [13,14], whereas LEE expression is both decreased and increased in response to rpoS mutation, depending on growth conditions [28,30–32]. Not surprisingly, rpoS mutants are impaired in their ability to survive passage in both murine and bovine.
models of infection [33]. $\sigma^S$ is tightly regulated at multiple levels of control [34], and the factors that dictate rpoS/$\sigma^S$ expression indirectly influence acid resistance, the LEE, and EHEC pathogenesis.

Recently, another alternative sigma factor, sigma N ($\sigma^N$), has been shown to control structural and regulatory genes of both acid resistance and the LEE in EHEC serotype O157:H7 [35]. When bound to RNA polymerase (RNAP), the RNAP-$\sigma^N$ holoenzyme (ER$^N$) 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 [36–39]. EHEC strains null for rpoN (encoding $\sigma^N$) 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 [35]. 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 $\sigma^N$-$\sigma^S$ regulatory pathway in E. coli [35].

There is precedent for such a pathway in Borrelia burgdorferi, in which a $\sigma^N$-$\sigma^S$ regulatory pathway controls the expression of membrane lipoproteins essential for transmission and pathogenesis [40–42]. In the B. burgdorferi model, $\sigma^N$ 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 $\sigma^N$ downregulates rpoS/$\sigma^S$ 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 [35]. In addition, a recent study reported increased levels and stability of $\sigma^N$ in an rpoN mutant of the nonpathogenic E. coli strain K-12 MG1655 [43]. This study further explores the regulatory interplay of $\sigma^N$ and $\sigma^S$, and uncovers mechanistic details about $\sigma^N$-$\sigma^S$ directed control of acid resistance and the LEE, and other genetic factors which contribute to the expression of this regulatory pathway.

Results

$\sigma^N$-$\sigma^S$ 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) [16,44], or during stationary phase growth in rich media (rpoS-GadX-GadW) [12], or rich media containing glucose (TrmE) [15]. 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 $\sigma^S$ negatively regulates GDAR through a $\sigma^S$-dependent pathway; namely, $\sigma^S$-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 TW14359ΔrpoN compared to TW14359 (p=0.001), as well as TW14359ΔrpoS (p=0.007), and TW14359ΔrpoNΔrpoS (p=0.005) (Fig. 1A). Adding to this, both gadX and gadW transcripts were upregulated in TW14359ΔrpoN (p<0.05), but not in TW14359ΔrpoS for gadX, or TW14359ΔrpoNΔrpoS 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. 1A); the presence of amplicons for trmE and ydeO was validated by gel electrophoresis. Thus, a rpoN mutation leads to increased expression of the GDAR-activating GadX-GadW pathway, agreeing with the rpoS-dependency of the phenotype.

In addition to GDAR, $\sigma^S$ regulates at least two more acid resistance systems in E. coli: the arginine-dependent acid resistance (ADAR) system [45], and the oxidative-dependent acid resistance (ODAR) system [33]. Both GDAR and ADAR systems protect the cell from acid by a proton scavenging mechanism that is facilitated by the conversion of glutamate to $\gamma$-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 [4]. 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 TW14359ΔrpoN relative to TW14359 and TW14359ΔrpoNΔrpoS (p<0.05) (Fig. 1B). However, adiC, encoding a putative regulator of adiA and adiC [46], was not altered in expression in either of the mutant backgrounds. Despite the

Figure 1. Transcript levels for acid resistance genes. Gene transcript levels as determined by qRT-PCR are plotted for genes of the GDAR system (panel A) and genes of the ADAR system (panel B). Mean transcript levels are normalized to the 16S rRNA gene m5S. Transcript levels are plotted against WT TW14359 (filled), TW14359ΔrpoN (empty), TW14359ΔrpoN ΔrpoS (hatched), and TW14359ΔrpoN ΔrpoS ΔadiY (stippled, gadX and gadE only) for panel A. Asterisks denote significant differences by Tukey's HSD following a significant F-test (n≥3, p<0.05 [*]; p<0.01 [**]). Error bars indicate standard error of the mean.

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increase in adaA and adaC expression in TW14359ΔrpoN, there was no corresponding increase in acid resistance by the ADAR mechanism (Table 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.

