σN-dependent control of acid resistance and the locus of enterocyte effacement in enterohemorrhagic Escherichia coli is activated by acetyl phosphate in a manner requiring flagellar regulator FlhDC and the σ5 antagonist FliZ

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Abstract
In enterohemorrhagic Escherichia coli (EHEC), sigma factor N (σN) regulates glutamate-dependent acid resistance (GDAR) and the locus of enterocyte effacement (LEE); discrete genetic systems that are 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-σN-dependent reduction in the activity of sigma factor S (σ5). This study elucidates pathway components and stimuli for σN-directed regulation of GDAR and the LEE in EHEC. Deletion of fliZ, the product of which reduces σ5 activity, phenocopied rpoN (σN) and ntrC null strains for GDAR and LEE control, acid resistance, and adherence. Upregulation of fliZ by NtrC-σN was shown to be indirect and required an intact flagellar regulator flhDC. Activation of flhDC by NtrC-σN and FlhDC-dependent regulation of GDAR and the LEE was dependent on rpoN -promoter flhDp2, and a newly described NtrC upstream activator sequence. Addition of ammonium chloride significantly altered expression of GDAR and LEE, acid resistance, and adherence, 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, abrogated NtrC-σN-dependent control of flhDC, fliZ, GDAR, and the LEE.

Introduction
Alternative sigma factor N (σN) when bound to RNA polymerase directs the transcription of genes for carbon and nitrogen metabolism, stress fitness, and regulation (Reitzer and Schneider 2001). In an increasing number of bacterial pathogens, σN also regulates genes for virulence and transmission, and is required for complete in vivo disease progression (Okada et al. 2008; Barchiesi et al. 2009; Albert-Weissenberger et al. 2010; Damron et al. 2012; Iyer and Hancock 2012; Mills et al. 2012; Sheng et al. 2012; Wang et al. 2012). For most pathogens, the mechanism underlying σN-dependent regulation of pathogenesis remains unknown; two exceptions to this include Borrelia burgdorferi, and to a lesser extent, enterohemorrhagic Escherichia coli (EHEC). In B. burgdorferi, the causative agent of Lyme borreliosis, σN activates the expression of genes encoding outer surface lipoproteins (OspA and OspC) essential for transmission from the tick vector to a mammalian host, and for establishment of infection (Pal et al. 2000; Hubner et al. 2001; Grimm et al. 2004). This Osp activation pathway requires another sigma factor, σ5, the transcription of which is directly activated from a σ5-promoter in what has been dubbed a σN-σ5 regulatory cascade (Smith et al. 2007; He et al. 2008). In EHEC serotype O157:H7, a food-borne pathogen attributed to outbreaks and sporadic cases of bloody diarrhea (hemorrhagic colitis) (Rangel et al. 2014).
control of \(\sigma^N\), acid resistance, and the LEE, and to examine the role for nitrogen availability in the stimulation of this pathway. The study identifies flhD and fliZ as new genetic determinants of this pathway and provides evidence that NtrC-\(\sigma^N\)-FlhDC-dependent activation of fliZ, the product of which modulates \(\sigma^N\) activity, is needed for regulation of GDAR and the LEE. Furthermore, the availability of acetyl phosphate, not ammonia, is shown to be an important factor for pathway activation.

**Experimental Procedures**

**Bacterial strains and growth conditions**

All strains and plasmids used in this study are listed in Table 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\textsubscript{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 mmol/L 3-morpholinopropane-1-sulfonic acid (MOPS) and containing 0.4% (w/v) glucose, or MOPS minimal medium. MOPS medium was prepared as described in Neidhardt et al.’s (1974) study, and contained 0.4% (w/v) glucose, 0.1% (w/v) L-glutamine. Cultures were grown for 18–20 h before inoculating into fresh DMEM or MOPS to a final OD\textsubscript{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.