σ5 has also been shown to upregulate and downregulate transcription of LEE genes in EHEC. For upregulation, σ5 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 [28]. It has also been reported that both the LEE3 and LEE5 operons possess σ5-responsive promoters [30]. For downregulation, σ5 is proposed to stimulate an unknown repressor of PchA, which is a positive regulator of ler [31,32,47]. The mutation of rpoN leads to the downregulation of LEE genes during exponential growth [35]. Since σ5 controls GDAR through a σ5-dependent pathway, it was predicted that σ5-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 LEE2), the effector chaperone cesT (on LEE5), and the translocated intimin receptor tir (on LEE5) were downregulated during exponential growth of TW14359ΔrpoN relative to TW14359 (p < 0.05) (Fig. 2A). In addition, transcript levels of ler (on LEE1) were reduced in TW14359ΔrpoN compared to TW14359 (p = 0.015) and TW14359ΔrpoS (p = 0.011) (Fig. 2B). Importantly, mutation of rpoS in TW14359ΔrpoN restored ler expression to levels consistent with TW14359ΔrpoS; ler expression was increased in rpoS null backgrounds relative to WT, but not significantly increased. These results indicate that σ5 positively regulates the LEE during exponential growth in an rpoS-dependent manner, and is consistent with the role of σ5 as a negative regulator of LEE expression via the PchA-Ler pathway [31,32,47].

Effect of rpoN Mutation on rpoS mRNA and σ5 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/σ5 [35]. The mutation of rpoN in E. coli strain K-12 MG1655 was recently shown to lead to increased σ5 levels and stability [43]. However, there are substantial differences at the genomic level between K-12 and EHEC O157:H7 strains [48]. As an important example, the TW14359 genome (and the genomes of many other EHEC strains), does not contain two of the thirteen σ5 enhancer-binding proteins found in K-12 and most other E. coli. This study thus aimed to validate the effect of rpoN mutation on σ5 levels and stability in the EHEC background and under the growth conditions that promote σ5-dependent control of GDAR and the LEE.

As anticipated, no difference was observed in the stability of rpoS mRNA between TW14359 and TW14359ΔrpoN (Fig. 3A). After 12 min of rifampin addition, rpoN 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 (TW14359ΔrpoN), which agrees with previous estimates [49,50]. Before addition of rifampin, however, levels of rpoS transcript were higher (1.5-fold) in TW14359ΔrpoN compared to TW14359, but not significantly higher. In agreement with experiments using strain MG1655, σ5 was more stable in TW14359ΔrpoN compared to TW14359, however absolute levels were not observed to be higher in TW14359ΔrpoN (Fig. 3B) as described for MG1655 [43].
In TW14359, σ^S was barely detectable after 4 min of tetracycline addition, but was detected for up to 12 min in TW14359ΔrpoN. The mean half-life for σ^S was estimated at 2.4 min for TW14359 and 5.5 min for TW14359ΔrpoN, 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 [29,51,32], and 10.5–30 min in stationary phase cultures [29,51]. These results reveal that in TW14359ΔrpoN, 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.

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^S is second only to the primary sigma factor, σ^70, whereas σ^2^ binding affinity lies at the bottom of this rank order [53,54]. In addition, the relative cellular abundance of each sigma factor influences gene expression through competition for RNAP [55]. During exponential growth, σ^N levels have been estimated at 10–16% those of σ^70, whereas σ^S is barely detectable [56–58]. 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^S 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^N promoters. This hypothesis might explain the σ^S stability, GDAR and LEE expression phenotype of TW14359ΔrpoN. To examine this possibility, a mutant version of the rpoN gene (rpoN^R456A) was constructed, the product of which can efficiently form E^N holoenzyme but cannot bind DNA to direct transcription from σ^N promoters [91,92]. If the increased stability of σ^S in TW14359ΔrpoN is solely the result of increased RNAP binding by σ^S, the expression of rpoN^R456A in TW14359ΔrpoN should reproduce WT levels of σ^S stability. This was not determined to be the case however, as the stability of σ^S in TW14359ΔrpoN is not only the result of increased RNAP binding by σ^S, but also the result of increased σ^N levels in comparison to TW14359 and TW14359ΔrpoNΔpRAM-2; pRAM-1 (Fig. 4A). The effect of rpoN^R456A expression on the GDAR and LEE expression phenotype of TW14359ΔrpoN was also examined. Transcript levels for the GDAR regulator gade, and the LEE regulator ler in TW14359ΔrpoN and TW14359ΔrpoNΔpRAM-2 did not differ from that of TW14359ΔrpoN, and both were increased in comparison to TW14359 and TW14359ΔrpoNΔpRAM-1, respectively (p<0.05) (Fig. 4B). Interestingly, survival by GDAR for TW14359ΔrpoNΔpRAM-2 was partially reduced compared to TW14359ΔrpoN, but remained substantially higher than TW14359 and TW14359ΔrpoNΔpRAM-1 (Table 1).

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 [29,51]. In strains mutated for clpP (the protease of ClpXP), σ^S is completely stable in exponential phase [51], however in exponential phase cultures of TW14359ΔrpoN, σ^S is still largely unstable (Fig. 3B), suggesting that there remains a sufficient amount of σ^S proteolysis. To reproduce the level of increased σ^S stability characteristic of TW14359ΔrpoN in the WT background, sub-inhibitory 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 TW14359ΔrpoN without the addition of 3, 4-DCI (Fig. 5A). Addition of 3, 4-DCI further increased σ^S levels in TW14359ΔrpoN 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 TW14359ΔrpoN 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 TW14359ΔrpoN (Table 1). Thus increased stability of σ^S alone cannot account for GDAR in TW14359ΔrpoN. The expression of LEE genes is known to be positively influenced by ClpP through its proteolytic effect on σ^S [31,32]. Consistent with this, 3, 4-DCI addition reduced expression from lepR456A-lec-ζ in TW14359 as indicated by a decrease in percent β-galactosidase activity relative to untreated controls (Fig. 5B). Since addition of 3, 4-DCI further increased σ^S stability in TW14359ΔrpoN, it was expected that this increase would correspond with a further decrease in ler.
expression. On the contrary, lerP430-lac expression did not differ in 3,4-DCI-treated TW14359DpRAM-2 cultures compared to untreated controls, and β-galactosidase activity was unchanged throughout growth compared to significantly reduced activity in TW14359 (p < 0.05) (Fig. 5B). These results reveal that although σN stability is sensitive to protease inhibition using 3, 4-DCI in TW14359DpRAM-2, 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).

Identification of the Enhancer-binding Protein Required for σN-directed Regulation of GDAR and the LEE

σN is a unique sigma factor in its requirement for enhancer-binding proteins (EBP) to initiate transcription [59]. If σN stability, GDAR and LEE expression in TW14359DpRAM-2 is dependent on σN-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 expression during exponential growth. Of the eleven mutants, only TW14359ΔglnG and TW14359ΔfhlA expressed GDAR comparable to levels observed for TW14359DpRAM-2 (Table 1). fhlA encodes a regulator of formate metabolism [60], and glnG (also gltG) encodes NfrC, a major regulator of nitrogen assimilation [61,62]. The impact of fhlA or nfrC mutation on LEE expression was then determined by transforming pRJM-1 containing lerP430-lac into both EBP isogenic backgrounds, TW14359DpRAM-2 and TW14359, and β-galactosidase activity was measured during exponential growth. Expression from lerP430-lac increased in TW14359 to levels observed for TW14359DpRAM-2 by Tukey’s HSD following a significant F-test (n > 3, p < 0.01 [**]).}

Figure 4. Effect of rpoN456A expression in TW14359ΔrpoN on σN stability, gadE and ler transcription. (Panel A): Representative western immunoblots for σN in TW14359 (WT), TW14359ΔrpoN complemented with rpoN (TW14359ΔrpoNpRAM-1), TW14359ΔrpoN (ΔrpoN), TW14359ΔrpoN complemented with rpoN456A (TW14359ΔrpoNpRAM-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 σN, and TW14359ΔrpoN (ΔrpoN) as a negative control. Equal gel loading was controlled for by westerns for GroEL. (Panel 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 rrnS. Asterisks denote significant differences between WT and TW14359ΔrpoNpRAM-1 when compared to TW14359ΔrpoN and TW14359ΔrpoNpRAM-2 by Tukey’s HSD following a significant F-test (n = 3, p < 0.05 [*]). Error bars indicate standard error of the mean. doi:10.1371/journal.pone.0046288.g004