**Procedures for genetic manipulation**

Nonpolar gene deletion mutants were constructed using the \(\lambda\) Red recombinease-assisted approach (Datsenko and Wanner 2000; Murphy and Campbellone 2003) and as described previously (Riordan et al. 2010). Primers used for the construction of deletion mutants are listed in Table S1. For overexpression of flhDC, a 932-bp polymerase chain reaction (PCR) fragment containing flhDC of strain TW14359 (nucleotide positions 2485400–2484469) was generated using primers flhDC-F/EcoRI and flhDC-R/XbaI. An EcoRI/XbaI digested fragment of the product was cloned into similarly digested arabinose-inducible expression vector pBAD22 (Guzman et al. 1995) to produce pRAM-3. pRAM-3 purified from DH5\(\alpha\) transformants was then used to transform TW14359 and derivative strains producing EcRAM-51 through EcRAM-53. For flhDC promoter expression studies, a 2942-bp XhoI/BamHI digested PCR fragment (nucleotide positions 2487394–2484453) was generated using primers flhD-1994/XhoI and flhC+595/BamHI. This fragment contained the flhDC open
Table 1. Strains and plasmids used in this study.

| Strain/Plasmid | Relevant characteristics | Source/Reference |
|----------------|--------------------------|------------------|
| DH5α           | Vector propagation, recA1 endA1 | Miroux and Walker (1996) |
| BL-21          | WT O157:H7 2006 outbreak, western US (NC_013008.1) | Manning et al. (2008) |
| TW14359        | WT2487152                  | This study       |
| EcRPF-6        | TW14359ArpoN               | Mitra et al. (2012) |
| EcRAM-26       | TW14359ΔntrC               | Mitra et al. (2012) |
| EcRAM-43       | TW14359ΔapoLΔgln+, suppressor mutant for Gln auxotrophy | This study |
| EcRAM-45       | EcRAM-43ΔglnA              | This study       |
| EcRAM-47       | TW14359ΔrrsA::kan KanR     | This study       |
| EcRAM-49       | TW14359ΔrpoN               | This study       |
| EcRAM-51       | EcRF-6 pRAM-3 AmpR         | This study       |
| EcRAM-52       | EcRAM-26 pRAM-3 AmpR       | This study       |
| EcRAM-53       | EcRAM-49 pRAM-3 AmpR       | This study       |
| EcRAM-58       | TW14359ΔflhDC             | This study       |
| EcRAM-59       | EcRAM-58 pRAM-4 AmpR       | This study       |
| EcRAM-60       | EcRAM-58 pRAM-5 AmpR       | This study       |
| EcRAM-61       | EcRAM-58 pRAM-6 AmpR       | This study       |
| EcRAM-63       | TW14359ΔackA              | This study       |
| EcRAM-66       | TW14359ΔflhDC             | This study       |
| EcRAM-68       | TW14359ΔflhDC             | This study       |
| Plasmid name   | pACYC177                  | Chang and Cohen (1978) |
| pET-24d        | IPTG-inducible His-tagging vector, KanR | Novagen |
| pBAD22         | Mid-copy arabinose-inducible cloning vector, AmpR | Guzman et al. (1995) |
| pSC-B          | High-copy cloning vector, KanR | StrataClone |
| pBAD-TA        | Mid-copy arabinose-inducible cloning vector, AmpR | Invitrogen |
| pRAM-1         | rpoN::pACYC177, AmpR KanR  | Mitra et al. (2012) |
| pRAM-3         | flhDC::pBAD22, AmpR       | 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, KanR | This study |
| pRAM-8         | fliZ::pBAD, AmpR           | This study       |
| pRAM-9         | ackA::pSC-B, AmpR KanR     | This study       |

Quantitative real-time PCR

RNA purification, cDNA synthesis, qRT-PCR cycling conditions, and data analysis for relative quantitation of gene expression followed previously described protocols (Riordan et al. 2010; Mitra et al. 2012; Morgan et al. 2013). Analysis was performed using a Realplex2 Mastercycler (Eppendorf, Hauppauge, NY). Cycle threshold (Ct) data were normalized to rrsA (16S rRNA gene) and normalized Ct values (ΔCt) were transformed to arbitrary gene expression units using 2-ΔCt/10^-8 as described by Livak and Schmittgen (2001). A previous method was used for the quantitation of flhD mRNA copy number (Bustin 2000). Briefly, a 154-bp PCR product containing flhD was generated using flhD+63 and flhD+216, column purified (Qiagen, Valencia, CA) and serially diluted in molecular grade water. Ct was measured for each dilution to generate a standard curve plotting Ct 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 Ct 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 analysis of variance (ANOVA) and Tukey’s HSD (α ≥ 3, α = 0.05) using R v. 2.13.0.
Protein extraction, purification, and procedures for western blots

Protein extraction, purification, and procedures for western blots followed a previously described protocol (Mitra et al. 2012; Morgan et al. 2013). Monoclonal antibodies for σ^S and GroEL were acquired from Neoclonne (Madison, WI) and Bio-Rad (Carlsbad, CA), respectively. Densitometry was used to estimate differences in protein levels using a ChemiDoc XR^S+ Imaging System and Image Lab 3.0 (Bio-Rad, Hercules, CA). Western blots were repeated a minimum of three times in independent trials.

Purification of NtrC

A 1425-bp Ncol/Xhol-digested PCR fragment generated using primers ntrC+F/Ncol and ntrC-R/Xhol was cloned into similarly digested pET-24d producing pRAM7 and replacing the ntrC stop codon with a C-terminal 6xHis 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 mmol/L Isopropyl β-D-1-thiogalactopyranoside for 16 h at 20°C (200 rpm). Cultures were harvested by centrifugation (5000g, 20 min) and 6xHis-NtrC was purified using a nickel Ni-NTA Protein Purification Kit (Qiagen) according to the manufacturer’s instruction.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (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-F/Ncol and flhD-R/XhoI for the flhD probe promoter, and glnA-311/Biotin and glnA-112/Biotin for the glnA promoter probe; biotin end-labeled Epstein-Barr nuclear antigen (EBNA) DNA was supplied with the kit. The flhD probe promoter probe (strain TW14359 ntrC knockout position 248734–2487242) contained a putative NtrC-binding site flanked by 0.1 kb. For the glnA probe 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 mmol/L KCl, 5 mmol/L MgCl₂, 1% (v/v) glycerol, 0.05% (v/v) NP-40, 50 ng/μL poly(dI-dC) copolymer competitor, 10x molar excess Bovine Serum Albumin (10 mg/mL), and 0, 2, 4, 8, or 16 μmol/L purified C-terminally labeled 6xHis-NtrC. Reactions were incubated for 40 min at 4°C, and were then separated by electrophoresis using 8% non-denaturing acrylamide gels prepared in 0.5x Tris-borate-EDTA buffer at 4°C for 80 min at 160 V, and DNA/protein complexes transferred to a nylon membrane (Fisher, Pittsburgh, PA). Membranes were UV cross-linked at 120,000 mL/cm² for 1 min and detected by chemiluminescence using the Biotin Detection System (Pierce) and a ChemiDoc XR^S+ Imaging System including Image Lab 3.0 (Bio-Rad, Hercules, CA).

Selection of suppressor mutants for glutamine auxotrophy

Spontaneous suppressor mutants for glutamine auxotrophy were selected in the TW14359ΔrpoN background by growth in MOPS minimal media without the addition of glutamine. Briefly, overnight cultures of TW14359ΔrpoN grown in MOPS media were inoculated into fresh MOPS containing 0.4% glucose and 0.1% NH₄Cl and grown at 37°C (200 rpm). The outgrowth of suppressor mutants (TW14359ΔrpoN 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 by Reitzer et al. (1987) 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.

Whole genome next-generation DNA sequencing and analysis

Genomic DNA was extracted from TW14359ΔrpoN and a single suppressor mutant of TW14359ΔrpoN (TW14359ΔrpoN 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, Grand Island, NY). Each DNA library was purified using the E-Gel® SizeSelect™ 2% Agarose system (Invitrogen), and the integrity and quantity of each was determined using a Bioanalyzer high-sensitivity DNA chip (Agilent). 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
were exported from the Ion Torrent Server and analyzed using the Genomics Suite software package (CLC Bio, Boston, MA). 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 TW14359ΔrpoN and TW14359ΔrpoN Gln+.

**Adherence assay**

Adherence to epithelial cells was determined following a previously described protocol (Morgan et al. 2013). 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 for 20 min. Giemsa-stained coverslips were examined at 1000× magnification by oil immersion, and microcolonies were scored as discrete clusters of five or more bacterial cells 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 1000× magnification by oil immersion, and microcolonies were scored as discrete clusters of five or more bacterial cells as previously defined (McKee and O’Brien 1995; Abe et al. 2002; Iyoda and Watanabe 2004). For each sample, a minimum of 10 viewing frames were observed and the average number of microcolonies were reported per 50 HT-29 cells. Microcolony counts were compared between strains by Tukey’s HSD following a significant F-test (n ≥ 3, α = 0.05) (R v. 2.13.0).