Figure 5. Effect of the serine protease inhibitor 3,4-DCI on σN stability and ler expression. (Panel A): Representative western immunoblot for σN stability in TW14359 (WT) and TW14359ΔrpoN (ΔrpoN) during exponential phase (Expo.) 4 min after the addition of tetracycline, and with or without 3,4-DCI, as well as in WT and TW14359ΔrpoN (ΔrpoN) during stationary phase (Stat.) with 3,4-DCI. Equal gel loading was controlled for by westerns for GroEL. (Panel B): Expression from lerP430-lac 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 TW14359ΔrpoN (squares). Asterisks denote significant differences between TW14359 and TW14359ΔrpoN at each OD600 by Welch’s t-test (n = 3, p < 0.05 [*]; p < 0.01 [**]). doi:10.1371/journal.pone.0046288.g005
at \( \text{OD}_{600} = 0.5 \), concordant with qRT-PCR data \((p = 0.008)\) (Figs. 2 and 6). Mutation of \( \text{phbD} \) had no apparent effect on \( \text{ler}^{P430-\text{lacZ}} \) expression, yet \( \text{ntrC} \) mutation reduced \( \text{ler}^{P430-\text{lacZ}} \) expression to 50% of WT at \( \text{OD}_{600} = 0.5 \) \((p = 0.006)\) to levels comparable with \( \text{TW14359} \Delta\text{rpoN} \) (Fig. 6). Thus the mutation of \( \text{ntrC} \) faithfully reproduces the GDAR and LEE expression phenotype of \( \text{TW14359} \Delta\text{rpoN} \). Interestingly, \( \sigma^N \) stability was increased in both EBP mutant backgrounds to the level of stability observed in \( \text{TW14359} \Delta\text{rpoN} \) (Fig. 7). These results reveal that mutation of \( \text{phbD} \) and \( \text{ntrC} \) similarly influence \( \sigma^N \) stability, yet only \( \text{ntrC} \) mutation phenocopies GDAR and LEE expression observed in \( \text{TW14359} \Delta\text{rpoN} \). A strain deleted for both \( \text{phbD} \) and \( \text{ntrC} \) was constructed to validate the dependence on \( \text{phbD} \) for NtrC-directed GDAR and LEE expression, but the mutant was too growth-impaired in DMEM to be phenotypically informative.

**Discussion**

The importance of \( \sigma^N \) in \( \text{E. coli} \) metabolism, particularly nitrogen metabolism, is undisputed. Strains mutated for \( \text{phbD} \) are growth-impaired under nitrogen-limiting conditions due to an inability to activate nitrogen regulatory response promoters. Mutation of \( \text{phbD} \) also clearly affects many genes in \( \text{E. coli} \) that are not directly tied to metabolism, but which are perhaps cued to the metabolic status of the cell through \( \sigma^S \), such as those involved in the regulation of motility [63,64], NO detoxification [65], and biofilm formation [66]. In the present study, the phenotype of acid resistance and LEE expression previously described for \( \text{phbD} \) mutants in \( \text{EHEC} \) [35], represents a case in which \( \sigma^N \)-dependent regulation is indirectly communicated through the downregulation of another sigma factor, \( \sigma^S \). The antagonistic interplay of \( \sigma^N \) and \( \sigma^S \) in the control of these discrete systems resembles that described on a genomic scale by Dong et al. [43], in which it was estimated that as many as 60% of \( \sigma^N \)-regulated genes are counter-regulated by \( \sigma^S \).