**Tests for acid resistance**

Acid resistance by the glutamate-dependent system was measured for exponential phase cultures grown in DMEM as previously described (Riordan et al. 2010; Mitra et al. 2012) with slight adaptations. Strains were grown in DMEM to OD₆₀₀ = 0.5 before inoculating (10⁶ CFU/mL final cell density) into E minimal glucose (EG) media containing 5.7 mmol/L 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.

**Results**

**NtrC-σ^N require fliZ for control of σ^S activity, GDAR, and the LEE**

Previous studies have revealed NtrC and σ^N negatively regulates GDAR and positively regulates the LEE by reducing the activity of alternative sigma factor S (σ^S) (Riordan et al. 2010; Mitra et al. 2012). 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 (Pratt and Silhavy 1998; Typas et al. 2007), whereas FliZ interferes with σ^S promoter-binding and transcription initiation, thus reducing σ^S-dependent transcription (Pesavento et al. 2008; Pesavento and Hengge 2012). During growth in DMEM (OD₆₀₀ = 0.5), both crl and fliZ expression were shown to be reduced in TW14359ΔntrC and TW14359ΔrpoN compared to TW14359 (P ≤ 0.05) (Fig. 1A), however, only TW14359ΔfliZ phenocopied TW14359ΔntrC and TW14359ΔrpoN for the control of GDAR and LEE genes (Fig. 1B). In TW14359Δacr, both gadE and gadB were increased in expression compared to TW14359 (P < 0.05), but less than for TW14359ΔrpoN, TW14359ΔntrC, and TW14359ΔfliZ, in which expression levels for all genes were nearly identical (P < 0.01) (Fig. 1B). The expression of LEE genes ler, tir, espA, and cesT did not differ between TW14359 and TW14359Δacr, but were uniformly reduced in TW14359ΔntrC, TW14359ΔrpoN, and TW14359ΔfliZ backgrounds (P < 0.05) (Fig. 1B). Both gadE and ler expressions were restored to near wild-type levels in fliZ complement strain TW14359ΔfliZpRAM-8 or by the deletion of rpoS in TW14359ΔfliZ (Fig. 1B). Consistent with the effect of fliZ deletion on gadE and gadB expression, CFU/mL of TW14359ΔfliZ recovered following exposure to acidified (pH 2) EG media for 1 h increased by 10- to 100-fold compared to TW14359, TW14359ΔfliZpRAM-8, and TW14359ΔfliZΔrpoS, yet remained ~10-fold less than that observed for TW14359ΔntrC and TW14359ΔrpoN (Fig. 1C). Furthermore, the ability to form microcolonies on HT-29 intestinal cells was decreased in TW14359ΔfliZ compared to TW14359 (P = 0.02), and matched that observed for TW14359ΔntrC and TW14359ΔrpoN (Fig. 1D). Thus, NtrC-σ^N positively regulate fliZ during exponential growth, the product of which is predicted, downregulates GDAR and upregulates the LEE by reducing the activity of extant σ^S.
**Requirement for flhDC in the activation of fliZ by NtrC-\(\sigma^N\)**

flZ is encoded as the second gene of a three gene operon (fliAZY), the transcription of which is directed from at least two promoters, fliA\(_P1\) and fliA\(_P2\). Neither of these promoters are \(\sigma^N\)-dependent, however, fliA\(_P1\) is activated by the regulator of flagellar biosynthesis and motility FlhDC, for which there is a predicted \(\sigma^N\)-dependent promoter, flhD\(_P2\) (Zhao et al. 2010). 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 flZ by NtrC-\(\sigma^N\) is a consequence of direct activation of flhDC transcription from this promoter.

In agreement with this, flhDC expression was similarly reduced in both TW14359\(\Delta\)ntrC and TW14359\(\Delta\)poN backgrounds compared to TW14359 during growth in DMEM (OD\(_{600}=0.5\) \(P<0.05\)) (Fig. 2A). Also, flhDC significantly decreased gadE levels and increased ler levels when overexpressed in TW14359\(\Delta\)ntrC and TW14359\(\Delta\)poN \(P<0.05\), but not in TW14359\(\Delta\)flZ (Fig. 2B). To define cis-elements of the flhDC promoter region important for NtrC-\(\sigma^N\)-dependent regulation, flhDC mRNA copy number was measured from three promoter fragments (Fig. 2C) cloned into arabinose-inducible vector pBAD22 and transformed into TW14359\(\Delta\)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 wild-type flhDC promoter fragment (Frag. I) \(P=0.004\) (Fig. 2C). flhDC copy number was further reduced when expressed from a fragment in which both the NtrC UAS and putative \(\sigma^N\) promoter flhD\(_P2\) were removed (Frag. III), but not significantly less than for Frag. II. Correspondingly, gadE expression increased \(P<0.01\) and ler expression decreased \(P<0.05\) in TW14359\(\Delta\)flhDC expressing either Frag. II or Frag. III when compared to Frag. I (Fig. 2C). Thus, the putative NtrC UAS site and \(\sigma^N\) promoter flhD\(_P2\) 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-\(\sigma^N\) dependent glutamine synthetase promoter, glaA\(_P2\) (Fig. 2D). No shift was observed for flhD or glnA promoter probes in the absence of 6xHis-NtrC, or for the negative control EBNA DNA probe (Fig. 2D).
Glutamine is not essential for NtrC-σN-dependent regulation of GDAR and the LEE

The preceding experiments reveal NtrC-σN to directly activate flhDC transcription, the product of which upregulates fltZ. FltZ, in-turn, reduces the activity of ρS and consequently, σS-dependent control of GDAR and LEE expression. While much is understood as to how σN regulates GDAR and the LEE (Sperandio et al. 1999; Foster 2004; Iyoda and Watanabe 2005; Laaberki et al. 2006), the mechanistic basis for activation of NtrC-σN-dependent control of these discrete genetic systems is as yet unknown. NtrC-σN 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 (reviewed in Reitzer and Schneider [2001]). 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 ρN promoter glnAP2 in a manner dependent on NtrC. As such, strains that are null for rpoN or ntrC cannot initiate transcription from glnAP2 and are auxotrophic for glutamine when nitrogen is limiting. The significance of glutamine metabolism to NtrC-σN-dependent control of GDAR and the LEE was thus examined by selecting a
suppressor mutant of glutamine auxotrophy in TW14359ΔrpoN and observing its effect on GDAR and LEE gene expression, acid resistance, and adherence. Growth of TW14359ΔrpoN 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. 3A). However, after 48 h the outgrowth of a prototrophic (Gln+) suppressor mutant (TW14359ΔrpoNGln+) was repeatedly observed in which wild-type growth in MOPS media was restored (Fig. 3A), and in which the expression of glnA was significantly increased compared to TW14359ΔrpoN during growth in DMEM (OD600 = 0.5) (P = 0.013) (Fig. 3B); glnA expression was still, however, slightly but significantly lower in TW14359ΔrpoNGln+ 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: glnAP1 and glnAP3 are σ70 promoters that are repressed by NtrC during nitrogen-limitation, whereas glnAP2 is a σN 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 derepression of glnAP1 and/or glnAP3, while mutations in the promoter(s) enhance transcription from glnAP1 or result in formation of a de novo σ70 consensus at glnAP2 (Reitzer et al. 1987). DNA sequencing of ntrC and the glnA promoter region did not reveal any of these described mutations in TW14359ΔrpoNGln+. Sequencing of the TW14359ΔrpoNGln+ genome, however, revealed a single adenine deletion in the ntrC ORF at nucleotide position 4,910,080 (accession NC_013008, NCBI), resulting in a frameshift 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 TW14359ΔrpoNGln+ (Fig. 3B), reflects derepression at the glnAP1 and glnAP3 promoters due to NtrC inactivation.