![Figure 6. Expression from \( \text{ler}^{P430-\text{lacZ}} \) in \( \sigma^N \) enhancer binding protein mutants. Mean expression from \( \text{ler}^{P430-\text{lacZ}} \) represented as \( \beta \)-galactosidase activity during exponential growth for \( \text{TW14359} \) (triangles), \( \text{TW14359} \Delta\text{phbD} \) (circles), \( \text{TW14359} \Delta\text{phbA} \) (squares), \( \text{TW14359} \Delta\text{ntrC} \) (diamonds) and empty vector pR5551 (hatched line). The asterisk denotes a significant difference for \( \text{TW14359} \Delta\text{phbD} \) and \( \text{TW14359} \Delta\text{ntrC} \) when compared to the remaining stains by Tukey’s HSD following a significant F-test \((n=3, p<0.05)\).](image)

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For control of acid resistance, \( \sigma^N \) negatively regulates the \( \sigma^S \)-directed Gad\( \text{X-GadW} \) pathway of glutamate-dependent acid resistance (GDAR) activation. This agrees with the dependence on \( \text{phbD} \) and \( \text{gadE} \) for acid resistance formerly described for \( \text{phbD} \) mutants [35], and with research showing that \( \sigma^N \) expression in a \( \Delta\text{gadW} \) background cannot induce the GDAR central regulator \( \text{gadE} \) [67]. In this regulatory circuit, \( \sigma^N \) drives the transcription of \( \text{gadX} \), the product of which then activates \( \text{gadE} \) transcription. Gad\( \text{X} \) also downregulates Gad\( \text{W} \), which is a negative regulator of \( \sigma^S \) [12]. As observed for GDAR, \( \sigma^N \) is clearly dependent on \( \text{phbD} \) for upregulation of the LEE, conforming to the role of \( \sigma^S \) as a negative regulator of LEE expression [31,32]. This \( \sigma^N-\sigma^S \) 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 \( \text{phbD} \) mutated \( \text{EHEC} \) [35] but is in the current study, may be explained by the increased sensitivity of qRT-PCR.

The GDAR and LEE expression phenotype of \( \text{TW14359} \Delta\text{phbD} \) correlates with an increase in \( \sigma^S \) stability similar to that described for K-12 [43], however no increase in \( \sigma^N \) levels was observed as was for K-12. This disparity in results could reflect genetic differences between K-12 and \( \text{TW14359} \), or differences in experimental growth conditions. For the latter, the M9 glucose media used by Dong et al. [43] should be strongly growth restrictive for \( \text{phbD} \) mutants, which are auxotrophic for glutamine and a positive control for \( \text{phbD} \) activity. Thus the \( \sigma^N \) stability of the remaining strains by Tukey’s HSD following a significant F-test \((n=3, p<0.05)\). For control of acid resistance, \( \sigma^N \) negatively regulates the \( \sigma^S \)-directed Gad\( \text{X-GadW} \) pathway of glutamate-dependent acid resistance (GDAR) activation. This agrees with the dependence on \( \text{phbD} \) and \( \text{gadE} \) for acid resistance formerly described for \( \text{phbD} \) mutants [35], and with research showing that \( \sigma^N \) expression in a \( \Delta\text{gadW} \) background cannot induce the GDAR central regulator \( \text{gadE} \) [67]. In this regulatory circuit, \( \sigma^N \) drives the transcription of \( \text{gadX} \), the product of which then activates \( \text{gadE} \) transcription. Gad\( \text{X} \) also downregulates Gad\( \text{W} \), which is a negative regulator of \( \sigma^S \) [12]. As observed for GDAR, \( \sigma^N \) is clearly dependent on \( \text{phbD} \) for upregulation of the LEE, conforming to the role of \( \sigma^S \) as a negative regulator of LEE expression [31,32]. This \( \sigma^N-\sigma^S \) 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 \( \text{phbD} \) mutated \( \text{EHEC} \) [35] but is in the current study, may be explained by the increased sensitivity of qRT-PCR.