Expression levels for gadE and ler did not differ between TW14359ΔrpoN and TW14359ΔrpoNGln+ during growth.
in DMEM (OD_{600} = 0.5), indicating that glutamine availability has no impact on GDAR and LEE gene regulation. Interestingly, however, CFU/mL recovered from acidified EG media were decreased by ~1000-fold for TW14359ΔrpoN in comparison to TW14359ArpoN (Fig. 3C). Deletion of glnA in TW14359ΔrpoN restored survival in acid comparable to that of TW14359ΔrpoN (Fig. 3C), suggesting that glutamine synthetase production plays an indirect role in EHEC acid resistance. Overexpression of glnA in TW14359ArpoN (strain TW14359ΔrpoNpRAM-9) similarly mitigated the acid resistance phenotype of TW14359ΔrpoN (Fig. 3C), clearly demonstrating a role for glnA in the complete acid resistance phenotype of TW14359ΔrpoN. Adding to this, CFU/mL recovered from acidified EG increased by ≥100-fold in TW14359ΔglnA compared to TW14359. Consistent with qRT-PCR data on ler (Fig. 3A), the number of microcolonies formed on HT-29 cells for all grounds (Fig. 4C) and correspondingly increased the ler expression in TW14359ArpoN (Fig. 4B). For the LEE, ammonium addition and NH_4Cl (1 g/L) (OD_{600} = 0.5), the expression of flhDC, fliZ, and ler was decreased and gadB increased in TW14359ΔrpoN in comparison to TW14359 (P < 0.05), but not in TW14359ΔrpoN. The addition of sodium acetate (2 g/L) to glycerol treatments restored flhDC, fliZ, and ler expression to levels observed for glucose in TW14359, but to have no effect on flhDC or fliZ expression in these backgrounds (Fig. 4E). This reduction in rpoS transcript levels correlated with a reduction in σ^S levels in all backgrounds with ammonium, however, σ^N levels were not as strongly reduced in TW14359ArpoN when compared to TW14359 or TW14359ΔrpoN (Fig. 4F).

Feng et al. (1992) 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 (McCleary and Stock 1994; Wolfe 2005). It was thus of interest to determine the effect of glucose and acetyl-P availability on NtrC-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 (1 g/L) (OD_{600} = 0.5), the expression of flhDC, fliZ, and ler was decreased and gadB increased in TW14359ΔrpoN and TW14359ΔrpoN when compared to TW14359 (P < 0.05) (Fig. 5A), similar to that observed during growth in DMEM media (Figs. 1A, B and 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 TW14359ΔrpoNpRAM-1 (P < 0.05), but not in TW14359ΔrpoN and TW14359ΔrpoN (Fig. 5A). Likewise, glycerol substitution increased gadB expression in TW14359 and TW14359ΔrpoNpRAM-1 (P < 0.05), but not in TW14359ΔrpoN and TW14359ΔrpoN. 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 not significantly increased when compared to glycerol treatments (Fig. 5A). In TW14359ΔrpoN and TW14359ΔrpoN, acetyl-P was observed to generally increase fliZ, flhDC, and ler expression, 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 expressions were measured in a

**:Acetyl phosphate stimulates the NtrC-σ^N-pathway controlling GDAR and LEE expression**

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 may prompt 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 TW14359ΔrpoN and TW14359ΔrpoN backgrounds.

While the addition of ammonium chloride (2 g/L NH_4Cl) was observed to slightly but not significantly increase GDAR gene (gadE and gadB) expression in TW14359, expression in TW14359ΔrpoN and TW14359ΔrpoN uniformly decreased (P < 0.05) (Fig. 4A). Correspondingly, ammonium addition reduced CFU/mL recovered for TW14359ΔrpoN and TW14359ΔrpoN by ~100- to 1000-fold but had no observable effect on CFU/mL recovered for TW14359 (Fig. 4B). For the LEE, ammonium addition increased ler, tir, espA, and cesT expression in all backgrounds (Fig. 4C) and correspondingly increased the 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 these 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 (Fig. 5I). Interestingly, growth in DMEM containing ammonium was observed to significantly reduce rpoS expression in TW14359, TW14359ΔrpoN, and TW14359ΔrpoN (P < 0.01), while having no impact on flhDC or fliZ expression in these backgrounds (Fig. 4E). This reduction in rpoS transcript levels correlated with a reduction in σ^S levels in all backgrounds with ammonium, however, σ^N levels were not as strongly reduced in TW14359ArpoN when compared to TW14359 or TW14359ΔrpoN (Fig. 4F).
strain null for acetate kinase (*ackA*), the product of which catalyzes the interconversion of acetate to acetyl-P (Rose et al. 1954). In TW14359ΔntrC, TW14359ΔrpoN, and TW14359ΔackA, *gadB* expression was significantly and uniformly increased when compared to TW14359 (*P < 0.01*) (Fig. 5B). Complementation with *ackA* (strain

Figure 4. Role for ammonium in the NtrC-σN-dependent pathway controlling GDAR and the LEE. (A) Expression levels for *gadE* (filled) and *gadB* (empty) without (−) and with (+) the addition of NH₄Cl plotted for wild-type and derivative strains; asterisks denote significant difference between treatments by t-test (*P < 0.05, **P < 0.01, n ≥ 3). (B) Representative colony-forming units (CFU/mL) on LBA for wild-type and derivative strains grown without (−NH₄) or with (+NH₄) NH₄Cl added to DMEM and following 1-h challenge in EG media (pH 7 vs. pH 2). (C) Expression levels for ler (black), tir (white), espA (stippled), and cesT (gray) for wild-type and derivative strains grown without (−NH₄) or with (+NH₄) NH₄Cl added to DMEM. (D) Counts for microcolonies on HT-29 cells plotted for wild-type and mutant derivative strains grown without (−NH₄) or with (+NH₄) NH₄Cl. Boxplots are as described for Figure 1D. (E) Expression levels of *flhDC*, *fliZ*, and *rpoS* plotted for wild type (black), ΔntrC (white), and ΔrpoN (stippled). (F) Representative western blot for σS and GroEL (control) in wild type, ΔrpoN, and ΔntrC grown without (−) or with (+) NH₄Cl added to DMEM. For A, C, and E, asterisks denote significant differences between treatments by t-test (*P < 0.05, **P < 0.01, n ≥ 3). For D, plots that differ in lowercase letter differ significantly by Tukey’s HSD following a significant F-test (*n ≥ 3, P < 0.05*). Error bars denote standard deviation. GDAR, glutamate-dependent acid resistance; LEE, locus of enterocyte effacement.
TW14359ΔackApRAM-8) restored gadB expression to wild-type levels. For ler, expression was similarly reduced in TW14359ΔackA, TW14359ΔntrC, and TW14359ΔrpoN when compared to TW14359 and TW14359ΔackApRAM-8 (P < 0.05). Together, these data provide evidence that regulation of GDAR and the LEE by NtrC-RN is insensitive to changes in nitrogen availability (i.e., glutamine/ammonium), but instead is influenced by the availability of acetyl-P.

Discussion

In the present study, NtrC and σN have been shown to positively regulate the expression of crl and fliZ, the products of which control the activity of σ5. It is predicted that of the two, only FliZ is a required component of the σN pathway controlling σ5, GDAR, and the LEE. What impact crl upregulation in TW14359ΔrpoN has on σ5, if any, is as yet unclear. Crl and FliZ play antagonistic roles in the regulation of σ5. Crl directly binds σ5 facilitating interaction with RNA polymerase and holoenzyme (Eσ5) formation (Bougdour et al. 2004), whereas FliZ acts downstream of Eσ5 formation, binding to the -10 box of σ5 promoters (Pesavento and Hengge 2012) precluding promoter recognition by Eσ5. Thus, FliZ may be dominant to Crl in σN-directed control of σ5 activity. Alternatively, Crl reduces σ5 stability in an RssB-dependent manner during all stages of growth (Typas et al. 2007). It is therefore plausible that the increased stability of σ5 in rpoN null backgrounds (Dong et al. 2011; Mitra et al. 2012) results from reduced crl expression. This is consistent with the observation that in TW14359ΔrpoN the GDAR and LEE expression phenotype cannot be reproduced by increasing σ5 stability alone (Mitra et al. 2012).