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Addition of the serine protease inhibitor 3,4-DCI was shown to result in increased \( \sigma^N \) stability in TW14359, and further increased \( \sigma^S \) stability in TW14359rpoN. This cumulative increase in \( \sigma^S \) stability in TW14359rpoN 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 \( \sigma^S \) stability by inhibiting ClpP. Regardless of which is true, increasing \( \sigma^S \) stability alone by interfering with proteolysis did not alter GDAR and LEE expression in TW14359rpoN, suggesting that the mechanistic basis of these phenotypes is distinct. Mutation of rpoN could lead to increased \( \sigma^S \) activity at promoters, or modulate its affinity for RNAP. For the former, both FlhZ and Fls RNA have been reported to reduce \( \sigma^S \) activity at selective promoters [68,69]. Interestingly, transcript levels of fljCZ were markedly upregulated in rpoN null K-12 [43], but not in EHEC [35]. For the latter, various proteins and small molecules are known to facilitate Ehrlichia holoenzyme formation, including Crl [70], Rsd [71], and ppGpp [72]. Currently, the involvement of any of these regulators in \( \sigma^N \)–\( \sigma^S \) control of GDAR and the LEE is unknown.

This study revealed that a strain mutated for ntrC, encoding nitrogen regulatory protein NtcC, is phenotypically similar to an rpoN mutant in regards to \( \sigma^S \) stability, GDAR and LEE expression. NtcC is a canonical \( \sigma^N \) 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 \( \sigma^S \)–24/–12 binding site [62,73,74]. 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 NtcC/\( \sigma^N \) driven promoter directly or indirectly downregulates \( \sigma^S \), which in-turn affects GDAR and LEE expression. Currently however, there is no experimental evidence to support a role for any of the known NtcC/\( \sigma^N \) regulated genes in this. Alternatively, NtcC could activate \( \sigma^S \) promoters independent of DNA binding, which may relax the site selectivity of NtcC/\( \sigma^N \) dependent transcription initiation. Examples of this have been described for Rrp2 of B. burgdorferi, and FlgR of Campylobacter jejuni, that activate \( \sigma^S \) promoters in the absence of known UAS sites for these EBP's by some unknown mechanism [75–77]. There is also a precedent for NtcC regulating transcription independent of \( \sigma^S \). NtcC binds to the core promoters of gldcP1 and gldcP2, repressing gldcG/gldcALG (glutamine synthetase operon) transcription by interfering with \( \sigma^N \)-dependent initiation [61]. Other E. coli promoters that are directly downregulated by NtcC have not however been described.

This study further identified FlhA as a putative EBP involved in the control of \( \sigma^S \) and GDAR, but not the LEE. FlhA 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 CO2 and H2 [78]. However, the fact that flhA 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 [78], together suggesting that the acid resistance conferred by flhA mutation is independent of this mechanism. Alternatively, mutation of flhA may lead to the accumulation of formic acid during growth on glucose (DMEM contains 4 g/l glucose) leading to acid-adapta-

Directed Gene Deletion and Site-specific Mutation

Gene deletion mutants were constructed using the \( \lambda \) Red recombinase-assisted approach [89,90] and as described [33]. Primers used for the deletion of \( \sigma^N \) EBP's, as well as rpoN and rpoS are provided in Table S1. For site-specific mutatio

Materials and Methods

Bacterial Strains and Culture Conditions

The strains and plasmids used in this study are listed in Table 2. Strains were stockeed 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) [86] containing 4 g/l glucose and 4 mM glutamine (pH 7.4) were used to inoculate fresh DMEM to a final OD600 = 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 [35]. The growth of strains in DMEM was monitored by taking OD600 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 [87,88].
Table 2. Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
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<tr>
<td>DH5α</td>
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<tr>
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<td>TW14359</td>
<td>WT 2006 outbreak, western U.S.</td>
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HindIII (Table S1). This fragment was ligated into ClaI/HindIII-digested pACYC177 to produce pRAM-1 (Table 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 S1) and Pfu Ultra™ high fidelity DNA polymerase (Agilent, Santa Clara, CA) to produce pRAM-2 (Table 2). The resultant σ² 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 [91,92]. pRAM-1, in addition to pRAM-2 purified from E. coli XL10-Gold® (Agilent) transformants, were transformed into strain TW14359 ΔrpoN as described [35]. Genetic constructs were validated by PCR, and restriction mapping, or by DNA sequencing and qRT-PCR.

Tests for Acid Resistance

Acid resistance by the glutamate- and arginine-dependent systems was measured as described [35] with slight adaptations. For the glutamate-dependent acid resistance mechanism, mid-exponential (OD₆₀₀ = 0.5) DMEM cultures were inoculated to 10⁶ 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.