The transcription of fliZ is largely determined by FlhDC, a global regulator of motility genes (Francez-Charlot et al. 2014).
2003). FlhD forms a heterodimer with FlhC, directly activating transcription of the flhA2Y operon from the \( \sigma^N \)-dependent promoter flhA2P. This study determined that flhDC was required for \( \sigma^S \)-directed regulation of GDAR and LEE genes in a manner that was dependent on an intact flhZ. Based on our results, it is predicted that NtrC and \( \sigma^N \) directly activate transcription of flhDC during exponential growth in DMEM (4 g/L glucose, with no NH\(_4\)\_3) requiring the putative \( \sigma^N \)-promoter flhD\(_P\), and a newly identified NtrC box at positions 2481732–2487152 (Fig. 6). This NtrC box is nearly identical to the predicted NtrC consensus (Ferro-Luzzi Ames and Nikaido 1985), differing by a single nucleotide in the dyad repeat region. Upregulation of FlhDC leads to increased transcription of flhZ (Francez-Charlot et al. 2003), the product of which decreases the activity of \( \sigma^S \) (Pesavento and Hengge 2012) (Fig. 6). This suggests that during exponential growth NtrC-\( \sigma^N \) keep the activity of extant \( \sigma^S \) in check by increasing FlhDC-dependent transcription of flhZ. One consequence of this reduced \( \sigma^S \) activity in EHEC is an increase in LEE expression (Riordan et al. 2010) and correspondingly, increased in vitro microcolony formation. This could occur by at least two discrete mechanisms: by upregulation of pchA or through the downregulation of gadE (Fig. 6). PchA is a LEE activator that is negatively regulated by \( \sigma^S \) (Iyoda and Watanabe 2005), whereas GadE represses the LEE and is activated by \( \sigma^S \) through upregulation of gadX (Ma et al. 2003). While the involvement of PchA in this pathway cannot be ruled out, only gadE and gadX expressions are significantly altered in the rpoN null background (Riordan et al. 2010). Even though FlhDC has been shown to effect adherence in E. coli, until now, the association has been negative. Leatham et al. (2005) 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 (Iyoda et al. 2006). As 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 (Iyoda et al. 2006; Morgan et al. 2013). Perhaps FlhDC is used by NtrC 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 \( \sigma^N \)-dependent regulation of the LEE (Mitra et al. 2012).

By reducing the activity of \( \sigma^S \), \( \sigma^N \) also helps to maintain a low level of GDAR gene expression during exponential growth (Fig. 6). However, unlike \( \sigma^N \)-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 \( \sigma^S \) 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 \( \gamma \)-amino butyric acid (GABA) (Fig. 6). In the absence of glutamate, GDAR is defective in protecting E. coli from acid stress (reviewed in Foster [2004]). It is this cellular glutamate that is the source of the corresponding mechanism. Specifically, under nitrogen-limiting conditions (ex. growth in DMEM), NtrC-\( \sigma^N \) activate transcription of glutamine synthetase (glnA), which catalyzes the conversion of glutamate (Glu) to glutamine (Gln) (Fig. 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 σN-regulated genes have been shown to be antagonistically controlled by σP in E. coli (Dong et al. 2011), 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-σN-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 σP promoters for nitrogen assimilation (Reitzer 2003). 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 reduces expression of the bundle-forming pilus genes bfpA and bfpT, and reduces T3S-secretion of the EspA, EspB, and EspC translocon proteins (Puente et al. 1996; Kenny et al. 1997; Martínez-Laguna et al. 1999). For ETEC, ammonium increased expression of the 987P fimbria genes fasH and fasA (Edwards and Schifferli 1997). Changes in 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 increases toward the distal small intestine (Toskes 1993; Edwards and Schifferli 1997; Martínez-Laguna et al. 1999). 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 (Martínez-Laguna et al. 1999). 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 noncognate phosphodonor in the σN pathway controlling GDAR and the LEE. Acetyl-P is a plausible candidate (Fig. 6), as it is a known NtrC phosphodonor (Feng et al. 1992; Atkinson and Ninfa 1998), 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), altered 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 σN 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 (Takamura and Nomura 1988; Pruss and Wolfe 1994). Correspondingly, control of gad and LEE genes by NtrC and σP is restricted to the mid-exponential phase of growth (Riordan et al. 2010; Mitra et al. 2012). Remarkably, acetyl-P also serves as a phosphodonor for Rrp2, a σN EBP found in B. burgdorferi and required for activation of the σN-σP pathway regulating virulence expression in this pathogen (Xu et al. 2010). Thus, the use of acetyl-P for autophosphorylation of σN 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 (Feng et al. 1992). 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-σN 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 (Wolfe 2005), suggesting that it may be used by NtrC to communicate various environmental cues to σN.

**Conflict of Interest**

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Effect of *ntrB* deletion on the expression of genes for GDAR and LEE control. Gene expression levels plotted for wild type (black), ΔrpoN (white), Δ*ntrC* (hatched), and Δ*ntrB* (gray). Asterisks denote significant difference between wild-type and respective mutants by *t*-test (*P* < 0.05, **P** < 0.01, *n* ≥ 3). Error bars denote standard deviation.

**Figure S2.** Growth curve for TW14359 and mutant derivative strains in DMEM. Optical density (OD<sub>600</sub>) is plotted for each strain as a function of time. Samples were taken every hour for 12 h. OD<sub>600</sub> measurements differed by less than 5% for each time point and strain.