Quantitative Real-time PCR (qRT-PCR)

Primer pairs for qRT-PCR are provided in Table S1. RNA purification, cDNA synthesis, qRT-PCR cycling conditions and data analysis followed previously described protocols [35,93].

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 σ², 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-σ² mAbs (Neoclon, Sigma-Aldrich).
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 containing 2% skim milk. Western blots were repeated a minimum of three times in independent trials.

σ5 and rpoS mRNA Stability

Cultures were grown to mid-exponential phase (OD600 = 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 (σ5 protein stability). RNA was purified and validated as described [93]. For rpoS mRNA stability, gene transcript levels were measured using qRT-PCR and primers rpoS+356 and rpoS446 (Table S1). Protein was extracted, and σ5 levels measured by western immunoblots. The half-life in minutes for rpoS mRNA and σ5 was extrapolated from gene transcript or protein levels, respectively, using linear regression analysis and as described [94]. The strength of linearity was estimated by the correlation coefficient (r2), and exceeded 0.85 (85%) for all analyses.

lacZ Transcriptional Fusions and β-galactosidase Assay

A 429-bp BamHI/EcoRI digested PCR fragment generated using primers ler-1/BamHI and ler-430/EcoRI (Table S1) and corresponding to nucleotide positions 4,679,303–4,679,731 in strain TW14359 was cloned into the similarly digested vector pRS551 [95] using T4-DNA ligase (Fisher) to create pRJM-1 (Table S1). 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 (lerP430-lacZ). pRJM-1 purified from DH5α transformants was used for transformation into various WT and mutant backgrounds. The lerP430-lacZ fusion was confirmed by PCR and sequencing. To measure β-galactosidase activity from lerP430-lacZ, 50 μl culture samples taken at OD600 = 0.25 (early exponential), OD600 = 0.5 (mid-exponential) and OD600 = 1.0 (late exponential) were immediately added to 950 μl Z-buffer (1 M KCl, 1 mM MgSO4, 0.05 M β-mercaptoethanol, 0.06 M Na2HPO4, 0.04 M NaH2PO4·H2O, 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 ortho-nitrophenol, the reaction was terminated by the addition of 0.5 ml Stop Solution (1 M Na2CO3) and samples were mixed and then centrifuged at 21,000 × g for 5 min before measuring β-galactosidase activity. β-galactosidase activity was converted to Miller Units as described [96].

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) [97]. The MIC for 3,4-DCI was at 60 μM for both WT and rpoS null backgrounds. The effect of 3,4-DCI addition to growing cultures on σ5 stability, GDAR and LEE expression was determined as described above. For σ5 stability, 3,4-DCI was added to cultures at mid-exponential phase (OD600 = 0.4) and incubated to OD600 = 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 OD600 = 0.4 as for σ5 stability, and then GDAR tested, or β-galactosidase activity measured from lerP430-lacZ as described above. Control cultures did not contain 3,4-DCI for all experiments.

Supporting Information

Figure S1 Growth of strains in Dulbecco’s Modified Eagle’s Medium (DMEM). Mean (n = 2) optical density 600 nm (OD600) plotted for TW14359 (empty squares), TW14359ΔrpoS (filled squares), TW14359ΔrpoSΔs (circles), TW14359ΔfhlA (plus signs), TW14359ΔntrC (triangles), and TW14359ΔrpoSΔntrCΔs (diamonds). Individual OD600 measurements for each strain varied by less than 5%. For lerP430-lacZ expression (Fig. 6), sampling was done for all strains except for TW14359ΔrpoS and TW14359ΔrpoSΔntrC at OD600 = 0.25, OD600 = 0.5, and OD600 = 1.0 approximately corresponding to early-, mid- and late-exponential phase, respectively. For all remaining experiments, sampling was done at OD600 = 0.5. (TIF)

Table S1 Primers used in this study.

(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: JTR AM. Performed the experiments: AM PAF JKM KWV SLV. Analyzed the data: JTR AM. Wrote the paper: JTR AM PAF.

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